PN-ABK121

76002

CONRAD INTERNATIONAL WORKSHOP

Nonsteroidal Gonadal Factors:
Physiological Roles and Possibilities in Contraceptive Development

January 6-8, 1988
Omni International Hotel
Norfolk, Virginia, USA

The Jones Institute
for Reproductive Medicine

Department of Obstetrics
and Gynecology

Eastern Virginia Medical School

DEDICATION
TO
CORNELIA P. CHANNING, Ph.D.
1938-1985

It is most appropriate that the first CONRAD International Workshop on Nonsteroidal Gonadal Factors be dedicated to the memory of our colleague, Dr. Cornelia Channing.

Nina Channing was a friend, mentor and collaborator to many who attended this Workshop, and touched their lives both as a scientist and human being.

Indeed, it was Dr. Channing's consummate fervor as a leader and pioneer in the area of gonadal regulators — especially inhibins and OMI — that has finally resulted in many of the accomplishments described at this Workshop.

Nothing would have given Nina more pleasure than to know that the isolation of some of her putative substances, especially inhibin, has finally been realized.

It is, therefore, an honor for the CONRAD Program to formally dedicate this Workshop to the memory of Cornelia P. Channing in the hope that her memory continues to shine in our hearts as we make progress in reproductive endocrinology, a subject to which she dedicated her life.

Proceedings of the First Contraceptive Research and Development (CONRAD) Program
International Workshop on Nonsteroidal Gonadal Factors
January 6 to 8, 1988, Norfolk, Virginia, U.S.A.
Funded by the Agency for International Development, Washington, D.C., U.S.A.

Copyright© 1988 by The Jones Institute Press.
All rights reserved. No part of this book may be reproduced or transmitted in any form by any means, electronic or mechanical, including photocopying, recording, or by any information storage and retrieval system, without permission in writing from the publisher.

The Jones Institute Press and the Agency for International Development divest themselves of any responsibility for injury and/or damage to persons or property as a matter of products liability, negligence or otherwise, or from any use or operation of any methods, products, instructions, or ideas contained in the material herein. No suggested test or procedure should be carried out unless, in the reader's judgment, its risk is justified. The veracity of the scientific procedures described are the responsibility of the authors.

Printed and bound in the United States of America.

THE JONES INSTITUTE PRESS
855 W. Brambleton Avenue
Norfolk, Virginia 23510

LIBRARY OF CONGRESS #88-81244

The CONRAD Workshop Series
Library of Congress Cataloging-in-Publication Data
Nonsteroidal gonadal factors.

CONRAD WORKSHOP SERIES EDITORS

Gary D. Hodgen, Ph.D.
Senior Director
The CONRAD Program

Henry L. Gabelnick, Ph.D.
Director, Extramural Programs and Product Development
The CONRAD Program

Nancy J. Alexander, Ph.D.
Director, Applied Fundamental Research
The CONRAD Program

Zev Rosenwaks, M.D.
Director, Clinical Programs
The CONRAD Program

David F. Archer, M.D.
Director, Clinical Research
The CONRAD Program

Anibal A. Acosta, M.D.
Director, Fellowships and Andrology
The CONRAD Program

Jeffrey M. Spieler, M.Sc.
Biologist
Research Division, Office of Population
Agency for International Development

Rose-Marie Bradley Jones
Production Editor

DEFINITION OF TERMS

Inhibin: A glycoprotein hormone, consisting of two dissimilar disulfide-linked subunits termed α and β which inhibits pituitary gonadotropin synthesis and/or secretion, preferentially that of FSH.

Follicle Regulatory Protein (FRP): A 15,000 kDa peptide derived from medium and small sized follicles which inhibits aromatase activity and gonadotropin dependent progesterone production by granulosa cells.

Anti-Mullerian Hormone (AMH) [also called Mullerian Inhibiting Substance (MIS)]: A glycoprotein hormone consisting of two identical disulfide linked 72K subunits which inhibits Mullerian duct development in the male fetus.

Follistatin: Glycosylated single chain proteins of M.W. 35,000 and 32,000, with identical N-amino acid terminus, which inhibit the release of pituitary FSH.

Activin: A glycoprotein hormone, consisting of two similar disulfide linked β subunits of inhibin A and/or B which preferentially stimulate FSH release *in vitro*. FRP was formerly used to designate an FSH releasing protein (activin).

Gonadrotropin Surge Inhibiting Factor (GnSIF): A putative hormone derived from follicular fluid which blocks the surge mode of LH and FSH secretion induced by estrogen positive feedback or GnRH.

Prorenin: Prorenin is the biological precursor of renin which is intimately involved in blood pressure and fluid and electrolyte homeostasis through the (pro)renin-angiotensin-aldosterone system. Prorenin is produced by the ovary in response to the LH surge and/or hCG administration and by the placenta (chorion laeve).

Oocyte Maturation Inhibitor (OMI): A low molecular weight (<2000 kDa) substances(s) capable of maintaining the developing oocyte at the diplotene stage (dictyate substage) of prophase one during the first meiotic division.

CONTENTS

1. **The Purification of Native Inhibin and Chemical Characterization** 1
   Darrell N. Ward

2. **Structure and Recombinant Expression of Human Inhibin and Activin** 19
   Anthony J. Mason

3. **Isolation and Characterization of Gonadal Polypeptides that Regulate the Secretion of Follicle-Stimulating Hormone** 30
   Nicholas P. Ling

4. **Distribution and Regulation of Inhibin Subunit mRNAs** 47
   Helene Meunier

5. **Structure and Function of Inhibin and Its Related Peptides** 56
   Kaoru Miyamoto

6. **Monitoring of Bioactive Follicle-Stimulating Hormone (FSH) in Normal and Abnormal Reproductive States** 74
   Aaron J.W. Hsueh

7. **Changes in the Serum Concentrations of Inhibin in Mammals** 91
   Yoshihisa Hasegawa

8. **Regulation of the Subunit mRNAs of Follicle-Stimulating Hormone by Inhibin, Estradiol, and Progesterone** 110
   William L. Miller

9. **Gonadal Inhibitors of Steroidogenesis and Gametogenesis** 125
   Paul Franchimont

10. **Inhibin—Regulation and Mechanism of Action** 137
    Henry G. Burger

11. **The Physiology of Inhibin in Women** 149
    Robert I. McLachlan

12. **How Does Inhibin Regulate LH and FSH Secretion? Studies in Perifused Rat Pituitary Cells** 168
    Stephen J. Winters

13. **Role of Inhibin-Related Peptides in the Human Placenta** 181
Felice Petraglia

14. **Identification of a Factor in Fractions of Sertoli Cell-Enriched Culture Medium Containing CMB-21 That Stimulates Androgen Production by Leydig Cells** 192
C. Yan Cheng and C. Wayne Bardin

15. **Ovarian Hyperstimulation in Monkeys: Evidence for a Gonadotropin Surge-Inhibiting Factor (GnSIF)** 202
Robert S. Schenken

16. **The Regulation of Pituitary Gonadotropin Secretion by Gonadotropin Surge-Inhibiting Factor (GnSIF) and Inhibin** 221
Douglas R. Danforth

17. **Experience with Follicle Regulatory Protein as an Inhibitor of Folliculogenesis and Spermatogenesis *In Vivo*** 235
Gere S. diZerega

18. **Anti-FSH Antibody as a Probe in Determining the Role of FSH in the Maintenance of Gonadal Function in the Primate** 249
N. Raghuveer Moudgal

19. **Follicular Regulation of Oocyte Maturation: Role of Oocyte Maturation Inhibitor** 260
Alexander Tsafriri

20. **Oocyte Maturation Inhibitor: Several Candidates for the Title** 265
Seymour Pomerantz

21. **Anti-Mullerian Hormone in the Ovary** 284
Nathalie Josso

22. **Mullerian Inhibiting Substance in the Ovary: Function and Mechanism of Action** 297
Patricia K. Donahoe

23. **Insulin-Like Growth Factor I: A Paradigm for Putative Intraovarian Regulators** 314
Eli Y. Adashi

24. **Growth Factors and Reproduction** 330
David W. Schomberg

25. **Prorenin and Reproductive Function** 339
Jean E. Scaley

26. **Ovarian Prorenin in Women Undergoing Ovarian Stimulation with Gonadotropins and During Pregnancy** 355
Joseph Itskovitz

CLOSING PANEL 364
C. Wayne Bardin
Sheldon J. Segal
Roy Hertz
Jeffrey M. Spieler
Robin Foldesy
Gabriel Bialy

WORKSHOP PARTICIPANTS 370

1

The Purification of Native Inhibin and Chemical Characterization

DARRELL N. WARD
KATHERINE K. HINES
WAYNE L. GORDON
GEORGE R. BOUSFIELD

The early history of the isolation of inhibin was complicated by disconsonant reports of the molecular size and suitable handling procedures (see reviews).[2,5,7,9,29] Moreover, the investigations utilizing male sources of inhibin were, in several instances, led to characterize products now known to be of doubtful relationship to inhibin.[13,17,19] The situation in the male is in a process of clarification and we will not comment further on inhibin isolation from male sources in this presentation.

In retrospect, the initial confusion concerning the properties of inhibin derived from ovarian follicular fluid related, in the main, to the fact that there were definite higher molecular weight forms of inhibin and that inhibin also tends to form ill-defined association complexes with other inert proteins in follicular fluid. Our lab wasted some time trying to isolate these complexes to be treated by dissociation as a final step.[4] We soon learned that was an impractical approach.

Adsorption and dissociation were used in early studies by several investigators,[2,3,6,14] but in 1985 several laboratories developed, within a short space of time, various purification schemes for both porcine and bovine follicular fluid that had in common the use of dissociation conditions at one or more stages in the fractionation. Most importantly, all agreed on an inhibin of a minimum size of approximately 32 kilodalton (kDa) proteins.[10,12,18,23,25]

NATIVE INHIBIN PURIFICATION

The approach to inhibin fractionation that we developed — and are still developing — in our laboratory, after we abandoned the association complex approach, is outlined in Figure 1. We looked first for a rapid and efficient method to separate the proteins in ovarian follicular fluid from the salts and large liquid volumes.

The acetone precipitation procedure of Huang and Miller[16] proved rapid and very efficient, i.e. 99-100 percent recovery of activity. The product precipitate is designated AP. The AP material we next extracted (50 mg/ml) at room temperature with 95% acetic acid

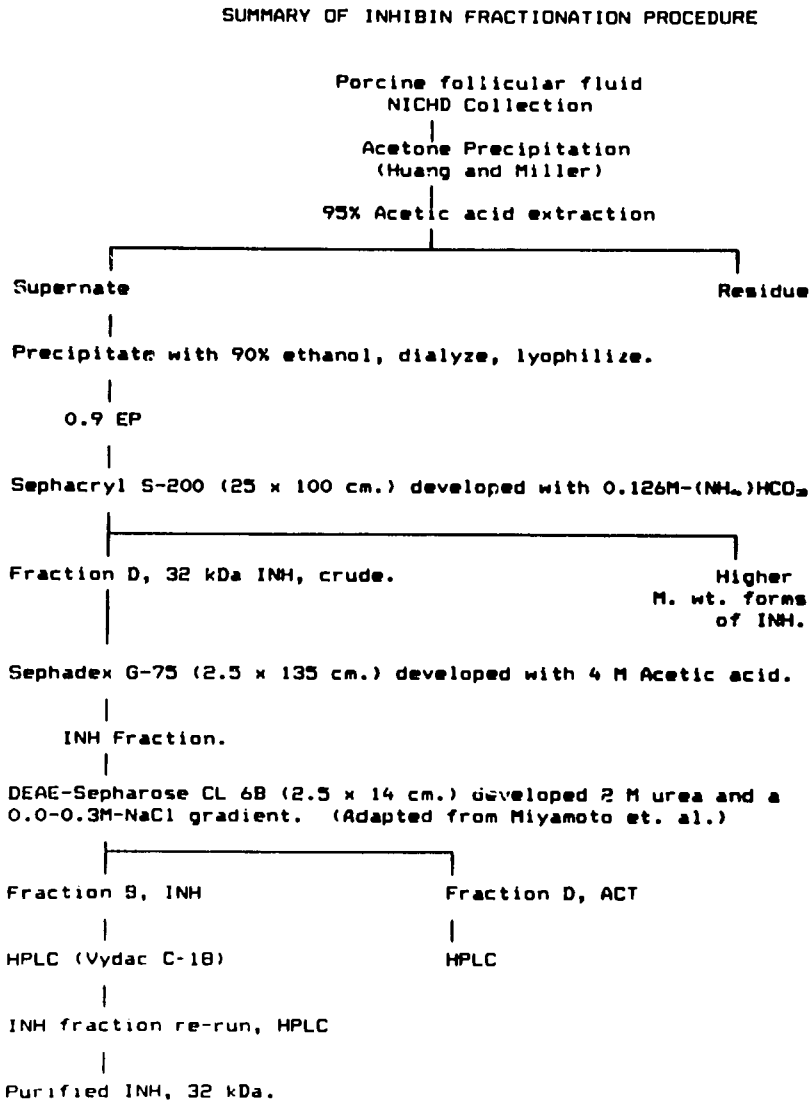


Figure 1. Outline for the 32 kDa porcine inhibin fractionation scheme employed in our laboratory.

overnight. This step provides a strong protein solubilizing action and at the same time inactivates many proteases or potential proteases.[12] The solubilized proteins were then precipitated in the cold with pre-chilled ethanol added slowly to obtain a 90% ethanol precipitate. The precipitate (EP) was the starting material for several variations of fractionation that we have applied. The procedure outlined in Figure 1 has been largely described in two publications from our lab.[12,13] The EP fraction accounts for 70-90% of the ac-

tivity in the original porcine follicular fluid, based on data acquired in a series of fractionations extending over the past four years.

We believe the strong acetic acid and the following precipitation separates the inhibin from proteins with which it tended to associate. At any rate, the material behaves as discrete forms of inhibin in our subsequent fractionation steps.

Our next step is chromatography on a Sephacryl S-200 column in a volatile buffer system (0.126M-ammonium bicarbonate, pH 8). Four major fractions are observed based on absorption at 280 nm. These are a high molecular weight fraction A which has no inhibin activity. Fraction B contains the higher molecular weight forms of inhibin. Fraction C contains intermediate molecular weight forms of inhibin mixed with considerable impurities similar to albumin. Fraction D contains the low molecular weight form of inhibin (32 kDa) at about the 1% level of purity. The inhibin activity in fractions B and C rechromatograph to the same area of the chromatographic pattern, thus they represent true molecular forms of inhibin similar to those so nicely characterized by Miyamoto and colleagues for bovine inhibin.[22] These higher molecular weight forms account for approximately 80-90% of the original inhibin activity. Thus, these larger forms of inhibin comprise a significant part of the inhibin activity in follicular fluid. As more studies arise comparing purified, or partially purified, 32 kDa inhibin to physiological studies that used crude follicular fluid, it may prove important to take into account these larger forms of inhibin and their conversion and rate of conversion to the 32 kDa form of inhibin.

For our present discussion we will follow only the 32 kDa form of inhibin in Fraction D from the Sephacryl S-200 column. We have found that this fraction chromatographs much better at the HPLC stage if we employ a Sephadex G-75 chromatography developed with 4 M acetic acid, as illustrated in Figure 1. Also, since the HPLC resolves the activin from inhibin very poorly we have found that a chromatographic step adapted from Miyamoto *et al.*[23] is useful, because their DEAE-Sepharose CL 6B provides a good separation of activin and inhibin activity. We insert this step as indicated in Figure 1.

Fraction B from the DEAE-Sepharose chromatography can be taken directly to a reverse phase chromatography with a 0.1% trifluoroacetic acid development via a gradient to acidified acetonitrile. However, the adjacent impurities overlap the inhibin peak the first time through the column and it is necessary to rechromatograph the inhibin at least once to get a homogeneous preparation. This is a costly procedure in terms of final product, and we are working on means of improving the product to load to the HPLC by an affinity chromatographic step, such as the lentil lectin column to be discussed below, or an immunoaffinity step. These latter steps are still under development.

The Sephacryl S-200 step has now been scaled up to a 25 x 100 cm. column operation to provide a chromatography pattern as in Figure 2. The fractions are now collected in large flasks, based on this pattern, and concentrated to small volume on a Minitan filter system. Fraction D contains the 32 kDa inhibin activity, which is processed on a Sephadex G-75 column. For this chromatography we use 500 mg fraction D, dissolved in 30 ml of 25% acetic acid, and applied to a 5 x 150 cm. column developed with the same solvent.

---

†The details of the pituitary cell culture assay we use and the reference preparation on which we base our units of inhibin have been described previously.

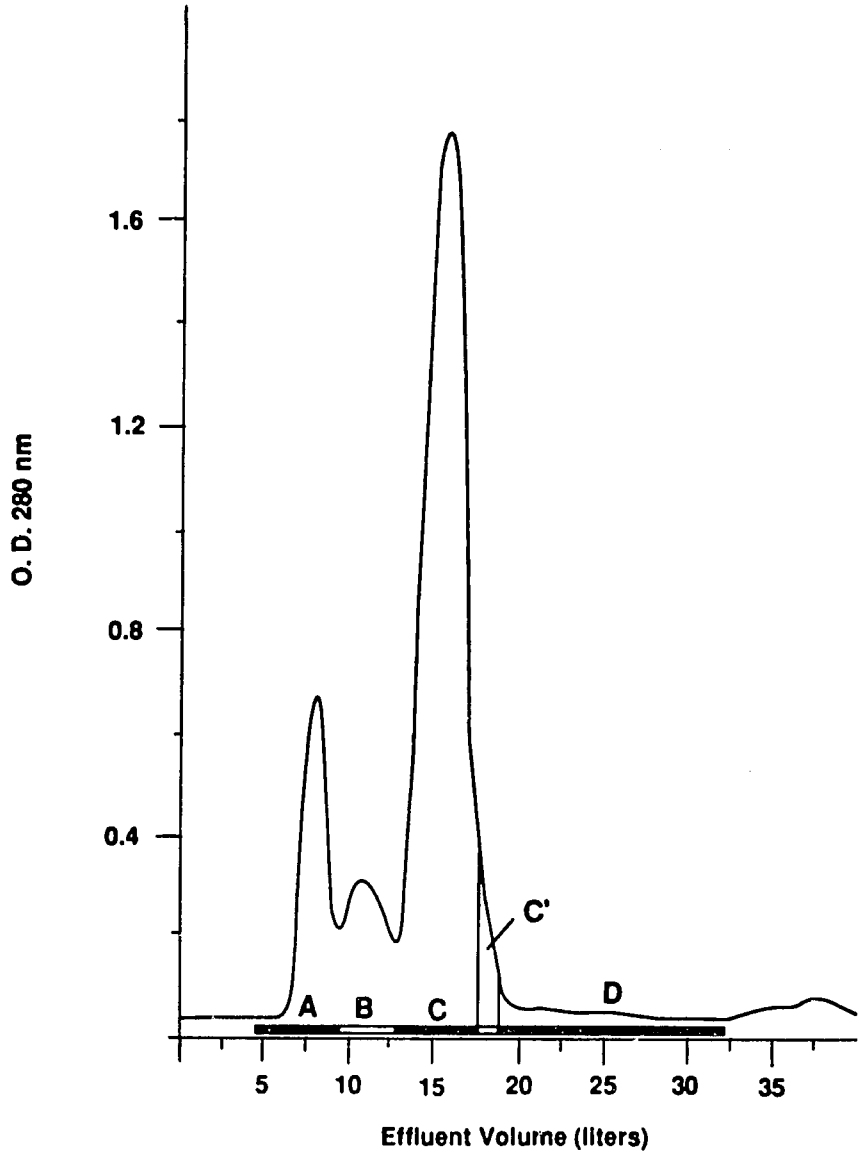


Figure 2. Chromatographic pattern obtained with Sepharose S-200 chromatography of the EP product (35 g) in 0.126 M NH_4HCO_3 on a 25 x 100 cm. column, 4°C. Fraction B contains high molecular weight forms of inhibin, Fraction C the intermediate molecular weight inhibin forms. The C′ Fraction is collected as an overlap area between the intermediate and 32 kDa form. Fraction D contains the bulk of the 32 kDa inhibin.

Inactive fractions A and B, followed by the inhibin activity as shown in the fraction C, stippled area, Figure 3, are obtained. The inhibin fraction from the Sephadex G-75 column treatment is then applied to the DEAE-Sephacryl column. The column is developed

as indicated in Figure 4. The inhibin is eluted in peak B, the activin in peak D. The indicated fractions are collected and worked up separately by (membrane concentration not performed), dialysis, and lyophilization. The fractions are then examined by reverse phase HPLC.

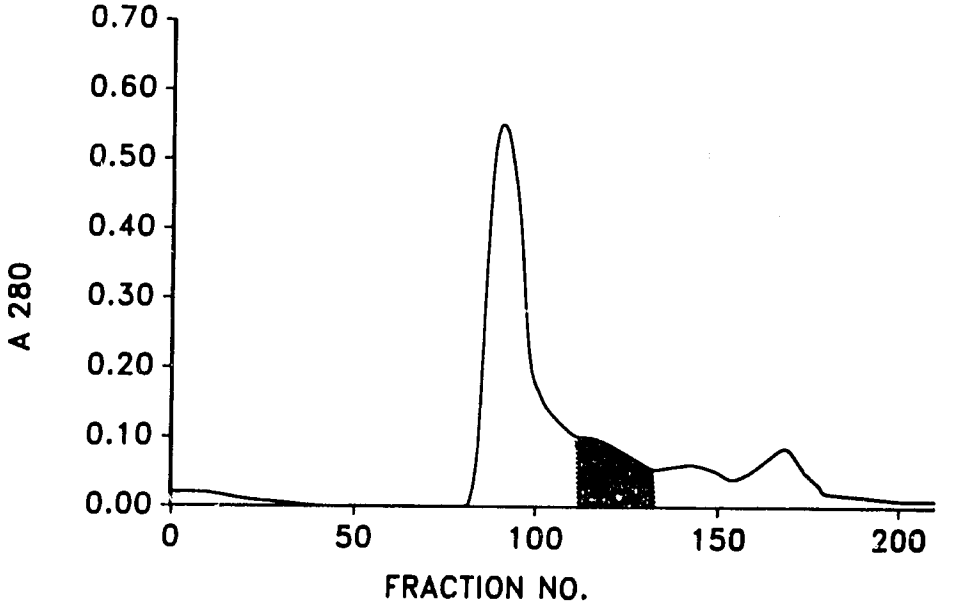


Figure 3. Sephadex G-75 chromatography of 500 mg Fraction D, on a 5 x 150 cm column developed with 25% acetic acid. The inhibin is in Fraction C, stippled area.

The HPLC chromatogram obtained with the inhibin-rich fraction B from the DEAE-Sephacryl chromatography provides a pattern as shown in Figure 5. The inhibin fraction is cut as indicated in the cross-hatched area. This fraction area is usually cut in two to three fractions (manual collection of the effluent), and the pattern of the SDS gels obtained is somewhat dictated by the reaction time of the collector. The indicated purity is about 50%. Rechromatography will raise this to about 95% purity, but the losses on the rechromatography are of the order of 50% and higher, since cuts are made to remove impurity as much as possible. With the gradient as indicated in Figure 5 the location of inhibin in the 55-57 minute elution point and at about 39% solvent B is very reproducible. This is very near the location of activin under these chromatographic conditions, as may be seen in Figure 6. Actually, activin should be slightly in advance of the inhibin, but if they are on the same chromatographic run, the inhibin will usually obscure the activin response in the assay of the column fractions.

Figure 7 summarizes the assay data for the first-pass HPLC fractions of inhibin (split peak) or activin (Figures 5 and 6, respectively.) The reference standard we routinely employ is also represented, but we have no reference prep for activin.

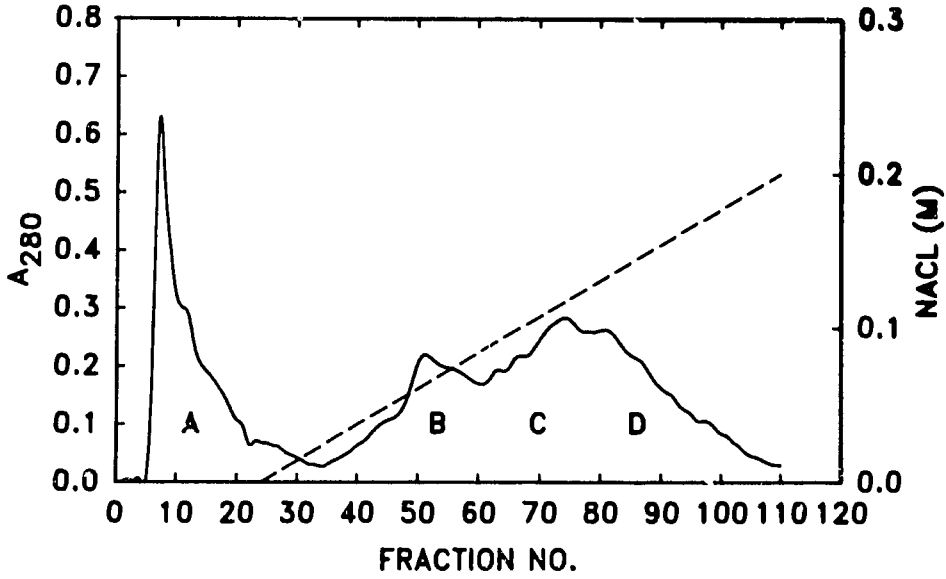


Figure 4. DEAE-Sepharose CL6B chromatograph pattern with 160 mg of the Sephadex G-75 Fraction C, applied in 3 ml of the initial development buffer, 0.013 M-Tris.HCl, 0.005% CHAPS, 8 M urea, pH 7.5. A gradient, 0-0.2 M-NaCl, was applied as indicated.

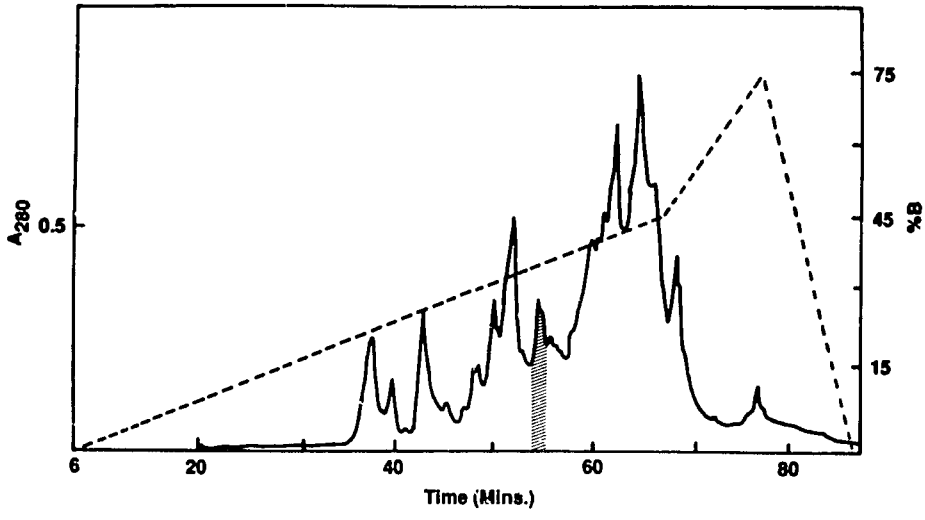


Figure 5. HPLC of Fraction B, Figure 4, on a reverse phase (C-18) Vydac column (1 x 25 cm), 33 mg in 0.95 ml 0.1% TFA. Column development: 10 minutes isocratic 0.1% TFA/H_2O (Solvent A), 10% solvent B (0.1% TFA in acetonitrile); 60 minutes linear gradient to 50% B; 10 minutes gradient to 75% B; 10 min gradient to 10% B. The inhibin fraction, crosshatched, contained 2.0 mg of inhibin of approximately 50% purity.

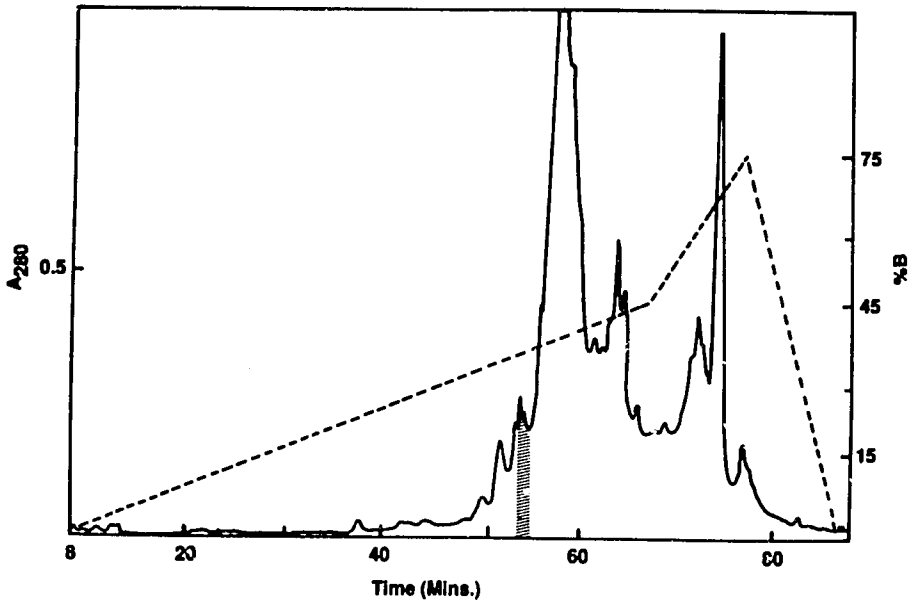


Figure 6. HPLC of Fraction D, Figure 4, 36 mg. Chromatographic conditions as for Figure 5. The activin fraction, crosshatched, contained 1.3 mg of partially purified activin. The degree of purity has not been established.

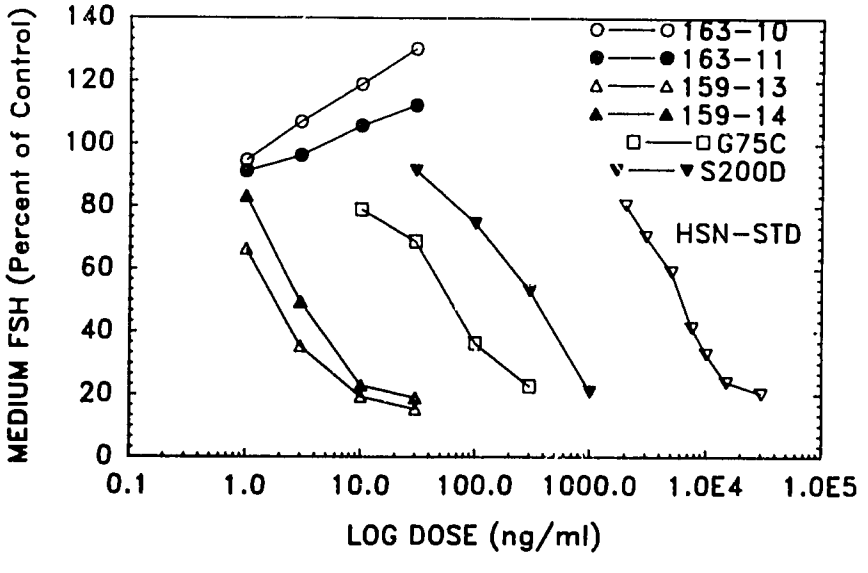


Figure 7. Summary of the pituitary cell culture assays on the fractions from the Sephacryl S-200 inhibin (S200-D), the Sephadex G-75 (G-75C), the leading edge (159-13) and trailing edge (159-14) from the inhibin fraction in Figure 5. The activin fraction from Figure 6, was assayed as a forward (163-10) and trailing (163-11) portion of the peak.

For many studies the inhibin at the first-pass HPLC stage of purification (i.e. approximately 50% pure) has been used, as we will demonstrate below. This is a concession to the losses we take at rechromatography of the first-pass HPLC inhibin fraction. These losses are of the order of 50%, plus the additional requirements for the analytical characterization and assay. After rechromatography on HPLC, our best preparations have an ID_{50} in the pituitary cell culture assay of 0.6 ng/ml, or better than 9000-fold purification over the starting follicular fluid. The first-pass HPLC inhibin fractions usually have an ID_{50} in the 1.2 to 2.5 ng/ml potency range.

After rechromatography the SDS-PAGE gel appears as in Figure 8, which shows on the left two lanes the trailing edge cut and on the right the leading edge cut from the re-run inhibin peak, each applied to the gel at two levels, here run under reducing conditions. The earlier eluting part of the inhibin peak was slightly the better of the two in this collection. Note the almost doublet appearance of the α subunit at the 20 kDa level and the lighter staining of the β subunit at about the 14 kDa level (here stained with Coomassie Blue).

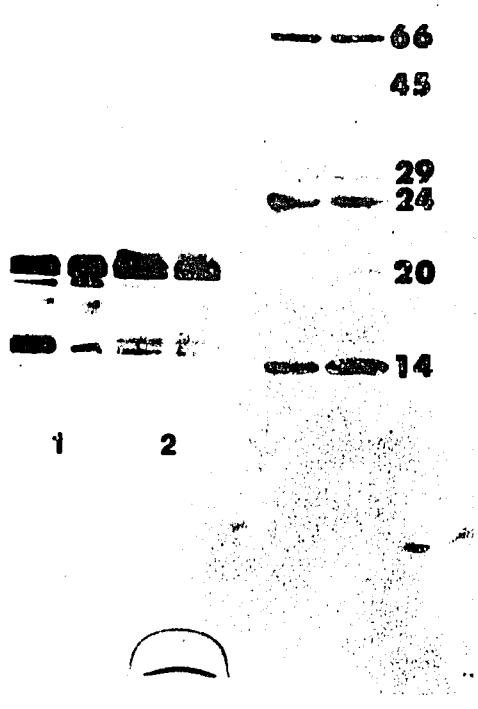


Figure 8. SDS-PAGE electrophoresis pattern (reducing conditions) from an HPLC rechromatographed fraction, with an ID_{50} of 0.6 ng/ml in the pituitary cell culture assay. Sample 1 represents 50 and 25 ug of the leading portion of the peak, and sample 2 the same for last half of the peak. The print has been overexposed to bring out the traces of impurities at 19, 17.5 and 13 kDa, most readily seen in Sample 1. The inhibin α subunit is at 20 kDa, a heavily staining doublet, and the inhibin β subunit, a lighter staining doublet at approximately 14.5 kDa. Marker proteins were in the two right lanes. Coomassie Blue stain.

FRACTIONATION COMPARISONS

We have tabulated some of the published reports on inhibin products obtained from various purification procedures. In this table if we have left a blank in any column it means the original paper did not present the data in a manner that would allow us to make the calculation, or, in the case of the high molecular weight forms, these were noted but without comment on what quantities were obtained (Table 1). Clearly, the highest overall yields have been reported by Miyamoto and colleagues.[23] Their procedures rely heavily on the use of high urea concentrations in most of their steps. High concentrations of acetic acid (4M to 16M) are used to dissolve and dissociate in the procedures of Robertson *et al.*,[25,26] Ling *et al.*,[18] and Gordon *et al.*,[12] while Rivier *et al.*[24] used 6M guanidine.HCl.

The procedure of Ling *et al.*[18] was unique in that it employed a reverse phase HPLC step using triethyl amine-phosphate ion pair development and this allowed them to identify an inhibin-A and inhibin-B in their low molecular weight fraction and possibly follistatin,[30] although the descriptive manuscripts on the isolation of follistatin are not available at the time of this writing. All the other procedures must either select for the inhibin-A form, isolate mixtures of the two, or else the inhibin-B is absent from follicular fluid of some species. Thus the isolation procedure devised by Ling *et al.*[18] was critical to the probes subsequently devised for the historic studies by Mason et al.,[20] and the wealth of recombinant DNA information that has followed.[6,21,27]

CARBOHYDRATE ANALYSES

There are several reports indicating that inhibin is a glycoprotein. For example, de Jong *et al.*[16] showed inhibin activity from follicular fluid was retained on a concanavalin A-Sepharose column. Subsequently, Miyamoto *et al.*[23] showed that their highly purified

TABLE I
COMPARISON OF PURIFICATION PARAMETERS FOR INHIBIN FROM OVARIAN FOLLICULAR FLUID
(32 kD form)

PARAMETER	MIYAMOTO	ROBERTSON	LING	RIVIER	OURS
Steps	5	3	7	10	5
i.d.50 (ng)	0.9	1-2	0.6	0.3	0.6
Fold purified over pFF1	8,000	5500	-	10,000	9,900
Yield/liter pFF1 (mg)	1.8	-	0.033	0.019	0.15
High mol. wt. forms in good yield (>32 kD)	Yes	Yes	-	-	Yes

preparations of porcine inhibin were completely bound to concanavalin A-Sepharose columns. Van Dijk *et al.*[28] extended the binding studies with bovine inhibin to lentil lectin-Sepharose columns, but observed poor recoveries.

Lentil lectin has a specificity similar to concanavalin A, but by virtue of its lower affinity for mannose residues it is reported to be more selective.[11] We have utilized this form of affinity chromatography with the inhibin fraction obtained from the Sephadex G-75 step. This material was poorly soluble in the buffer required for this chromatography (see insoluble fraction, Table 2). However, the soluble material chromatographed well and eluted sharply by α-methyl mannoside as shown in Figure 9. The purification achieved was about 5-fold, so this is a useful step if we succeed in modifying the developing buffer to increase the solubility of this fraction. Note that all the soluble activity was retained, indicating that there is no inhibin that lacks carbohydrate in this fraction.

Using preparations of inhibin obtained at our first-pass HPLC purification stage (*i.e.* about 50% purity) we have been able to study the carbohydrate composition of porcine inhibin and to demonstrate that the carbohydrate is limited to the porcine inhibin α subunit.

For our analysis of the neutral and amino sugars in inhibin we turned to the Dionex BioLC system with the pulsed amperometric detector. This unit allows the detection of the neutral and amino sugars developed on an anion exchange column with 20 mM NaOH. The sugars are detected directly without post-column derivatization. The chromatographic pattern obtained at the one nanomole level is shown in Figure 10. The operating range for our inhibin samples has been from this range down to 100 picomoles. The analytical conditions employed are essentially those developed by Hardy *et al.*[15] For our sialic acid determinations we used a colorimetric method.[1]

It was first essential to show that any carbohydrate in the preparations was due to inhibin and not glycosylated impurities. This was done by means of the periodate-Schiff reaction on SDS-gels of the samples, as shown in Figure 11. The only staining observed was on the α subunit, here visible in the 20 kDa area of the gel, since the samples were run under reducing conditions. The periodate-Schiff rose-red stain was very obviously limited to the doublet band observed at the α subunit area, and as visualized on subsequent staining with Coomassie Blue staining, as shown on the right of the figure. This result indicates

TABLE 2
RECOVERY SUMMARY FROM LENTIL LECTIN EXPERIMENT

Fraction	Weight (mg)	ID_{50} (ng/ml)	Specific Activity (Units/mg)	Total Activity (Units)	Percent Recovery (Activity)
G-75C (Start)	43.4 mg	50	40	3906	100
LL-A	22.0	>1000	--	--	--
LL-B	4.0	10	450	1800	46
Insoluble	14.4	35	130	1872	48

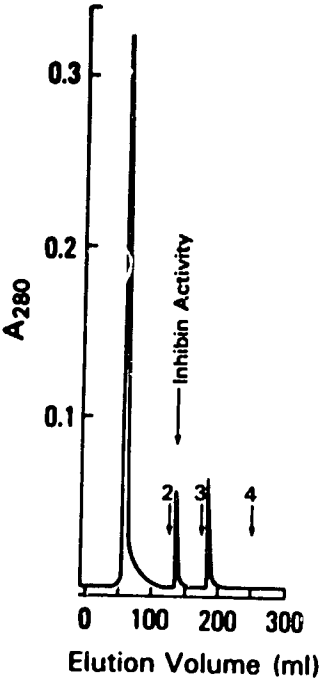


Figure 9. Chromatography of 44 mg of the Sephadex G-75 Fraction C material on a 1 x 15 cm column of Lentil Lectin Sepharose. The inhibin activity was eluted in peak 2 with 0.3 M-α methyl mannoside. For further details of the chromatography see Table 2, and text.

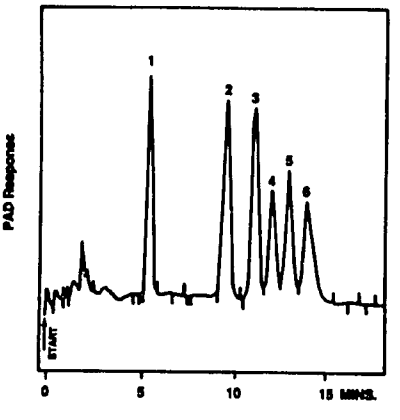


Figure 10. Anion exchange chromatography of neutral and amino sugars on a BioLC chromatographic unit, using pulsed amperometric detection, 1 nanomole of each of the following sugars: 1.fucose, 2.galactosamine, 3.glucosamine, 4.galactose, 5.glucose, 6.mannose. The column was developed at ambient temperature with 20 mmol NaOH, 1 ml/min. Column, Dionex HOIC-AS6.

Figure 11. SDS-PAGE gel electrophoresis patterns for the carbohydrate studies on inhibin. Lane 1, BRL marker proteins. Lane 2, semi-pure inhibin preparation, 60 ug (see text), stained with periodate-Schiff reagent. Lane 3, gel from lane 2 restained with Coomassie Blue. Lane 4, Sample run under non-reducing conditions, stained with Coomassie Blue. Electrophoresis was run under reducing conditions for all other samples. Note only the inhibin α subunit doublet, 20 kDa, showed a positive reaction with the periodate-Schiff reagent. Except for the inhibin β subunit, 14.5 kDa, the remaining bands represent impurities in this first-pass HPLC inhibin preparation. In Lane 4 the inhibin is the heavy-staining doublet band at 32 kDa.

that all of the carbohydrate is on the α subunit. Since the cDNA-deduced sequence of porcine inhibin[20] had one N-linked glycosylation site for the 32 kDa form, position 36 of the α subunit, and none for either β subunit, this indicates there are no O-linked glycosylation sites on the β subunits. That cannot be determined for the α subunit on the basis of these data, but it seems likely that the one available N-linked position is probably glycosylated.

Knowing no carbohydrate was detectable on the impurities present in these samples, as measured by the periodate-Schiff reaction, we analyzed these preparations directly on the likely assumption that all the carbohydrate present was attributable to inhibin. The analytical values obtained for two such preparations are shown in Table 3, which contains the observed value and corrected value. The corrected value adjusts for the theoretical moles of inhibin in the sample on the basis of the specific activity of the preparation.

TABLE 3
CARBOHYDRATE ANALYSIS FOR INHIBIN

Sugar Component	Nanomoles Per Sample (Observed)		Nanomoles per Nanomole of Inhibin[a]	
	Sample 1	Sample 2	Sample 1	Sample 2
Fucose	0.139 ± 0.006	0.158 ± 0.004	0.57	1.02
Galactosamine	0.120 ± 0.024	0.109 ± 0.011	0.49	0.70
Glucosamine	0.606 ± 0.045	0.579 ± 0.012	2.48	3.72
Galactose	0.554 ± 0.064	0.515 ± 0.035	2.27	3.31
Glucose	0.553 ± 0.045	0.899 ± 0.064	2.26	5.78
Mannose	0.421 ± 0.049	0.495 ± 0.043	1.72	3.18
Sialic Acid	N.D.	(0.417 to 0.424)[b]	N.D.	2.70

[a]These values have been corrected for the non-glycosylated impurities present. The correction is based on a specific activity of 2200 (Sample 1) and 1400 (Sample 2), assuming pure inhibin has a specific activity of 9000 in our assay system.

[b]Colorimetric method[1], duplicate determination.

N.D. = Not determined.

The values obtained (Table 3) are suggestive of typical N-linked carbohydrate values with the surprising exception of the glucose present. We also examined a third preparation, which also qualitatively had glucose present, but the sample was too small to establish reliable ratios among the sugars. Thus, in three separate preparations we have observed glucose in the carbohydrate composition of porcine inhibin, suggesting it is a routine component of the carbohydrate in the product isolated from follicular fluid. At this stage we recognize the caveat that this must be further substantiated with more highly purified preparations of inhibin, and such studies are underway. Nonetheless, this has prompted speculation that the carbohydrate on inhibin may in some way be unique. High glucose is found in the commonly observed glycosylation via the dolichol phosphate pathway, at the so-called high mannose stage in the ER, but this is trimmed in processing via the Golgi apparatus to the so-called complex carbohydrate form, at which stage the fucose and sialic acid is added. So the mixed forms here observed suggest possible early and late stages in the "conventional" glycoprotein biosynthesis. Perhaps the inhibin in the follicular fluid is a mixture of material released prematurely from the ER, and more mature forms of the glycoprotein. Further study will be required to determine this. Except for the glucose, however, the observed ratios in Table 3 are close to the mature complex form, not the high mannose form.

We have initiated studies on the deglycosylation of inhibin to learn whether this is an essential element for inhibin action.

DISULFIDE STUDIES

We tried one more reaction with the first-pass HPLC inhibin product, and that was direct -SH alkylation, or reduction and alkylation studies. There has been no evidence that inhibin had a free -SH group, but our first study was a control on the second. We tried alkylation with ^{14}C-labeled iodoacetic acid on the inhibin without reduction. The product showed no label incorporated as studied by radioautography after SDS-gel electrophoresis (non-

reducing conditions). From that we conclude that all the half-cystines in inhibin are involved in -S-S- bond formation. We can also conclude that none of the impurities in these preparations have free -SH groups, either. When we did the experiment with a reduction of disulfide bonds by a 10-fold excess of dithiothreitol, the incorporation was as expected, as shown in Figure 12, with heavy labeling of both the 20 kDa and 14 kDa forms of the subunits. It is also very clear that almost all the impurities in this fraction had cystine disulfide bonds that were labeled. The only thing we can really learn from this is that the half-cystine placements in inhibin will be very demanding of the purification procedure in order to provide sufficient purified inhibin to conduct the studies. Thus, we should continue to improve the efficiency of the overall purification procedure in order to characterize native inhibin more extensively.

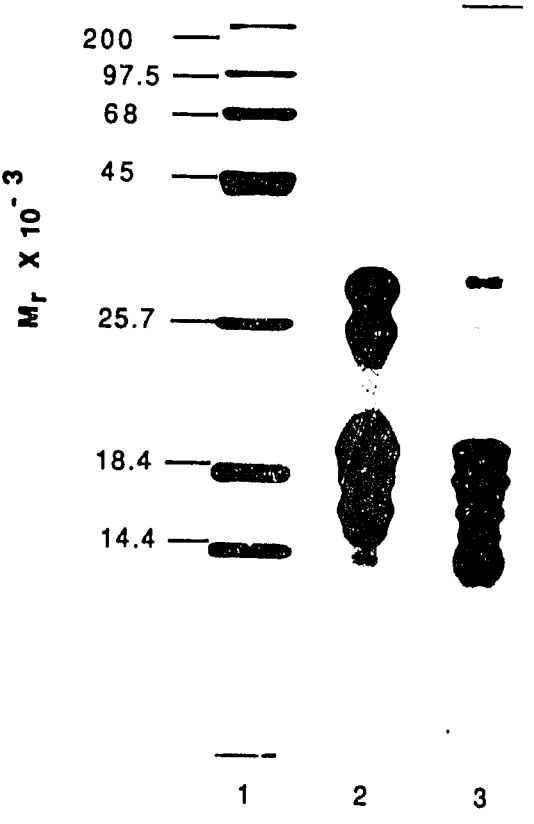


Figure 12. SDS-PAGE electrophoresis pattern from the reduction and alkylation of a first-pass HPLC inhibin fraction. Lane 1, molecular weight markers; Lane 2, radioautograph from 78 ug of reduced and alkylated (^{14}C-iodoacetate); Lane 3, same as lane 2, but with Coomassie stain. The α and β subunits are heavily alkylated, as are most of the impurities in this fraction.

ACKNOWLEDGMENTS

We are indebted to the Contraceptive Development Branch, NICHD, and Dr. Gabriel Bialy for provision of the porcine follicular fluid used in our studies. Our research has been supported by research grants from NICHD (HD-18210), NIDKD (DK-09801), and the Robert A. Welch Foundation (G-147). We appreciate the expert technical assistance provided by Nancy S. Jones, Sam S. Burchfield, Hyun S. Nahm, and Eric Whiting for some of the studies described.

REFERENCES

1. AMINOFF D: Methods for the quantitative estimation of N-acetyl-neuraminic acid and their application to hydrolysates of sialomucoics. Biochem J 81: 384, 1961
2. BAKER HWG, EDDIE LW, HIGGENSON, HUDSON B, NIALL HD: Studies on the purification of ovine inhibin. Ann NY Acad Sci 383: 329, 1982
3. BAKER HWG, EDDIE LW, HUDSON B, NIALL HD: Variability in the apparent molecular weight of inhibin. Aust J Biol Sci 38:327, 1985
4. CHANNING CP, LIU W-K, GORDON WL, XUE Y-F, WARD DN: Porcine inhibin: Initial fractionation as a high molecular weight complex. Arch Androl 13:219, 1984
5. CHANNING CP, GORDON WL, LIU W-K, WARD DN: Minireview: Physiology and biochemistry of ovarian inhibin. Proc Soc Exptl Biol Med 178:339, 1985
6. DE JONG FH, JANSEN EHJM, VAN DER MOLEN HJ: Purification and characterization of inhibin. In FRANCHIMONT P, CHANNING CP (eds): Intragonadal Regulation of Reproduction, p 229. New York, Academic Press, 1981
7. DE JONG FH, ROBERTSON DM: Review — Inhibin: 1985 update on action and purification. Molec Cell Endocrinol 42:95, 1985
8. FORAGE RG, RING JM, BROWN RW, MCINERNEY BV, COBON GS, GREGSON RP, ROBERTSON DM, MORGAN FJ, HEARN MTW, FINDLAY JK, WETTENHALL REH, BURGER HG, DE KRETSER DM: Cloning and sequence analysis of cDNA species coding for the two subunits of inhibin from bovine follicular fluid. Proc Natl Acad Sci USA 83:3091, 1986
9. FRANCHIMONT P, VERSTRAELEN-PROYARD J, HAZEE-HAELSTEIN MT, Ch., DEMOULIN RA, BOURGUIGNON JP, HUSTIN J: Inhibin: From concept to reality. Vitamins Hormones 37:243, 1979
10. FUKUDA M, MIYAMOTI K, HASEGAWA Y, NOMURA M, IGARASHI M, KANGAWA K, MATSUO H: Isolation of bovine follicular fluid inhibin of about 32 kDa. Molec Cell Endocrinol 44:55, 1986
11. GOLDSTEIN IJ, PORETZ RD: Isolation, physicochemical characterization, and carbohydrate-binding specificity of lectins. In: LIENER IE, SHARON N, GOLDSTEIN IJ (eds): Molecular Biology: The Lectins, Properties, Functions, and Applications in Biology and Medicine, p 33. New York, Academic Press, Inc., 1986
12. GORDON WL, LIU W-K, WARD DN: Inhibin fractionation: A comparison of human and porcine follicular fluid, with particular reference to protease activation. Biol Reprod 35:209, 1986
13. GORDON WL, LIU W-K, AKIYAMA K, TSUDA R, HARA M, SCHMID K, WARD DN: Beta-microseminoprotein (a-MSP) is not an inhibin. Biol Reprod 36:835, 1987
14. GRADY RR, CHARLESWORTH MC, SCHWARTZ NB: Characterization of the FSH-suppressing activity in follicular fluid. Recent Prog Hormone Res 38:409, 1982
15. HARDY MR, TOWNSEND RR, LEE YC: Glycoconjugate monosaccharide analysis using anion exchange chromatography with pulsed amperomatric detection (Abstr. #1). Society for Complex Carbohydrates Annual Meeting, Nov. 5-7, 1987
16. HUANG ES-R, MILLER WL: Porcine ovarian inhibin preparations sensitize cultured ovine gonadotropes to luteinizing hormone-releasing hormone. Endocrinology 115:513, 1984

17. KOHAN S, FROYSA B, CEDERLUND E, FAIRWELL T, LERNER R, JOHANSSON J, KHAN S, RITZEN M, JORNVALL H, CEKAN S, DICZFALUSY E: Peptides of postulated inhibin activity: Lack of in vitro inhibin activity of a 94-residue peptide isolated from human seminal plasma, and of a synthetic replicate of its C-terminal 28-residue. FEBS Letrs 199:242, 1986
18. LING N, YING S-Y, UENO N, ESCH F, DENOROY L, GUILLEMIN R: Isolation and partial characterization of a Mr 32,000 protein with inhibin activity from porcine follicular fluid. Proc Natl Acad Sci USA 82:7217, 1985
19. LIU L, BOOTH J, MERRIMAM GR, BARNES KM, SHERINS RJ, LORIUX DL, CUTLER, JR GB: Evidence that synthetic 31-amino acid inhibin-like peptide lacks inhibin activity. Endoc Res 11:191, 1985
20. MASON AJ, HAYFLICK JS, LING N, ESCH F, UENO N, YING S-Y, GUILLEMIN R, NIALL H, SEEBURG PH: Complementary DNA sequences of ovarian follicular fluid inhibin show precursor structure and homology with transforming growth factor-β. Nature 659:19, 1985
21. MASON AJ, NIALL HD, SEEBURG PH: Structure of two human ovarian inhibins. Biochem Biophys Res Commun 135:957, 1986
22. MIYAMOTO K, HASEGAWA Y, FUKUDA M, IGARASHI M. Demonstration of high molecular weight forms of inhibin in bovine follicular fluid (bFF) by using monoclonal antibodies to bFF 32K inhibin. Biochem Biophys Res Commun 136:1103, 1986
23. MIYAMOTO K, HASEGAWA Y, FUKUDA M, NOMURA M, IGARASHI M, KANAGAWA K, MATSUO H. Isolation of porcine follicular fluid inhibin of 32K daltons. Biochem Biophys Res Commun 129:396, 1985
24. RIVIER J, SPIESS J, MCCLINTOCK R, VAUGHAN J, VALE W. Purification and partial characterization of inhibin from porcine follicular fluid. Biochem Biophys Res Commun 133:120, 1985
25. ROBERTSON DM, DE VOS FL, FOULDS LM, MCLACHLAN RI, BURGER HG, MORGAN FJ, HEARN MTW, DE KRETSER DM: Isolation of a 31 kDa form of inhibin from bovine follicular fluid. Molec Cell Endocrinol 44:271, 1986
26. ROBERTSON DM, FOULDS LM, LEVERSHA L, MORGAN FJ, HEARN MTW, BURGER HG, WETTENHALL REH, DE KRETSER DM: Isolation of inhibin from bovine follicular fluid. Biochem Biophys Res Commun 126:220, 1985
27. STEWART AG, MILBORROW HM, RING JM, CROWTHER CE, FORAGE RG: Human inhibin genes: Genomic characterization and sequencing. FEBS Letrs 206:329, 1986
28. VAN DIJK S, STEENBERGEN C, DE JONG FH, VAN DER MOLEN HJ: Comparison between inhibin from human and bovine ovarian follicular fluid using fast protein liquid chromatography. Molec Cell Endocrinol 42:245, 1985
29. WARD DN, GLENN SD, LIU W-K, GORDON WL: Chemistry and physiology of gonadal peptides. In GREENWALD GS, TERRANOVA PF (eds): Factors Regulating Ovarian Function, p 141. New York, Raven Press, 1983
30. YING S-Y, BECKER A, SWANSON G, TAN P, LING N, ESCH F, UENO N, SHIMASAKI S, GUILLEMIN R: Follistatin specifically inhibits pituitary follicle stimulating hormone release *in vitro*. Biochem Biophys Res Commun 149:133, 1987

DISCUSSION

CHAIRPERSON: VIRGINIA C. UPTON, Ph.D.

DR. HOFFMAN: Have there been any attempts to try to take the high molecular weight forms and manipulate those to increase the yield at a lower molecular weight (e.g. 32 kDa) form?

DR. WARD: We are just beginning studies on these higher molecular weight forms ourselves. I believe that Dr. Miyamoto has done much more with the higher molecular weight forms than any other group. I think that your suggestion is practical, but conditions for doing that have not been established, to my knowledge. It also is not known how the large forms are glycosylated, etc.

I would like to mention one thing concerning the glucose in inhibin, which is quite an unusual finding as the glycoproteins go. Referring to the HPLC run we did on inhibin, as an additional control for whether or not the glucose obtained was an artifact from some of the fractionation procedures, we established that another glycoprotein fraction, the activity of which is not yet known, comes off a few minutes ahead of the inhibin. This is a glycoprotein that has undergone all of the same fractionation procedures as inhibin itself. It had all of the sugars in that analysis, but lacked glucose. So, other glycopeptides in this same batch, or fractionation, so to speak, did not contain glucose. We believe this suggests that the glucose is an interesting component of the carbohydrate in inhibin.

DR. SHETH: Are you mostly using the pituitary cell culture system to assay your preparation? Can you give us some information on the assay?

DR. WARD: Yes. We have published our studies on the assay. We actually developed this assay while Cornelia Channing was working with us on her sabbatical. The assay is used to monitor inhibin purification. It is quite selective for FSH response to inhibin preparations. Our culture conditions include insulin, estradiol and progesterone in the assay medium. Therefore, you have elevated levels of FSH and LH to work with. We do not see any effect of the inhibin preparations on LH levels, which remain constant.

DR. MASON: What did you learn about the disulfide linkages in the studies you mentioned but did not have time to present?

DR. WARD: We have tried alkylation studies using [14C] iodoacetic acid. As a control, in our studies we did direct alkylation without reduction, and we found no iodoacetate incorporation as measured by radioautography. That is not surprising as I did not previously think there was any evidence for free -SH groups in inhibin. But we now have direct evidence that this is the case. We then went ahead to do dithiothreitol reductions and incorporation into the alpha and beta subunits, and we got the expected incorporation.

The main conclusion that I think we can draw from our disulfide study is that it is going to take a large quantity of the native inhibin in order to do these characterization studies. Therefore, one of the conclusions in my chapter is that we should continue to develop more efficient purification procedures in order to do these native characterizations.

DR. RAJ: I did not notice if you mentioned *in vivo* testing. Have you given your preparation, at any stage of purification, to rats, mice, or any other animals? If so, have you observed FSH reduction with or without any LH reduction?

DR. WARD: Yes, we have. Some of our earlier studies were in collaboration with Nina Schwartz. We used her assay to monitor many early stage fractions, but not yet for the final stage purification.

DR. SHETH: What are the relative proportions of activin and inhibin in your preparation?

DR. WARD: That is a difficult question to answer. We use as a reference material the same crude follicular fluid preparation that we have used as our lab standard for many years.

However, as you know, inhibin suppresses the activin action, and so you do not see activin in this early stage of preparation. Until you separate them, you cannot detect activin. So, in terms of proportions, I could not give you a number.

DR. UPTON: First, I would like to know if you use a preservative in your columns? Second, how long did it take, a week or so, to run those columns to obtain the large quantities you reported?

DR. WARD: Actually, it does not take quite that long. But these are big columns, so you can generate big volumes in a hurry. These columns are all run in the cold room for the Sephacryl studies and the G-75. We do not routinely use any preservative at all. The columns are maintained in the cold, and the recoveries of inhibin activity are very good through all the stages except the last stage, HPLC. Of course, many of the solvents we are using are rather obnoxious for bacteria, etcetera, to grow. Thus, there is a sort of nondeliberate built-in preservative.

DR. POMERANTZ: I wonder if you would talk a moment about the Dionex column you are using for directly observing the carbohydrates.

DR. WARD: The column is called Dionex BioLC. It is an anion—exchange column. You develop it with 20mM sodium hydroxide. You have to have the Dionex hardware so that everything does not get corroded in that analysis. NaOH is very corrosive. Actually, at such a high pH you are ionizing the hydroxyls on your sugars and chromatographing on that basis. The real beauty of the Dionex system is its pulse amperometric detection system.

DR. MANBERG: I was wondering if you could comment on the source of the follicular fluid that you use in large volumes. Also, have you seen any variability with new supplies?

DR. WARD: There is some variability, even sometimes in terms of color. Some of it is more greenish looking; some is more amber. All of our material is obtained through the courtesy of Dr. Bialy's collection program at NICHD, and we do fully acknowledge this great source of support that has been available through NICHD.

2

Structure and Recombinant Expression of Human Inhibin and Activin

ANTHONY J. MASON

The development and maintenance of mammalian reproduction is due to the interplay of many hormones originating from the hypothalamic-pituitary-gonadal axis. The central players in this complex system are the pituitary gonadotropins, LH and FSH. The synthesis and release of both of these hormones is controlled by a combination of hypothalamic and gonadal factors. The recent characterization of two gonadal peptide hormones, activin and inhibin, which regulate the release of FSH has added a new dimension to the complexity of this system.

Recently four independent laboratories have isolated and partially characterized two forms of porcine[7,12,17] and one form of bovine follicular fluid inhibin.[16] The two forms of the inhibin molecule have been shown to be 32 kilodalton (kDa) proteins comprised of a common 18 kDa α subunit disulphide-linked to different 14 kDa β subunits (βA and βB). In contrast, the two forms of activin that have been isolated consist of a disulphide linked homodimer of the βA subunit of inhibin[18] and a disulphide linked heterodimer of the βA and βB subunits of inhibin.[8]

STRUCTURE OF HUMAN INHIBIN

Once preliminary sequence information was obtained from the three porcine inhibin subunits,[7] oligonucleotide nucleotide probes were employed to isolate cDNA clones for the α, βA, and βB subunits from a porcine ovarian cDNA library. Upon sequencing, the detailed structure of each of the subunit precursors was elucidated.[9] Similarly, the full length structure of the bovine α subunit and a partial structure of the βA subunit were determined.[6]

In order to determine the structure of the human inhibin subunits, porcine cDNA fragments were used to screen a human ovarian cDNA library for homologous sequences. Clones which hybridized at high stringency with the porcine probes were analyzed in more detail. By piecing together sequences from a variety of cDNA clones we were able to predict for the first time the complete amino sequences of the α (366 amino acids), βA (426 amino acids) and βB (407 amino acids) precursors of human inhibin.[10] The DNA sequences and predicted amino acid sequences are shown in Figures 1-3. All of these sequences have been confirmed by the sequence analysis of human genomic clones (unpublished data).

Each of the mature human subunits resides at the C-terminus of a much larger precursor. Presumably the subunits are released by proteolytic processing at the basic residues

```
   1 TGGGGAAGACTGGGTGAGAAGGGTAGAAGAGGGTGGGTGTGGGATGGGGAGGGGAGAGTGGAAAGGCCCTGGGCAGACCCTGGCAGAAGGGGCACGGGGC

                                                1                                   10
                                                Met Val Leu His Leu Leu Leu Phe Leu Leu Leu Thr Pro Gln Gly
 101 AGGGTGTGAGTTCCCCACTAGCAGGGCCAGGTGAGCT      ATG GTG CTG CAC CTA CTG CTC TTC TTG CTG CTG ACC CCA CAG GGT

                    20                                      30                                          40
     Gly His Ser Cys Gln Gly Leu Glu Leu Ala Arg Glu Leu Val Leu Ala Lys Val Arg Ala Leu Phe Leu Asp Ala
 183 GGG CAC AGC TGC CAG GGG CTG GAG CTG GCC CGG GAA CTT GTT CTG GCC AAG GTG AGG GCC CTG TTC TTG GAT GCC

                                        50                                          60
     Leu Gly Pro Pro Ala Val Thr Arg Glu Gly Gly Asp Pro Gly Val Arg Arg Leu Pro Arg Arg His Ala Leu Gly
 258 TTG GGG CCC CCC GCG GTG ACC AGG GAA GGT GGG GAC CCT GGA GTC AGG CGG CTG CCC CGA AGA CAT GCC CTG GGG

                    70                                      80                                          90
     Gly Phe Thr His Arg Gly Ser Glu Pro Glu Glu Glu Glu Asp Val Ser Gln Ala Ile Leu Phe Pro Ala Thr Asp
 333 GGC TTC ACA CAC AGG GGC TCT GAG CCC GAG GAA GAG GAG GAT GTC TCC CAA GCC ATC CTT TTC CCA GCC ACA GAT

                                        100                                         110
     Ala Ser Cys Glu Asp Lys Ser Ala Ala Arg Gly Leu Ala Gln Glu Ala Glu Glu Gly Leu Phe Arg Tyr Met Phe
 408 GCC AGC TGT GAG GAC AAG TCA GCT GCC AGA GGG CTG GCC CAG GAG GCT GAG GAG GGC CTC TTC AGA TAC ATG TTC

                    120                                     130                                         140
     Arg Pro Ser Gln His Thr Arg Ser Arg Gln Val Thr Ser Ala Gln Leu Trp Phe His Thr Gly Leu Asp Arg Gln
 483 CGG CCA TCC CAG CAT ACA CGC AGC CGC CAG GTG ACT TCA GCC CAG CTG TGG TTC CAC ACC GGG CTG GAC AGG CAG

                                        150                                         160
     Gly Thr Ala Ala Ser Asn Ser Ser Glu Pro Leu Leu Gly Leu Leu Ala Leu Ser Pro Gly Gly Pro Val Ala Val
 558 GGC ACA GCA GCC TCC AAT AGC TCT GAG CCC CTG CTA GGC CTG CTG GCA CTG TCA CCG GGA GGA CCC GTG GCT GTG

                    170                                     180                                         190
     Pro Met Ser Leu Gly His Ala Pro Pro His Trp Ala Val Leu His Leu Ala Thr Ser Ala Leu Ser Leu Leu Thr
 633 CCC ATG TCT TTG GGC CAT GCT CCC CCT CAC TGG GCC GTG CTG CAC CTG GCC ACC TCT GCT CTC TCT CTG CTG ACC

                                        200                                         210
     His Pro Val Leu Val Leu Leu Leu Arg Cys Pro Leu Cys Thr Cys Ser Ala Arg Pro Glu Ala Thr Pro Phe Leu
 708 CAC CCC GTC CTG GTG CTG CTG CTG CGC TGT CCC CTC TGT ACC TGC TCA GCC CGG CCT GAG GCC ACG CCC TTC CTG

                    220                                     230                 α subunit               240
     Val Ala His Thr Arg Thr Arg Pro Pro Ser Gly Gly Glu Arg Ala Arg Arg Ser Thr Pro Leu Met Ser Trp Pro
 783 GTG GCC CAC ACT CGG ACC AGA CCA CCC AGT GGA GGG GAG AGA GCC CGA CGC TCA ACT CCC CTG ATG TCC TGG CCT

                                        250                                         260
     Trp Ser Pro Ser Ala Leu Arg Leu Leu Gln Arg Pro Pro Glu Glu Pro Ala Ala His Ala Asn Cys His Arg Val
 858 TGG TCT CCC TCT GCT CTG CGC CTG CTG CAG AGG CCT CCG GAG GAA CCG GCT GCC CAT GCC AAC TGC CAC AGA GTA

                    270                                     280                                         290
     Ala Leu Asn Ile Ser Phe Gln Glu Leu Gly Trp Glu Arg Trp Ile Val Tyr Pro Pro Ser Phe Ile Phe His Tyr
 933 GCA CTG AAC ATC TCC TTC CAG GAG CTG GGC TGG GAA CGG TGG ATC GTG TAC CCT CCC AGT TTC ATC TTC CAC TAC

                                        300                                         310
     Cys His Gly Gly Cys Gly Leu His Ile Pro Pro Asn Leu Ser Leu Pro Val Pro Gly Ala Pro Pro Thr Pro Ala
1008 TGT CAT GGT GGT TGT GGG CTG CAC ATC CCA CCA AAC CTG TCC CTT CCA GTC CCT GGG GCT CCC CCT ACC CCA GCC

                    320                                     330                                         340
     Gln Pro Tyr Ser Leu Leu Pro Gly Ala Gln Pro Cys Cys Ala Ala Leu Pro Gly Thr Met Arg Pro Leu His Val
1083 CAG CCC TAC TCC TTG CTG CCA GGG GCC CAG CCC TGC TGT GCT GCT CTC CCA GGG ACC ATG AGG CCC CTA CAT GTC

                                        350                                         360
     Arg Thr Thr Ser Asp Gly Gly Tyr Ser Phe Lys Tyr Glu Thr Val Pro Asn Leu Leu Thr Gln His Cys Ala Cys
1158 CGC ACC ACC TCG GAT GGA GGT TAC TCT TTC AAG TAT GAG ACA GTG CCC AAC CTT CTC ACG CAG CAC TGT GCT TGT

     366
     Ile OC*
1233 ATC TAA  GGGTGGGGGGTCTTCCTTCTTAATCCCATGGCTGGTGGCCACGCCCCCACCATCATCAGCTGGGAGGAAAGGCAGAGTTGGGAAATAGATG

1330 GCTCCCACTCCTCCCTCCTTTCACTTCTCTGCCTATGGGCTACCCTCCCCACCCCACTTCTATCTCAATAAAGAACACAGTGCATATGAAAAAAAAAAAA

1430 AA
```

Figure 1. DNA sequence and deduced amino acid sequence of the α-chain precursor of human inhibin. Potential glycosylation sites are overlined. The likely signal sequence cleavage site is indicated by an arrow. This sequence was derived from both cDNA and genomic clones.

which precede the NH_2-terminal residues of each subunit. The α-subunit precursor contains three possible N-linked glycosylation sites of which two are present in the mature sequence. Both β precursors have only one N-linked glycosylation, each of which resides in the prohormone domain. The seven cysteine residues present in the mature a sequence can be aligned with 7 of the 9 cysteines present in each cf the mature β subunit sequences.

This suggests that these three are evolutionary related. In addition, we noted that the βA and βB sequences share approximately 70% homology and also have a significant degree of structural and primary sequence homology with human transforming growth factor-

```
   1 TGCTCCCTGACAGCCACAAACCTACAGCACTGACTGCATTCAGAGAGGAACCTGCAAACAAAACTTCACAGAAAACTTTTTGTTCTTGTTCCAGAGAATT

 101 TGCTGAAGAGGAGAAGGAAAAAAAAAAACACCAAAAAAAAAAAATAAAAAAATCCACACACACAAAAAACCTGCGCGTGAGGGGGGAGGAAAAGCAGGGCCT

                                          1                                                   10
                                          Met Pro Leu Leu Trp Leu Arg Gly Phe Leu Leu Ala Ser Cys Trp
 201 TTTAAAAAGGCAATCACAACAACTTTTGCTGCCAGG    ATG CCC TTG CTT TGG CTG AGA GGA TTT CTG TTG GCA AGT TGC TGG

                     20 ↓                                    30                                      40
     Ile Ile Val Arg Ser Ser Pro Thr Pro Gly Ser Glu Gly His Ser Ala Ala Pro Asp Cys Pro Ser Cys Ala Leu
 282 ATT ATA GTG AGG AGT TCC CCC ACC CCA GGA TCC GAG GGG CAC AGC GCG GCC CCC GAC TGT CCG TCC TGT GCG CTG

                                          50                                                  60
     Ala Ala Leu Pro Lys Asp Val Pro Asn Ser Gln Pro Glu Met Val Glu Ala Val Lys Lys His Ile Leu Asn Met
 357 GCC GCC CTC CCA AAG GAT GTA CCC AAC TCT CAG CCA GAG ATG GTG GAG GCC GTC AAG AAG CAC ATT TTA AAC ATG

                     70                                      80                                      90
     Leu His Leu Lys Lys Arg Pro Asp Val Thr Gln Pro Val Pro Lys Ala Ala Leu Leu Asn Ala Ile Arg Lys Leu
 432 CTG CAC TTG AAG AAG AGA CCC GAT GTC ACC CAG CCG GTA CCC AAG GCG GCG CTT CTG AAC GCG ATC AGA AAG CTT

                                          100                                                 110
     His Val Gly Lys Val Gly Glu Asn Gly Tyr Val Glu Ile Glu Asp Asp Ile Gly Arg Arg Ala Glu Met Asn Glu
 507 CAT GTG GGC AAA GTC GGG GAG AAC GGG TAT GTG GAG ATA GAG GAT GAC ATT GGA AGG AGG GCA GAA ATG AAT GAA

                     120                                     130                                     140
     Leu Met Glu Gln Thr Ser Glu Ile Ile Thr Phe Ala Glu Ser Gly Thr Ala Arg Lys Thr Leu His Phe Glu Ile
 582 CTT ATG GAG CAG ACC TCG GAG ATC ATC ACG TTT GCC GAG TCA GGA ACA GCC AGG AAG ACG CTG CAC TTC GAG ATT

                                          150                                                 160
     Ser Lys Glu Gly Ser Asp Leu Ser Val Val Glu Arg Ala Glu Val Trp Leu Phe Leu Lys Val Pro Lys Ala Asn
 657 TCC AAG GAA GGC AGT GAC CTG TCA GTG GTG GAG CGT GCA GAA GTC TGG CTC TTC CTA AAA GTC CCC AAG GCC AAC

                     170                                     180                                     190
     Arg Thr Arg Thr Lys Val Thr Ile Arg Leu Phe Gln Gln Gln Lys His Pro Gln Gly Ser Leu Asp Thr Gly Glu
 732 AGG ACG AGG ACC AAA GTC ACC ATC CGC CTC TTC CAG CAG CAG AAG CAC CCG CAG GGC AGC TTG GAC ACA GGG GAA

                                          200                                                 210
     Glu Ala Glu Glu Val Gly Leu Lys Gly Glu Arg Ser Glu Leu Leu Leu Ser Glu Lys Val Val Asp Ala Arg Lys
 807 GAG GCC GAG GAA GTG GGC TTA AAG GGG GAG AGG AGT GAA CTG TTG CTC TCT GAA AAA GTA GTA GAC GCT CGG AAG

                     220                                     230                                     240
     Ser Thr Trp His Val Phe Pro Val Ser Ser Ser Ile Gln Arg Leu Leu Asp Gln Gly Lys Ser Ser Leu Asp Val
 882 AGC ACC TGG CAT GTC TTC CCT GTC TCC AGC AGC ATC CAG CGG TTG CTG GAC CAG GGC AAG AGC TCC CTG GAC GTT

                                          250                                                 260
     Arg Ile Ala Cys Glu Gln Cys Gln Glu Ser Gly Ala Ser Leu Val Leu Leu Gly Lys Lys Lys Lys Lys Glu Glu
 957 CGG ATT GCC TGT GAG CAG TGC CAG GAG AGT GGC GCC AGC TTG GTT CTC CTG GGC AAG AAG AAG AAG AAA GAA GAG

                     270                                     280                                     290
     Glu Gly Glu Gly Lys Lys Lys Gly Gly Gly Glu Gly Gly Ala Gly Ala Asp Glu Glu Lys Glu Gln Ser His Arg
1032 GAG GGG GAA GGG AAA AAG AAG GGC GGA GGT GAA GGT GGG GCA GGA GCA GAT GAG GAA AAG GAG CAG TCG CAC AGA

                                          300                                                 310 βA subunit
     Pro Phe Leu Met Leu Gln Ala Arg Gln Ser Glu Asp His Pro His Arg Arg Arg Arg Arg Gly Leu Glu Cys Asp
1107 CCT TTC CTC ATG CTG CAG GCC CGG CAG TCT GAA GAC CAC CCT CAT CGC CGG CGT CGG CGG GGC TTG GAG TGT GAT

                     320                                     330                                     340
     Gly Lys Val Asn Ile Cys Cys Lys Lys Gln Phe Phe Val Ser Phe Lys Asp Ile Gly Trp Asn Asp Trp Ile Ile
1182 GGC AAG GTC AAC ATC TGC TGT AAG AAA CAG TTC TTT GTC AGT TTC AAG GAC ATC GGC TGG AAT GAC TGG ATC ATT

                                          350                                                 360
     Ala Pro Ser Gly Tyr His Ala Asn Tyr Cys Glu Gly Glu Cys Pro Ser His Ile Ala Gly Thr Ser Gly Ser Ser
1257 GCT CCC TCT GGC TAT CAT GCC AAC TAC TGC GAG GGT GAG TGC CCG AGC CAT ATA GCA GGC ACG TCC GGG TCC TCA

                     370                                     380                                     390
     Leu Ser Phe His Ser Thr Val Ile Asn His Tyr Arg Met Arg Gly His Ser Pro Phe Ala Asn Leu Lys Ser Cys
1332 CTG TCC TTC CAC TCA ACA GTC ATC AAC CAC TAC CGC ATG CGG GGC CAT AGC CCC TTT GCC AAC CTC AAA TCG TGC

                                          400                                                 410
     Cys Val Pro Thr Lys Leu Arg Pro Met Ser Met Leu Tyr Tyr Asp Asp Gly Gln Asn Ile Ile Lys Lys Asp Ile
1407 TGT GTG CCC ACC AAG CTG AGA CCC ATG TCC ATG TTG TAC TAT GAT GAT GGT CAA AAC ATC ATC AAA AAG GAC ATT

                     420                                     426
     Gln Asn Met Ile Val Glu Glu Cys Gly Cys Ser AM*
1482 CAG AAC ATG ATC GTG GAG GAG TGT GGG TGC TCA TAG AGTTGCCCAGCCCAGGGGGAAAGGGAGCAAGAGTTGTCCAGAGAAGACAGTG

1570 GCAAAATGAAGAAATTTTTAAGGTTTCTGAGTTAACCAGAAAAATAGAAATTAAAAACAAAACA
```

Figure 2. DNA and amino acid sequence of the βA-chain precursor of human inhibin. Potential N-linked glycosylation sites are overlined; and the most likely signal sequence cleavage site is indicated by an arrow. The DNA sequence was derived from cDNA and genomic clones.

β9. Since our initial observation of this related gene family, a diverse range of functions have been added to the transforming growth factor family-β. These include Mullerian-inhibiting substance[1], two new forms of TGF-β,[11] a Drosophila pattern formation gene product decapentaplegic (DPP), and a Xenopus egg differentiation factor Vg1.[19] All of these proteins display conservation of the cysteine residue array at the C-terminus of their precursors.

The primary structure of the inhibin subunits has been determined for human, bovine, porcine, and rat.[4,6,9,10] Comparison of these sequences shows that the α subunit is ap-

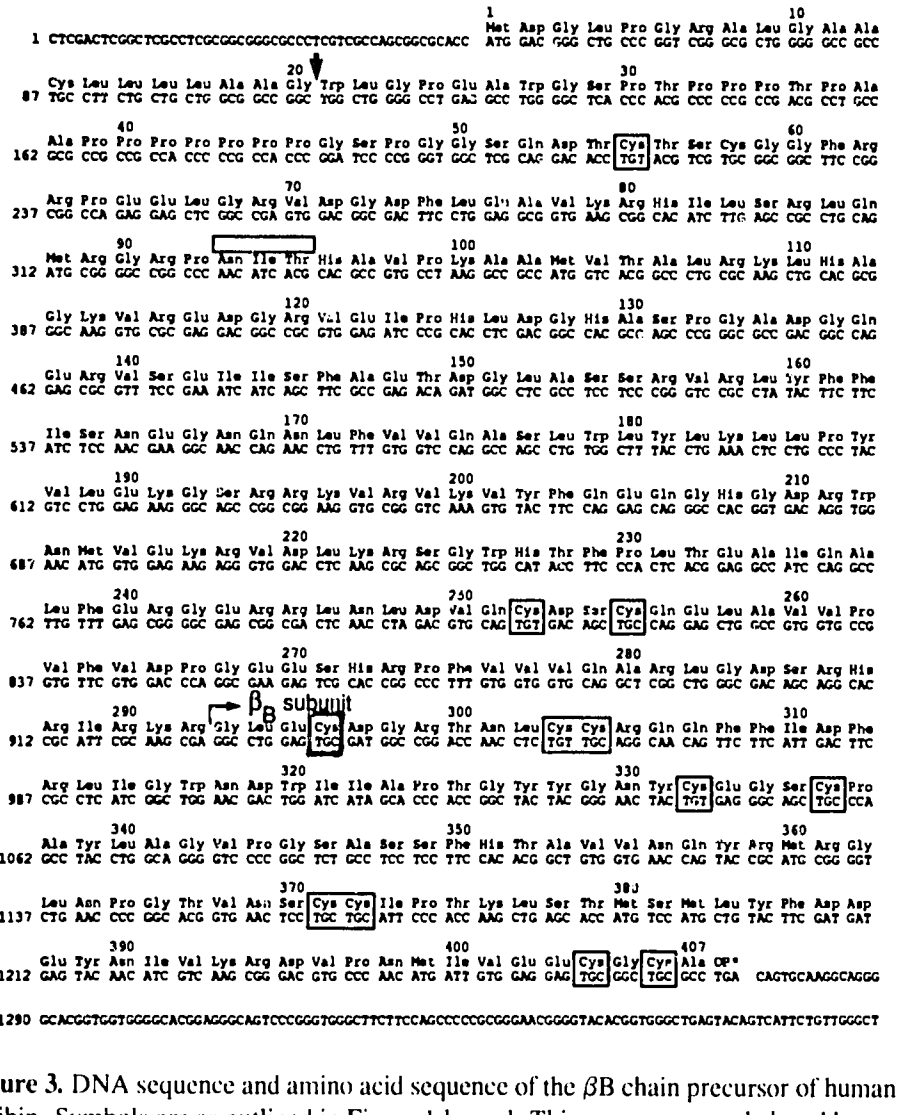


Figure 3. DNA sequence and amino acid sequence of the βB chain precursor of human inhibin. Symbols are as outlined in Figure 1 legend. This sequence was deduced by sequencing cDNA and genomic clones.

proximately 80% conserved while the β subunit sequences are almost completely conserved (>95% homology). This high degree of conservation suggests that the biological functions of these molecules are highly dependent on their primary structure. The recent observation that the βA activin molecule is an erythroid differentiation factor[5] suggests that members of the inhibin family may be involved in many diverse biological functions.

We determined the structure of each of the inhibin subunit genes. Each gene is comprised of only two exons separated by a 2.1 kb intron for the α gene, a 10 kb intron for the βA gene and a 2.8 kb intron in the case of the βB gene. Similar results have been reported by Stewart *et al.*[17] The human a gene is located on the distal portion of the long arm of chromosome 2, the βA gene is on chromosome 7 while the βB is situated near the centromere on the short arm of chromosome 2 (D. Barton and U. Francke, unpublished observations).

RECOMBINANT EXPRESSION OF INHIBIN AND ACTIVIN

By using the cloned cDNAs for the inhibin precursors we attempted to synthesize biologically active inhibin and activin in mammalian tissue culture cells. Our initial experiments with Chinese Hamster ovary cells (CHO) and SV40 expression vectors demonstrated that such a system could produce biologically active inhibin and activin. Thus CHO cells, transfected with an expression plasmid containing both the α and βA subunit sequences under their own SV40 promoters, secreted biologically active inhibin. Similarly, CHO cells, transfected with an SV40-βA construct, secreted βA dimers which were biologically active in the *in vitro* pituitary assay.[2] Activin and inhibin proved to be extremely difficult to purify from CHO supernatants due to aggregation and inefficient processing of the precursors by the CHO cells.

In order to overcome these problems we screened a large number of cell types to find one that would more efficiently process the inhibin precursor. The following studies were performed with a mammalian kidney cell line[3] which possessed the ability to express the inhibin precursors at a much higher level and could also more efficiently process and assemble the subunits.

The existence of both the α and β subunit messages in the same cells in the ovary poses the problem of how to regulate the production of either αB dimers or β dimers. When one transfects tissue cells with an equal ratio of an a subunit expression vector and a βA subunit vector, approximately equimolar amounts of both activin and inhibin are secreted (see Fig. 4, Lane A). This is not due to individual cells taking up on the βA subunit plasmid, since when cells are transfected with a vector containing both subunits a similar result is observed. This suggested that in order to only obtain inhibin $\alpha\beta$A dimers, the a subunit protein needs to be in excess as seen in gonadal tissue.[9] In order to test this hypothesis we transfected cells with varying ratios of the α expression plasmid to a constant amount of the βA expression vector. In this way we were able to define the optimum ratio of a subunit to βA subunit needed to produce only inhibin $\alpha\beta$A dimers. The secretion pattern of such an inhibin producing cell line (#1042) is shown in Lanes 3 and 7 of Figure 5. This cell line produces a large excess of free α subunit, at least 2 and maybe 3 isoforms of 30-32 kDa inhibin, 58 kDa inhibin and an ~100 kDa form of inhibin. Such isoforms of inhibin have been observed from both ovine and bovine sources.[15]

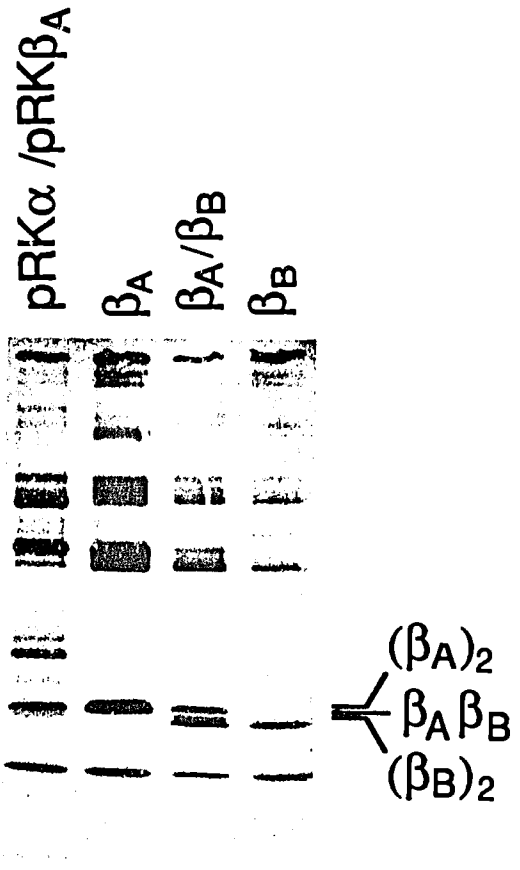


Figure 4. SDS-PAGE of the supernatants of mammalian kidney cells transiently transfected with: Lane 1, 5 fg of α-subunit vector and 5 fg of βA subunit vector; Lane 2, 5fg of βA subunit vector; Lane 3, 5 fg of βA subunit vector and 5 fg of βB subunit vector; Lane 4:, 5 fg of βB subunit vector.

Using the same cell line and expression vectors we are also able to produce large quantities of biologically active βA activin. Figure 6 shows the secreted products from two independent cell lines (#619,740) that are producing 24 kDa βA dimers of activin (Lanes 1,2), which upon reduction split into 14.5 kDa monomers (lanes 3 and 4). The two activins that have been isolated from natural sources are the homodimer of the βA subunit and the heterodimer of the βA and βB subunits.[8,18] The possibility exists that a homodimer of the βB subunit may also stimulate FSH secretion from pituitary cells. In order to investigate this possibility we transfected cells with an expression vector containing the complete βB precursor sequence (see Fig. 3). These cells secreted a βB dimer of 22 kDa in size (see Fig. 4, Lane 4), which displayed biological activity of similar potency to

recombinant βA activin. This for the first time shows that a βB homodimer can also be classified as an activin. It remains to be seen whether such a molecule exists *in vivo*.

When cells are transfected with an equimolar ratio of a βA and βB expression vector, the three distinct activin molecules are produced. The homodimers of βA and βB are produced as well as the heterodimer, in a ratio of approximately 2:2:1. One of several possibilities for this result is that each subunit may possess a greater affinity for itself than for the non-identical monomer.

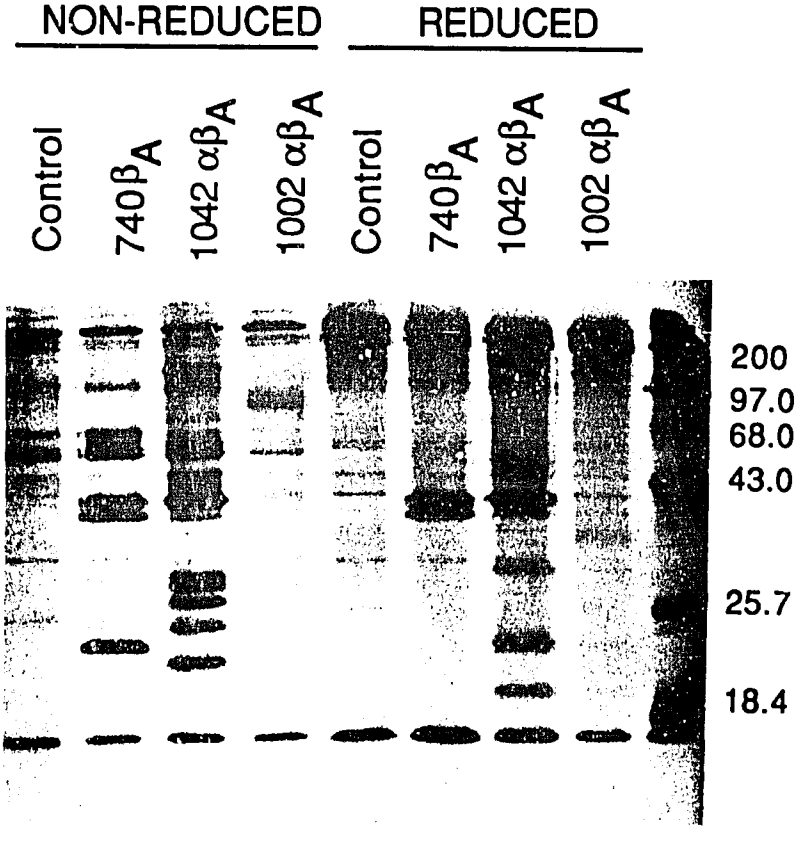


Figure 5. SDS-PAGE of the 35S-labelled supernatants of stable inhibin producing clones (#1042, 1022) and activin βA producing clones (#740), run under reducing and non-reducing conditions. The molecular weights of the size markers are indicated.

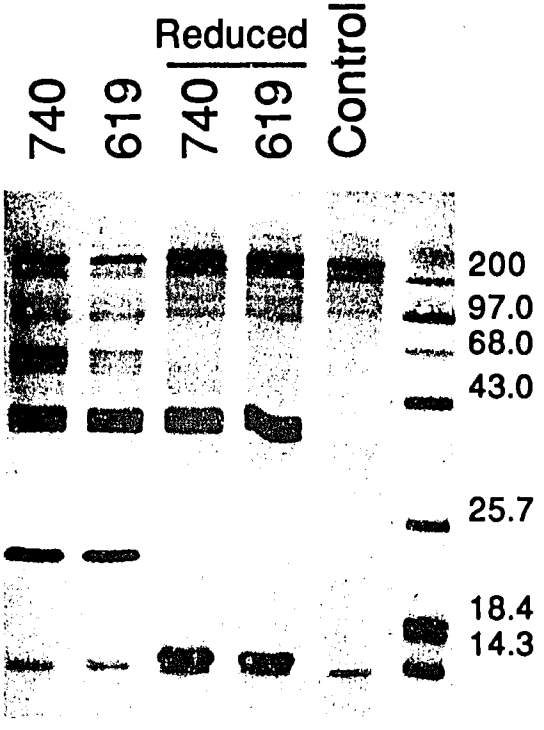


Figure 6. SDS-PAGE under reducing and non-reducing conditions of 35S-labeled supernatants from two activin βA producing clones (#740 and #619). The size of the markers is as indicated.

ACKNOWLEDGMENTS

I wish to thank my colleagues Karoly Nikolics, Ralph Schwall, Peter Seeburg, Hugh Niall, Lucy Rhee, Mark Renz, and Eva Szonyi for their various contributions to this work.

REFERENCES

1. CATE RL, MATTALIANO RJ, HESSION C, TIZARD R, FARBER NM, CHEUNG A, NINFA EG, FREYL AZ, GASH DJ, CHOW EP, FISHER RA, BERTONIS JM, TORRES G, WALLNER BP, RAMACHANDRAN KL, RAGIN RC, MANAGRIARO TF, MACLAUGHLIN DT, AND DONAHUE PK: Isolation of the bovine and human genes for Mullerian inhibiting substance and expression of the gene in animal cells. Cell 45:685, 1986
2. CHANNING CP, GORDON WL, LIU W-K, WARD DN: Physiology and biochemistry of ovarian inhibin. Proc Soc Exp Biol Med 178:339, 1985
3. EATON DL, WOOD WI, EATON D, HASS PE, HOLLINGSHEAD P, WION K, MATHER J, LAWN RM, VEHAR GA, GORMAN C: Construction and characterization of an active Factor VIII variant lacking the central one third of the molecule. Biochemistry 25:8343, 1986

4. ESCH FS, SHIMMASAKI S, COOKSEY K, MERCADO M, MASON AJ, YING S-Y, UENO N, LING N: Complementary deoxyribonucleic acid (cDNA) cloning and DNA sequence analysis of rat ovarian inhibins. Molecular Endocrinology 1:388-396, 1987
5. ETO Y, TSUJI T, TAKEZAWA M, TAKANO S, YOKOGAWA Y, SHIBAI H: Purification and characterization of erythroid differentiation factor (EDF) isolated from human leukemia cell line THP-1. Biochem Biophys Res Commun 142:1095, 1987
6. FORAGE RG, RING JM, BROWN RW, MCINERNEY BV, COBON GS, GREGSON RP, ROBERTSON DM, MORGAN FJ, HEARN MTW, FINDLAY JK, WETTENHALL REH, BURGER HG, DEKRETSER DM: Cloning and sequence analysis of cDNA species coding for the two subunits of inhibin from bovine follicular fluid. Proc Natl Acad Sci USA 83:3091, 1986
7. LING N, YING S-Y, UENO N, ESCH F, DENOROY L, GUILLEMIN R: Isolation and partial characterization of a M_r 32,000 protein with inhibin activity from porcine follicular fluid. Proc Natl Acad Sci USA 82:7217, 1985
8. LING N, YING S-Y, UENO N, SHIMASAKI S, ESCH F, HOTTA M, GUILLEMIN R: Pituitary FSH is released by a heterodimer of the β subunits from the two forms of inhibin. Nature 779, 1986
9. MASON AJ, HAYFLICK JS, LING N, ESCH F, UENO N, YING SY, GUILLEMIN R, NIALL H, AND SEEBURG PH: Complementary DNA sequences of ovarian follicular fluid inhibin show precursor structure and homology with transforming growth factor-β. Nature 318:659, 1985
10. MASON AJ, NIALL HD, SEEBURG PH: Structure of two human ovarian inhibins. Biochem Biophys Res Commun 135:957, 1986
11. MASSAGUE J: The TGF-β family of growth and differentiation factors. Cell 49:437, 1987
12. MIYAMOTO K, HASEGAWA Y, FUKUDA M, NOMURA M, IGARASHI M, KANGAWA K, MATSUO H: Isolation of porcine follicular fluid inhibin of 32K daltons. Biochem Biophys Res Commun 129:396, 1985
13. PADGETT RW, STJOHSTON D, GELBART WM: A transcript from a drosophila pattern gene predicts a protein homologous to the transforming growth factor-β family. Nature 325:81, 1987
14. RIVIER J, SPIESS J, MCCLINTOCK R, VAUGHAN J., VALE W: Purification and partial characterization of inhibin from porcine follicular fluid. Biochem Biophys Res Commun 133:120, 1985
15. ROBERTSON D, DEVOS F, MCLACHLAN RI, SALAMONSEN LA, HEARN MTW, MORGAN FJ, FINDLAY JK, BURGER HG, DE KRETSER DM: Purification of inhibin and its isoforms. In Inhibin-Non-Steroidal Regulation of Follicle Stimulating Hormone Secretion. BURGER HG, DeKRETSER DM, FINDLAY JK, IGARASHI M (eds), New York, Raven Press, p 18, 1987
16. ROBERTSON DM, FOULDS LM, LEVERSHA L, MORGAN FJ, HEARN MTW, BURGER HG, WETTENHALL REH, DEKRETSER DM: Isolation of inhibin from bovine follicular fluid. Biochem Biophys Res Commun 126:220, 1985
17. STEWART AG, MILBORROW HM, RING JM, CROWTHER CE, FORAGE RG: Human inhibin genes. Genomic characterization and sequencing. Febs 206:329-333, 1986.
18. VALE W, RIVIER J, VAUGHAN J, MCCLINTOCK R, CORRIGAN A, WOO W, KARR D, SPIESS J: Purification and characterization of an FSH releasing protein from porcine ovarian follicular fluid. Nature 776, 1986
19. WEEKS DL, MELTON DA: A maternal mRNA localized to the vegetal hemisphere in Xenopus eggs codes for a growth factor related to TGF-β. Cell 51, 861, 1987

DISCUSSION

CHAIRPERSON: PAUL J. MANBERG, Ph.D.

DR. CROWLEY: Do you have any idea how the body regulates the subunit ratio? Is it at the level of expression?

DR. MASON: I think the studies done thus far suggest that FSH has a great effect on the expression of the α subunit and a small effect on the expression of the β subunit.

DR. CROWLEY: I appreciate the effort that has gone into the lovely work you described. I have gone through similar problems. Have you attempted to express the α and β subunits separately and then subsequently combined them?

DR. MASON: No.

DR. SAIRAM: From the outset, have you found the dimers of α in the natural fluid, or only in your expression experiments? And if so, what kind of activity is seen?

DR. MASON: You may get a little bit of α subunit dimer formation. In our bioassay it has no activity, neither does it appear to act as an antagonist of either inhibin or activin.

DR. MIYAMOTO: I have one comment on that question. We also produced cell lines that have initiated only α cDNA, and we found that only an α monomer is produced in that cell culture assay. It has inhibin activity by itself, but this is only a preliminary result. We have much more work to do.

DR. WARD: I noticed that even your expression cells presented here were producing a sort of a doublet form of the α subunit, and you mentioned this was attributable to the carbohydrate moiety. Have you studied the aspect of the carbohydrate in detail that is responsible for this?

DR. MASON: We have changed the asparagine residues to glutamine residues, and found that the doublet is resolved into one band.

DR. SHETH: I would like to show two slides of the synthetic peptides. These are fragments of porcine β-inhibin for which we have determined the structure. I would like to talk about this amino acid at the bottom. The next slide shows that, when assayed, we get a twofold FSH rise compared to about 20 or 30% obtained with activin. With some modification this peptide shows inhibin activity.

DR. MASON: We and others get about a 100% rise in FSH levels with activin, not the 20 to 30% rise that you just mentioned. I would like to ask Dr. Sheth what bioassay systems he used to measure the activin.

DR. SHETH: We injected the inhibin preparation once daily for three days. Following the last injection, we bled the animals and the serum was assayed.

DR. MASON: Does it work in any other assays?

DR. SHETH: Yes, we try to work with *in vitro* as well as *in vivo* assays. We are checking the preparation also in mice and hamsters.

DR. MASON: Have you used dispersed pituitary cells?

DR. SHETH: We have not yet.

DR. MANBERG: Have you characterized the activin in *in vivo* models?

DR. MASON: No.

DR. BIALY: I have several questions regarding NIH. Have you been able to compare the relative potencies of the natural and synthetic material? Secondly, what do you mean by producing "substantial" amounts of materials? And, thirdly, the question you have been hoping for, when will it be available for distribution?

DR. MASON: I will answer the second question first. "Substantial" means large. But we are talking about making greater than 10 mg, which is a significant amount. The relative potencies are being compared in collaboration with people at the SALK Institute. The ED-50 that we get with the synthetic material compares fairly well with the natural material. To answer your last question, we hope to have the activin available to give out to some people towards the end of February and the inhibin towards the end of March this year. We would like to conduct some animal studies. This material will not be suitable for use in human studies because it will not have gone through all the preclinical toxicology testing, *etc.* that is required. Actually, we want to find out what it does in an animal before we go to the trouble and expense of making material that is suitable for human studies.

DR. MANBERG: Is this material properly glycosolated? Have you looked at any glycosolation?

DR. MASON: We have not compared the synthetic with natural material. But it is definitely glycosolated. We do not seem to get any material that is not glycosolated.

3

Isolation and Characterization of Gonadal Polypeptides that Regulate the Secretion of Follicle Stimulating Hormone

NICHOLAS LING
NAOTO UENO
FREDERICK ESCH
SHUNICHI SHIMASAKI
MAKOTA KOGA
SHAO-YAO YING
ROGER GUILLEMIN

The granulosa cells of the ovary secrete into the follicular fluid many proteins which can modify the secretion of pituitary follicle stimulating hormone (FSH). Two such proteins, named inhibins A and B, which specifically inhibit the secretion of pituitary FSH, have recently been isolated from porcine[6,12,13] and bovine follicular fluid.[5,15] The inhibins are heterodimeric proteins of relative molecular mass (M_r) 32,000, composed of a common glycosylated α subunit of M_r 18,000 and one of two homologous, but distinct β subunits of M_r 14,000 (inhibin A [5] $\alpha\beta_B$ and inhibin B [5] $\alpha\beta_B$). The common α subunit can also exist in a larger molecular form of M_r 44,000 and in combination with the β subunit of inhibin A to form an inhibin of M_r 56,000.[14]

Using partial amino acid sequence information, the messages encoding the α, β_A and β_B subunits of porcine inhibins were cloned from an ovarian cDNA library[11], as were the α and β_A subunits of bovine inhibin.[4] DNA sequence analyses showed that all of the subunits were initially synthesized as large precursor proteins with the mature subunits residing at the C-terminal portion of the precursors. Surprisingly, the two β subunits, besides being homologous to each other, were found to be structurally related to a homodimeric protein, transforming growth factor β,[11] which has totally different biological activities. Moreover, in addition to the inhibins, two FSH-releasing proteins of apparent M_r 24,000, named activin A or FRP and activin AB, were also isolated from porcine follicular fluid.[9,10,17] The former was characterized as a homodimer, comprised of two β subunits of inhibin A ($\beta_A\beta_A$) linked by disulfide bridge(s), while the latter is a heterodimer comprised of the β subunits of inhibins A and B ($\beta A\beta B$). Thus, depending on the combina-

This research was supported by NICHD contract NOI-HD-6-2944, Program project grants HD-09690 and DK-18811 from the National Institutes of Health, and a grant from the Robert J. and Helen Kleberg C. Foundation.

tion of the gene products, molecules with totally different biological properties could be derived from a limited number of genes (Fig. 1).

In our original work on the isolation of the two M_r 32,000 inhibins from porcine follicular fluid, we had noted a side fraction migrating at the beginning of the first reversed-phase high performance liquid chromatography (RP-HPLC) chromatogram, ahead of the activins and inhibins, which could also specifically suppress pituitary FSH secretion in the inhibin bioassay. The fractions containing this activity have now been purified to homogeneity to yield two glycosylated single-chain proteins of M_r 35,000 and 32,000, respectively.[16] The two proteins have similar amino acid compositions and identical N-terminal amino acid sequences, but structurally they are totally unrelated to the inhibins and were named follistatins (FS). In this paper, we present the purification and isolation of these two novel FSH release-inhibiting proteins from porcine follicular fluid, as well as the molecular cloning of their precursors.

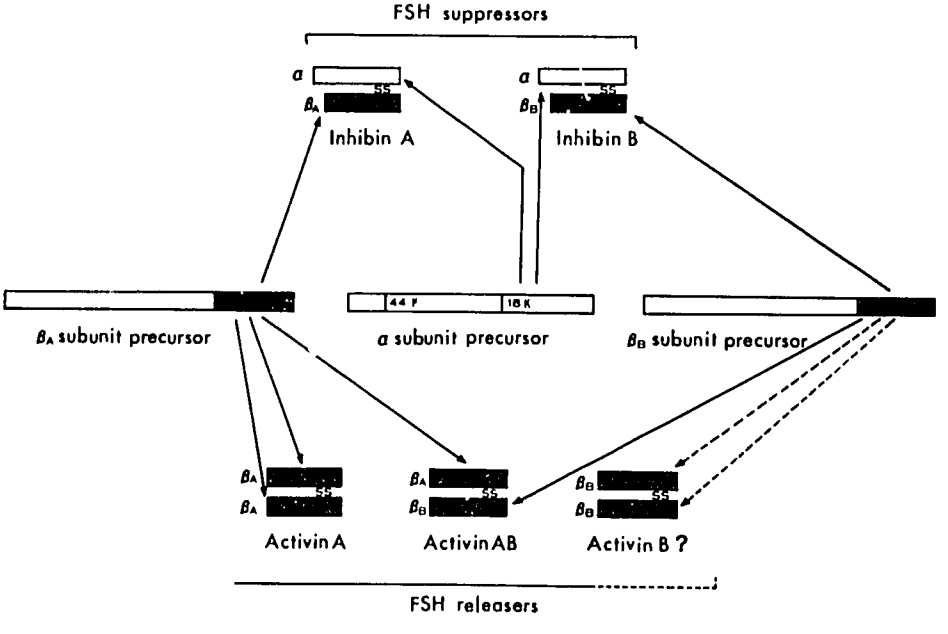


Figure 1. Multiple arrangement of gene products from the α, β_A and β_B subunit precursors of inhibins A and B through disulfide bridging to yield FSH releasers and suppressors. 18K and 14K denote the M_r 18,000 and 44,000 mature α subunits, respectively. The broken lines indicate a possible product which has not been isolated and characterized.

PURIFICATION AND ISOLATION OF FS

The starting material for the purification of FS was a side fraction derived from the purification of the porcine ovarian inhibins and activins.[8-10] Briefly, porcine follicular fluid was processed batchwise through heparin-Sepharose affinity chromatography and the adsorbed proteins eluted with 1 M NaCl (Fig. 2a). After dialysis in 30% acetic acid (v/v), the affinity purified proteins were further size-fractionated on a Sephacryl S-200 column in 30% acetic acid (Fig. 2b). The column fractions were monitored by an *in vitro* bioassay using immature female rat anterior pituitary cells in monolayer culture.[8] Active fractions were

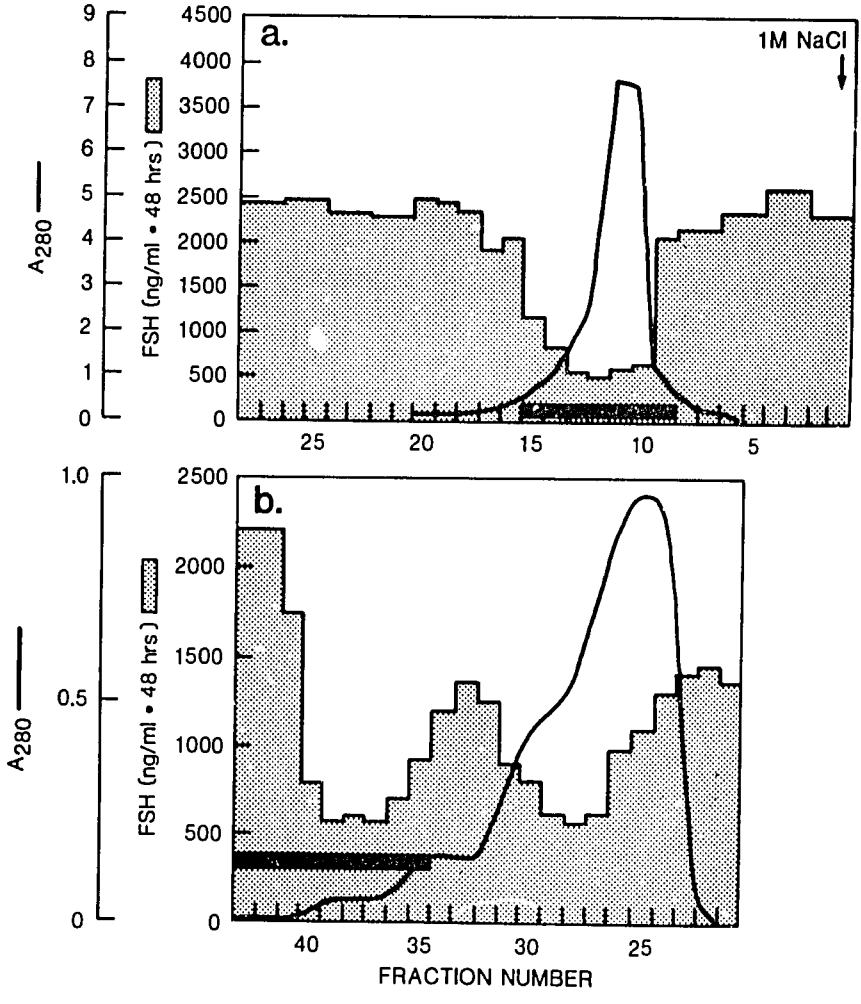


Figure 2. a) Heparin-Sepharose affinity chromatography of porcine follicular fluid proteins, showing the profile of the 1 M NaCl/10 mM Tris • Cl, pH 7, eluate from the column. b) Sephacryl S-200 gel filtration in 30% acetic acid (v/v) of the inhibin and activin proteins recovered from the heparin-Sepharose column [solid bar in a)].

identified by their ability to either inhibit or increase the basal secretion of FSH, but not that of LH, into the culture medium in 48 hours. Fractions containing the M_r 32,000 inhibins and FSH-releasing activities from the column (fractions 35-43 in Fig. 2b) were pooled and lyophilized. The lyophilized material (~40 mg) was dissolved in 40 ml of 0.2 N acetic acid and filtered through a Millex-HA 0.45 fm filter (Millipore, Bedford MA). The filtrate was applied directly to a 10 X 250 mm Vydac 5-μm C_4 column (The Separation Group, Hesperia CA). After all the filtrate had been loaded, the column was washed with the triethylammonium phosphate (Et_3NHP) solvent system until the UV absorption reached baseline. The inhibins and activins were separated with a linear gradient of 27 to 36% acetonitrile in the Et_3NHP solvent system in 90 minutes at a flow of 3 ml/min. Three zones of FSH release-inhibiting activity corresponding to FS, inhibin A and inhibin B were detected as were two zones of FSH-releasing activity designated as activin A and activin AB, as shown in Figure 3a. The inhibins and activins have been purified to homogeneity and their primary structures characterized as reported previously.[1,7,10,11] The remaining FSH release-inhibiting zone (fractions 3 and 4, designated as FS in Fig. 3a) was pooled and, after mixing with an equal volume of 0.2 N acetic acid, applied directly onto a 10 X 250 mm Vydac 5-μm C_4 column as before.[1] The active material was eluted from the column with a linear gradient of 21 to 30% acetonitrile in the trifluoracetic acid (TFA) solvent system in 90 minutes at a flow of 3 ml/min as shown in Figure 3b. Fractions with FSH release-inhibiting activity (fractions 19-23) detected by the bioassay were pooled and, after removing the acetonitrile in a Speed-Vac concentrator (Savant, Hicksville NY) and adjusting to pH 6.5 with 0.1 N NH_4OH, were pumped directly onto a 7.5 X 75 mm Spherogel-TSK 10-μm DEAE-5PW column (Toyo Soda, Tokyo JAPAN). After loading, the column was washed with buffer A (0.01 M Na_2HPO_4, pH 6.5) until the UV absorption reached baseline. The FS activity was separated with a linear gradient of 0 to 0.3 M NaCl in buffer A in 90 minutes at a flow of 1 ml/min as shown in Figure 3c. The active material eluting in fractions 19 to 22 was pooled and, after diluting to four times its original volume with 0.2 N acetic acid, further purified on a 10 X 250 mm Vydac 5-μm C4 column likewise and eluted with a linear gradient of 21 to 30% acetonitrile in the TFA solvent system in 90 min at a flow of 1 ml/min as shown in Figure 3d. The active material eluting in fractions 33 to 35 (peak I) was pooled and diluted to twice its original volume and rechromatographed on a 10 X 250 mm Vydac 5-μm phenyl column with a linear gradient of 18 to 27% acetonitrile in the Et_3NHP solvent system in 90 min at a flow of 1 ml/min as shown in Figure 3e. Finally, the active material in fractions 34-36 was pooled and after dilution with 0.2 N acetic acid, concentrated by chromatography on a 4.6 X 250 mm Aquapore 10-μm RP-300 column, using a linear gradient of 20 to 80% acetonitrile in the TFA solvent system in 60 min at a flow of 0.5 ml/min as shown in Figure 3f. Altogether, 400 μg of FS were isolated from 18 liters of follicular fluid.

STRUCTURAL CHARACTERIZATION OF FS

On $NaDodSO_4$/PAGE analysis under non-reducing conditions, FS showed a single band migrating at apparent M_r 35,000 after coomassie blue staining as shown in Figure 4a. In contrast to inhibins A and B, FS exhibited a single band that migrated at apparent M_r 42,000 under reducing conditions (Fig. 4b), indicating that it is comprised of a single polypep-

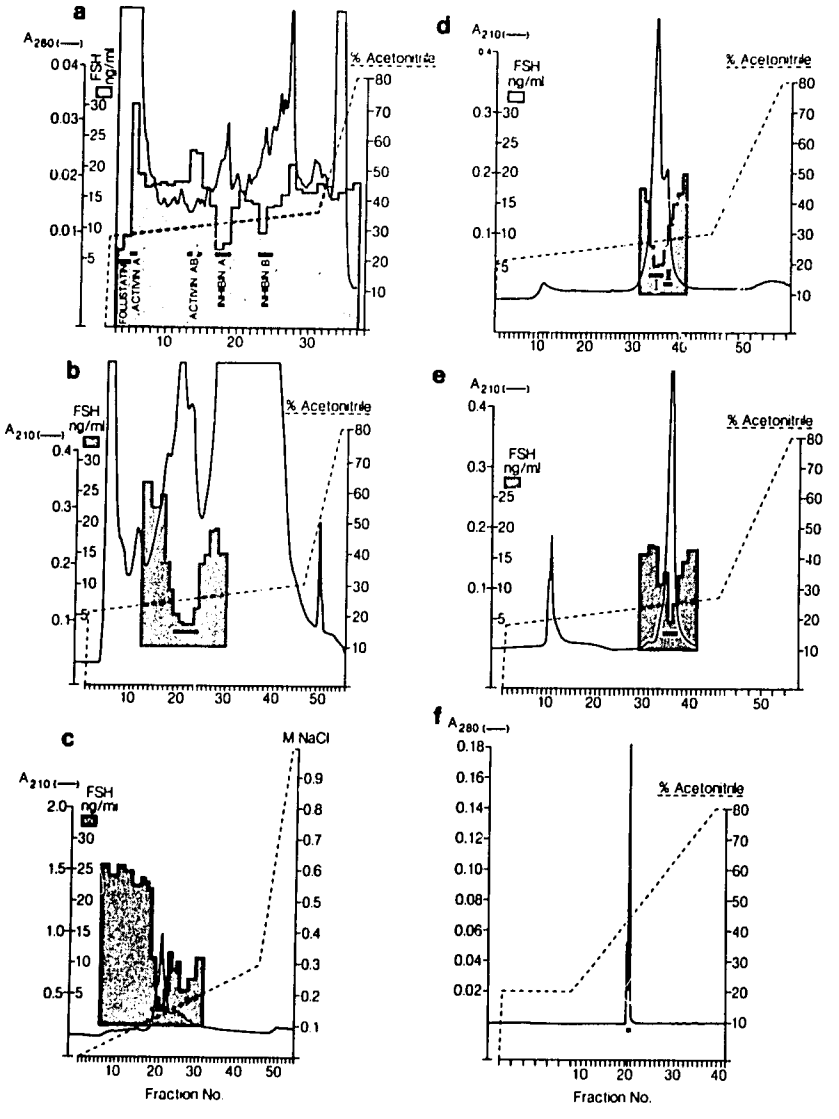


Figure 3. HPLC purification of FS. a) The active material denoted by the solid bar in Fig. 2b was chromatographed on a Vydac C_4 column and eluted with the indicated gradient of acetonitrile in the Et_3NHP system. Three zones of FSH suppressing activity (FS, inhibin A and inhibin B) and two zones of FSH releasing activity (activin A and activin AB) were recovered. b) The active zone corresponding to FS was pooled and further purified on a Vydac C_4 column with the indicated gradient of acetonitrile in the TFA system. c) The material denoted by the solid bar in Figure 2b was pooled and, after removing the acetonitrile in a Speed-Vac concentrator, applied to a TSK Spherogel DEAE-5PW column and eluted with the indicated gradient of NaCl in 10 mM Na_2HPO_4 buffer, pH 6.5. d) Active fractions [solid bar in c)] recovered from the ion-exchange HPLC were pooled and further purified on a Vydac C_4 column with the indicated gradient of acetonitrile in

tide chain. Microsequence analyses of FS revealed its N-terminal as Gly-Asn-Cys-Trp-Leu-Arg-Gln-Ala-Lys-Asn-Gly-Arg-Cys-Gln-Val-Leu-Tyr-Lys-Thr-Glu-Leu-Ser-Lys-Glu. In addition, 63% of the labeled FS was retained on a concanavalin A—Sepharose 4B affinity column which could be displaced with 0.2 M α-methyl-D-mannopyranoside, indicating that FS is glycosylated.[16]

Using similar RP-HPLC conditions (data not shown), a M_r 32,000 form (Fig. 4c) of FS, with an identical N-terminal amino acid sequence as the M_r 35,000 FS, was isolated from fractions 36-37 of Figure 3d (peak II). Its amino acid composition is related to the M_r 35,000 form, and upon reduction, this protein migrated as a single band at apparent M_r 40,000 on $NaDodSO_4$/PAGE (Fig. 4d).

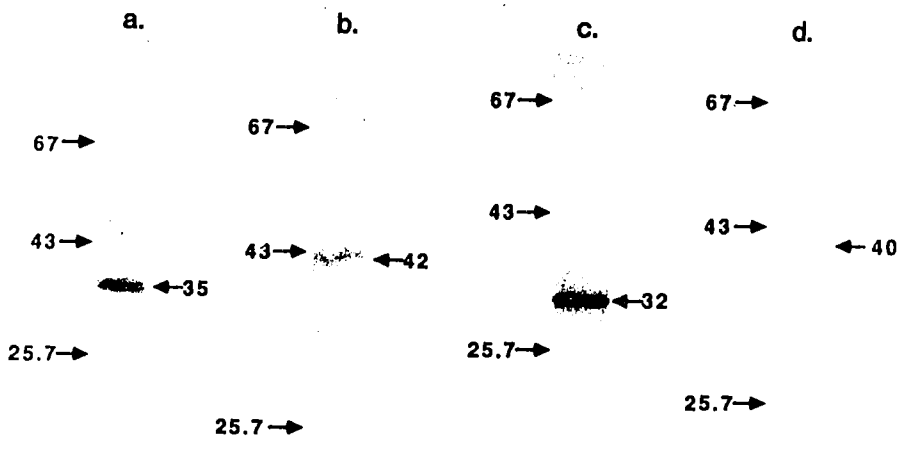


Figure 4. $NaDoSO_4$/PAGE analyses of the purified 35K FS from peak I of Figure 3d under nonreducing a) and reducing b) conditions, as well as the purified 32K FS from peak II of Figure 3d under nonreducing c) and reducing d) conditions. Migrating positions of the molecular weight standards are indicated at the left side of each gel. Numbers represent M_r X 10^{-3}.

---

the TFA system. Two peaks of FSH suppressing activity, I and II, denoted by the solid bars, were detected. e) The active material corresponding to peak I in (d) was pooled and chromatographed on a Vydac phenyl column with the indicated gradient of acetonitrile in the Et_3NHP system. f) The material denoted by the solid bar in (e) was pooled and concentrated by chromatography on an Aquapore RP-300 column for characterization, using the indicated gradient of acetonitrile in the TFA system.

Six M_r 35,000 FS preparations comprising ~6.4 nmol of polypeptides were reduced with dithiothreitol, alkylated with ^{14}[C]-iodoocetamide and desalted by RP-HPLC to yield ~2.8 nmol ^{14}[C]-carboxyamidomethylated FS as estimated by amino acid analyses.[2] This material was digested with trypsin and the digestion fragments purified by RP-HPLC as indicated in Figure 5. The resulting FS tryptic fragments were characterized by both amino acid analysis and gas-phase microsequencing as shown in Table 1. In total, 11 tryptic peptides were characterized in addition to the N-terminal sequence, resulting in the assignment of 186 amino acids representing ~60% of the mature hormone. The availability of this large quantity of protein sequence data was a particularly valuable adjunct to cDNA cloning and DNA sequencing in the ultimate determination of the primary structure of the protein.

CLONING AND DNA SEQUENCE ANALYSIS OF FS

Two long synthetic oligonucleotide probes were designed, using amino acid sequences identified in the FS tryptic fragments, FS(36-48) and FS(174-185).[2] The specificity of these probes was initially established with porcine ovarian RNA blots where both probes hybridized to a 1.9 kb RNA species (data not shown). Subsequently, 1.5 X 10^6 clones from an oligo-dT-primed porcine ovarian cDNA library in λgt10 were screened with these probes and thirteen clones hybridizing to both probes were identified and purified by rescreening. The cDNA inserts in 12 of the 13 clones could be excised with EcoRI and were subcloned into M13mp19 phage for sequence analyses.[2]

The results of the DNA sequence analyses of five FS cDNA clones with inserts ranging in size from 1.1 to 2.2 kb are shown in Figure 6. Only sequence data common to multiple clones, amounting to 1070 bp for clones FS12, FS5, and FS18, and 1049 bp for clones FS4

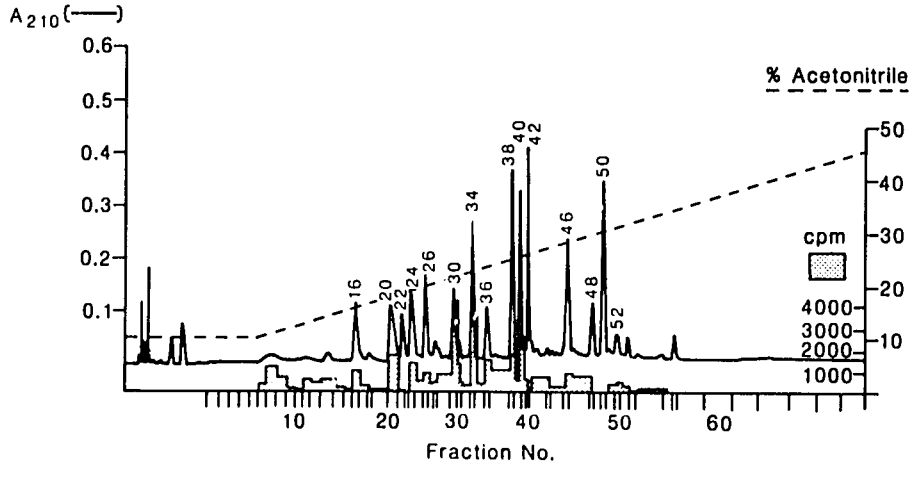


Figure 5. RP-HPLC purification of the peptide fragments obtained from trypsin digestion of the [^{14}C]-carboxyamidomethylated 35K FS. The peptides were separated on a 2.1 X 100 mm Brownlee RP-300 cartridge using the indicated gradient of acetonitrile in the TFA system.

Table 1. Protein Microsequencing Results from the ^{14}[C]-Carboxyamidomethylated Tryptic Fragments of 35K Follistatin (FS)

RP-HPLC Fraction No[c]	Peptide Fragment	Structure			
		5	10	15	20
50	FS(32-48)	LSTSW	TEEDV	NDNTL	FK
52	FS(49-63)	WMIFN	GGAPN	CIPCK	
34	FS(87-99)	CVCAP	DCS[a]NI	TWK	
38	FS(141-159)	DVFCP	GSSTC	VVDQT	NNAY
40	FS(166-185)	ICPEP	TSSEQ	YLCGN	DGVTY
22	FS(201-209)	SIGLA	YEGK		
46	FS(227-232)	CLWDF	K		
30	FS(238-249)	CSLCD	ELCPE	SK	
24	FS(250-269)	SEEPV	CASD[ab]X	ATYAS	ECAMK
36	FS(270-282)	EAACS	SGVLL	EVK	
16	FS(283-300)	HSGSC	NSISE	DTEEE	EED

a Potential Asn-linked glycosylation site

b No identifiable >PhNCS-amino acid released at this cycle. Residue identified as Asx by combining microsequencing and amino acid analysis data for this peptide. Typical Asn-linked glycosylation sequence, Asn-X-(Ser, Thr), suggests that this Asx is glycosylated.

c The No. refers to the chromatographic fractions in Fig. 5.

and FS6, are presented in Figure 7 and those consensus regions of the clones are diagrammatically indicated in Figure 6. All FS cDNA clones contain a putative initiation Met at nucleotides 18-20 which is located within a sequence favored for eukaryotic initiation sites,[7] A/G-X-X-A-U-G-G. Clones FS12, FS5 and FS18 contain an open reading frame of 1032 bases encoding a precursor protein of 344 amino acids (preFS-344) while clones FS4 and FS6 contain the same 5'-nucleotide sequence down to base 970 where a termination codon interrupts the precursor coding sequence after 317 amino acids (preFS-317) and begins a 3'-untranslated region unrelated to the 3'-noncoding region of clone FS12, FS5 and FS18. The larger 2.2 kb insert found in cDNA clone FS18 (Fig. 6) was caused by the insertion of two introns between nucleotides 515 and 516, and 740 and 741, as shown in Figure 7. The insertion probably occurred during cDNA synthesis. The origin of the two different termination signals for the FS clones is probably derived from alternative splicing of the precursor mRNA.

The first 29 residues of all FS precursors constitute a typical signal sequence followed immediately by the N-terminus of the mature proteins as established by gas-phase microsequencing of both forms of the intact FS.[12] However, the exact lengths of the different forms of the mature proteins are unknown. No C-terminal sequence information could be ob-

tained with the limited quantities of purified natural product available and amino acid analyses data on polypeptides of this size are not sufficiently discriminating to identify the C-terminal residue. The two groups of clones in Figure 6 encode mature FS sequences of 315 (FS12, FS5 and FS18) and 288 (FS4 and FS6) amino acids, respectively. The corresponding calculated molecular masses of 34.8K and 31.6K, respectively, are in good agreement with $NaDodSO_4$/PAGE estimates of M_r 35,000 and M_r 32,000 for the two dif-

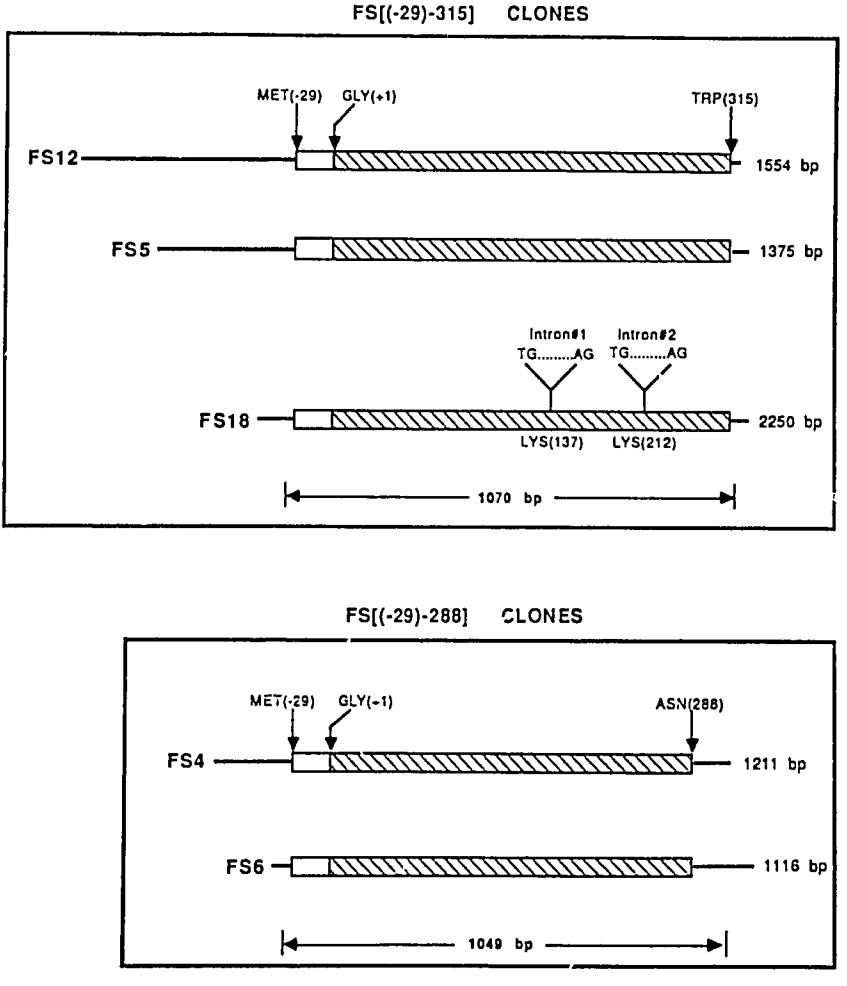


Figure 6. Schematic representation of two populations of FS cDNA clones, FS[(-29)-315] and FS[(-29)-288], identified in the porcine ovarian cDNA library. Coding sequences are boxed and the mature coding sequences boxed and slashed. The untranslated sequences are represented with a line. The actual length of each of the characterized cDNA clones is indicated on the right as well as those sections of the clones whose consensus sequence is presented in Figure 7 (1070 bp of clones FS12, FS5 and FS18 and 1049 bp of clones FS4 and FS6). The location of the two introns in clone FS18 are indicated.

```
                  -29                          -20
   1  CCCGCGCCTGACCCCAGAATGGTCCGTCCCAAGCACCAGCCCGGCGGGGCTATGCCTCCTG   60
                          MetValArgProLysHisGlnProGlyGlyLeuCysLeuLeu

                  -10                          -1 +1
  61  CTGCTGCTGCTCTGCCAGTTCATGGAGGACCGCAGCGCCCAGGCTGGGAATTGCTGGCTC   120
      LeuLeuLeuLeuCysGlnPheMetGluAspArgSerAlaGlnAlaGlyAsnCysTrpLeu

                  10                           20
 121  CGCCAAGCAAAAAACC[illegible]GGTGCCAGGTCCTGTATAAAACCGAACTGAGCAAGGAGGAG   180
      ArgGlnAlaLysAsnGlyArgCysGlnValLeuTyrLysThrGluLeuSerLysGluGlu

                  30                           40
 181  TGCTGCAGCACCGGCCGCCTGAGCACCTCCTGGACTGAGGAGGACGTAAATGACAACACA   240
      CysCysSerThrGlyArgLeuSerThrSerTrpThrGluGluAspValAsnAspAsnThr

                  50                           60
 241  CTTTTCAAGTGGATGATTTTCAATGGAGGTGCCCCCAACTGCATCCCATGTAAAGAAACG   300
      LeuPheLysTrpMetIlePheAsnGlyGlyAlaProAsnCysIleProCysLysGluThr

                  70                           80
 301  TGCGAGAACGTGGACTGTGGGCCCGGGAAAAAATGCCGAATGAACAAGAAGAACAAACCC   360
      CysGluAsnValAspCysGlyProGlyLysLysCysArgMetAsnLysLysAsnLysPro

                  90                           100
 361  CGCTGCGTCTGCGCCCCGGATTGTTCTAACATCACCTGGAAAGGCCCAGTCTGTGGGCTG   420
      ArgCysValCysAlaProAspCysSerAsnIleThrTrpLysGlyProValCysGlyLeu

                  110                          120
 421  GATGGGAAAACCTACCGCAACGAATGTGCTCTCCTCAAGGCCAGATGTAAAGAACAGCCG   480
      AspGlyLysThrTyrArgAsnGluCysAlaLeuLeuLysAlaArgCysLysGluGlnPro

                                   INTRON #1
                  130                          140
 481  GAACTGGAAGTCCAGTACCAAGGCAAATGTAAAAAGACCTGTCGGGATGTTTTCTGTCCA   540
      GluLeuGluValGlnTyrGlnGlyLysCysLysLysThrCysArgAspValPheCysPro

                  150                          160
 541  GGCAGCTCCACATGTGTGGTGGACCAGACTAATAATGCCTACTGTGTGACATGTAACCGC   600
      GlySerSerThrCysValValAspGlnThrAsnAsnAlaTyrCysValThrCysAsnArg

                  170                          180
 601  ATTTGCCCAGAGCCCACCTCCTCAGAACAGTATCTCTGTGGGAATGATGGAGTGACCTAC   660
      IleCysProGluProThrSerSerGluGlnTyrLeuCysGlyAsnAspGlyValThrTyr

                  190                          200
 661  TCCAGTGCCTGTCACCTGAGAAAGGCTACCTGCCTACTGGGCAGATCTATTGGATTGGCC   720
      SerSerAlaCysHisLeuArgLysAlaThrCysLeuLeuGlyArgSerIleGlyLeuAla

                   INTRON #2
                  210                          220
 721  TATGAGGGAAAGTGTATCAAAGCAAAGTCCTGTGAAGACATCCAGTGCACTGGTGGAAAA   780
      TyrGluGlyLysCysIleLysAlaLysSerCysGluAspIleGlnCysThrGlyGlyLys

                  230                          240
 781  AAGTGCTTATGGGATTTCAAGGTTGGCAGAGGCCGTTGTTCCCTCTGCGATGAGCTGTGC   840
      LysCysLeuTrpAspPheLysValGlyArgGlyArgCysSerLeuCysAspGluLeuCys

                  250                          260
 841  CCTGAGAGTAAGTCTGAGGAGCCTGTCTGTGCCAGTGACAATGCCACCTACGCCAGTGAG   900
      ProGluSerLysSerGluGluProValCysAlaSerAspAsnAlaThrTyrAlaSerGlu

                  270                          280
 901  TGTGCCATGAAGGAAGCGGCCTGTTCCTCAGGTGTGCTGCTGGAAGTAAAGCACTCTGGA   960
      CysAlaMetLysGluAlaAlaCysSerSerGlyValLeuLeuGluValLysHisSerGly

                  290                          300
 961  TCTTGCAACTCCATTTCAGAAGACACCGAGGAGGAGGAGGAATGAAGACCAGGACTAC   1020
      SerCysAsnSerIleSerGluAspThrGluGluGluGluGluAspGluAspGlnAspTyr

FS4,6          TGAATCTGCCCGTAAAACCTGAGCCATTGATTCTTCAGAACTTTCTGCAGT
               Ter

                  310          315
1021  AGCTTTCCTATATCTTCTATTCTAGAGTGGTAAACCCTCCACCAATTTTC   1070
      SerPheProIleSerSerIleLeuGluTrpTer

FS4,6          TTTTGACTTCATAGATAATGTTTTTAA   1049
```

Figure 7. Nucleotide and predicted amino acid sequences of the porcine FS cDNA inserts. Nucleotides are numbered at the left and the right and amino acids throughout. The cDNA sequence of the clones are identical from nucleotides 1-970, but clones FS12, FS5 and FS18 diverge from FS4 and FS6 at base no. 971, resulting in the truncation of the coding sequences of FS4 and FS6 at that point. The putative signal peptide comprises FS(-29 to -1); the eukaryotic translation initiation site consensus sequence is marked with a jagged underline. The mature FS sequence is *italicized* and the tryptic peptides characterized by

ferent FS species. Additionally, the mature M_r 35,000 FS must minimally contain FS(1-300) based on the characterization of the tryptic fragment FS(283-300) in Table 1.

DNA sequencing identified two potential Asn-linked glycosylation sites in the mature hormone at Asn(95) and Asn(259). Sequence analysis of the tryptic fragments containing these residues [FS(87-99) and FS(250-269), respectively, in Table 1] clearly identified the residue at FS(95) as > PhNCS-Asn but no identifiable > PhNCS-amino acid was released at FS(259). The residue at FS(259) was subsequently identified at the protein level as Asn by combining microsequencing and amino acid analysis data obtained for the tryptic fragment FS(250-269), suggesting that Asn(259) is carbohydrate-bound as noted in Figure 7.

Inspection of the mature FS sequence reveals several notable features (see Fig. 7). 1) FS is extremely cysteine-rich, containing 36 cysteines in the N-terminal 287 amino acids, suggesting that the mature protein is highly cross-linked with disulfide bridges. 2) Mature preFS-317 terminates at Asn(288) while mature preFS-344 possesses an extra acidic C-terminal sequence in FS(292-304), Glu-Asp-Thr-Glu-Glu-Glu-Glu-Glu-Asp-Glu-Asp-Gln-Asp, which probably "dangles" outside the highly cross-linked protein sphere and is available for binding to positively charged elements. 3) The N-terminal 288 amino acids of FS are organized into four domains corresponding to FS(3-63), FS(65-136), FS(138-211), and FS (215-288). Three of the four domains (II, III, IV) are homologous to each other as well as to human epidermal growth factor[6] and human pancreatic secretory trypsin inhibitor,[1] when aligned for maximum homology as shown in Figure 8.

The physiological significance of FS is under investigation. Preliminary studies with the two porcine proteins[18] showed that they can specifically inhibit the secretion of FSH in rat anterior pituitary primary culture with a potency of approximately one third of that of inhibin A on a molar basis (Fig. 9a). In addition, the inhibitory effect is additive to that of inhibin to produce the same maximal inhibition of FSH release exerted by either inhibin or FS alone (Fig. 9b). Moreover, pituitary cells exposed to FS showed significantly less depletion of intracellular FSH than those treated with inhibin (Fig. 9c), indicating that FS may act primarily on the suppression of FSH release rather than on both release and synthesis, as is the case with inhibin.[14,15] Further physiological studies must await the purification of a large quantity of this material from follicular fluid or its production by gene expression. With the unexpected finding that the activins of ovarian origin are the same molecules as those produced by the human leukemia cell-line, THP-1, inducing differentiation of the erythroblasts,[3] it is quite possible that the FS proteins may be similarly involved as regulating factors in physiological systems unrelated to ovarian-pituitary functions.

---

microsequencing are underlined once. Amino acid sequences employed for the design of synthetic oligonucleotide probes are underlined twice while the putative Asn-linked glycosylated site, Asn(259) is printed in bold. The location of two introns found in clone FS18 is indicated.

Figure 8. Alignment of amino acid sequences of the four domains of FS, human pancreatic secretory trypsin inhibitor (hPSTI) and human epidermal growth factor (hEGF). Amino acids are shown in one letter code and identical residues are shaded. Numbers refer to the amino acid positions in the mature protein sequence.

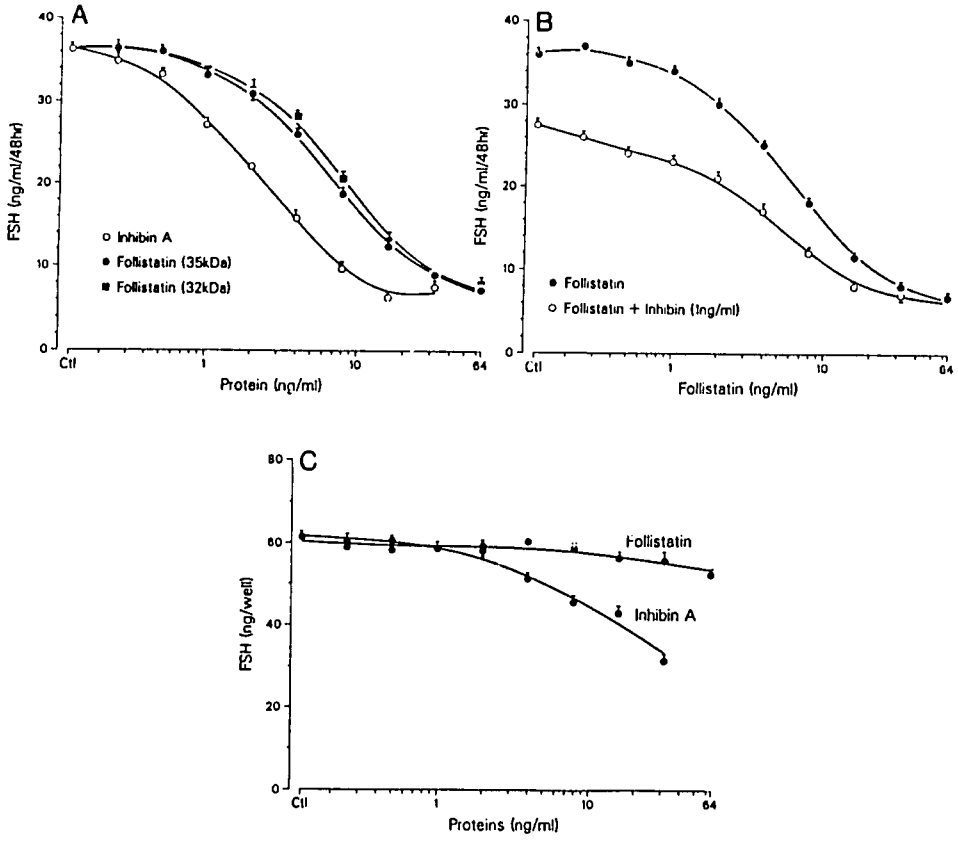


Figure 9. The effects of FS and inhibin on FSH secretion and synthesis in rat anterior pituitary cells. a) Dose-response curves of inhibin A, 35K FS and 32K FS on the suppression of spontaneous FSH release. b) Additive effect of FS and inhibin on the suppression of FSH release. Multiple doses of 35K FS alone and in the presence of a constant dose of inhibin were added to the cells. The open circles show additivity at the lower doses of FS plus inhibin, but identical Emax for FS alone or in combination with inhibin in the high doses. c) The effects of inhibin and FS on pituitary FSH content. Inhibin clearly suppresses the intracellular content of FSH whereas FS is much less effective.

ACKNOWLEDGMENT

We would like to thank D. Angeles, A. Becker, F. Castillo, K. Cooksey, A. Koba, M. Mercado, M. Regno, R. Schroeder, G. Swanson, P. Tan for technical assistance; D. Higgins and B. Gayer for preparing the manuscript.

REFERENCES

1. BARTELT DC, SHAPANKA R, GREENE LJ: The primary structure of the human pancreatic secretory trypsin inhibitor. Arch Biochem Biophys 179:189, 1977
2. ESCH F, SHIMASAKI S, MERCADO M, COOKSEY K, LING N, YING SY, UENO N, GUILLEMIN R: Structural characterization of follistatin: A novel follicle-stimulating hormone release-inhibiting polypeptide from the gonad. Mol Endocrinol 1:849, 1987
3. ETO Y, TSUJI T, TAKEGAWA M, TAKANO S, KOKOGAWA Y, SHIBAI H: Purification and characterization of erythroid differentiation factor (EDF) isolated from human leukemia cell line THP-1. Biochem Biophys Res Commun 142:1095, 1987
4. FORAGE RG, RING JM, BROWN KW, MC INERNEY BV, COBON GS, GREGSON RP, ROBERTSON DM, MORGAN FJ, HEARN MTW, FINDLAY JK, WETTENHALL REH, BURGER HG, DE KRETSER DM: Cloning and sequence analysis of cDNA species coding for the two subunits of inhibin from bovine follicular fluid. Proc Natl Acad Sci USA 83:3091, 1986
5. FUKUDA M, MIYAMOTO K, HASEGAWA Y, NOMURA M, IGARASHI M, KANGAWA K, MATSUO H: Isolation of bovine follicular fluid inhibin of about 32kDa. Mol Cell Endocrinol 44:55, 1986
6. GREGORY H: Isolation and structure of urogastrone and its relationship to epidermal growth factor. Nature 257:325, 1975
7. KOZAK M: Possible role of flanking nucleotides in recognition of the AUG initiator codon by eukaryotic ribosomes. Nucleic Acids Res 9:5233, 1981
8. LING N, YING SY, UENO N, ESCH F, DENOROY L, GUILLEMIN R: Isolation and partial characterization of a M_r 32,000 protein with inhibin activity from porcine follicular fluid. Proc Natl Acad Sci USA 82:7217, 1985
9. LING N, YING SY, UENO N, SHIMASAKI S, ESCH F, HOTTA M, GUILLEMIN R: Pituitary FSH is released by a heterodimer of the β-subunits from the two forms of inhibin. Nature 321:779, 1986
10. LING N, YING SY, UENO N, SHIMASAKI S, ESCH F, HOTTA M, GUILLEMIN R: A homodimer of the β-subunits of inhibin A stimulates the secretion of pituitary follicle stimulating hormone. Biochem Biophys Res Commun 138:1129, 1986
11. MASON AJ, HAYFLICK JS, LING N, ESCH F, UENO N, YING SY, GUILLEMIN R, NIALL H, SEEBURG PH: Complementary DNA sequences of ovarian follicular fluid inhibin show precursor structure and homology with transforming growth factor β. Nature 318:659, 1985
12. MIYAMOTO K, HASEGAWA Y, FUKUDA M, NOMURA M, IGARASHI M, KANGAWA K, MATSUO H: Isolation of porcine follicular fluid inhibin of 32K daltons. Biochem Biophys Res Commun 129:396, 1985
13. RIVIER J, SPIESS J, MC CLINTOCK R, VAUGHAN J, VALE W: Purification and partial characterization of inhibin from porcine follicular fluid. Biochem Biophys Res Commun 133:120, 1985
14. ROBERTSON DM, FOULDS LM, LEVERSHA L, MORGAN FJ, HEARN MTW, BURGER HG, WETTENHALL REH, DE KRETSER DM: Isolation of inhibin from bovine follicular fluid. Biochem Biophys Res Commun 126:220, 1985
15. ROBERTSON DM, DE VOS FL, FOULDS LM, MC LACHLAN RI, BURGER HG, MORGAN FJ, HEARN MTW, DE KRETSER DM: Isolation of a 31 kDa form of inhibin from bovine follicular fluid. Mol Cell Endocrinol 44:27, 1986
16. UENO N, LING N, YING SY, ESCH F, SHIMASAKI S, GUILLEMIN R: Isolation and partial characterization of follistatin: A single-chain M_r 35,000 monomeric protein that inhibits the release of follicle-stimulating hormone. Proc Natl Acad Sci USA 84:8282, 1987
17. VALE W, RIVIER J, VAUGHAN J, MC CLINTOCK R, CORRIGAN A, WOO W, KARR D, SPIESS J: Purification and characterization of an FSH releasing protein from porcine ovarian follicular fluid. Nature 321:776, 1986

18. YING SY, BECKER A, SWANSON G, TAN P, LING N, ESCH F, UENO N, SHIMASAKI S, GUILLEMIN R: Follistatin specifically inhibits pituitary follicle stimulating hormone release *in vitro*. Biochem Biophys Res Commun 149:133, 1987

DISCUSSION

CHAIRPERSON: PAUL J. MANBERG, Ph.D.

DR. KEEPING: Have you been able to show production of follistatin in granulosa cells *in vitro?*

DR. LING: No, because we do not have the antibody to measure this substance. If you use the bioassay, you do not know what you are measuring. But production of the antibody is very difficult. We have prepared many synthetic peptide fragments, but they never produced an antibody that can read the native molecule.

DR. MILLER: Can you iodinate follistatin and obtain biologic activity?

DR. LING: Yes, we have demonstrated that.

DR. MILLER: Is that done with chloramine-T?

DR. LING: No, we use the lactoperoxidase method and iodinated follistatin is a very stable molecule.

DR. BARDIN: Previously you said that you had seen this molecule in the testis. Have you done any more work on that?

DR. LING: Yes, we did. We have done Northern analysis of rat testes and found that the message is there. And we have also cloned the human follistatin from a testicular cDNA library derived from a 50-year-old normal man. So this molecule is produced in both the male and female.

DR. BARDIN: Has anyone looked at the bioactivity of either inhibin or follistatin in purified pituitary cells (i.e. purified gonadotropins) or the binding of these peptides to pituitary cells?

DR. LING: With regard to the bioactivity of inhibin or follistatin in purified gonadotropes, I do not believe that has been reported because it is very difficult to prepare a pure population of gonadotropes from the heterogeneous mixture of pituitary cells. As far as the binding studies are concerned, many research groups have tried to iodinate inhibin and to establish a binding assay with pituitary cells. As far as I know, nobody has succeeded.

We have undertaken some preliminary studies with the binding assay of follistatin, but so far we do not have any good data. All I can say is that you can prepare a stable iodinated follistatin molecule.

DR. MASON: What happens when you add follistatin and activin together in the inhibin bioassay?

DR. LING: We have not done this yet. In order to see if follistatin will antagonize the effect of activin, we need to have activin, which I hope we could obtain from you soon.

DR. WARD: I have not had a chance to study your sequence information yet. Does it have any homology with the subunits of the inhibins?

DR. LING: The cloning paper on follistatin will come out in the November issue of *Molecular Endocrinology.* There is absolutely no sequence homology between follistatin and the α,β_A, and β_B chains of the inhibins. They are completely different.

DR. MANBERG: You mentioned earlier that you looked at several fragments when you were trying to develop the RIA. Do you have any smaller fragments that retain the biological activity?

DR. LING: No, none of them has activity.

DR. MANBERG: So you need the full structure?

DR. LING: Yes. Apparently you do need the full structure to have the biological activity. However, I think this molecule should be a simple compound to prepare using biotechnology.

DR. MANBERG: What has been the problem in the development of the RIA?

DR. LING: The problem is due mainly to the lack of the native molecule because we used so much material on the characterization that we do not really have any more substance available for immunization. We are using mainly the synthetic fragments at this moment for immunization because we can synthesize peptides in our lab. We have made a lot of synthetic fragments.

So far our luck is not that good in terms of eliciting an antibody that can read the native molecule.

DR. MANBERG: Is there any sequence homology with the human seminal plasma inhibins?

DR. LING: No. Absolutely none. The only sequence homology with follistatin is what I have said. There is some homology with human pancreatic secretory trypsin inhibitor (PSTI) and epidermal growth factor (EGF).

DR. MANBERG: Earlier there was a question regarding the relative amounts of these various inhibin-like peptides. Do you have any feel for the relative amount of follistatin?

DR. LING: I cannot say anything about the high molecular weight inhibin-like substances, because we totally discount those in our purification scheme. But within the 32K molecular weight zone, to my best estimation, there is as much follistatin as there is inhibin. In other words, there is a lot of follistatin in follicular fluid. And to dispel your fear about any more surprises, I can say that there is no more inhibin-like molecule, at least in the 32K molecular weight region, so there will not be any more surprises. We have searched all through the column fractions and follistatin is the only one remaining that we found.

DR. WARD: Would you care to explain why you did the additivity experiment between inhibin and follistatin and what it means?

DR. LING: Well, I should not answer that question because I am not a biologist. My simple interpretation is that since we do not know what the receptor looks like, we cannot say that they act at the same receptor. All I can say is that they probably involve the same intracellular mechanism in terms of inhibiting the FSH release. However, I do not know how the signal is transduced across the cell membrane to the cytoplasm of the cell because we have never been able to demonstrate receptors for these types of proteins at the pituitary level. And I am not aware of anybody else doing this. In contrast, activin receptors have been demonstrated in pituitary cells and also in lymphocytes at the recent Neuroscience Meeting, but not for inhibin. So the bioassay data would imply that they probably involve the same or a similar intracellular mechanism. That is as far as I can venture.

Inhibin and follistatin seem to act in a similar way. You see the same maximal inhibition of FSH whether they are used alone in combination.

DR. WARD: Have you tested any other substances that showed homology with follistatin for FSH inhibiting activity?

DR. LING: No, but I doubt very much that they would have biological activity. As a matter of fact, I believe that EGF has been assayed in pituitary cells and it has no effect. I do not know about PSTI.

DR. MASON: Do you still find activin activities with TGFβ? The reason I ask is that TGFβ-like material is in follicular fluid, but you cannot isolate any TGFβ with activin activity. And also, Wylie Vale has not been able to demonstrate activin activity with TGFβ and neither have we with recombinant TGFβ.

DR. LING: I think that question should be answered by Shao-Yao Ying, because I do not do the bioassay. What I learned is that the bioactivity always depends on who does the bioassay. I can answer the chemistry questions, but for the bioassay I cannot, because it seems like an art.

DR. MASON: Do you really think that TGFβ has activin activity? How do you know that the follistatin has inhibin activity if you do not trust your bioassay?

DR. LING: At least we got agreement between different groups, because we are not the only group that found the activity with follistatin. As I mentioned before, the group in Australia also saw the same thing, apparently, a long time ago, and they were just hesitant to publish it. They did finally publish the paper two weeks ago in *Biochem Biophys Res Commun.* As far as I can remember, I think Jock Findlay did publish or said something in the Tokyo Inhibin Symposium that they did see FSH releasing activity with TFGβ. So apparently we seem to agree with the Australian group, as always. But as I said, the bioassay is more like an art. There is a lot of mystery with regard to what secret ingredients you put into the cell culture.

DR. MANBERG: Have you ever tested to see how long the follistatin will remain active if you expose it to plasma?

DR. LING: We have not. All I can say is that follistatin is very stable in terms of temperature, refrigeration, and lyophilization. You cannot destroy it, because we isolated this molecule actually from side fractions from our early days of inhibin purification and kept them in the freezer. The activity was not destroyed during the storage. The stability is probably explained by all the cysteines in the molecule which bound it tightly together.

4

Distribution and Regulation of Inhibin Subunit mRNAs

HÉLÈNE MEUNIER
CATHERINE RIVIER
AARON J W HSUEH
WYLIE VALE

Inhibin is a glycoprotein hormone which has been isolated from bovine and porcine follicular fluids and from ovine rete testis fluid. In the ovary, inhibin is produced mainly by granulosa cells, while in the testis Sertoli cells are known to secrete this glycoprotein. Inhibin is a potent inhibitor of basal FSH release from the pituitary gland, and may play a role in the control of both male and female gonadal functions. Recent studies conducted in several species have shown that inhibin is constituted of two subunits, α and β, linked by disulfide bridges.[3,5,10-12,17] Two related β subunits have been identified (βA and βB) and, as observed for the α subunit, these subunits are encoded by separate genes. Interestingly, dimers of the β-subunits (βAβA and βAβB; Activin-A and Activin-AB) were found to be biologically active, and exert an opposite effect to that of the inhibin $\alpha\beta$ heterodimer on FSH secretion.[9,16] Thus, the elucidation of the primary structure of inhibin, as well as the isolation and determination of the nucleic acid sequences for each of the inhibin subunits have provided us with invaluable means to study the role of this molecule under various physiological conditions.

We have employed the S1 nuclease analysis method to investigate in greater detail the patterns of expression of inhibin subunits.[14] This technique is based on the ability of sample RNA's to protect small, single-stranded cDNA probes of predetermined sizes for α-, βA- and βB-subunits from degradation by the S1 nuclease enzyme. The amounts of inhibin-RNA's were estimated by measuring the concentration of each RNA-protected cDNA probe on acrylamide gel after a 2 hour digestion with S1 nuclease.

REGULATION OF INHIBIN SUBUNIT EXPRESSION IN THE RAT OVARY

We know from previous studies[12] that FSH stimulates inhibin α-subunit expression in the rat ovary. However, little is known about the effect of FSH on inhibin β subunits. We have investigated changes in inhibin α-, βA- and βB-subunit expression in the immature female

rats using the RNA-protection method described above. As seen in Figure 1, PMSG, which exibits FSH-like activity in the rat, increases ovarian levels of inhibin α subunit 5-fold, while levels of βA- and βB-subunit RNAs are increased 10- and 8-fold, respectively. Diethylstilbestrol (DES) causes similar changes in α-subunit RNA levels. The levels of αA- and αB-subunit RNAs are increased 5- and 20-fold, respectively, in the presence of the estrogen. The effect of PMSG was also analyzed in the mature cycling female rat (data not shown) and PMSG was found to induce changes similar to the those observed in the immature female rat. This strongly suggests that FSH and estrogen both stimulate inhibin messenger RNAs *in vivo* and do so by inducing slightly different patterns of expression.

Changes in inhibin α- and β-subunits were also monitored using a well defined granulosa cell culture system[7]. Cells were incubated for 24 hours with FSH or with forskolin, an activator of adenyl cyclase. Figure 2 shows that *in vitro* stimulation of granulosa cells by FSH leads to a 7- and 10-fold increase in levels of inhibin α and βA subunit RNAs, while forskolin stimulates levels, respectively, of 10- and 15-fold. These results agree with previous observations showing that FSH and forskolin enhance inhibin production in granulosa cells[1] and suggest that FSH stimulation of inhibin α- and βA-subunit expression occurs via a cyclic AMP dependent pathway.

The $\alpha\beta$ and $\beta\beta$ dimers of inhibin subunits have been shown to exert opposite paracrine effects on LH-stimulated androstenedione production by theca cells.[8] Therefore, endogenous stimulation of inhibin subunits expression in the ovary is likely to induce a series of physiological responses, both locally and through direct and indirect actions mediated at the pituitary level.

TESTICULAR LEVELS OF INHIBIN α, βA AND βB RNAs DURING SEXUAL MATURATION

In the male rat, earlier observations suggested that the role of inhibin may be restricted to the first six weeks of age.[6] Because we also observed that relatively low levels of inhibin subunits RNAs were present in the mature testis, we decided to investigate the possibility that the expression of inhibin subunits might be age-dependent. As shown in Figure 3, inhibin α-, βA- and βB-RNA levels in the testis were 4- to 10-fold higher in 8 to 15 day-old animals when compared to mature (90 day-old) animals. Although the relative abundance of all three inhibin subunit RNAs decreased with age, it is clear that the βA-RNA levels declined most rapidly. This suggests that regulation of inhibin βA- and βB-subunit expression is controlled in an independent manner during maturation of the rat testis, and may explain, in part, why the βB mRNA could not be detected by RNA gel blot analysis in the mature rat testis.[3] Using a different approach, we observed that bioactive and immunodetectable inhibin in testicular extracts and in plasma decrease in a parallel fashion in rats of increasing age. The production of bioactive inhibin by Sertoli cells has recently been confirmed[2] by direct measurement of inhibin α subunit in Sertoli cell culture medium. From these observations we conclude that Sertoli cells produce higher levels of inhibin α- and β-subunits in the early stage of testicular maturation and eventually reach very low levels in the mature animal.

As demonstrated in the ovary, the $\alpha\beta$ and $\beta\beta$ dimers of inhibin mediate, in an opposite fashion, the LH-stimulated steroid production in testis cell cultures prepared from neonatal

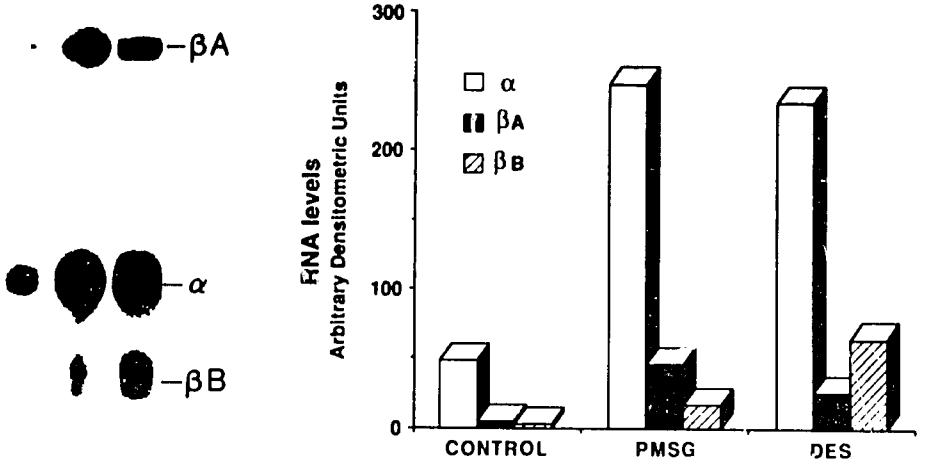


Figure 1. Effects of pregnant mare gonadotropin (PMSG) and diethylstibestrol on levels of inhibin α, βA and βB RNAs in the immature female ovary. Twenty one-day-old female rats were injected with PMSG (200 IU/Kg BW), and ovaries removed after 48 hours. Twenty one-day old female rats were implanted with a DES capsule and ovaries were removed three days later. RNA was prepared by LiCl method and 2.5 μg of RNA was submitted to S1 nuclease analysis. On the left is one autoradiogram showing the respective position of the cDNA probes. Autoradiograms of several exposures were scanned via densitometer and the integrations were corrected according to the specific activity of each probe. On the right, the RNA levels are expressed as arbitrary densitometric units.

and hypophysectomized adult rats.[8] The higher levels of inhibin α- and β-RNA in the testes of very young rats suggests that inhibin subunits may regulate pituitary and gonadal functions at an early stage in the sexual development of the male rat.

INHIBIN SUBUNIT RNAs ARE PRESENT IN DIVERSE TISSUES

We took advantage of the sensitive S1 nuclease analysis to investigate a series of tissues from mature rats for expression of inhibin α- and β-subunit RNAs (Fig. 4). As we already know, inhibin α-subunit RNA was present in relatively high levels in the mature rat ovary, while the βA and βB-subunit RNAs were about 6- to 10-fold less abundant. Inhibin α-RNA was detected in lower levels in the mature testis, whereas the βA- and βB-subunit RNAs were about 50 and 15 times less abundant than the α-chain. Using this analysis, we found that inhibin α-subunit was expressed in other tissues such as the placenta, pituitary, adrenal, bone marrow, spleen, kidney, thymus, brain and spinal cord. Various levels of βA-subunit RNA were also detected in term placenta, bone marrow, spleen, brain and spinal cord, whereas low levels of βB-subunit RNA were detected in the placenta, pituitary, adrenal gland and brain. It is interesting to note that in the placenta, the βA-subunit RNA

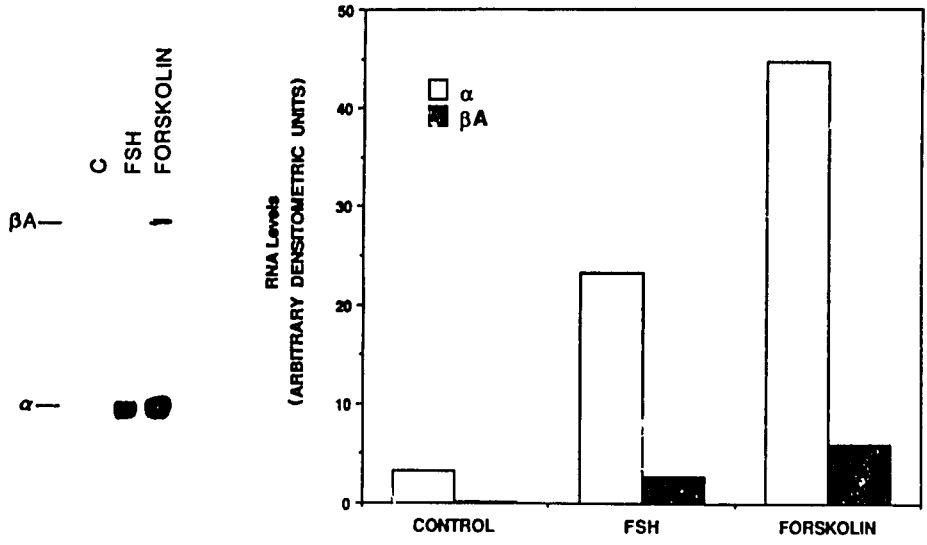


Figure 2. FSH- and forskolin-induced increase in inhibin α- and βA-subunit RNA levels in cultured rat granulosa cells. Granulosa cells were obtained from 26-day-old female rats implanted with DES-containing silastic capsule (at day 21). Total RNA was prepared from cell cultures by the LiCl method, after 24-hour treatment with FSH (30 ng/ml) and forskolin (10 μM). RNA was analyzed and quantitated as described in the legend to Figure 1. On the left is the autoradiogram, and on the right, the RNA levels for the α and βA subunits after densitometric evaluation.

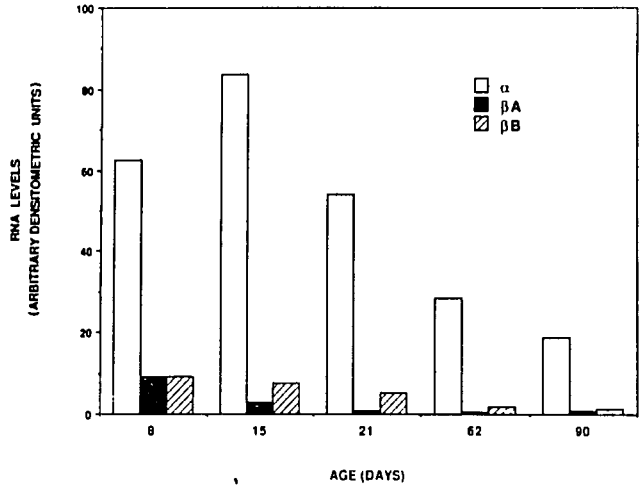


Figure 3. Expression of inhibin subunits in the testis during sexual maturation. Total RNA was prepared from rat testes at the indicated ages, using the guanidine isothiocyanate (GTC) method. Forty micrograms of total RNA from each time point was analyzed and quantitated by densitometry as described in the legend to Figure 1.

is much more abundant than the α- and βB-RNAs. With respect to the absolute RNA content, the βA-subunit RNA is present in larger quantity in term placenta than in the mature ovary. We and others have shown[13,15] that bioactive inhibin is in fact produced by human placenta cells in culture. The interaction between inhibin and other placental hormones is discussed in more details in a separate chapter. Our finding that βA RNA is present in relatively high levels in rat term placenta suggests that in addition to the hormonal action of the inhibin αβ heterodimer, homodimers of the βA subunit may play an active role in the control of growth and developmental functions in this tissue.

Another example of preferential βA-subunit expression is seen in the bone marrow of male rats. As shown in Figure 4, significant levels of βA RNA were detected in the bone marrow, whereas levels of α and βB RNAs remained very low or below the detectable limit. Expression of the βA-subunit in this tissue is supported by the recent work of Eto and

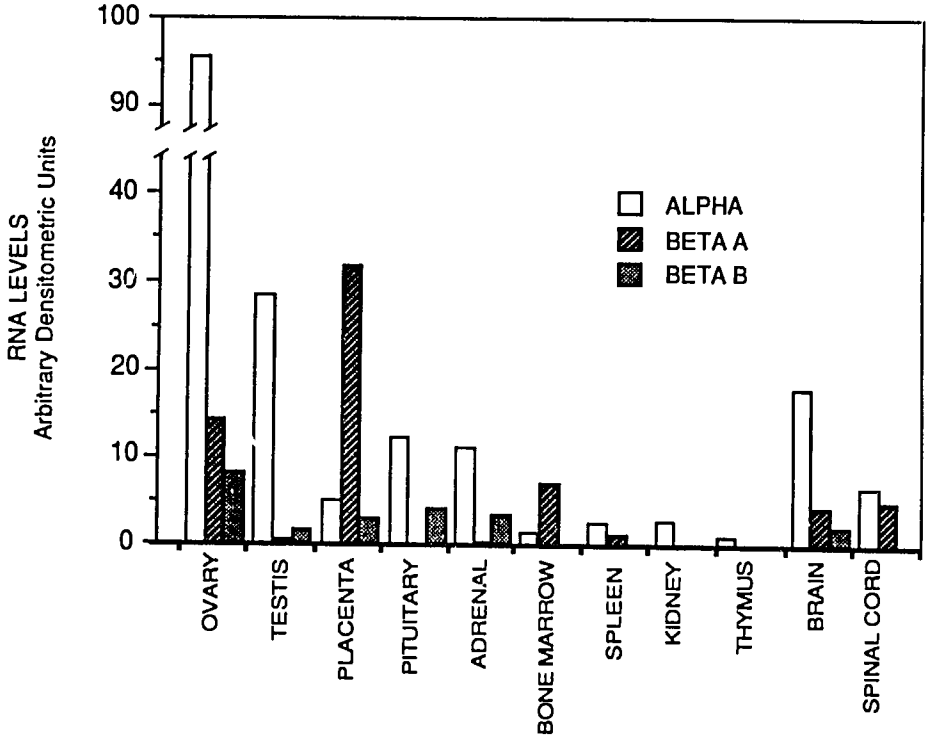


Figure 4. Levels of inhibin α, βA and βB RNAs in various tissues. Total RNA was prepared from mature male and female animals (and analyzed as described in legend to Figure 3). The following tissues from female rats were used: ovary, term placenta, pituitary, adrenal, spleen, kidney, brain, spinal cord, and, mammary gland. The following tissues from male rats were used: testis, bone marrow, thymus, liver and pancreas. Mammary gland, liver and pancreas did not contribute signals above background level and therefore are not included in this figure. These results are representative of at least three experiments.

collaborators[4] who isolated from a human leukemia cell line a factor which stimulates a mouse Friend cell line to differentiate into erythrocytes. Upon partial sequence analysis of the N-terminal, this factor was found to be identical in amino acid composition to the inhibin βA-subunit. In a different context, we have shown that both activin and inhibin modulate in opposite ways the induction of haemoglobin accumulation in a human erythroleukaemic cell line, K562, and the proliferation of erythroid progenitor cells in human bone marrow cultures.[18] Thus, the above findings strongly suggest that healthy bone marrow produces βA homodimer molecules that in turn exert a local effect to promote differentiation of stem cells, an effect which *in vivo* could be antagonized by inhibin either produced locally in low amounts or produced by other tissues such as the gonads.

Another interesting observation made in this study is that inhibin α, βA and βB RNAs are present in relatively low levels in the rat brain. In previous experiments, inhibin α, βA and βB mRNAs have remained undetectable by RNA gel blot analysis in the rat brain. The fact that we are now able to detect these RNAs can be explained by the increased specificity and higher efficiency of hybridizations of cDNA probes using the S1 nuclease method of analysis. Because inhibin α, βA and βB mRNAs are expressed in the brain and in the spinal cord, we speculate that these subunits may exert regulatory functions in the central and peripheral nervous systems. In the same figure we show that the α- and βB-subunit RNAs were also present in the piuitary gland, suggesting the inhibin subunits may exert autocrine and paracrine effects on the pituitary gland. Finally, the presence of inhibin α and βA RNAs in the adrenal gland suggests that inhibin may be involved in some stress-related responses.

Inhibin subunit mRNAs are clearly present in a variety of tissues where they appear to follow very different expression patterns. Whether the relative proportion of each inhibin RNA is indicative of which major $\alpha\beta$ or $\beta\beta$ dimer species is produced in a specific tissue under given physiological conditions still remains to be determined. Nevertheless, based on the above observations, we propose that inhibin subunits may exert hormonal actions in discrete tissues remote from their site of production and exert local effects as growth/differentiation factors.

REFERENCES

1. BICSAK TA, TUCKER EM, CAPPEL S, VAUGHAN J, RIVIER J, HSUEH AJW: Hormonal regulation of granulosa cell inhibin biosynthesis. Endocrinol 119:2711, 1986
2. BICSAK TA, VALE W, VAUGHAN J, TUCKER EM, CAPPEL S, HSUEH AJW: Hormonal regulation of inhibin production by Sertoli cells. Mol Cell Endocrinol 49:211, 1987
3. ESCH FS, SHIMASAKI S, COOKSEY K, MERCADO M, MASON AJ, YING S-Y, UENO N, LING N: Complementary deoxyribonucleic acid (cDNA) cloning and DNA sequence analysis of rat ovarian inhibins. Molec Endocrinol 1:388, 1987
4. ETO Y, TSUJI T, TAKEZAWA M, TAKANO S, YOKOGAWA Y, SHIBAI H: Purification and characterization of erythroid differentiation factor (EDF) isolated from human leukemia cell line THP-1. Biochem Biophys Res Commun 142:1095, 1987
5. FORAGE RG, RING JM, BROWN RW, McINERNEY BV, COBON GS, GREGSON RP, ROBERTSON DM, MORGAN FJ, HEARN MTW, FINDLAY JK, WETTENHALL REH, BURGER HG, deKRESTER DM: Cloning and sequence analysis of cDNA species coding for the two subunits of inhibin from bovine follicular fluid. Proc Natl Acad Sci USA 83:3091, 1986

6. HERMANS WP, vanLEEUWEN ECM, DEBETS MH, deJONG FH: Involvement of inhibin in the regulation of follicle-stimulating hormone concentrations in prepubertal and adult, male and female rats. J Endocrinol 86:79, 1980
7. HSUEH AJW, WANG C, ERICKSON GF: Direct inhibitory effect of gonadotropin-releasing hormone upon follicle-stimulating hormone induction of luteinizing hormone receptor and aromatase activity in rat granulosa cells. Endocrinol 106:1697, 1980
8. HSUEH AJW, DAHL KD, VAUGHAN J, TUCKER EM, RIVIER J, BARDIN W, VALE W: Heterodimers and homodimers of inhibin subunits have different paracrine actions in the modulation of luteinizing hormone-stimulated androgen synthesis. Proc Natl Acad Sci USA 84:5082, 1987
9. LING N, YING S-Y, UENO N, SHIMASAKI S, ESCH F, HOTTA M, GUILLEMIN R: Pituitary FSH is released by a heterodimer of the β-subunits from the two forms of inhibin. Nature 321:779, 1986
10. MASON AJ, HAYFLICK JS, LING N, ESCH F, UENO N, YING S-Y, GUILLEMIN R, NIALL H, Seeburg PH: Complementary DNA sequences of ovarian follicular fluid inhibin show precursor structure and homology with transforming growth factor-β. Nature 318: 659, 1985
11. MASON AJ, NIALL HD, SEEBURG PH: Structure of two human ovarian inhibin. Biochem Biophys Res Commun 135:957, 1986
12. MAYO KE, CERELLI GM, SPIESS J, RIVIER J, ROSENFELD MG, EVANS RM, VALE W: Inhibin A-subunit cDNAs from porcine ovary and human placenta. Proc Natl Acad Sci USA 83:5849, 1986
13. McLACHLAN RRI, HEALY DL, ROBERTSON DM, BURGER HG, deKRESTER DM: The human placenta: A novel source of inhibin. Biochem Biophys Res Commun 140:485, 1986
14. MEUNIER H, RIVIER C, EVANS RM, VALE W: Gonadal and extragonadal expression of inhibin α, βA and βB subunits in various tissues predicts diverse functions. Proc Natl Acad Sci USA 85:247, 1988
15. PETRAGLIA F, SAWCHENKO P, LIM ATW, RIVIER J, VALE W: Localization, secretion and action of inhibin in human placenta. Science 237:187, 1987
16. VALE W, RIVIER J, VAUGHAN J, McCLINTOCK R, CORRIGAN A, WOO W, KARR D, SPIESS J: Purification and characterization of an FSH-releasing protein from porcine ovarian follicular fluid. Nature 321:776, 1986
17. WOODRUFF TK, MEUNIER H, JONES PBC, HSUEH AJW, MAYO KE: Rat inhibin: Molecular cloning of α- and β-subunit complementary deoxyribonucleic acids and expression in the ovary. Molec Endocrinol 1:561, 1987
18. YU J, SHAO L, LEMAS V, YU AL, VAUGHAN J, RIVIER J, VALE W: Importance of FSH-releasing protein and inhibin in erythrodifferentiation Nature 330:765, 1987

DISCUSSION:

CHAIRPERSON: ALAN CORBIN, Ph.D.

DR. HUTCHISON: Have you done autoradiographic studies to see whether a functional corpus luteum expresses inhibin α-subunit? You mentioned that a fully degenerated corpus luteum did not.

DR. MEUNIER: We have looked at inhibin α-subunit expression by *in situ* hybridization in old and fresh corpora lutea. In degenerated corpora lutea, silver grains were sparse and did not contributed to a significantly increased signal. A slightly larger number of silver grains were found in fresh (30 hour) corpora lutea. No specific hybridization could be detected in 50 hour, or older, corpora lutea.

DR. HUTCHISON: Are the granulosa cells luteinized in your culture? So autoradiographical *in vitro* luteinized granulosa cells are expressing α subunit?

DR. MEUNIER: The granulosa cells we used in our studies were treated immediately after being put in culture, and harvested within 48 hours. They are not, therefore, luteinized cells.

DR. BARDIN: What is your speculation on why there is so much excess α both basally and FSH-stimulated? Everywhere you look with the S1 nuclease you show a tremendous differential in excess of α.

DR. MEUNIER: We generally observed α-subunit RNA in excess; however, it is not the rule. All I can do at this time is speculate. One possible explanation is that the β-subunit for the α is lower. In this case, an excess of α could dictate formation of the α- and β-subunit dimers; whereas, when the α-subunit is absent or produced in low levels, the β-β dimer association might be favored.

Another possibility is that the β-subunit RNAs are less stable than the α-subunit RNAs leading to their rapid degradation.

DR. BARDIN: That was going to be my next question. How do you determine the stability of S1 nuclease of the β-subunit?

DR. MEUNIER: Well, in fact, the reason we decided to use the S1 nuclease analysis is that by the conventional technique, that is the RNA gel blot analysis, we found it very difficult to quantitate levels of β-subunit RNAs. On RNA gel blots, the β-subunit mRNA appeared to be degraded, although we know from studies with the α-subunit that the RNA was in excellent condition. Perhaps, the β-subunit mRNAs are not as stable as the α, or are processed rapidly.

The S1 nuclease analysis allows us to look at a short but very specific portion of the inhibin messages. Thus we can quantitate the RNA levels of this fragment of about 30 bp at a given time, with a considerable decrease in background.

DR. PAULSEN: Did your *in vitro* studies on the effect of diethylstilbestrol demonstrate a direct or indirect effect?

DR. MEUNIER: The results I have shown describing the stimulatory effect of diethylstilbestrol (DES) on inhibin expression were observed 72 hours after the implantation of a DES releasing capsule in intact immature female rats. We are now pursuing further studies, using hypophysectomized animals, to determine whether we are seeing a direct or indirect effect of estrogens on ovarian inhibin expression.

DR. PAULSEN: Since it is well known that diethylstilbestrol stimulates granulosa cells or proliferation in the hypophysectomized immature female rat, I was not clear if your inhibin α expression units were per cell. What was your denominator in your granulosa cell culture?

DR. MEUNIER: The effect of diethylstilbestrol on ovarian inhibin expression was studied using the intact immature rat as a model. Total RNA was extracted from ovaries of treated

and untreated animals, and later normalized to a fixed concentration before S1 nuclease analysis. The denominator is a constant amount of total RNA from each sample, in this case 2.5 μg of total RNA, and the variable is the specific levels of inhibin subunits expressed under the conditions described.

DR. DONAHOE: Could you describe the probe that you have used for your *in situ* hybridization, and how long it took you to develop it?

DR. MEUNIER: The probe we used for in situ hybridization to inhibin α-subunit mRNA was relatively short, approximately 300 nucleotides long. This probe was derived from the C-terminal portion of a full length cDNA encoding the rat α precursor molecule.

DR. DONAHOE: How long did it take you to develop your films?

DR. MEUNIER: Emulsion-coated slides were exposed for four days and then developed. Some information could be derived after 3 to 5-day exposures using Kodak XAR-5 autoradiograms. Our studies were conducted using 35-S probes. A shorter exposure time is generally required for probes labelled with 32-P.

QUESTION: What units did you use to measure RNA levels?

DR. MEUNIER: With regard to the question about the units, the units representing the levels of RNA in various tissues are 'arbitrary densitometric units'. We have chosen to express it in this way because the integration units obtained after scanning vary according to the exposure time, although the distribution pattern remains the same. RNA concentration was calculated for each sample by taking two different optical density readings of serial dilutions. RNA was normalized to a fixed concentration, for example 2.5μg, and submitted to S1 nuclease analysis.

5

Structure and Function of Inhibin and Its Related Peptides

KAORU MIYAMOTO
YOSHIHISA HASEGAWA
MASAKI FUKUDA
MASAO IGARASHI

Since 1932 when the existence of inhibin in mammalian gonads was first postulated, various attempts to identify the hormone had been made by a number of researchers. Recently, four groups including ourselves were successful at isolating and characterizing the hormone from porcine and bovine follicular fluids.[5,9,14,19,20] Inhibin forms isolated so far were 32 KDa and 56 KDa molecules, both comprised of dimeric subunits held together by disulfide bridges. Two types of 32 KDa inhibin were also isolated from porcine follicular fluid and each shown to be a dimer composed of a common α-chain (20 K α-subunit) and one of two distinct, but highly homologous β-chains (14 K β A and β B subunits). A 56 KDa inhibin isolated from bovine follicular fluid was composed of a 44 K α-subunit and 14 K β A subunit. Furthermore a homodimer of β A subunit and a heterodimer of βA and β B subunits have been isolated from porcine follicular fluid preparations. Such β-dimers have been reported to possess FSH-releasing activity *in vitro*.[10,25] cDNA structures of α, β A, and β B subunits have been determined.[4,11,12,13] The DNA sequencing showed that all subunits are encoded in different mRNAs and are synthesized as large precursor molecules and each precursor contains several potential proteolytic processing sites (*i.e.* clusters of two or more basic residues). Possibly proteolytic processing at different sites in combination with various disulfide bridge formations may cause a diversity of inhibin-related molecules composed of such subunits. Actually we have reported the existence of several inhibin forms of different molecular weight in both porcine and bovine follicular fluids.[14,15]

In the first part of this chapter, we will describe the characterization of several forms of inhibin in bovine follicular fluid by using monoclonal antibodies specific to each α- and β- subunit.

Although the chemical structures of inhibin and its related peptides have been well characterized, little is known about their action mechanisms. Now opposite actions of inhibin and β-dimers have been reported regarding FSH secretion[10,25] as well as androgen production from Leydig cells or theca cells[7] *in vitro*. Both inhibin and β-dimers seem to act on the pituitary to preferentially alter the FSH secretion. It is well known, however, that most gonadotropes in the rat pituitary have secretory granules of both LH and FSH in the same cells. Therefore, it is desirable to show an appropriate model that can explain how such peptides control FSH secretion without altering LH secretion. In the latter part

of this chapter, the action mechanism of inhibin on rat pituitary cells is examined by comparing it with that of cycloheximide. A possible model for LH and FSH secretion from the gonadotrope will be postulated. In addition, it is notable that concentrations of β-dimers in the follicular fluid seem quite low as compared with that of inhibin. This raises a critical question as to whether the β-dimers are actually working on the pituitary to regulate FSH secretion *in vivo*. For *in vivo* study, a fairly large quantity of the hormones must be prepared. An immunoaffinity chromatography using a monoclonal antibody to bovine 32 KDa inhibin, that is used for the characterization of multiple inhibin forms, enables one to isolate bovine inhibin on a large scale. Regarding β-dimers, on the other hand, Etoh *et al.*[3] reported that a tumor cell line derived from human leucocyte is producing β A-homodimer. The authors were also successful at producing recombinant β A-dimer on a large scale. In this report, effects of inhibin and β A-dimer on gonadotropin secretion *in vivo* by using these substances will also be described.

CHARACTERIZATION OF MULTIPLE INHIBIN FORMS IN BOVINE FOLLICULAR FLUID

Gel chromatographic separation of bovine follicular fluid revealed that inhibin activity was found in fractions ranging from more than 100 KDa to 30 KDa. The smallest inhibin form, namely 32 KDa inhibin, was purified to homogeneity as listed in Table 1. The 32 KDa inhibin thus purified is composed of 20 K α-subunit and 13K β-subunit that are linked by disulfide bridges. N-terminal sequencing of the two subunit components revealed that the isolated hormone is composed of α and βA chains.

Monoclonal antibodies specific to the α subunit and the β subunit, respectively, have been prepared by using the purified 32 KDa bovine inhibin as an antigen. As shown in Figure 1, an immunoblotting analysis revealed that one of the antibodies named 256H specifically recognizes the α-subunit while another antibody, 44H, recognizes the β-subunit. Both the antibodies recognize the intact 32 KDa inhibin as well.

Table 1. Purification of Inhibin from 1L bovine follicular fluid

	protein (mg)	ED_{50}(ng/ml)	total units (10^3)	purification factor	over-all recovery (%)
bovine follicular fluid	69570	6400	10870	1	100
Matrex Gel Red-A	10352	1050	9859	6.09	91
Phenyl Sepharose	2967	320	9273	20.0	85
Sephacryl S-200					
fr.25-28 (80K)	2428	1250	1942	5.36	17.8
fr.29-32 (55K)	420	140	3005	47.8	27.6
fr.36-40 (32K)	113.6	42	2704	159	24.8
DEAE Sepharose CL6B (32K fraction)					
fr.45-52	6.8	4.5	1510	1422	13.9
fr.56-59	2.0	13	150	492	1.4
RP-HPLC (32K-DEAE fr.45-52)	1.42	1.2	1183	5330	10.8

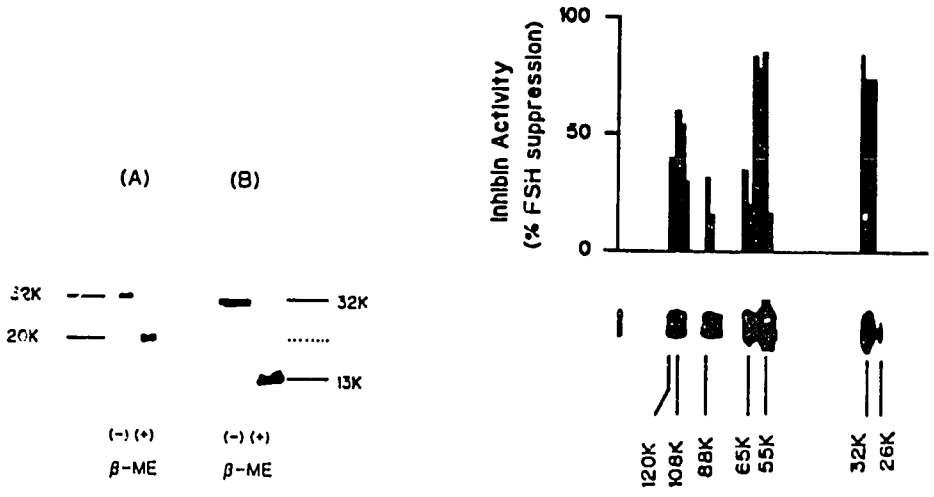


Figure 1. SDS-PAGE of bFF 32 KDa inhibin immunostained by monoclonal antibodies (left). Proteins were transferred to a nitrocellulose membrane and immunostained by (A) 256H and (B) 44H.

SDS-PAGE of an inhibin preparation obtained after the immunoaffinity chromatography (right). Proteins were immunostained by 256H. Inhibin activity of the respective bands is indicated by black bars.[15]

The antibody 256H is also used as a ligand for an immunoaffinity chromatography. Bovine follicular fluid could directly be applied on an immunoaffinity column and inhibin activity could be recovered by eluting the column with an acidic buffer. Under ideal conditions, as shown in Figure 2, almost all the inhibin activity (approximately 98-99%) in bovine follicular fluid bound to the resin coupled with the α-subunit specific antibody. Practically no inhibin or FSH-releasing activity remained in the flow through fraction. This suggests that inhibin activity in bovine follicular fluid can almost entirely be accounted for by molecules that have a common immunoreactive site for the α-subunit. It is also conceivable that FSH-releasing activity, namely activin, in bovine follicular fluid may be very low, since β-dimers must be found in the flow through fraction after the present immunoaffinity chromatography. Alternatively, activity of the β-dimers may possibly be hidden by β-dimer binding proteins in the follicular fluid, or some other proteins having inhibin-like activity in the flow through fraction may cancel the β-dimer's activity. Ueno *et al.*[24] recently reported the presence of a protein having FSH-suppressing activity distinct from inhibin in porcine follicular fluid, named follistatin. Further studies have to be done to quantify the amount of β-dimers and follistatin in the follicular fluid.

Because almost all the inhibin activity could be recovered by the immunoaffinity chromatography, diversity of inhibin molecules in bovine follicular fluid was examined by using the inhibin preparation obtained from the immunoaffinity chromatography. The inhibin preparation absorbed on and eluted from the column was then separated on a SDS-

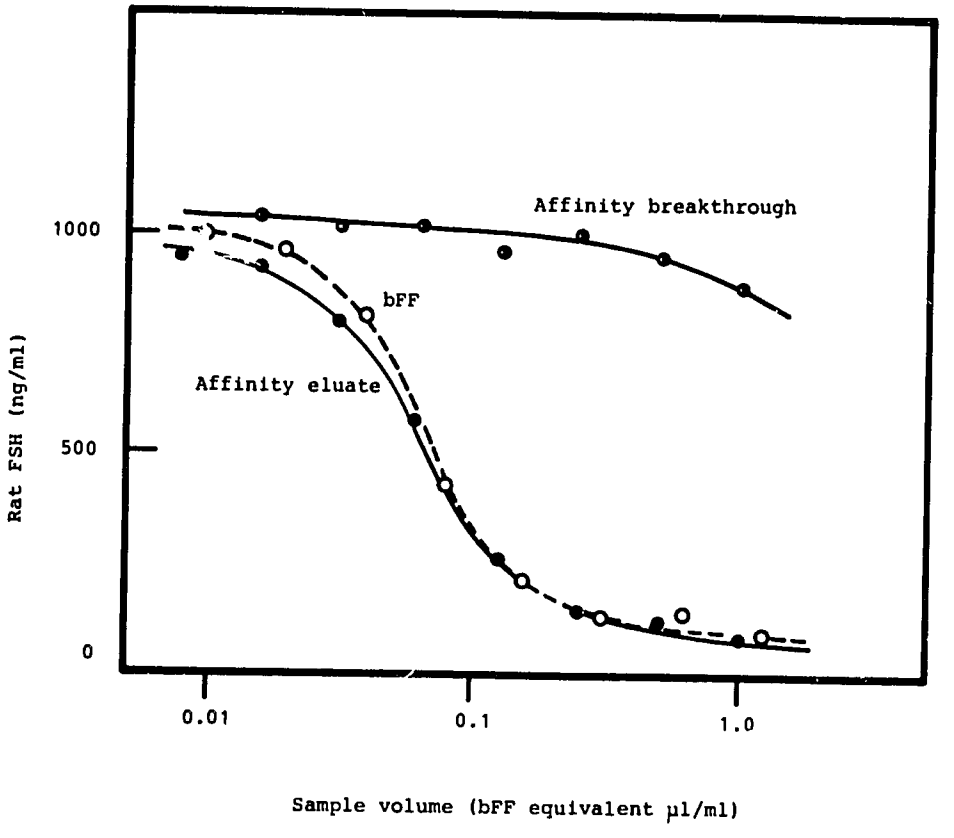


Figure 2. Inhibin activity of fractions obtained after the immunoaffinity chromatography using α-chain specific antibody 256H. (-O-) bovine follicular fluid before applying to the immunoaffinity column. (-◐-) flow through fraction from the immunoaffinity column. (-●-) fraction absorbed on the column. Volumes of the fractions used for the bioassay were corrected so that the activity is quantitatively compared to each other. Volumes are expressed as nl equivalent to bFF.

PAGE under non-reducing conditions. The gel was subjected to an immunoblotting procedure using antibody 256H. At the same time, gel slices were extracted and the extracts were subjected to an *in vitro* inhibin bioassay using rat anterior pituitary cell culture system. As shown in Figure 1, biological activity of inhibin was found in several regions on the SDS-PAGE. Figure 1 also shows that at least seven immunoreactive bands were stained by the antibody 256H. Amongst them the band corresponding to 26 KDa seems to have no biological activity, while six other bands seem to have an intrinsic inhibin activity. However, those high molecular weight inhibin forms might not have inhibin activity, since those forms are possibly processed to 32 KDa form, that is really active, during the assay of such large inhibin forms. Further studies should be done to clarify the problem.

These apparently bioactive and immunoreactive proteins were further analyzed by two dimensional SDS-PAGEs. Multiple inhibin forms were first separated as above on a gel under non-reducing conditions. The gel was then incubated in a solution containing 2% β-mercaptoethanol for 1 hour at 37°C. After the incubation, the reduced gel strip was placed onto a 10% SDS-slab gel and then electrophoresed to the second direction. Figure 3 shows immunoreactive spots when stained with both or either of the antibodies 256H and 44H. By a pattern stained with both of the antibodies, it is evident that 65 KDa, 55 KDa, and 32 KDa inhibins are composed of two polypeptide subunits; *i.e.* 20 K α-subunit and 13 K β-subunit for 32 KDa inhibin, 44K α-subunit and 13 K β-subunit for 55 KDa inhibin and 57 K α-subunit and 13 K β-subunit for 65 KDa inhibin, respectively. On the other hand,

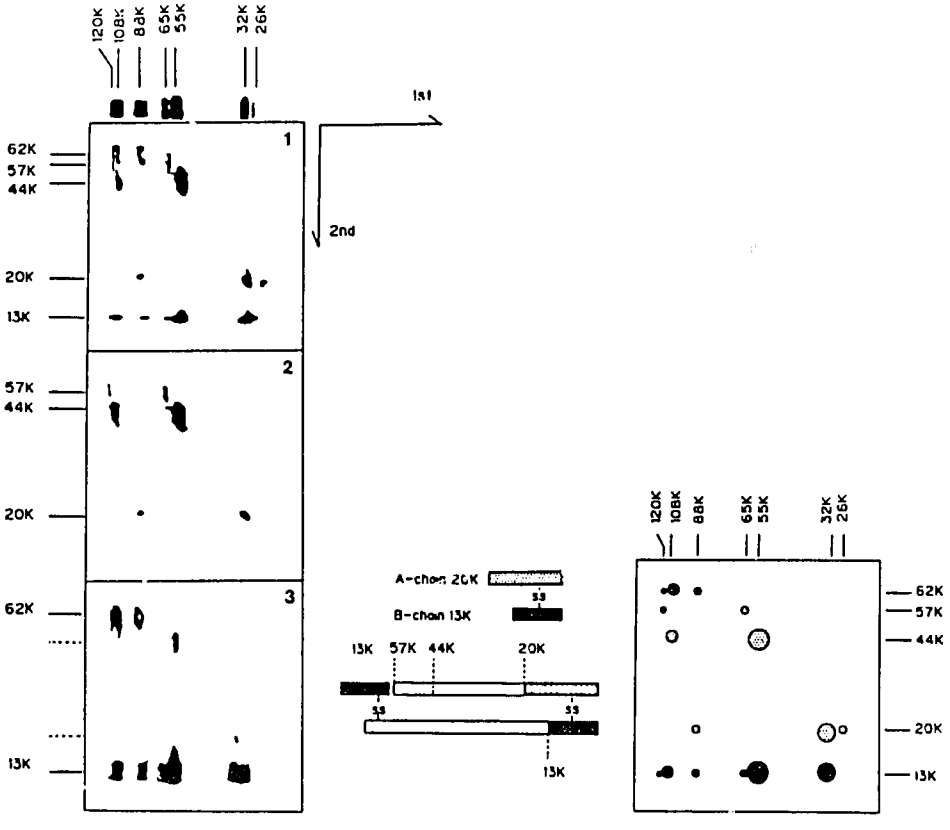


Figure 3. (left) Two-dimensional SDS-PAGEs of the inhibin preparation from the immunoaffinity chromatography. Polypeptides were transferred to nitrocellulose membranes and immunostained with 1) both 256H and 44H, 2) 256H alone, and 3) 44H alone.

(right) Schematical drawing of the immunoreactive spots on the two dimensional SDS-PAGEs. Black circles are polypeptide spots related to β-subunit and dotted circles indicate polypeptides related to α-subunit. Hypothetical arrangement of the subunits for the largest inhibin form, 120 KDa inhibin, is also shown.[15]

inhibin forms of higher molecular weights, 120 KDa, 108 KDa, and 88 KDa, inhibins, are composed of three polypeptide subunits, *i.e.*, 88 KDa inhibin made up of 62 K β-subunit, 20 K α-subunit and 13 K β-subunit, and 108 KDa inhibin of 62 K β-subunit, 44 α-subunit and 13 K β-subunit, and 120 KDa inhibin of 62 K β-subunit, 57 K α-subunit and 13 K β-subunit, respectively.

A summary of the results follows: 1) Three inhibin forms of smaller molecular weights are made up of two subunits. The 57 K, 44 K, and 20 K polypeptides are probably corresponding to molecules that are differently processed from the α-subunit precursor. 2) Three inhibin forms of higher molecular weights are composed of three polypeptide subunits. In these inhibins, 62 K β-subunit precursor is linked to the smaller inhibin forms, 65 KDa, 55 KDa, and 32 KDa inhibins, to form 120 KDa, 108, KDa, and 88 KDa inhibins, respectively. The 62 K polypeptide is possibly a precursor of β-subunit.

Based on the above findings, a possible structure of inhibin can be drawn as shown in Figure 3, for the largest form of inhibin. On the hypothetical inhibin structure, we assume that all inhibin forms are derived from the largest form. The organization of inhibin subunits in follicles seems so complicated that several inhibin forms are simultaneously present in the follicular fluid. In addition, β-dimers could not be produced by the process proposed here. Therefore, a process for the formation of β-dimers may be different from that of inhibin. Isolation and characterization of such high molecular weight inhibin forms is essential to verify the organization of inhibin molecules.

ACTION MECHANISM OF INHIBIN

Action mechanism of inhibin on the pituitary was examined *in vitro* by using purified pFF 32 KDa inhibin and a potent inhibitor of protein biosynthesis, cycloheximide. To examine effects of inhibin or cycloheximide on the basal secretion of FSH and LH, the following experimental procedure was used. Dispersed pituitary cells were prepared by the method described previously[9] and 100 μl aliquots of the cell suspensions were placed into each well of 96-well plates. PFF 32 KDa inhibin or cycloheximide in 100 μl medium over a wide dose range was then added to each well immediately after cell-plating. At the end of 72 hour incubation, 100 μl and 50 μl aliquots of the resulting incubation media were subjected to RIA for FSH and LH, respectively, to estimate the basal secretion.

For the determination of cell contents of gonadotropins, cells were lysed by the addition of 200 μl of 1% BSA containing 0.2% Triton X-100 to the cultures described above after removal of the incubation media. The cell lysates were then assayed by RIA for LH and FSH, respectively.

As shown in Figure 4, during the first three days of incubation, purified pFF 32 KDa inhibin suppressed basal FSH secretion but did not suppress basal LH secretion. At the same time, the amount of FSH in the cells (cell contents) was also reduced by the inhibin treatment, whereas the amount of LH in the cells was not affected. It is evident, therefore, that the total amount of FSH was severely suppressed by the inhibin treatment, indicating that inhibin acts to suppress biosynthesis of FSH. On the other hand biosynthesis of LH does not seem to be affected by inhibin. This is consistent with earlier observations reported by several investigators,[1,2,22] though they used crude inhibin preparations for their experiments. To verify such reaction characteristics of inhibin, the effects of protein biosyn-

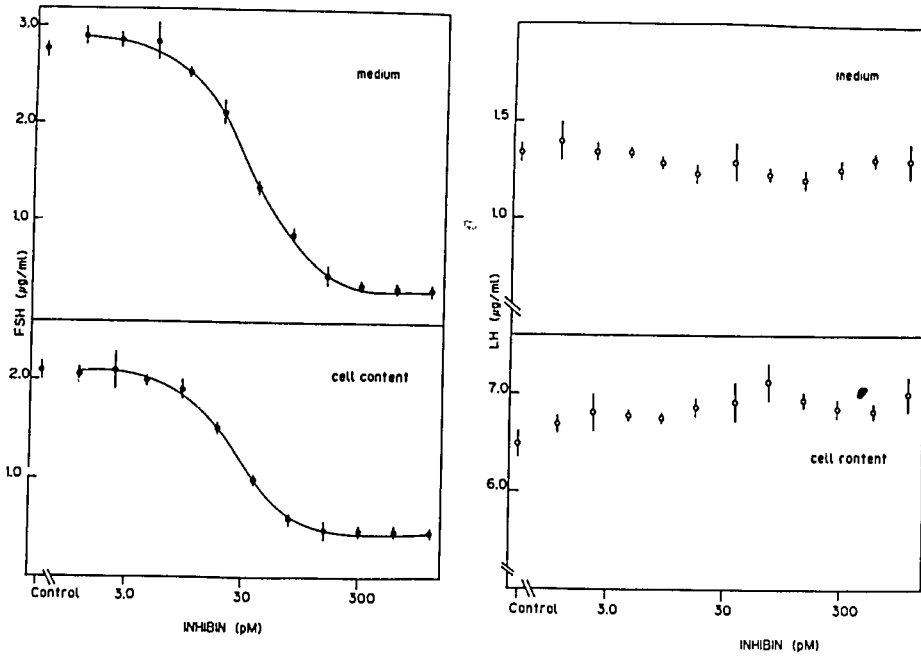


Figure 4. Effects of inhibin on basal FSH and LH secretion and on FSH and LH intracellular contents. The values are expressed as mean ± SD of quadruplicate determinations.[6]

thesis inhibitor, cycloheximide, on the basal gonadotropin secretion were next examined. Surprisingly, quite similar to inhibin, cycloheximide suppressed only FSH but not LH regarding the basal secretion and cell contents as shown in Figure 5. This indicates that suppression of FSH is likely to be a consequence of the reduction of intracellular storage of the hormone. On the other hand, neither inhibin nor cycloheximide could affect biosynthesis of LH, since total amount of LH was not reduced by such treatments during the culture period. It should be noted that a general inhibitor of protein biosynthesis selectively suppressed FSH secretion from the pituitary cells in appearance.

On the other hand, the LHRH-stimulated release of both FSH and LH could be suppressed by the treatment with purified pFF 32 KDa inhibin as shown in Figure 6, supporting previous observations in which crude inhibin preparations were used.[1,22] Because the crude inhibin preparation had suppressed LHRH-stimulated release of LH as well as FSH, it has been argued whether or not the suppression of LHRH-stimulated release of LH is due to another active substance(s) distinct from inhibin in these crude preparations. Our present observation clearly indicates that the suppression of LHRH stimulated release of LH is one of the intrinsic actions of inhibin on the pituitary cells. As shown in Figure 7, it should be emphasized again that cycloheximide also mimicked the inhibin actions on both FSH and LH under LHRH-stimulated conditions.

With regard to FSH secretion, since the total amount of FSH is severely reduced by the pretreatment with inhibin or cycloheximide during the preculture period, the amplitude

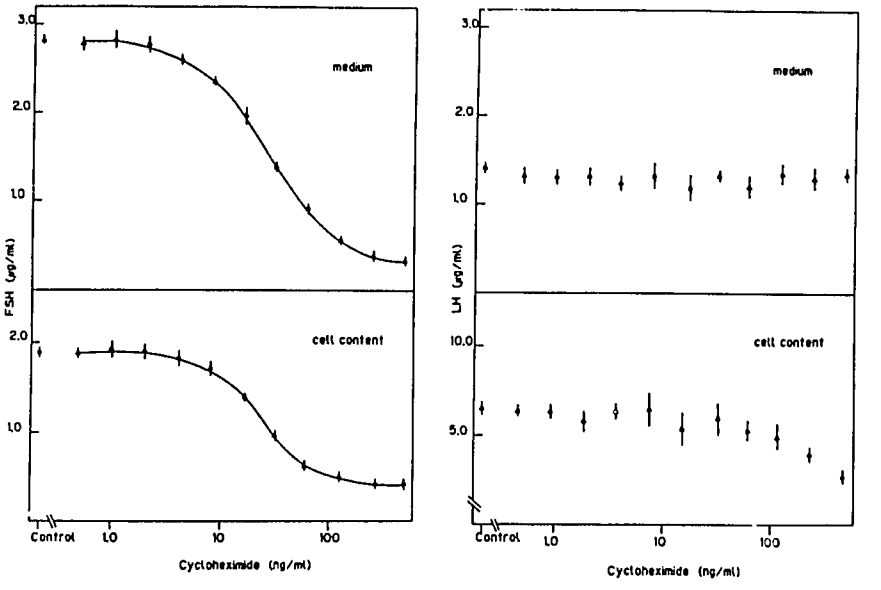


Figure 5. Effects of cycloheximide on basal FSH and LH secretion and on FSH and LH intracellular contents. The values are expressed as mean ± SD of quadruplicate determinations.[6]

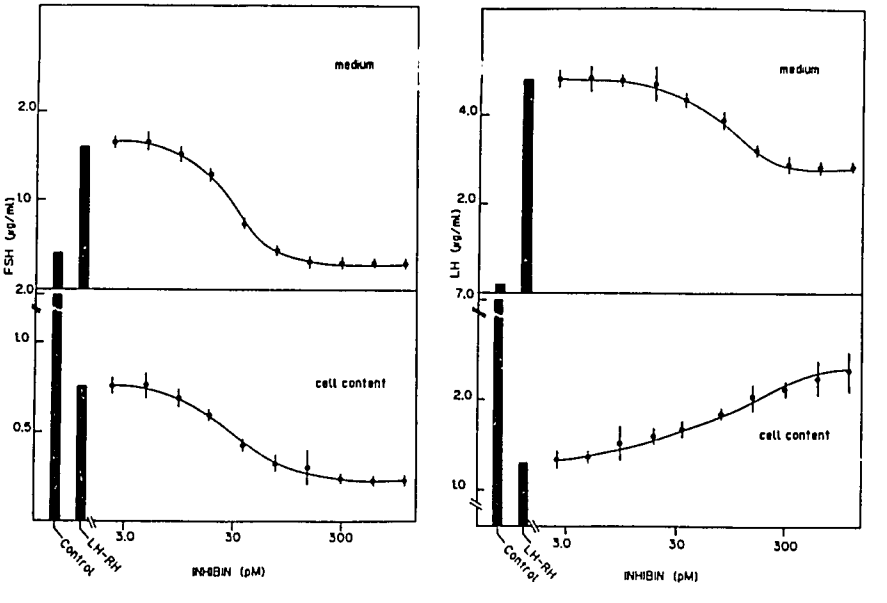


Figure 6. The response of FSH and LH secretion by pituitary cells in culture to a 6 hr incubation with 3.0 ng/ml of LHRH and increasing concentrations of inhibin. The cells had been preincubated for 72 hr in identical concentrations of inhibin. The FSH and LH intracellular contents after the 6 hr incubation are also shown. The values are expressed as the mean ± SD of quadruplicate determinations.[6]

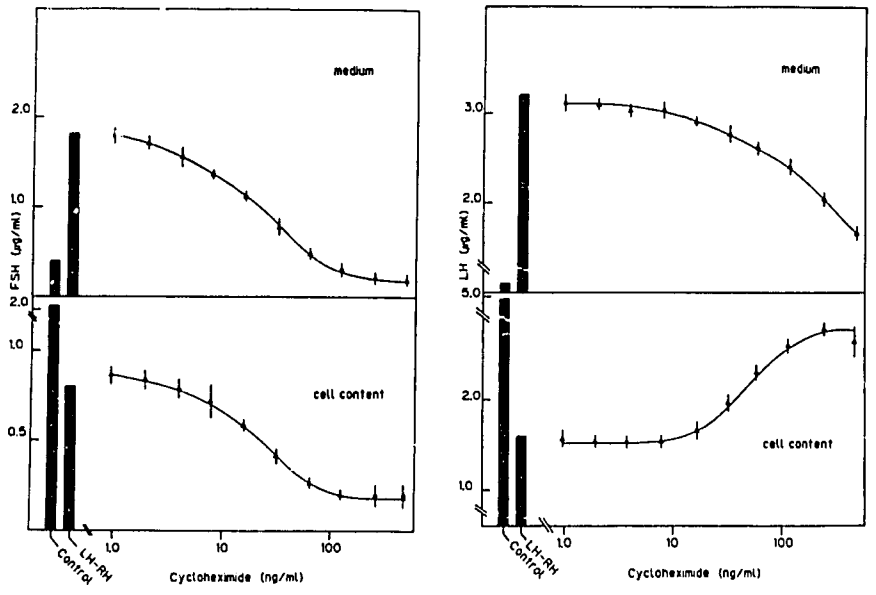


Figure 7. The response of FSH and LH secretion by pituitary cells in culture to a 6 hr incubation with 3.0 ng/ml of LHRH and increasing concentrations of cycloheximide. The cells had been precultured for 72 hr in identical concentrations of cycloheximide. The FSH and LH intracellular contents after the 6 hr incubation are also shown. The values are expressed as the mean ± SD of quadruplicate determinations.[6]

of the LHRH-stimulated release of FSH must be lowered because of depression of the hormone. On the other hand, in sharp contrast to FSH, neither cycloheximide nor inhibin could alter the total amount of LH as clearly shown in Figures 6 and 7. Therefore, it seems unlikely that direct inhibition of LH biosynthesis by inhibin or cycloheximide is a major factor causing the suppression of the LHRH-stimulated release of LH. Rather it is likely that inhibin acts on the cellular machinery through which LHRH exerts the action on LH secretion, because the increment of cellular LH concentrations was observed along with the suppression of the LH release into the medium. As in the case of the cycloheximide treatment, suppression of biosynthesis of certain cellular components may account for such a suppression of LHRH-stimulated release of LH.

As shown in Figure 8, although a calcium ionophore, A23187, and a cyclic AMP analog, dibutyl cyclic AMP and a stimulator of adenyl cyclase, cholera toxin, are all effective in stimulating the secretion of LH and FSH from the pituitary cells, only TPA (PMA), a potent protein kinase C activator, could stimulate LH or FSH secretion to the same extent as LHRH in our culture system during the 6 hour incubation. It is well known that TPA exerts the action through activating protein kinase C in the cells.[17,18,23] Inhibin also suppressed the TPA-stimulated release of both FSH and LH in a dose-dependent manner, indicating that inhibin exerts the action, at least in part, by acting on the C-kinase system in the gonadotrope.

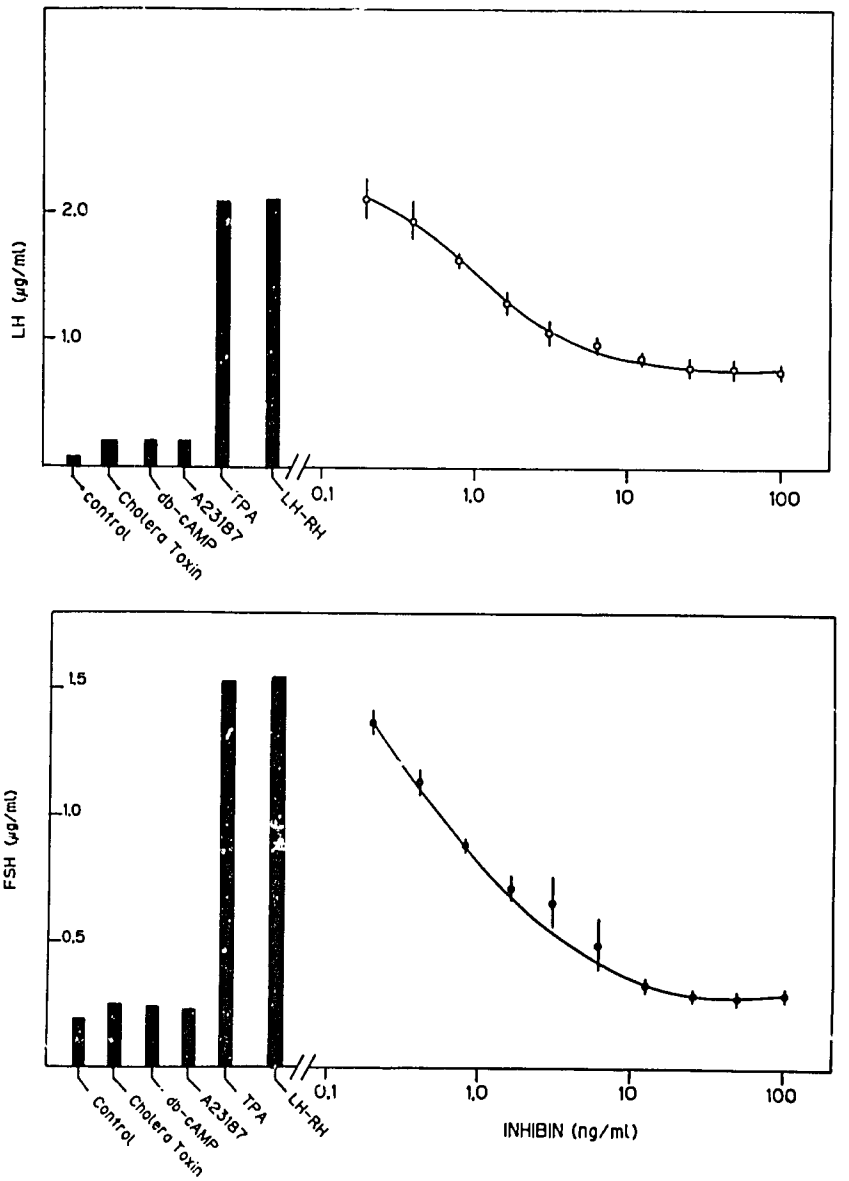


Figure 8. The response of FSH and LH secretion by pituitary cells in culture to a 6 hr incubation with 10 nM of TPA and increasing concentrations of inhibin. The cells had been preincubated for 72 hr in identical concentrations of inhibin. The effects of cholera toxin (10 μg/ml), dibutyl cyclic AMP (1.0 mM) and A23187 (1.0 mM) on FSH and LH secretion are also shown. The values are expressed as the mean ± SD of quadruplicate determinations.[6]

Consequently, the following conclusions can be drawn from these experiments:

1) The purified 32 KDa inhibin suppresses FSH but not LH with regard to the basal secretion. However, inhibin actually suppresses both FSH and LH in the LHRH-stimulated release of FSH and LH.
2) Cycloheximide mimics all the inhibin actions on the gonadotropin secretion, suggesting that the difference between LH and FSH with regard to the response to inhibin or cycloheximide may arise from the marked difference between these hormones in susceptibility to biosynthetic inhibition.

A MODEL FOR LH AND FSH SECRETION FROM PITUITARY GLAND

Accumulating evidence indicates that discrete changes in serum FSH and LH levels occur frequently under certain physiological and experimental conditions.[21] However, there is also considerable evidence which indicates that both LH and FSH are produced by the same gonadotropes with minor exceptions.[8,16] It is also well known that LHRH is much more effective in releasing LH than FSH. Yet, inhibin favors acting on FSH rather than on LH. Based on the *in vitro* experiments described above, a working hypothesis for the regulation of gonadotropin secretion from pituitary cells is schematically drawn in Figure 9, which may afford some explanation for such discrete patterns of LH and FSH secretion from the same cells. As previously discussed, LH is much less sensitive to biosynthetic inhibition than FSH. It is also likely that most of the synthesized LH is stored in the secretory granules and is not able to be released without stimulation by LHRH, because the amount of LH in the cells is much greater than that released into the medium under non-stimulated

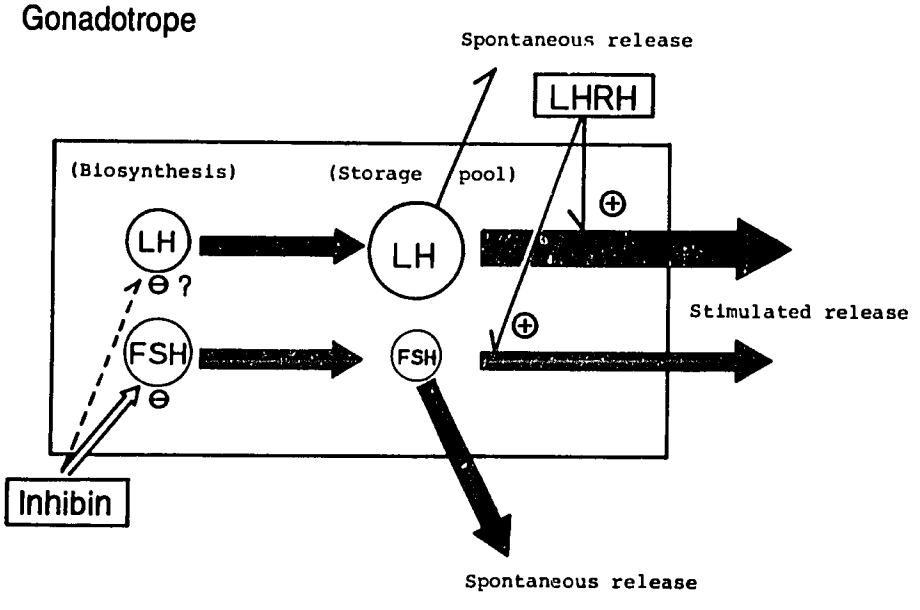


Figure 9. A model for LH and FSH secretion from gonadotrope.

conditions. On the other hand, it is likely that FSH is more sensitive to biosynthetic inhibition and a considerable part of the synthesized FSH is easily released into the medium without any stimulation. This is supported by the fact that the amount of FSH released into the medium exceeds that in the cells within a 72 hour incubation period under non-stimulated conditions.

By such a model as Figure 9, factors that promote or attenuate biosynthetic rates of proteins in gonadotropes appear to act preferentially on FSH. This is supported by the fact that a general protein biosynthesis inhibitor acts specifically on FSH regarding gonadotropin secretion. On the other hand, factors that stimulate release of hormones from their secretory granules, for example LHRH or potassium ion, much more effectively release LH than FSH. In fact, FSH secretion is stimulated to only 2 to 3 times that of the basal levels even with the maximal doses of LHRH. This is probably because the basal levels of FSH are relatively high and the amount of stored FSH is relatively small. LH secretion, on the other hand, can be stimulated to even 20 to 30 times that of the basal levels, since the basal LH levels are quite low compared to the amount of LH stored in the cellular pool.

Although our hypothetical model is too simple to explain complicated patterns of gonadotropin secretions, it is likely that differences in biosynthetic and storage characteristics between LH and FSH themselves play an important role in the discrete changes in the serum concentrations of LH and FSH observed under certain physiological and experimental conditions.

ACTIONS OF βA-DIMER ON GONADOTROPIN SECRETION

As expected from the discussion above, a factor that stimulates preferentially the release of FSH in appearance must be promoting FSH biosynthesis. Actually, as shown in Figure 10, the β A-dimer activin A absolutely stimulated FSH biosynthesis during the 72 hour incubation period. The FSH releasing activity of β-dimers may be a consequence of a function of the peptides in the promotion of biosynthesis of FSH (and possibly other proteins in gonadotropes). The concept is supported by the fact that β-dimers lack acute effects on the gonadotropin secretion. However, one should be very careful in using the *in vitro* FSH releasing assay, because anything that has an ability to alter biosynthetic rates of proteins in gonadotrope may possibly be a specific FSH regulator in appearance in the *in vitro* system. For example, we have found strong FSH-releasing activity in human cord serum in our *in vitro* bioassay. However, the active substance in human cord serum turned out to be progesterone. Actually, synthetic progesterone has strong FSH releasing activity in our bioassay. No one believes progesterone is one of FSH releasing substances, because it has no FSH releasing activity *in vivo*. Likewise, we believe that FSH releasing activity of β A-dimer, that is clearly observed *in vitro*, should be identified *in vivo*. Fortunately, milligram orders of recombinant β A-dimer became available and an *in vivo* experiment was carried out to test the substance. As shown in Figure 11, a single injection of 50 μg per rat of β A-dimer could not increase serum FSH levels in adult male rats. The lower part of the Figure 11 shows effects of purified bovine inhibin at a dose of 10 μg per rat on the suppression of serum FSH levels in castrated male rats. Inhibin actually suppressed serum FSH levels significantly *in vivo*, indicating clearly that inhibin is a specific inhibitory regulator of FSH.

It should also be pointed out that the amount of β A-dimer in bovine follicular fluid seems quite low as compared with that of inhibin. Recent work regarding local actions of β-dimers revealed that they have modulatory effects on LH-stimulated androgen reproduction by theca cells or Leydig cells. Beta-dimers also are reported to attenuate cytodifferentiation

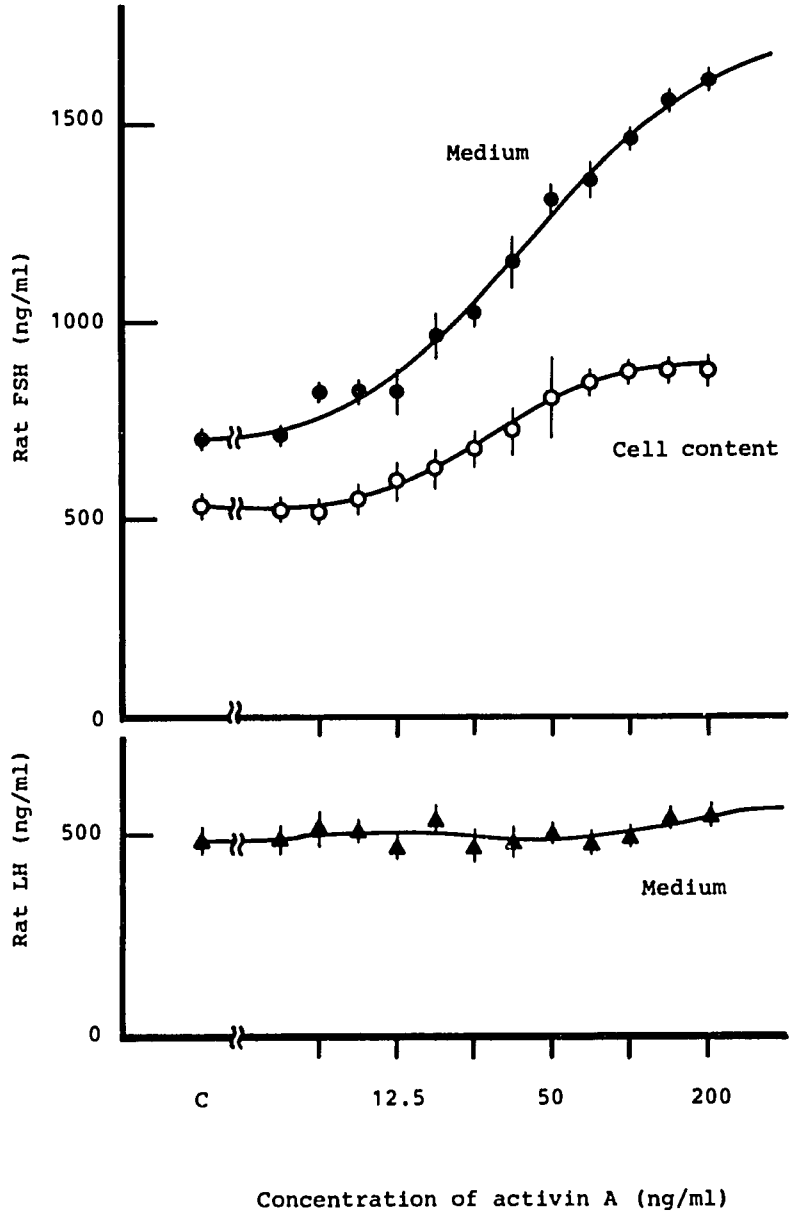


Figure 10. Effects of β A-dimer on basal FSH and LH secretion and on the FSH intracellular contents *in vitro*.

of granulosa cells induced by FSH. Functions of the β-dimers *in vivo* might be their local actions rather than the FSH-releasing activity.

Further studies *in vivo* have to be done to clarify the real function of β-dimers. Production of recombinant inhibin related peptides may be beneficial to such studies.

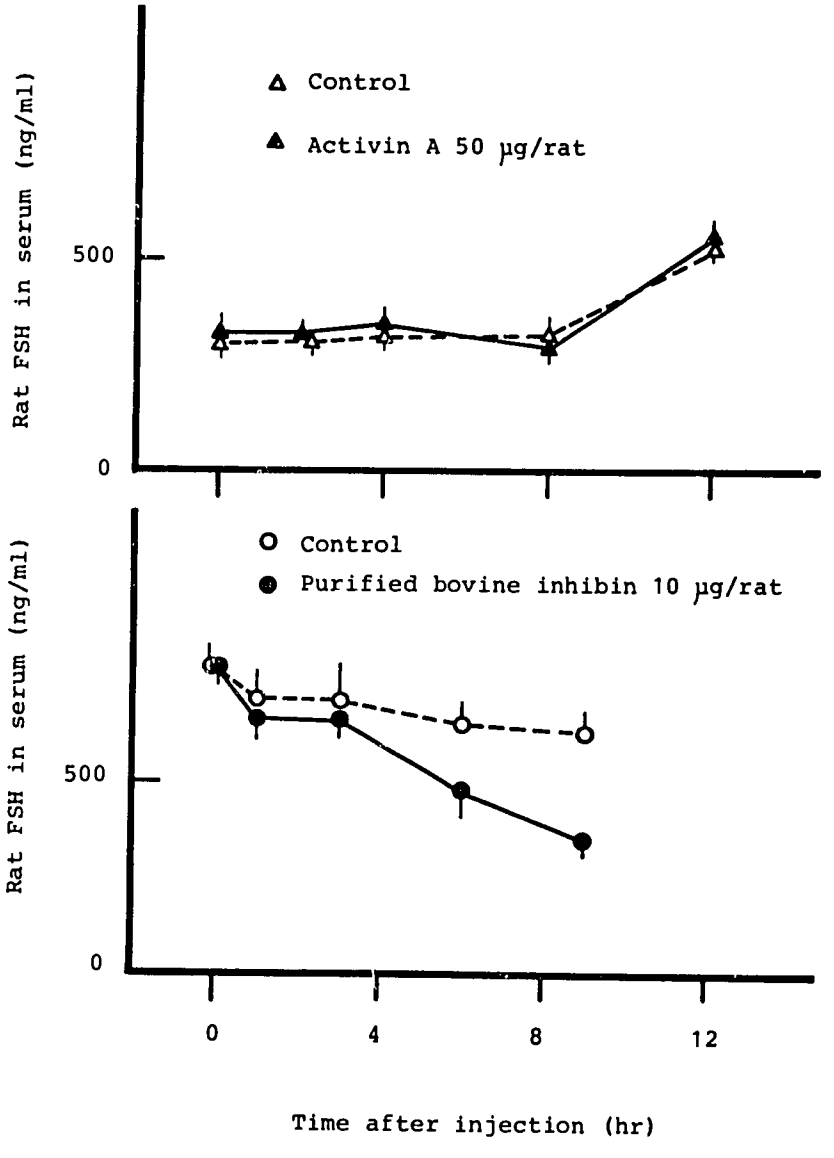


Figure 11. Effects of β A-dimer and bFF inhibin on FSH secretion *in vivo*. (upper) Changes in the serum FSH levels after a single injection of 50 μg/rat of β A-dimer (activin A) to adult male rats. (lower) Effects of a single injection of 10 μg/rat of bFF inhibin on serum FSH levels of castrated adult male rats.

ACKNOWLEDGMENTS

The collaboration of Dr. K. Kangawa and H. Matsuo, Miyazaki Medical College, is gratefully appreciated. We wish to thank Dr. Y. Etoh and H. Shibai, Ajinomoto Co. for kindly providing recombinant β A-dimer. We also wish to thank Dr. A. F. Parlow and the National Pituitary and Hormone Distribution Program, NIDDK, for rat gonadotropin RIA kits.

REFERENCES

1. BAKER HWG, EDDIE LW, HIGGINSON RE, HUDSON B, KEOGH EJ, NIALL HD: Assay of inhibin. In FRANCHIMONT P, CHANNING CP (eds): Intragonadal Regulation of Reproduction, p 193. London, Academic Press 1983
2. DE JONG FH, SMITH SD, VAN DER MOLEN HJ: Bioassay of inhibin-like activity using pituitary cells *in vitro*. J Endocrinol 80:91, 1979
3. ETO Y, TSUJI T, TAKEZAWA M, TAKANO S, YOKOGAWA Y, SHIBAI H: Purification and characterization of erythroid differentiation factor (EDF) isolated from human leukemia cell line THP-1. Biochem Biophys Res Commun 142:1095, 1987
4. FORAGE RG, RING JM, BROWN RW, McINERY BV, COBON GS, GREGSON RP, ROBERTSON DM, MORGAN FJ, HEARN MTW, FINDLEY JK, WETTENHALL, BURGER HG, De KRETSER DM: Cloning and sequence analysis of cDNA species coding for the two subunits of inhibin from bovine follicular fluid. Proc Natl Acad Sci USA 83:3091, 1986
5. FUKUDA M, MIYAMOTO K, HASEGAWA Y, NOMURA M, IGARASHI M, KANGAWA K, MATSUO H: Isolation of bovine follicular fluid inhibin of about 32K Da. Mol Cell Endocrinol 44:55, 1986
6. FUKUDA M, MIYAMOTO K, HASEGAW Y, IBUKI Y, IGARASHI M: Action mechanism of inhibin *in vitro:* Cyclhoeximide mimics inhibin actions on pituitary cells. Mol Cell Endocrinol 51;41, 1987
7. HSUEH AJW, DAHL KD, VAUGHAN J, TUCKER E, RIVIER J, BARDIN CW, VALE W: Heterodimers and homodimers of inhibin subunits have different paracrine action in the modulation of luteinizing hormone-stimulated androgen biosynthesis. Proc Natl Acad Sci USA 84:5082, 1987
8. INOUE K, KUROSUMI K: Ultrastructural immunocytochemical location of LH and FSH in the pituitary of the untreated male rat. Cell Tissue Res 235:77, 1984
9. LING N, YING S-Y, UENO N, ESCH F, DONOROY L, GUILLEMIN R: Isolation and partial characterization of a Mr 32,000 protein with inhibin activity from porcine follicular fluid. Proc Natl Acad USA 82:7212, 1985
10. LING N, YING S-Y, UENO N, SHIMASAKI S, ESCH F, HOTTA M, GUILLEMIN R: Pituitary FSH is released by a heterodimer of the β-subunits from the two forms of inhibin. Nature 321:779, 1986
11. MASON AJ, HAYFLCK JS, LING N, ESCH F, UENO N, YING S-Y, GUILLEMIN R, NIALL H, SEEBURG PH: Complementary DNA sequences of ovarian follicular fluid inhibin show precursor structure and homology with transforming growth factor. Nature 318:659, 1985
12. MASON AJ, NIALL HD, SEEBURG PH: Structure of two human ovarian inhibins. Biochem Biophys Res Commun. 135:957, 1986
13. MAYO K, CERELLI GM, SPIESS J, RIVIER J, ROSENFELD MG, EVANS RM, VALE W: Inhibin A-subunit cDNAs from porcine ovary and human placenta. Proc Natl Acad Sci USA 83:5849, 1986
14. MIYAMOTO K, HASEGAWA Y, FUKUDA M, NOMURA M, IGARASHI M, KANGAWA K, MATSUO H: Isolation of porcine follicular fluid inhibin of 32K Daltons. Biochem Biophys Res Commun 129:396, 1985
15. MIYAMOTO K, HASEGAWA Y, FUKUDA M, IGARASHI M: Demonstration of high molecular weight forms of inhibin in bovine follicular fluid (bFF) by using monoclonal antibodies to bFF 32K inhibin. Biochem Biophys Res Commun 136:1103, 1986
16. NAKANE PK: Classification of anterior pituitary cell types with immunoenzyme histochemistry. J Histochem Cytochem 18:9, 1970
17. NAOR Z, CATT K: Mechanism of action of gonadotropin-releasing hormone. J Biol Chem 256:2226, 1981
18. NIEDEL JE, KUHN LJ, VANDERBARK CR: Phorbol diester receptor copurifies with protein kinase C. Proc Natl Acad Sci USA 80:36, 1983

19. RIVIER J, SPIESS J, McCLINTOCK R, VAUGHAN J, VALE W: Purification and characterization of inhibin form porcine follicular fluid. Biochem Biophys Res Commun 133:120, 1985
20. ROBERTSON DM, FOULDS LM, LEVERSHA L, MORGAN FJ, HEARN MTW, BURGER HG, WETTENHALL REH, De KRETSER DM: Isolation of inhibin from follicular fluid. Biochem Biophys Res Commun 126:220, 1985
21. SCHWARZ NB: Selective control of FSH secretion. In McCANN SM, DHINDA DS (eds): Role of Peptides and Proteins in Control of Reproduction, p 193. New York, Elsevier Biomedical, 1983
22. SCOTT RS, BURGER HG, QUIGG H: A sensitive and rapid *in vitro* bioassay for inhibin. Endocrinology 107:1536, 1980
23. SMITH MA, VALE WW: Superfusion of rat anterior pituitary cells attached to cytodex beads: Validation of a technique. Endocrinology 107:1425, 1980
24. UENO N, LING N, YING S-Y, ESCH F, SHIMASAKI S, GUILLEMIN R: Isolation and partial characterization of follistatin: A single-chain Mr 35,000 monomeric protein that inhibits the release of follicle-stimulating hormone. Proc Natl Acad Sci USA in press, 1987
25. VALE W, RIVIER H, VAUGHAN J, McCLINTOCK R, CORRIGAN A, WOO W, KARR D, SPIESS J: Purification and characterization of an FSH-releasing protein from porcine ovarian follicular fluid. Nature 321:776, 1986

DISCUSSION

CHAIRPERSON: ALAN CORBIN, Ph.D.

DR. CORBIN: Perhaps Dr. Miyamoto could review briefly the slides that he did not have time to present.

DR. MIYAMOTO: I was going to show the multiple inhibin forms in bovine follicular fluid. They are all derived from the α and β subunit but are processed differently. Secondly, the concentration of β dimers in bovine follicular fluid seems very low compared with that of inhibin. Actions of inhibin, or β dimers, on the secretion of gonadotropin seems to be a consequence of the inhibition or the promotion of biosynthesis of proteins in gonadotropes. Finally, under certain conditions, β dimer is not effective in releasing FSH *in vivo*. This might be suggestive of certain local actions of the β dimers rather than an effect on pituitary cells.

DR. HSUEH: Based on your hypothesis that cycloheximide is doing something very similar to inhibin, have you tested the possibility that, for example, if you look at the α subunits of FSH and LH in the same system, what happened to it in terms of response to either cycloheximide or inhibin?

DR. MIYAMOTO: We did not test the α or β subunit levels, so I am not able to comment on that.

DR. DANFORTH: Were you using inhibin A or inhibin B for the studies?

DR. MIYAMOTO: We used inhibin A.

DR. HUTCHISON: In the *in vivo* rat studies, were you injecting activin, was that IN, IP, IV? What was it?

DR. MIYAMOTO: IV injection.

DR. PETRAGLIA: When you speculate on the possible effect of inhibin on the LH release induced by GnRH, can we hypothesize that the effect of inhibin is on the release more than in the synthesis of LH? You showed results that the action could be on the release of the storage pool of LH.

DR. MIYAMOTO: Yes. Very little decrease in the storage pool was observed by the inhibin or cycloheximide treatment. I do not know why. The storage pool of LH is very large so that the biosynthetic inhibition of LH might not be obvious, even if the inhibition actually takes place by the inhibin or cycloheximide treatment.

DR. PETRAGLIA: So, we can say that the inhibin acts on the release?

DR. MIYAMOTO: Yes, it does. But the conditions are very limited, i.e. under LHRH-stimulated conditions.

DR. PETRAGLIA: To what extent was total protein synthesis inhibited?

DR. MIYAMOTO: It is rather difficult to check it, because inhibin is specific to gonadotropes and the population of gonadotropes in the pituitary is very low. Since we use the total pituitary system, the extent of inhibition is very difficult to detect.

DR. PETRAGLIA: How did you determine the dose of cycloheximide then?

DR. MIYAMOTO: We dosed until the cells were affected.

DR. CROWLEY: I would just offer some evidence in support of your idea that there is an LHRH independent mechanism of FSH regulation. Dr. Hall in our group has examined a pure GNRH antagonist in a normal woman in the early follicular phase over an allowed order of doses. We got a very nice dose response in LH inhibition, but we blocked LHRH. However, we have minimal to no effect upon FSH release in this human model. This fits in with the results of several other investigators showing, at the very least, a differential effect or blockade of LHRH receptor on LH over FSH. Similar results have been obtained with GnRH antiserum, but over a narrower range of doses. I think there is a growing body of evidence to support your theory of GnRH independent regulation of FSH.

DR. BURGER: If I could just make one additional comment which amplifies that further. If you look at the model of the hypothalamic-pituitary disconnected sheep which Ian Clark has developed, where the influence of hypothalamus on the pituitary is separated, and you pulse that animal with GnRH, in the ovariectomized animal you maintain normal castrate levels of LH and FSH. However, if you stop the GnRH pulses, LH levels become undetectable within six hours after the GnRH stimulus is removed and FSH levels remain at the castrate concentration for at least 30 hours afterwards and then decline only very slowly They can then be lowered further with inhibin in those circumstances.

So, I think that a number of us are developing the notion that in many circumstances FSH is a constitutive product of the gonadatrope which is really rather independent of GnRH input. On the other hand, LH secretion depends very closely on GnRH and, therefore, there are different stimulatory and different inhibitory mechanisms.

DR. PAULSEN: My question is to Dr. Burger. Have you corrected for the half-life of the hormone, because when you inject FSH in the human being in a steady state it takes much longer for FSH to disappear with a single dose, and LH goes down very rapidly. So, I am not sure that what you have just stated has supported the differential effect on inhibition.

DR. BURGER: I think the detectability and maintenance of concentration is long beyond the half-life of FSH in that animal.

6

Monitoring of Bioactive Follicle-Stimulating Hormone (FSH) in Normal and Abnormal Reproductive States

KRISTINE D. DAHL
AARON J. W. HSUEH

Gonadotropin-releasing hormone (GnRH) is secreted in a pulsatile manner by the hypothalamus to activate and maintain follicle-stimulating hormone (FSH) and luteinizing hormone (LH) synthesis and release.[15] The gonadotropins (FSH and LH) are released into the general circulation and are transported to the ovary and testes, where they are responsible for the regulation of gametogenesis and gonadal steroidogenesis. Precise measurements of FSH and LH were not possible prior to the development of specific radioimmunoassays (RIA).[19] Since that time, serum levels of immunoreactive FSH and LH have been characterized during puberty, and in various normal and disease states.[5,25,30]

Although essentially all of the available data on FSH levels in body fluids have been obtained by RIA, these data do not necessarily reflect bioactivity. Some modified forms of FSH may not cross-react with the antibody but still retain their bioactivity, whereas some biologically inactive FSH (*e.g.*, deglycosylated forms) may still bind the FSH antibody. Marked disparities between bio- and immuno- estimates of both crude and purified hormone preparations have been observed.[1,24,26] The anterior pituitary is capable of secreting several forms of FSH with differing bio- and immuno-potencies. Animal studies examining pituitary content of FSH revealed that various molecular forms of FSH existed, and that these forms could be altered by changing the hormone milieu.[3,4,22] This molecular heterogeneity of FSH suggested that the interactions of FSH with the gonads are not only dependent on the amount of FSH released from the anterior pituitary, but potentially also on the biopotency of the FSH. However, the role of this FSH pleomorphism in physiologic situations is not clear due, in part, to the lack of an appropriate bioassay sensitive enough to detect circulating bioactive FSH.

Our studies of the endocrine, paracrine, and autocrine action of various hormones on granulosa cell differentiation resulted in the development of a sensitive and specific *in vitro* bioassay for FSH.[11] This chapter describes the implementation of the Granulosa cell Aromatase Bioassay (GAB) for the measurement of bioactive FSH levels in various physiological and pathological conditions.

HUMAN MENSTRUAL CYCLE STUDIES

To demonstate the validity of the GAB assay in measuring bio-FSH levels in human serum, granulosa cells were cultured with increasing concentrations of highly purified human FSH-3 or a crude pituitary gonadotropin preparation (LER-907) to obtain a standard curve (Fig. 1).[12] We have tested the effect of pretreatment with increasing doses of polyethylene glycol (PEG) in gonadotropin-free serum to minimize the serum inhibitors and to insure total recovery of FSH. Pretreatment of human serum with 12% PEG minimized the serum inhibitory effect with an estimated 98% recovery of exogenously added FSH.[11] All cultures, including the standard curves, were balanced with PEG-treated gonadotropin-free serum (from oral contraceptive pill users) to obtain a final concentration of 4%. The human FSH preparation (LER-907) had a working range in the assay between 0.25 to 2.0 mIU per culture. Our subsequent data are presented in units based on the biological potency of LER-907 for comparison with RIA results. Thus, all serum samples were pretreated with 12% PEG to minimize serum inhibitory factors.

Treatment of granulosa cells with increasing aliquots of PEG-pretreated serum from women in various physiologic and pathophysiologic states resulted in dose-response curves

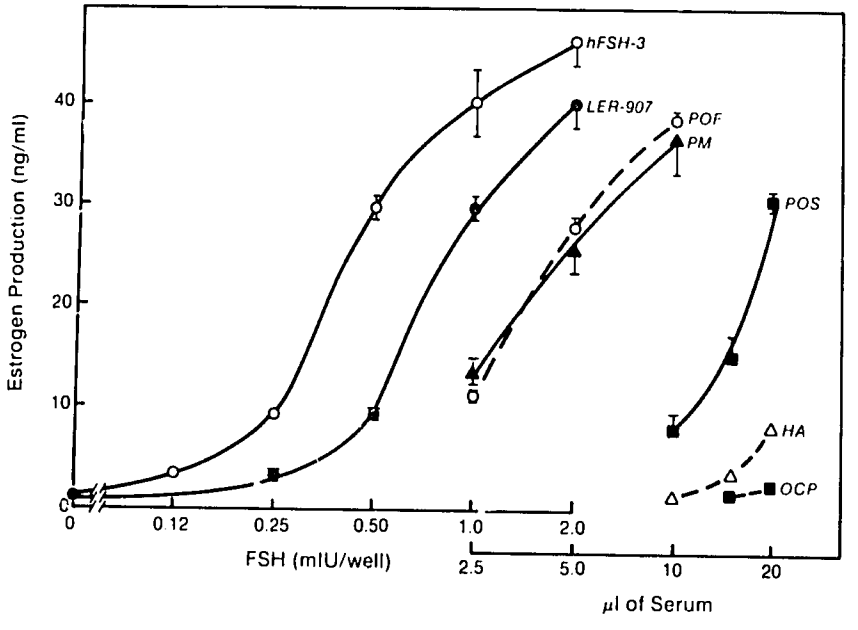


Figure 1. Dose-dependent stimulation of estrogen production by FSH preparations and by serum from women in various physiologic and pathophysiologic states.

Granulosa cells were cultured as described for the GAB assay method with increasing concentrations of human pituitary FSH preparations (hFSH-3 or LER-907), or with increasing aliquots of PEG-pretreated human serum (POF = premature ovarian failure; PM = postmenopause; POS = preovulatory surge; HA = hypothalamic amenorrhea; OCP = oral contraceptive pill users).

parallel to the standard curve (Fig. 1). In contrast, serum from oral contraceptive pill users (OCP) did not stimulate estrogen production at the highest aliquot used. In patients with hypothalamic amenorrhea (HA), the largest aliquot was within the sensitivity range of the assay, and in women during the preovulatory surge (POS), aliquots of 10 and 20 μl resulted in substantial estrogen production. Furthermore, in postmenopausal women (PM) or patients with premature ovarian failure (POF), aliquots of 2.5 to 10 μl were within the working range of the standard curve. For each serum sample, 3 to 4 aliquots of serum in triplicate cell cultures were used. Only samples that yielded dose-response curves parallel to the standard curve were used for the calculation of FSH potency. Mean levels of FSH bioactivity in these clinical states were consistent with the clinical presentation, undetectable in oral contraceptive pill users, intermediate in patients with hypothalamic amenorrhea, and highest in postmenopausal women and patients with premature ovarian failure.

Daily serum samples from seven women throughout ovulatory cycles were assayed for FSH bioactivity or immunoreactivity (Fig. 2). For measurement of FSH bioactivity, all samples were treated with 12% PEG and aliquots of 5, 10, 15, and 20 μl were used. For the purpose of data consolidation and analysis, the day of the LH surge was designated as day 0. The mean serum immuno-FSH levels in the seven women were plotted around the mid-cycle LH peak and revealed a profile in keeping with that found in previous reports.[19,23] The serum bio-FSH profile from the same seven women was very similar to the immuno-FSH profile (Fig. 2). The corresponding B/I ratios ranged from 1.4 to 3.4, with a mean of 2.5 $\pm$ 0.1. The correlation coefficient of the mean values throughout the

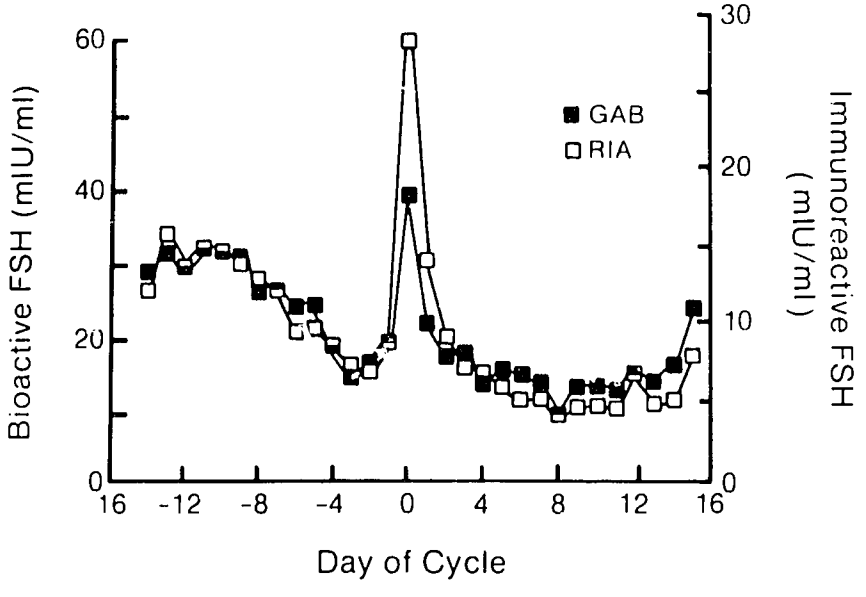


Figure 2. Mean levels of bioactive and immunoreactive FSH throughout the menstrual cycle.

Mean levels of bioactive and immunoreactive FSH throughout the menstrual cycle in 7 regularly cycling women, with data centered around the midcycle LH surge.

menstrual cycle obtained by the bioassay and RIA was 0.91 ($P < 0.001$). Thus, the GAB assay detects FSH bioactivity in all women tested, and, in general, the results parallel levels measured by RIA.

GNRH AGONIST AND ANTAGONIST TREATMENT

Synthetic derivatives of GnRH with agonistic and antagonistic properties have been developed and tested in several clinical studies including suppression of prostatic cancer growth and the development of male contraceptives.[17,21,23,31] Chronic administration of GnRH agonistic analogs leads to suppression of gonadal function through a complex mechanism of "pituitary desensitization".[17,18,21] Several studies have focused on qualitative changes in circulating LH because of the discrepancy between serum LH and testosterone levels.[2,9,18] Serum immuno-LH and FSH levels are only modestly affected, whereas serum bio-LH levels are dramatically reduced. Consequently, the B/I ratio of LH, an index of the biopotency, decreased by approximately 90%. However, GnRH agonist treatment did not result in reliable suppression of spermatogenesis. When GnRH antagonists are administered to normal men, there is a decrease in both bio- and immuno-LH, and endogenous testosterone is decreased to castrate levels, but there is only a minimal decrease in immuno-FSH. Therefore, there are disparate effects of GnRH agonists and antagonists on FSH and spermatogenesis. Most of the questions raised in the studies described above specifically relate to whether immuno-FSH data reflect bio-FSH, or if the disparate results are due to the differential regulation of circulating bioactive FSH. Therefore, we examined the effects of GnRH analogs on bioactive FSH levels in various endocrine states.

GNRH ANTAGONIST TREATMENT IN MEN

Although GnRH antagonists clearly suppress circulating LH levels, only minor decreases in FSH levels were detected using RIA. In men, immuno-FSH levels in serum decreased only 20 to 30%.[31] We measured serum bio-FSH levels in 5 normal men after a single injection (20 mg) of a potent GnRH antagonist, RS-69439, [N-Ac-D-Nal(2)[1],d-pCl-Phe[2],D-Trp[3],D-h(Arg(Et_2))6,D-Ala]-GnRH. Although only minimal suppression of immuno-FSH was detected, pronounced inhibition (79-89%) of bio-FSH levels occurred (Fig. 3).[7] Concomitantly, the ratio of bio- to immuno-FSH levels decreased drastically after the antagonist administration. These data reinforced earlier expectations that GnRH antagonists might be potential male contraceptives and provided the first report of changes in circulating bio- to immuno-FSH levels.

The circulating half-life of this GnRH antagonist is about 48 hours. Early studies demonstrated that GnRH antagonists have no effect on granulosa cell aromatase induction by FSH. To discount possible interference of antagonists on the bioassay, a concentration (10^{-6} M) greater than the estimated circulating levels of this peptide was added to the cultures. Therefore, the decrease of bio-FSH levels was not due to the effects of circulating GnRH antagonist in the bioassay.

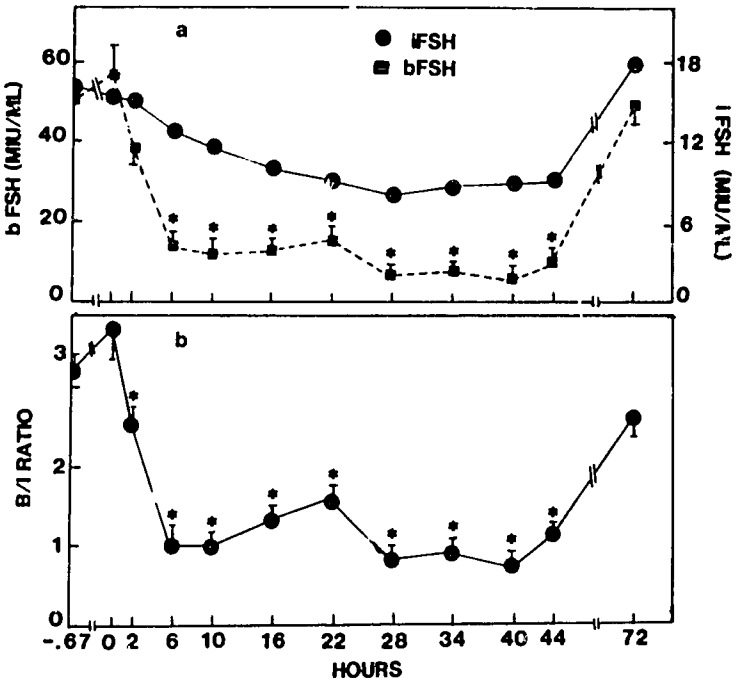


Figure 3. Effects of GnRH antagonist treatment on bioactive and immunoreactive FSH in men.

Effects of a subcutaneous injection of RS-69439 on bio(b)- and immuno(i)- FSH (a) and on the B/I ratio (b) in 4 normal men. Levels differing significantly from the pretreatment concentrations during the peptide treatment are marked with asterisks (*, $p < 0.05$).

GNRH ANTAGONIST TREATMENT IN WOMEN

Since treatment with a GnRH antagonist resulted in changes in the bio- to immuno-ratio of FSH in normal male subjects, we tested the effect of treatment with a GnRH antagonist (4F, Ac-Δ^{3}-Pro^{1},p-F-D-Phe^{2},D-Trp^{36}, GnRH) on female patients. Our results indicate that constant intravenous infusion to hypergonadotropic hypogonadal women resulted in a more pronounced decline of bio-FSH levels (62%) than immuno-FSH levels (30%) with a significant decrease in the B/I ratio for FSH (Fig. 4)[14] indicating substantial microheterogeneity of these molecules. Infusion of 4F-antagonist in the midfollicular phase of normal cycling women revealed a similar pattern of suppression of bio-FSH and immuno-FSH. When 4F-antagonist was administered subcutaneously for three days to midfollicular phase women, bio-FSH response was biphasic with the maximal decrease on the second day, followed by a recovery on the third day (Fig. 5). Correspondingly, there was a precipitous decline in serum estradiol (apparent demise of the dominant follicle), followed by a progressive rise in estradiol levels. Thus, in contrast to the immunoreactive FSH levels, measurement of bioactive FSH more clearly reflects the biologic action of FSH on the folli-

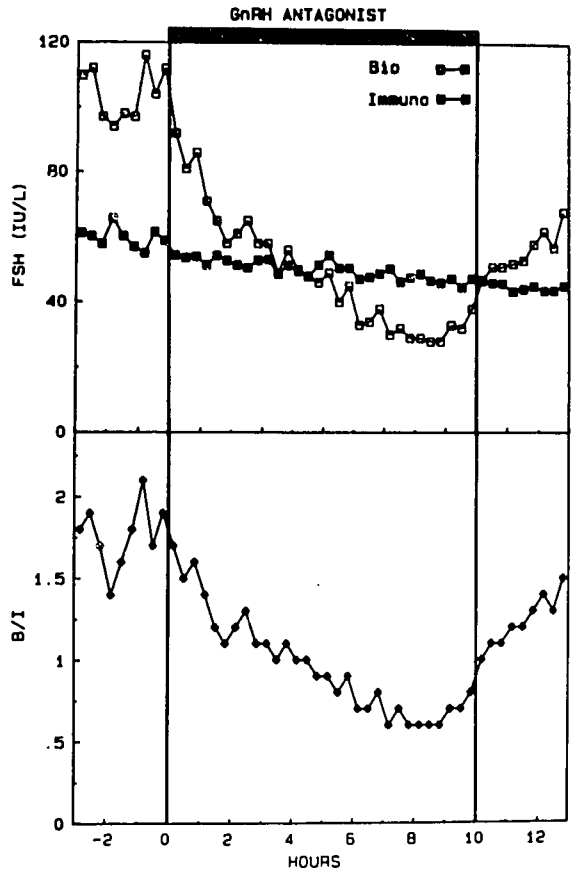


Figure 4. Effects of GnRH antagonist treatment on bioactive and immunoreactive FSH in a postmenopausal woman.

Serum bioactive and immunoreactive FSH (a) and B/I ratios (b) were measured at 20 minute intervals after a 10 hour infusion of the 4F-antagonist (30 μg/kg/h) in a postmenopausal woman.

cle in response to GnRH antagonist treatment in women, and reinforces the importance of measuring bio-FSH levels in different physiological, pathophysiological, and pharmacological conditions.

GNRH AGONIST TREATMENT IN NORMAL MEN

Normal men were treated with a potent GnRH agonist (D-Trp^6, Pro^9-NEt-GnRH), 500 μl/day for 16 weeks by sc infusion and testosterone ethanate (TE), 100 mg im every 2 weeks, or GnRH agonist for 20 weeks by sc injection and TE every 2 weeks from week 10 to 20. In the first study, both bio- and immuno-FSH declined approximately 57% with no signifi-

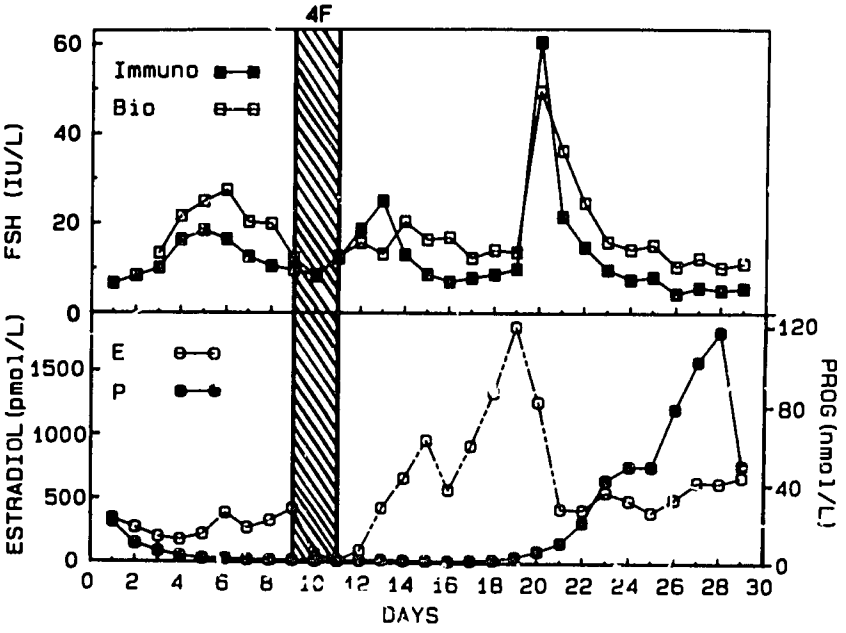


Figure 5. Effects of GnRH antagonist treatment during the mid-follicular phase.

Bioactive and immunoreactive FSH levels, and ovarian estradiol and progesterone levels were measured daily after the administration of the 4F-antagonist (80 μg/kc sc twice daily) for 3 days during the mid-follicular phase.

cant change in the B/I ratio (Fig. 6).[20] During the second study, both bio- and immuno-FSH followed a triphasic pattern, decreasing immediately after initiation of treatment, then progressively increasing to a peak at week 10 and then, after addition of TE to this regimen, decreasing again.[20] The B/I changes, however, were not statistically significant except that immuno-FSH levels were higher than baseline at week 10. Therefore, in contrast to the results observed after GnRH antagonist treatment, chronic GnRH agonist treatment does not alter FSH B/I ratios.

GNRH AGONIST TREATMENT IN MEN WITH PROSTRATE CANCER

To further study the effect of GnRH agonist treatment on FSH heterogeneity, bio- and immuno-FSH levels were measured in serum of five prostrate cancer patients during a six month treatment with a GnRH agonist analog (buserlin, 600 μg intranasally x 3/day), and up to 12 weeks after subsequent orchidectomy (Fig. 7).[10] After treatment with the agonist, both bio- and immuno-FSH transiently increased for 1 to 3 days. The increase in bioactivity was more pronounced and the B/I ratio increased at two weeks. With advancing time of treatment, immuno-FSH declined below pretreatment levels in 5 days, and stayed suppressed for the rest of the treatment. In contrast, FSH bioactivity did not decrease significantly below the treatment level during the 6 month treatment period,

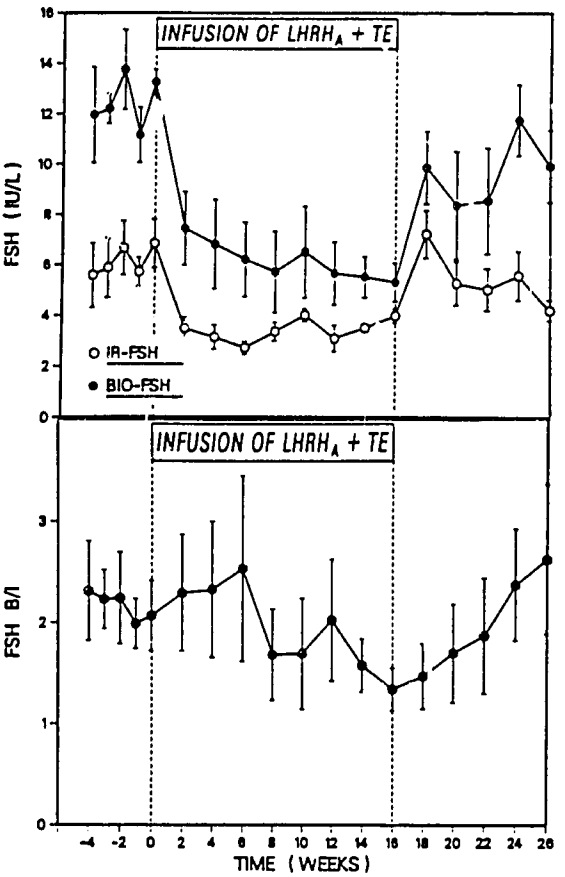


Figure 6. Effects of daily infusion of GnRH agonist treatment in normal men.

Bioactive (bio-) and immunoreactive (IR-) FSH (a) and B/I ratios (b) were measured after the administration of GNRH agonist by subcutaneous infusion (11.8 μg/34 minutes) and testosterone replacement (100 mg) for 16 weeks.

although the B/I ratio returned slowly towards pretreatment levels. After orchidectomy, the FSH activities showed dramatic increases, and the B/I ratio rose transiently from 1.5 to 7 in two weeks. In conclusion, the bio-FSH levels increased more acutely than immuno-FSH after GnRH agonist treatment and orchidectomy. A clear decline during long-term agonist treatment is only seen in immuno-FSH, which is in contrast to the concomitant decline in serum LH bioactivity reported earlier. The persistence of FSH bioactivity may partly explain the inconsistent effects of GnRH agonist treatments on the suppression of spermatogenesis.

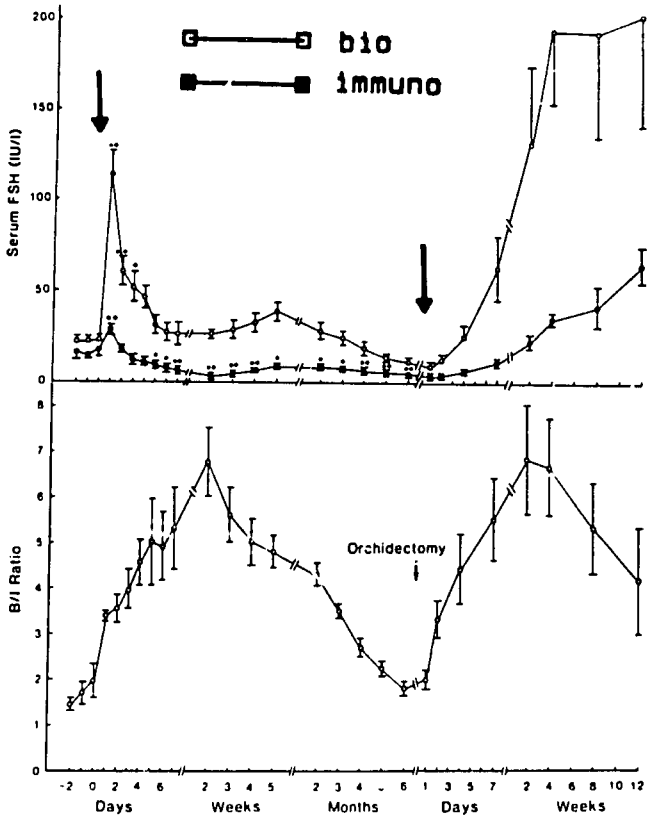


Figure 7. Effects of GnRH agonist before and after orchidectomy in men with prostrate cancer.

Serum bioactive (bio-) and immunoreactive (immuno-) FSH (a) levels and B/I ratios (b) were measured in patients treated for 6 months with the GnRH agonist buserlin, and for 12 weeks following orchidectomy at 6 months after the initiation of agonist therapy. Levels differing significantly from the pretreatment concentrations during the peptide treatment are marked with asterisks (*, $p < 0.05$; **, $p < 0.01$).

MOLECULAR BASIS FOR DECREASES IN THE B/I RATIOS OF FSH IN GNRH ANTAGONIST-TREATED PATIENTS

The chromatofocusing method separates proteins based on their isoelectric points.[29] This technique has been shown to be useful in identifying microheterogeneity of FSH molecules.[4] We have set up this method, in conjunction with gel filtration (separation of proteins by molecular weight), to analyze FSH heterogeneity when changes in the B/I ratio of FSH are detected.

The lower B/I ratios, and thus decreasing bio-potency of FSH, after GnRH antagonist injection may reflect the presence of different molecular species of FSH. Since FSH is

a glycoprotein, changes in FSH activity could be due to alterations in the degree of glycosylation.[4,8] The rapid decline in both bio- and immuno-FSH levels suggest that the GnRH antagonist may decrease the GnRH-induced FSH biosynthesis and/or release, inhibit the processing of FSH in the late stages of glycosylation, or alter the metabolism of FSH. It is also possible that the GnRH antagonist changes the tertiary structure of the FSH molecule, thereby changing its ability to bind to the receptor but not to the antibodies. However, the variation in the B/I ratio was not due to differential changes in the secretion of alpha and beta subunits because the RIA only measures intact FSH. Deglycosylated FSH has been shown to behave like antagonists in FSH bioassays.[27] It is possible that treatment with GnRH antagonists results in the secretion of deglycosylated or partially glycosylated FSH which decreases the action of intact FSH in the same sample. Therefore, we investigated the differences in FSH isoforms in postmenopausal women before and after GnRH antagonist treatment.

The FSH isoforms before and after GnRH antagonist treatment were separated based on their isoelectric properties using a chromatofocusing column with a pH gradient of 7 to 4 (Fig. 8, inset).[6] Each fraction was analyzed both by RIA (upper panel) and bioassay

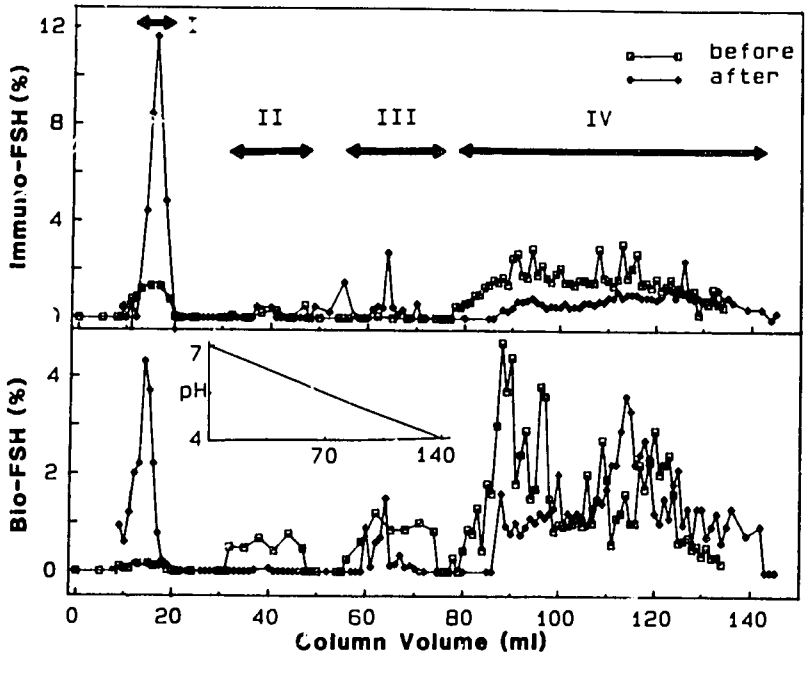


Figure 8. Chromatofocusing analysis of serum samples from hypogonadal women before and after GnRH antagonist infusion.

Chromatofocusing analysis of serum samples from hypogonadal women before (open squares) and after (closed diamonds) GnRH antagonist infusion, with the use of a pH gradient of 7 to 4 (inset). Fractionated samples were assayed (A) by RIA, and (B) by a granulosa cell aromatase bioassay. Results are expressed as the percent of total activity eluted from the column.

(lower panel). Four major hormone peaks were distinguished. Peak IV contained the majority of FSH isoforms based on both assays. Before treatment, peaks II and III contained the most bioactive FSH forms. After GnRH antagonist treatment, a substantial increase in the percentage of the most basic peak I was apparent, accompanied by decreases in the remaining peaks. The FSH B/I ratios were 0.1, 8, 57, and 2 for peaks I, II, III, IV, respectively, indicating that peak I was the least bioactive. The low B/I ratio in peak I suggested that these isoforms may be a mixture of FSH molecules with both agonist and antagonist activities.

IDENTIFICATION OF CIRCULATING FSH ANTAGONIST ISOFORMS

To estimate the molecular weight (Mr) of the newly formed FSH isoforms, peak I fractions, obtained from GnRH antagonist treated patients were pooled and added to an Ultrogel column which separates proteins by Mr. The applied sample displayed a wide range of Mr from 45,000 to 25,000 as assessed by RIA and the bioassay. However, the B/I ratios varied. Fractions with Mr and B/I ratios lower than intact FSH were pooled and run on a chromatofocusing column with a pH gradient from 11 to 7. The resulting fractions were again analyzed by RIA and bioassay (Fig. 9, upper panel). Fractions from pH 9.6 to 9.4 contained the majority of the immuno-FSH, but these isoforms were not bioactive. To investigate possible antagonistic effects of these FSH isoforms, column fractions were added together with 2 mIU/ml of human FSH in the bioassay. Samples with pH 9.6 to 9.4 inhibited FSH-stimulated estrogen production (Fig. 9, lower panel; open squares), but fractions with higher or lower pH were ineffective. A dose-dependent inhibition of estrogen production by the pH 9.5, but not by the pH 10.1, fraction was found (Fig. 9, inset). To rule out nonspecific inhibitory effects, the same fractions were added in conjunction with forskolin (10^{-5} M), which bypasses the FSH receptor and directly activates adenyl cyclases to increase estrogen biosynthesis in the granulosa cells. There was no inhibition of forskolin-stimulated estrogen production by any fractions (Fig. 9, lower panel; crosses), suggesting that the suppression of FSH action by these fractions is probably manifest at steps before adenyl cyclase activation.

To further demonstrate that the antagonistic effect of the samples with pH 9.6-9.4 is due to FSH-like molecules, immunoneutralization experiments were performed. As shown in Figure 10, treatment of cultured granulosa cells with antibodies against human FSH (Ab) or pooled fractions pH 9.6-9.4 (iso-FSH) alone did not increase estrogen production, whereas a near-saturating dose (1 ng/ml) of ovine FSH increased estrogen production. Because the Ab is species-specific, no significant decrease in estrogen biosynthesis was detected when Ab was added in conjunction with ovine FSH. When the iso-FSH was added with ovine FSH, there was a 86% inhibition of estrogen production. However, when Ab (1:1 or 1:5 dilutions) was added together with ovine FSH and the human iso-FSH, Ab blocked the inhibitory effects of iso-FSH in a dose-dependent manner. Therefore, the FSH antagonist isoforms were preferentially bound by the antibodies such that their receptor-specific inhibitory effects were blocked.

This is the first demonstration of a naturally-occurring circulating antagonistic isohormone. In tissue extracts of a mouse tumor, a TSH antagonist immunologically related to TSH has previously been demonstrated, but this isoform has not been found in the cir-

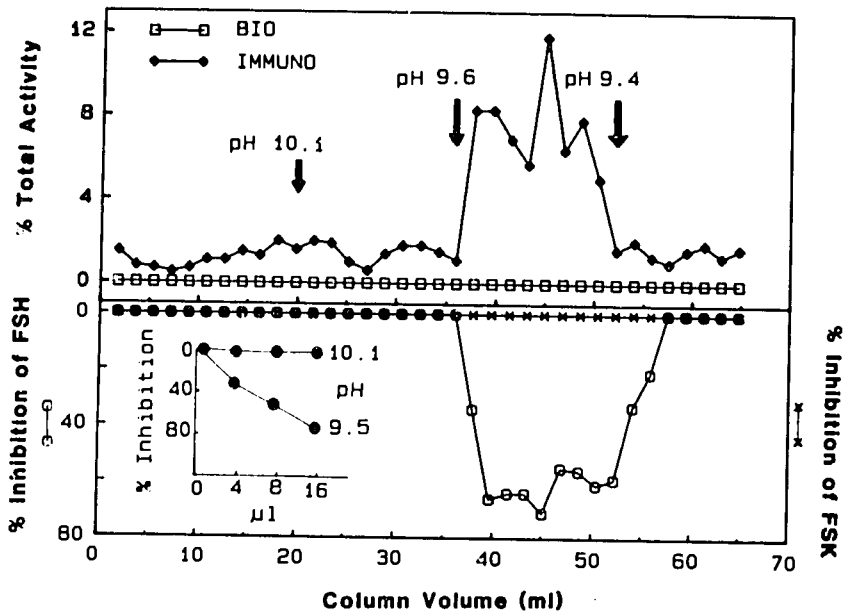


Figure 9. Chromatofocusing analysis of GnRH antagonist-treated patient samples with the use of a pH gradient of 11 to 8.

FSH isoforms with Mr and B/I ratio lower than those of intact FSH from an Ultrogel AcA 54 column were applied to the chromatofocusing column. (A) Column fractions were assayed by RIA, and by bioassay and shown as percent of total activity. (B) Antagonistic effects of FSH isoforms on FSH-stimulated (open squares) and forskolin-stimulated (crosses) estrogen production and dose-dependent inhibition of FSH-stimulated estrogen production by FSH isoforms (inset). Results were expressed as percent inhibition of estrogen production induced by FSH or forskolin (FSK).

culation.[13] Although fractionated follicular fluid contains FSH antagonist activities,[28] these uncharacterized factors are probably not FSH isoforms because no associated FSH immunoreactivity was detected.

Due to the lack of a sensitive FSH bioassay, earlier studies used radioligand receptor assays to assess FSH activity.[4] The receptor assay, however, can not differentiate between agonistic and antagonistic isoforms of FSH. To facilitate the separation of heterogeneous agonistic and antagonistic FSH isoforms, we used chromatofocusing techniques to separate molecules with different pI values, followed by estimation of bioactivity using a sensitive *in vitro* FSH bioassay. It is clear that treatment with GnRH antagonist causes the release of new FSH isoforms (pI 9.6-9.4) with potent FSH antagonistic properties.

In conclusion, present *in vitro* granulosa cell aromatase bioassay provides the tool to measure FSH bioactivity in diverse physiological and pharmacological states. We observed increases in the B/I ratio of FSH in GnRH agonist-treated patients with prostate cancer and decreases in the B/I ratio after GnRH antagonist treatment in both men and women. Chromatofocusing analysis of serum from GnRH antagonist-treated patients demonstrates

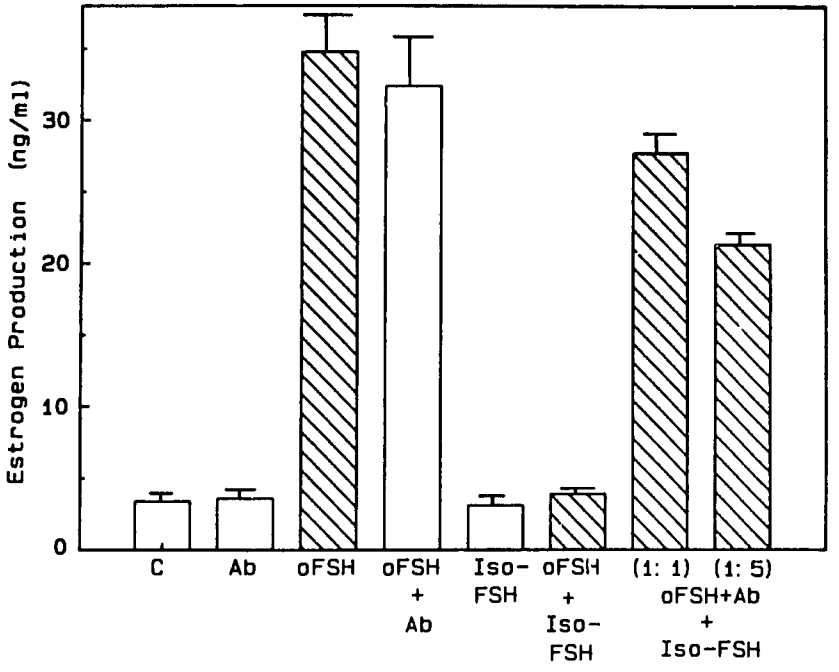


Figure 10. Immunoneutralization of the FSH isoforms with human FSH antibodies.

Immunoneutralization of the antagonistic effects of FSH isoforms (pH 9.6 to 9.4) with antibodies against human FSH. Cultured granulosa cells were treated with ovine FSH (o-FSH), antibodies against human FSH (Ab) (immunoglobulin G at 750 μg/ml, and pooled fractions pH 9.6 to 9.4 from the chromatofocusing column alone (iso-FSH) or in combination. The last two columns indicate incubations with ovine FSH, iso-FSH, and two different dilutions of the antibody. Estrogen production by granulosa cells is shown. Similar results were obtained in 3 separate experiments.

a naturally occurring circulating "anti-hormone" and provides the basis to elucidate the role of hormone antagonists in various physiological, pharmacological, and pathophysiological states. Further characterization and eventual purification of these FSH isoforms could aid our understanding of the glycosylation and action of FSH as well as provide clinical approaches for "anti-hormone" therapies. In addition to GnRH antagonists, the observed FSH antagonist isoforms may provide prototypes for the design of contraceptives.

REFERENCES

1. ALBERT A, ROSENBERG E, ROSS GT, PAULSEN CA, RYAN RJ: Report of the National Pituitary Agency collaborative study on the radioimmunoassay of FSH and LH. J Clin Endocrinol Metab 240:E125, 1968
2. BHASIN S, ROBINSON R, PETERSON M, STEINER BS, HANDELSMAN DJ, RAJFER J, HEBER D, SWERDLOFF RS: Molecular heterogeneity and biological activity of LH after GnRH agonist treatment. Fertil Steril 42:318, 1984
3. BOGDANOVE EM, CAMPBELL GT, BLAIR ED, MULA ME, MILLER AE, GROSSMAN GH: Gonad-pituitary feedback involves qualitative change: Androgens alter the type of FSH secreted by the rat pituitary. Endocrinology 95:219, 1974
4. CHAPPEL SC, ULLOA AGUIRRE A, COUTIFARIS C: Biosynthesis and secretion of follicle-stimulating hormone. Endocr Rev 4:179, 1983
5. CROWLEY WF, FILICORI M, SPRATT DI, SANTORO MF: The physiology of gonadotropin-releasing hormone (GnRH) secretion in men and women. Recent Prog Horm Res 41:473, 1985
6. DAHL KD, BICSAK TA, HSUEH AJW: Naturally occurring anti-hormones: Secretion of FSH antagonists by women treated with a GnRH analog. Science 239:72, 1988
7. DAHL KD, PAVLOU SN, KOVACS WJ, HSUEH AJW: The changing ratio of serum bioactive to immunoreactive follicle-stimulating hormone with a potent gonadotropin-releasing hormone antagonist. J Clin Endocrinol Metab 63:792, 1986
8. DIEBEL N, YAMAMOTO M, BOGDANOVE E: Discrepencies between radioimmunoassays and bioassays for rat FSH: evidence that androgen treatment and withdrawal can alter bioassay-immunoassay ratios. Endocrinology 92:1660, 1973
9. EVANS RM, DOELLE GC, LINDNER J, BRADLEY V, RABIN D: A luteinizing hormone-releasing hormone agonist decreases biological activity and modifies chromatographic behavior of luteinizing hormone in man. J Clin Invest 73:262, 1984
10. HUHTANIEMI IT, DAHL KD, RANNIKKO S, HSUEH AJW: Serum bioactive and immunoreactive follicle-stimulating hormone in prostatic cancer patients during GnRH agonist treatment and after orchidectomy. J Clin Endocrinol Metab 66:308, 1988
11. JIA X-C, HSUEH AJW: Granulosa cell aromatase bioassay (GAB) for follicle-stimulating hormone (FSH). Endocrinology 119:1570, 1986
12. JIA X-C, KESSEL B, YEN SSC, TUCKER EM, HSUEH AJW: Serum bioactive follicle-stimulating hormone during the menstrual cycle and in hyper- and hypo-gonadotropic states: Application of a sensitive granulosa cell aromatase bioassay (GAB). J Clin Endocrinol Metab 62:1243, 1986
13. JOSHI LR, WEINTRAUB BD: Naturally occurring forms of thyrotropin with low bioactivity and altered carbohydrate content act as competitive antagonists to more bioactive forms. Endocrinology 113:2145, 1983
14. KESSEL B, DAHL KD, KAZER RR, LIU C-H, RIVIER J, VALE W, HSUEH AJW, YEN SSC: The dependency of bioactive FSH secretion on gonadotropin-releasing hormone in hypogonadal and cycling women. J Clin Endocrinol Metab 66:361, 1988
15. KNOBIL E, PLANT T, WILDT L, BELCHETZ P, MARSHALL G: Control of the rhesus monkey menstrual cycle: permissive role of hypothalamic gonadotropin-releasing hormone. Science 207:1371, 1980
16. LABRIE F, DUPONT A, BELANGER A, ST-ARNAUD R, GIGUERE M, LACOURCIERE Y, ERMOND J, MONFETTE G: Treatment of prostate cancer with gonadotropin-releasing hormone agonists. Endocr Rev 7:67, 1986
17. LINDE R, DOELLE GC, ALEXANDER N, KIRCHNER F, VALE W, RIVIER J, RABIN D: Reversible inhibition of testicular steroidogenesis and spermatogenesis by a potent gonadotropin releasing hormone in normal men. N Engl J Med 305:663, 1981
18. MELDRUM DR, TSAO Z, MONROE SE, BRAUNSTEIN GD, SLADEK J, LU JKH, VALE W, RIVIER J, JUDD HL, CHANG RJ: Stimulation of LH fragments with reduced bioactivity following GnRH agonist administration in women. J Clin Endocrinol Metab 58:755, 1984
19. MIDGLEY RA: Radioimmunoassay for human follicle-stimulating hormone. J Clin Endocrinol Metab 27:295, 1967
20. PAVLOU SN, DAHL KD, WAKEFIELD G, RIVIER J, VALE W, HSUEH AJW, LINDNER J: Maintenance of the ratio of serum bioactive to immunoreactive FSH in normal men during chronic administration of a LHRH agonist. J Clin Endocrinol Metab (in press)

21. PAVLOU SN, INTERLANDI JW, WAKEFIELD G, RIVIER J, VALE W, RABIN D: Heterogeneity of sperm density profiles following 16-week therapy with continuous infusion of high dose LHRH analog plus testosterone. J Androl 7:228, 1986
22. PECKHAM WD, KNOBIL E: Qualitative changes in the pituitary gonadotropins of the male rhesus monkey following castration. Endocrinology 98:1061, 1976
23. RIVIER C, RIVIER J, VALE W: GnRH antagonists: Physiological and contraceptive applications in the female rat. In ZATICHNI GI, SHELTON JD, SCIARRA JJ (eds): Contraceptives. p 13, Philadelphia, Harper and Row, 1981
24. ROSENBERG E, SOLOD EA, ALBERT A: Luteinizing hormone activity of human pituitary gonadotropin as determined by the ventral prostate weight and the ovarian ascorbic acid depletion methods of assay. J Clin Endocrinol Metab 24:714, 1971
25. ROSS GT, CARGILLE CM, LIPSETT MB, RAYFORD PL, MARSHALL JR, STROTT CA, RODBARD D: Pituitary and gonadal hormone in women during spontaneous and induced ovulation cycles. Recent Prog Horm Res 26:1, 1970
26. RYAN KJ: Chemistry of pituitary gonadotropins. In: DICZFALUSY E (ed): Karolinska Symposium Research Methods in Reproductive Endocrinology, Acta Endocrinol [Suppl] 12:300, 1969
27. SAIRAM MR, BHARGARI GN: Deglycosylation of ovine pituitary lutropin subunits: Effects of subunit interaction and hormone activity. Arch Biochem Biophys 204:199, 1980
28. SLUSS PM, SCHNEYER AL, FRANKE MA, REICHERT LE JR: Porcine follicular fluid contains both follicle-stimulating hormone agonist and antagonist activities. Endocrinology 120:1477, 1987
29. SLUYTERMAN LAE, ELGERSMA O: Chromatofocusing: isoelectric focusing on ion exchange columns. I. general principles. J Chromatogr 150:17, 1978
30. STYNE DM, GRUMBACH MM: Puberty in the male and female: its physiology and disorders. In YEN SSC, JAFFE RB (eds): Reproductive Endocrinology, Pathophysiology, and Clinical Management. Philadelphia, Saunders, p 313, 1986
31. VICKERY BH: Comparisons of potential utility of LHRH agonists and antagonists for fertility control. J Steroid Biochem 23:779, 1985

DISCUSSION:

CHAIRPERSON: ALAN CORBIN, Ph.D.

DR. BURGER: You introduced the last part of your data by referring to studies of FSH activity in the urine of prepubertal children, but you did not tell us anything about what you found in your FSH bioassay.

DR. HSUEH: We have about two years of urine samples collected, but it takes time to analyze them and we have not completed the analysis

DR. CROWLEY: The FSH antagonist is very interesting. Have you had the opportunity to look at any patients with premature ovarian failure? There is a subset of these patients that have retained follicles in their ovary and apparently are not reacting to the high levels of FSH in circulation. This would be a logical circumstance in which the FSH antagonist would potentially play an important role.

DR. HSUEH: We have only analyzed samples from four patients. We presented results from these patients in the original publication of the bioassay (reference 12). We just do not have a large enough population of patients in order to, as you say, properly subgroup them.

DR. SAIRAM: I was pleased to see that you have found some evidence for the hypothesis that we advanced following years of work with the deglycosylated gonadotrophic hormones *in vitro*. They are really very effective and specific antagonists.

Have you tested the specificity of the pH 9.5 component which is a FSH antagonist? Does it do anything to LH action?

DR. HSUEH: We did not test that because the FSH bioassay does not respond to LH. We are limited by the amount of material we can obtain, so we have not looked at it in the LH bioassay.

DR. KHAN: I would like to comment on this high pH alkaline FSH that you found. We found similar FSH in pituitaries of castrated monkeys, both in males and females, but surprisingly we had a high biological activity in that fraction corresponding very well with immunoactivity.

The bioactivity was determined by rat Sertoli cell aromatase assay in this case. Would you like to comment on that?

DR. HSUEH: I do not think we are looking at the same molecule. Basically, when we see the pH shift, we are just looking at the removal of sialic acid residues, and many other carbohydrate groups still remain. As long as one removes the terminal sialic acids, you are going to get the same shift in pH. So the pH nine region can represent many different FSH forms.

I do not think we can really compare your study and this study. You can partially remove sialic acid residues, and shift the pH to around the six region. The molecules are much more bioactive *in vitro*, but less active *in vivo*. Once you have two galactose molecules exposed, they are picked up by liver cells.

DR. KHAN: The molecule that was found in the pituitary of castrated monkeys was not found in the intact animals. I think that the molecule is related to the endocrine condition of the monkey.

DR. SHETH: Did you check the ratio effect of inhibin on biologically versus immunologically active FSH?

DR. HSUEH: We do not have enough inhibin to do this type of study. This issue has been raised before.

DR. WINTERS: I was troubled in the studies on aged men. I was surprised that the FSH levels in your bioassay did not rise. I was also surprised that when you treated the men with the GnRH agonist, FSH bioassay levels rose and the immunoassay levels fell. This seems to be different from the data you went on to discuss later. How do you explain those observations?

DR. HSUEH: I do not understand why you are surprised by these data.

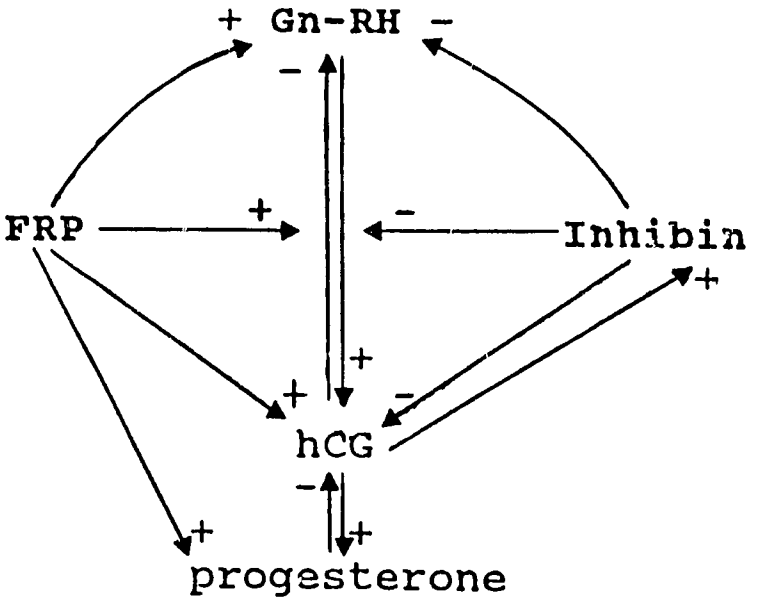


Figure 4. Possible role of inhibin and TSH-related peptides in the mechanism of control of hCG secretion from human placenta.

the implantation of a blastocyst, or of FRP to improve the implantation rate in humans, may be hypothesized.

ACKNOWLEDGMENTS

This research was supported by NIH grants HD13527 and AM26741, and was conducted in part by the Clayton Foundation for Research, California Division. F. Petraglia was partially supported by the Consiglio Nazionale delle Ricerche (350.542). W. Vale is a Senior Clayton Investigator. We thank Dr. Rivier for supplying the peptides, J. Vaughan, A Corrigan, G. Yamamoto, R. Kaizer, B. Galyean, and J. Dykert for their technical assistance.

REFERENCES

1. BANDIVDEKAR AH, VARADKAR AM, SHETH AR: Endocrine control of inhibin biosynthesis by human placenta. Biol Res Pregnancy Perinatol 6:128, 1985
2. BELISLE S, GUEVIN JF, BELIABARBA D, LEHOUX JG: Luteinizing hormone-releasing hormone binds to enriched human placental membranes and stimulates *in vitro* the synthesis of bioactive human chorionic gonadotropin. J. Clin Endocrinol Metab 59:119, 1984
3. BICKSAK TA, TUCKER EM, CAPPEL S, VAUGHN J, RIVIER J, VALE W, HSUEH AJW: Hormonal regulation of granulosa cell inhibition biosynthesis. Endocrinology 119:2711, 1986
4. BILLINGHAM RE: Transplantation immunity and the maternal-fetal relation. N Engl J Med 270:667, 1964

5. BRAUSTEIN GD, GRODIN JM, VAITUKAITIS JL, ROSS GT: Secretory rates of human chorionic gonadotropin by normal trophoblast. Am J Obstet Gynecol 115:447, 1973
6. CARITIS SN, HIRSH RP, ZELEZNIK AJ: Adrenergic stimulation of placental progesterone production. J Clin Endocrinol Metab 56:696, 1983
7. COLE LA, RESTREPO-CANDELO H, LAVY G, DECHERNEY A: hCG free B-subunit an early marker of outcome of *in vitro* fertilization clinical pregnancies. J Clin Endocrinol Metab 64:1328, 1987
8. CONTRACTOR SF, DAVIES H: Effect of human chorionic somatomammotrophin and human chorionic gonadotropin on phytohemagglutinin-induced lymphocyte transformation. Nature (New Biol) 243:284, 1973
9. COSIGNANI PG, TROJSI L, ATTANASIO AEM, FINZI GCL: Value of hCG and hCS measurement in clinical practice. Obstet Gynecol 44:673, 1974
10. CURRIE AJ, FRASER HM, SHARPE RM: Human placental receptors for luteinizing hormone-releasing hormone. Biochem Biophys Res Commun 99:332, 1981
11. FINN R, ST HILL CA, GOVAN AJ, GOVAN AJ, RALFS IG, GURNEY FJ, DENYE V: Immunological responses in pregnancy and survival of fetal homograft. Br Med J [Clin Res] 3:150, 1972
12. FORAGE GG, RING JM, BROWN RW, MCINERNEY BV, COBON GS, GREGSON RP, ROBERTSON DM, MORGAN FJ, HEARN MT, FINDLAY JK, WETTENHALL REH, BURGER HR, DEKRESTER DM: Cloning and sequence analysis of cDNA species coding for the two subunits of inhibin from bovine follicular fluid. Proc Natl Acad Sci USA 83:3091, 1986
13. HANING RV, CHOI AJ, KIGGINS AJ, KUZMA DL, SUMMERVILIE JW: Effects of dibutyryl adenosine 3',5'-monophosphate, luteinizing hormone-releasing hormone and aromatase inhibitor on simultaneous outputs of progesterone, 17b-estradiol and human chorionic gonadotropin by term placental explants. J Clin Endocrinol Metab 55:213, 1982
14. HOCHBERG Z, WEISS J, RICHMAN RA: Inhibin-like activity in extracts of rabbit placentae. Placenta 2:259, 1981
15. HUSSA RO, STORY MT, PATTILLO RA: Cyclic adenosine memophosaphate stimulates secretion of human chorionic gonadotropin and estrogens by human trophoblast *in vitro*. J Clin Endocrinol Metab 38:338, 1974
16. JAFFE RB, LEE PA, MIDGLEY AR: Serum gonadotropins before, at the inception of, and following human pregnancy. J Clin Endocrinol Metab 29:1281, 1969
17. JAFFE RB: Endocrine physiology of the fetus and fetoplacental unit. In YEN SSC, JAFFE RB (eds): Reproductive Endocrinology, p 737. Philadelphia, Saunders, 1986
18. JEPPSON S, RANNEVIK G, LIEDHOLM P, THORELL JI: Basal and LRH-stimulated secretion of FSH during early pregnancy. Am J Obstet Gynecol 127:32, 1977
19. KHODR GS, SILER-KHODR TM: The effect of luteinizing hormone-releasing factor on human chorionic gonadotropin secretion. Fertil Steril 30:301, 1978
20. KHODR GS, SILER-KHODR TM: Placental luteinizing hormone releasing factor and its synthesis. Science 207:315, 1980
21. LING N, YING SY, UENO N, ESCH F, DENOROY L, GUILLEMIN R: Isolation and partial characterization of a Mr 32,000 protein with inhibin from porcine follicular fluid. Proc Natl Acad Sci USA 82:7217, 1985
22. LING N, YING SY, UENO N, SHIMASAKI S, ESCH F, HOTTA M, GUILLEMIN R: Pituitary FSH is released by an heterodimer of the b-subunits from the two forms of inhibin. Nature 321:779, 1986
23. LING N, YING SY, UENO N, SHIMASAKI S, ESCH F, HOTTA M, GUILLEMIN R: A homodimer of b-subunits on inhibin A stimulates the secretion of pituitary follicle stimulating hormone. Biochem Biophys Res Commun 138:1129, 1986
24. MARSHALL JR, HAMMOND CB, ROSS GT, JACOBSON A, RAYFORD PL, ODELL WD: Plasma and urinary chorionic gonadotropin during human pregnancy. Obstet Gynecol 32:760, 1968
25. MASON AJ, HAYFLICK JS, LING N, ESCH F, UENO N, YING SY, GUILLEMIN R, NIALL H, SEEBURG PH: Complementary DNA sequences of ovarian follicular fluid inhibin show precursor structure and homology with transforming growth factor-b. Nature 318:659, 1985
26. MAYO KE, CERELLI GM, SPIEES J, RIVIER J, ROSENFELD MG, EVANS RM, VALE W: Inhibin a-subunit cDNAs from porcine ovary and human placenta. Proc Natl Acad Sci USA 83:5849, 1986
27. MCLACHLAN RI, HEALY DL, ROBERTSON DM, BURGER HG, DEKRETSER DM: The human placenta: a novel source of inhibin. Biochem Biophys Res Commun 140:485, 1986
28. MEUNIER H, EVANS E, VALE W: Control of inhibin α- and β-chain gene expression. Proc Natl Acad Sci USA, 1988

29. MILEWICH L, HENDRICKS TS, GRAHAM JE, GANT NF, SCHWARZ BE, MACDONALD PC: Adenylate cyclase from term human placenta and its regulation. Placenta 3:165, 1982
30. MISHELL DR, THORNEYCROFT IH, NAGATA Y, MURATA T, NAKAMURA RM: Serum gonadotropin and steroid patterns in early human gestation. Am J Obstet Gynecol 117:631, 1973
31. MIYAKE A, SAKUMOTO T, AONO T, KAWAMURA Y, MAEDA T, KURACHI K: Changes in luteinizing hormone-releasing hormone in human placenta throughout pregnancy. Obstet Gynecol 60:444, 1982
32. MIYAMOTO K, HASEGAWA Y, FUKUDA M, NOMURA M, IGANASHI M, KANGAWA V, MATSUO H: Isolation of porcine follicular fluid inhibin of 32K Dalton. Biochem Biophys Res Commun 129:399, 1985
33. OSATHANONDT R, TULCHINSKY D: Placental polypeptide hormones. In TULCHINSKY D, RYAN KJ (eds): Maternal-Fetal Endocrinology, p 17. Philadelphia, Saunders, 1980
34. PETRAGLIA F, LIM ATW, VALE W: Adenosine 3', 5'-monophosphate, prostaglandin and epinephrine stimulate the secretion of immunoreactive gonadotropin-releasing hormone from cultured human placental cells. J Clin Endocrinol Metab 65:198
35. PETRAGLIA F, SAWCHENKO P, LIM ATW, RIVIER J, VALE W: Localization, secretion and action of inhibin in human placenta. Science 237:187
36. PETRAGLIA F, SAWCHENKO PE, RIVIER J, VALE W: Evidence for local stimulation of ACTH secretion by corticotropin-releasing factor in human placenta. Nature 328:717, 1987
37. PETRAGLIA F, VAUGHAN J, VALE W: Hetero- and homo-dimers of inhibin subunits modulate peptide and steroid secretion from cultured human placental cells. [submitted] 1988
38. PURTILO DT, HALLGREN HM, YUNIS EJ: Depressed maternal lymphocyte response to phytohemagglutinin in human pregnancy. Lancet 1:769, 1972
39. RIVIER J, Vale W: Inhibin measurement and role in the immature female rat. Endocrinology 120:1688, 1987
40. RIVIER J, SPIESS J, MCCLINTOCK R, VAUGHAN J, VALE W: Purification and partial characterization of inhibin from porcine follicular fluid. Biochem Biophys Res Commun 133:120, 1985
41. ROBERTSON DM, FOULDS LM, LEVERSHA L, MORGAN FJ, HEARN HTW, BURGER HG, WETTENHALL REH, DEKRETSER DM: Isolation of inhibin from bovine follicular fluid. Biochem Biophys Res Commun 126:220, 1985
42. SARKAR DK: Gonadotropin-releasing hormone-like immunoreactivity in rat placenta. Neuroendocrinology 44:397, 1986
43. SATOH K, RYAN KJ: Adenylcyclase in the human placenta. Biochem Biophy Acta 244:618, 1971
44. SATOH K, RAYN KJ: Prostaglandins and their effects on human placental adenylcyclase. J Clin Invest 51:456, 1972
45. SAVARD K, MARSCH JM, RICE BF: Gonadotropins and ovarian steroidogenesis. Recent Prog Horm Res 21:285, 1965
46. SEEBURG PH, ADELMAN JP: Characterization of cDNA for precursor of human luteinizing hormone-releasing hormone. Nature 311:666, 1984
47. SEPPALA M, WAHLSTROM T, LEHTOVIRTA P, LEE JN, LEPPALUOTO J: Immunohistochemical demonstration of luteinizing hormone-releasing factor-like material in human syncytiotrophoblast and trophoblastic tumors. Clin Endocrinol 12:441, 1980
48. SILER-KHODR TM, KUEHL TJ, VICKERY BH: Effects of a gonadotropin-releasing hormone antagonist on hormonal levels in the pregnant baboon and on fetal outcome. Fertil Steril 41:448, 1984
49. SILER-KHODR TM, KHODR GS, RHODE J, VICKERY BH, NESTOR JJ: Gestational age-related inhibition of placental hCG, α-hCG and steroid hormone release *in vitro* by a GnRH antagonist. Placenta 8:1, 1987
50. SIMPSON ER, MACDONALD PC: Endocrine physiology of the placenta. Annu Rev Physiol 43:163, 1981
51. TAN L, ROUSSEAU P: The chemical identity of immunoreactive LHRH-like peptide biosynthesized in the human placenta. Biochem Biophys Res Commun 109:1061, 1982
52. TSURUHARA T, VAN HALL EV, DUFAU ML, CATT KJ: Ovarian binding of intact and desialylated hCG *in vivo* and *in vitro*. Endocrinology 91:296, 1972
53. VALE W, RIVIER J, VAUGHAN J, MCCLINTOCK R, CORRIGAN A, WOO W, KARR D, SPIESS J: Purification and characterization of an FSH releasing protein form porcine ovarian follicular fluid. Nature 321:776, 1986
54. WATKINS WB, YEN SSC: Somatostatin in cytotrophoblast of the immature human placenta: localization by immuneperoxidase cytochemistry. J Clin Endocrinol Metab 5:969, 1980

55. WHITSETT JA, JOHNSON CL, NOGUCHI A, DAROVEC-BECKERMAN C, COSTELLO M: Beta-adrenergic receptors and catecholamine-sensitive adenylate cyclase of human placenta. J Clin Endocrinol Metab 50:27, 1980
56. YOSHIMITI, STROTT CA, MARSHALL JR, LIPSETT MB: Corpus luteum function in early pregnancy. J Clin Endocrinol Metab 29:225, 1969

DISCUSSION

CHAIRPERSON: DO WON HAHN, Ph.D.

DR. ZILBERTSTEIN: Dr. Petraglia, on which day of culture did you measure the hCG or whatever you did subculture?

DR. PETRAGLIA: The studies for inhibin secretion were carried out one week after the preparation of the cells.

DR. ZILBERTSTEIN: Did your cells come from the syncytiotrophoblast?

DR. PETRAGLIA: There is much recent evidence showing progressive changes of cytotrophoblast to syncytiotrophoblast cells, but you still have both cell populations after one week of culture.

DR. ZILBERSTEIN: At least from our experience, it would be from 24 to 72 hours. Not all, but most of the cells are syncytiotrophoblastic. Do you also suggest that the inhibin from the cytotrophoblast regulates the syncytiotrophoblast which is producing hCG?

DR. PETRAGLIA: Regarding the first question, there are other reports showing that the changes of the cell population in placental cultures depends on the culture medium. There is no drastic change from one population to another.

Regarding the second question, we may suggest either a paracrine effect of inhibins going from one cell to another without going into the blood, or that inhibins are secreted into the blood and syncytiotrophoblast cells to modulate the hCG release. Both situations may exist.

DR. SHETH: Since your preliminary work was done with term placenta, have you seen the effect of human placental lactogen, which is more important at the time on placental inhibin concentrations?

DR. PETRAGLIA: We have not found any effect on human placental lactogen on inhibin secretion from cultured placental cells.

QUESTION: What is the level of hCG production after a week of culturing?

DR. PETRAGLIA: Twenty mM.

14

Identification of a Factor in Fractions of Sertoli Cell-Enriched Culture Medium Containing CMB-21 that Stimulates Androgen Production by Leydig Cells

C. YAN CHENG
PATRICIA L. MORRIS
PREMENDU P. MATHUR
ALLEEN MARSHALL
JOSEPHINE GRIMA
TERRY T. TURNER
SERGE CARREAU
KENNETH S. K. TUNG
C. WAYNE BARDIN

The study of testicular physiology has been severely limited in the past because of the lack of specific markers to monitor testicular function under normal and pathophysiologic conditions. When it was demonstrated that Sertoli cells secrete a large number of proteins into the seminiferous tubular lumen, many studies were performed in an attempt to isolate these proteins and use them to monitor testicular functions (for review see Bardin *et al.*).[1]

Recently, our laboratory developed a novel approach for studying proteins contained in the medium prepared from Sertoli cells of 20-day-old Sprague-Dawley rats.[9] Sertoli cell-enriched culture media were pooled (up to 8 liters) and fractionated by anion-exchange HPLC in a preparative HPLC column (Mono Q, HR10/10, 10 x 100 mm i.d.); proteins that bound to the column were eluted using a linear salt gradient. The elution profile obtained was virtually identical to that of an analytical column.[6,7,9] An aliquot from each fraction was subsequently resolved by SDS-PAGE, and proteins were visualized with silver nitrate. Using this approach, we have identified over 30 proteins in the Sertoli cell-enriched culture medium. Many but not all of these proteins are stimulated by FSH, testosterone, or a combination of FSH plus testosterone.[9] Several of these proteins have been purified and have specific immunoassays established: these include testibumin (CMB-1),[7,8] CMB-2,[14] CMB-22 and CMB-23.[6] However, their physiologic functions in the seminiferous epithelium are not yet known.

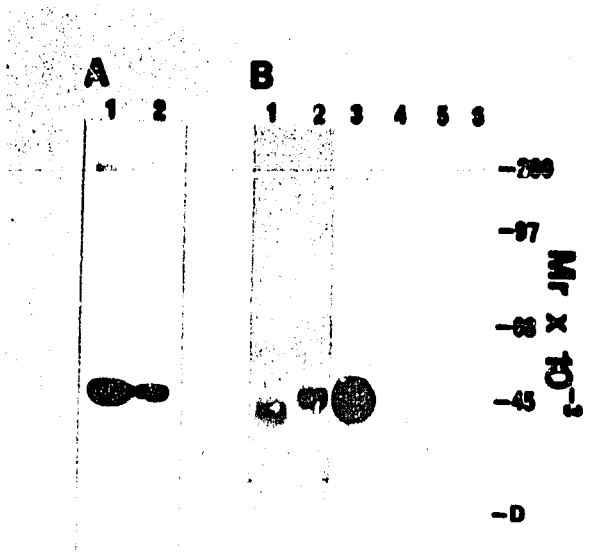
SERTOLI CELL PROTEINS THAT MODULATE LEYDIG CELL FUNCTIONS

It has been known for years that testicular Leydig cells and Sertoli cells are the primary targets of two pituitary hormones, LH and FSH, respectively. The binding of LH to its receptors on Leydig cells elicits a series of biochemical processes that lead to the production of androgens. The physiologic consequences of the coupling of FSH to its receptors in the Sertoli cells [17,20,26] are facilitation of spermatogenesis[4] and regulation of Sertoli cell proliferation during fetal life.[16] *In vivo* studies using hypophysectomized immature rats showed that administration of FSH induced increases in the number of LH receptors and in Leydig cell steroidogenesis.[5,21,23] These observations suggest that FSH plays a role in regulating Leydig cell function. We and others have speculated that FSH induces Sertoli cells to produce a paracrine factor(s) that modulates Leydig cell function. Studies from various laboratories have indicated that media from both Sertoli cell-enriched cultures and seminiferous tubules contain such a factor(s) which stimulates Leydig cell steroidogenesis.[2,13,18,19,25]

EARLY STUDIES ON PURIFICATION OF RAT CMB-21 AND ITS STRUCTURAL AND IMMUNOLOGIC HOMOLOG, RAM TESTICULAR CLUSTERIN

Preliminary investigations from this laboratory indicated that when proteins contained in Sertoli cell-enriched culture medium were fractionated by anion-exchange HPLC, fractions eluted between 0.2 and 0.3 M NaCl contained a factor(s) that modulates Leydig cell steroidogenesis. A protein designated CMB-21 was isolated from these fractions using sequential HPLC columns involving anion-exchange, chromatofocusing, and gel permeation. The highly purified CMB-21 stained with silver nitrate which has an apparent M_r of 43,000 daltons on SDS-polyacrylamide gel under reducing conditions is shown in Figure 1A. These fractions of highly purified CMB-21 contained Leydig cell stimulating activity (see below).

CMB-21 was electro-eluted from SDS-polyacrylamide gel slices, and a monospecific polyclonal antibody was prepared against this protein in rabbit. Use of this antibody for immunoblots showed that CMB-21 shares immunodeterminants with ram clusterin (Fig. 1B) which was isolated from a pool of ram rete testis fluid.[10] Ram testicular clusterin is a heterogeneous glycoprotein of M_r 80,000 daltons consisting of two non-identical subunits with the same apparent molecular weights (M_r 40,000 daltons) on SDS-polyacrylamide gels run under reducing conditions.[9] *In vitro* this protein can be assayed by its ability to aggregate Sertoli cells or erythrocytes from various species. Based on these observations, it was suggested that this protein may be involved in the cell-cell interactions within the seminiferous epithelium.[11] *In vitro* bioassay performed using rat erythrocytes and purified CMB-21 indicated that this protein also has biologic activity similar to that of ram clusterin. Since highly purified CMB-21 has similar *in vitro* biologic activity of ram clusterin (Cheng and Mathur, unpublished observations), we speculate that CMB-21 in rat may be the equivalent of clusterin in the ram. However, it is of note that antibodies prepared against ram clusterin did not cross-react with rat CMB-21 (Cheng, unpublished observations). Also, it is of interest to note that mouse Sertoli cell-enriched culture medium also contains a protein that shares epitopes with rat CMB-21 (Fig. 1B, Fig. 2A). When CMB-21



Figures Fig. 1A, B. Analysis of purified CMB-21 showing its cross-reactivity with Sertoli cell proteins of other species by immunoblots. (A) Purified CMB-21 eluted from gel permeation HPLC was resolved on a 10% T SDS-polyacrylamide gel and stained with silver nitrate. Lanes 1 and 2 represent fractions covered the protein peak of CMB-21 eluted from the HPLC column. (B) Immunblots of crude mouse Sertoli cell-enriched culture medium (lane 1 contained about 3 ug total protein), crude rat Sertoli cell-enriched culture medium (lanes 2 and 3 contained about 3 and 30 ug total protein, respectively), purified ram testicular clusterin (lanes 4 and 5 contained about 2 and 5 ug total protein), and 5 ug each of BSA and ovalbumin in lane 6.

was resolved by SDS-PAGE under non-reducing conditions (Fig. 2B), it had an apparent molecular weight of about 90,000 indicating that this protein is a dimer consisting of two monomers (Fig. 2A,B). However, mouse DMB-21 displayed two immunoreactive bands under non-reducing conditions which might represent the molecular variants of this protein because of its carbohydrate heterogeneity (Fig. 2B).

N-TERMINAL SEQUENCE ANALYSIS OF CMB-21 AND ITS COMPARISON WITH RAM TESTICULAR CLUSTERIN

The N-terminal amino acid sequences for both CMB-21 and ram testicular clusterin were compared. As noted above, ram testicular clusterin consists of two dissimilar subunits; the N-terminal sequences for the two subunits have been determined using o-phthalaldehyde.[10] When one of the clusterin subunits was compared with the N-terminal sequence of CMB-21, 9 of 20 amino acids determined were identical.

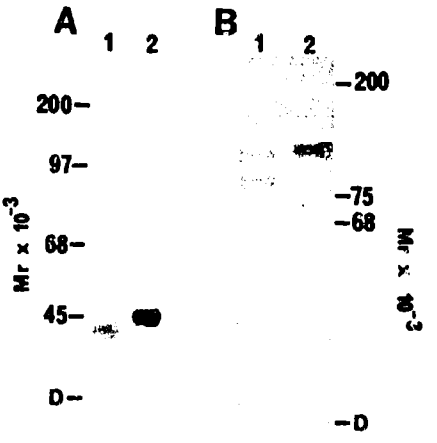


Figure 2A, B. Analysis of CMB-21 by SDS-PAGE under reducing (A) and non-reducing (B) conditions. (A) Mouse (lane 1) and rat (lane 2) primary Sertoli cell-enriched culture media fractionated by anion-exchange HPLC were resolved by SDS-PAGE in which the proteins (total protein content was about 1 ug per lane) were reduced by 2-mercaptoethanol containing in the SDS-sample buffer (0.125M Tris, pH 6.8, at 22C containing 1% SDS, 1.6% 2-mercaptoethanol). (B) Mouse (lane 1) and rat (lane 2) Sertoli cell-enriched culture media fractionated by anion-exchange HPLC (total protein content was about 1 ug per lane) were treated with SDS-sample buffer without 2-mercaptoethanol and resolved by SDS-PAGE. Following electrophoresis, proteins on the SDS-polyacrylamide gels were electrophoretically transferred onto nitrocellulose paper and immunologically stained using anti-CMB-21 antiserum.

In addition to their N-terminal sequence analysis, similarities between CMB-21 and clusterin were evident from the clusterin bioassay and from immunological studies. The highly purified CMB-21 preparations were then assayed for activity which stimulates Leydig cell steroidogenesis and it was shown that these preparations increased testosterone production. However, ram clusterin had no apparent effect on the Leydig cell steroidogenesis. These latter observations suggested that CMB-21 *per se* in view of its similarity to clusterin may not be the protein which stimulated Leydig cells.

TISSUE DISTRIBUTION OF CMB-21

We measured the concentrations of CMB-21 in different biological fluids using immunoblots as shown in Figure 3. This protein is present in reproductive tract fluids including those from rete testis, seminiferous tubule, caput and cauda epididymis. The im-

munoreactive CMB-21 in these fluids displayed two molecular forms with apparent molecular weights slightly different from the one detected in the Sertoli cell-enriched culture medium (Fig. 3). It is of note that immunoreactive CMB-21 in serum also differs from that identified in the reproductive tract fluids. In addition, the immunoreactive CMB-21 in serum is present at a concentration of about 2.5 nmol/ml and its concentration in the interstitial fluid is about 1/5 of that of serum. Since most protein hormones are active in the nM range, we were again led to the conclusion that CMB-21 may not possess Leydig cell stimulating activity.

IDENTIFICATION OF A FACTOR IN THE HIGHLY PURIFIED CMB-21 PREPARATION THAT MODULATES LEYDIG CELL STEROIDOGENESIS

Since highly purified CMB-21 preparations contain a factor that modulates Leydig cell function, we have attempted to isolate it from this material. Fractions obtained from anion-exchange HPLC containing the factor(s) which stimulates Leydig cell steroidogenesis were pooled, concentrated, and fractionated onto a Vydac C4 reverse-phase HPLC column (Fig.

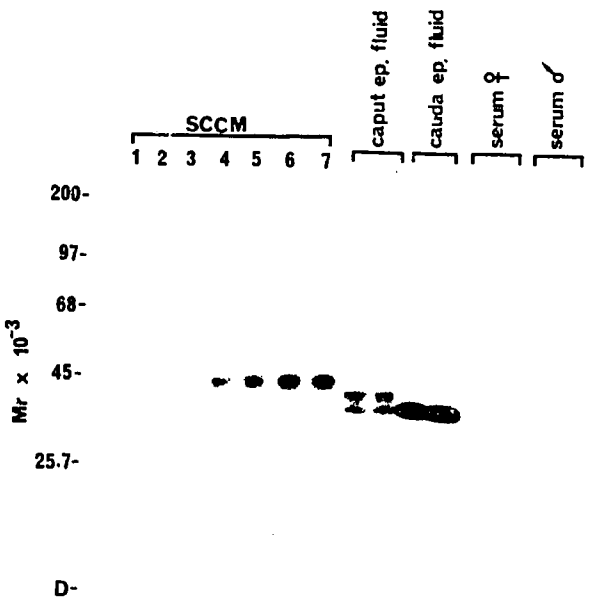


Figure 3. The relative distribution of CMB-21 in Sertoli cell-enriched culture medium, reproductive tract fluids, and serum demonstrated by immunoblots. Lanes 1-7 represent increasing amounts of crude Sertoli cell-enriched culture medium, along with caput epididymal fluid (about 10 ug total protein per lane), cauda epididymal fluid (about 10 ug total protein per lane), male (0.5 ul serum consisting of 30 ug total protein per lane) and female rat serum (0.5 ul serum consisting of 30 ug total protein per lane).

4A). Proteins were eluted using a linear gradient of acetonitrile, and a total of 12 protein peaks were seen. An aliquot from each of these fractions were assayed for Leydig cell stimulating activity using an *in vitro* bioassay system.

Leydig cell-enriched primary cultures were prepared from testes of 20-day-old Sprague-Dawley rats using established procedures.[15] Briefly, testes were decapsulated in F12/DME serum-free medium and treated with 0.05% collagenase/dispase in the presence of 0.005% soybean trypsin inhibitor. A single cell suspension enriched for Leydig cells was obtained by filtration and plated at a concentration of about 0.5 x 10^6 cells/ml. After 3 hours, an aliquot from each column fraction was added onto the well containing 1 ml Leydig cell-enriched culture. Spent media were collected 21 hours later and androstenedione was measured by radioimmunoassay. Control incubations were performed using 10 and 50 pg/ml of hCG and buffer without Sertoli cell proteins.

When each of the fractions shown in Figure 4A was bioassayed for Leydig cell stimulating activity, at least three peaks of activity designated I, II, and III were observed. Fractions

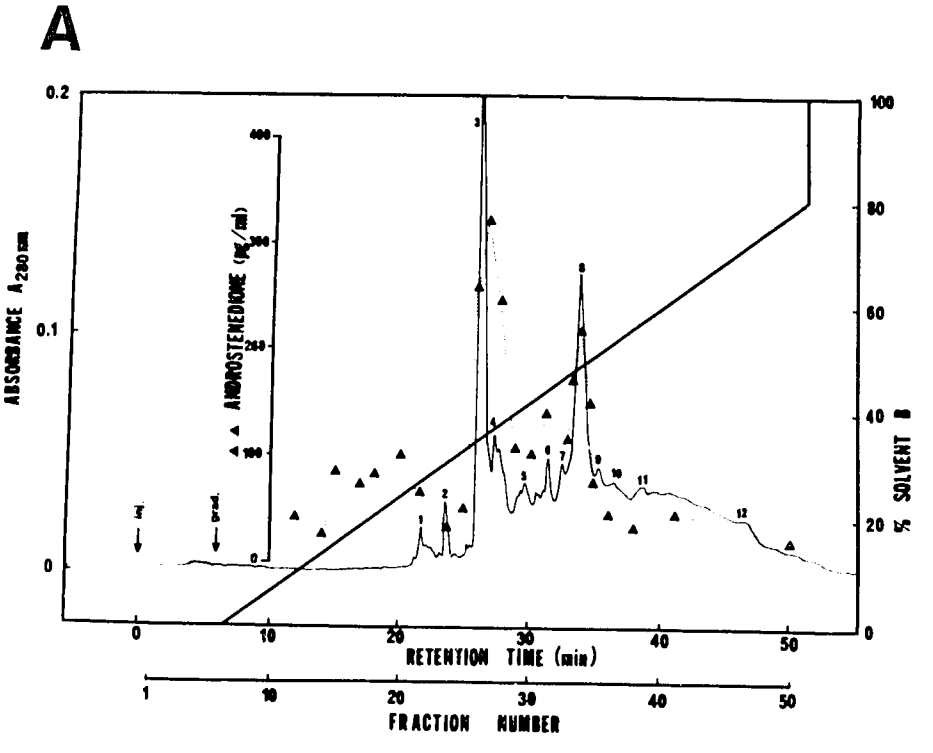
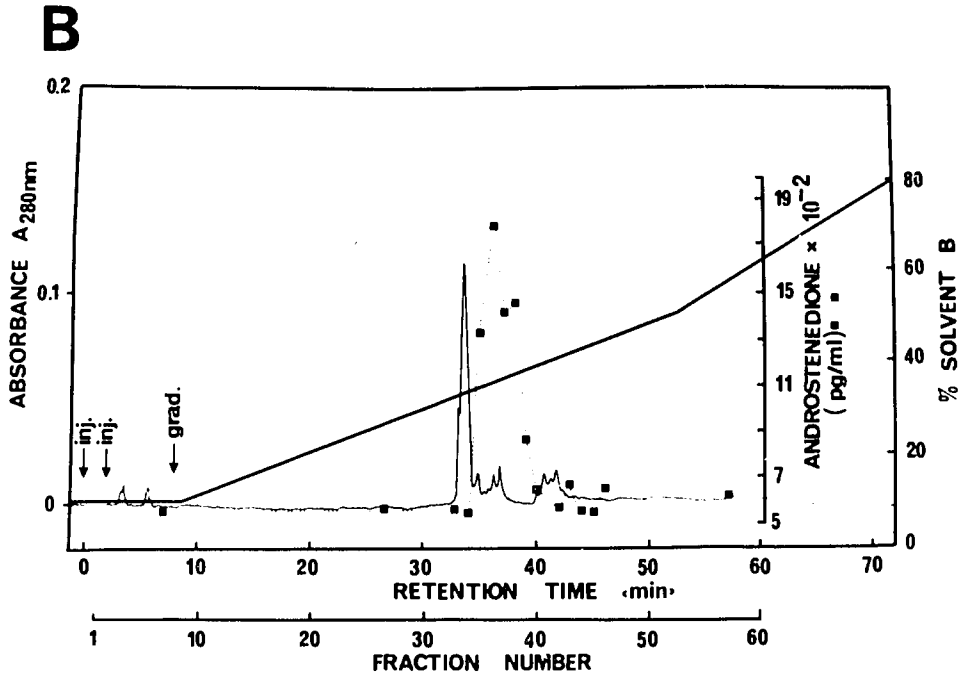


Figure 4A,B. Purification of factors in the Sertoli cell-enriched culture medium that modulates Leydig cell steroidogenesis. (A) Samples obtained from anion-exchange HPLC which contained activities that modulated Leydig cell functions were pooled and fractionated on a Vydac C4 reverse-phase HPLC column (4.6 x 250 mm i.d.). Proteins were eluted using a linear gradient of solvent B (95% acetonitrile/5% water, v/v, containing 0.1% TFA). Solvent A was 5% acetonitrile/95% water, v/v, containing 0.1% TFA. An aliquot

under Peak II were selected for subsequent studies because they had the most potent stimulating activity; in addition, fractions from Peak III were contaminated with CMB-21. Fractions under Peak II were pooled, lyophilized, and loaded onto a Vydac phenyl reverse-phase HPLC column (4.6 x 250 mm i.d.) and eluted using a linear acetonitrile gradient in TFA as shown in Figure 4B. It was shown that the Leydig cell stimulating factor resided between fractions 35 and 38 (Fig. 4B). The molecular identity of this factor is not yet known; work is now in progress to isolate this protein and determine its N-terminal amino acid sequence.



from each fraction was withdrawn and assayed for bioactivity using primary Leydig cell-enriched cultures. (B) Fractions under Peak II obtained from Fig. 4A were lyophilized and loaded onto a Vydac phenyl reverse-phase HPLC column (4.6 x 250 mm i.d.) using the same buffer system as shown in Fig. 4A. Bioactivity of each column fraction was determined by measuring the amount of androstenedione produced by primary Leydig cell-enriched cultures.

CONCLUSION

A testosterone- and FSH-responsive protein designated CMB-21 was isolated from Sertoli cell-enriched culture medium. This protein shares common epitopes and structural features with ram testicular clusterin. Highly purified CMB-21 but not clusterin contains

a factor that stimulates testosterone secretion by Leydig cells. The Leydig cell stimulating factor was separated from CMB-21 by reverse-phase HPLC. This novel peptide may be a short-loop regulator of Leydig cell function that is secreted by Sertoli cells.

ACKNOWLEDGMENTS

This work was supported by NIH grant HD 13541 to CYC and CWB, NIH grant HD 16149 to PLM, NIH grant HD 09490 TT and NIH grant HD 21953-02 to KSKT.

REFERENCES

1. BARDIN CW, CHENG CY, MUSTO NA, GUNSALUS GL: The Sertoli cell. In: The Physiology of Reproduction, Eds. E. KNOBIL, J.D. NEILL, New York, Raven Press, p 933, 1988
2. BENAHMED M, TABONE E, GRENOT C, SANCHEZ P, CHAUVIN MA, MORERA AM: Paracrine control of Leydig cell activity by FSH dependent proteins from Sertoli cells: an *in vitro* study. J Steroid Biochem 24:311, 1986
3. BLASCHUK O, BURDZY K, FRITZ IB: Purification and characterization of a cell-aggregating factor (clusterin), the major glycoprotein in ram rete testis fluid. J Biol Chem 258:7714-7720 1983
4. BREMNER WJ, MATSUMOTO AM, SUSSMAN AM, PAULSEN CA: Follicle-stimulating hormone and human spermatogenesis. J Clin Invest 68:1044-1052, 1981
5. CHEN YDI, PAYNE AH, KELCH RP: FSH stimulating of Leydig cell function in the hypophysectomized immature rat. Proc Soc Exp Biol Med 153:473-475, 1976
6. CHENG CY, BARDIN CW: Identification of two testosterone-responsive testicular proteins in Sertoli cell-enriched culture medium whose secretion is suppressed by cells of the intact seminiferous tubule. J Biol Chem 262:12768-12779, 1987
7. CHENG CY, BARDIN CW: Rat testicular testibumin is a protein responsive to follicle-stimulating hormone and testosterone that shares immunodeterminants with alhumin. Biochemistry 25:5276-5288, 1986
8. CHENG CY, GRIMA J, LEE WM, BARDIN CW: The distribution of rat testibumin in the male reproductive tract. Biol Reprod 37:875-885, 1987
9. CHENG CY, MATHER JP, BYER AL, BARDIN CW: Identification of hormonally responsive proteins in primary Sertoli cell culture medium by high performance liquid chromatography. Endocrinology 118:480-488, 1986
10. CHENG CY, MATHUR PP, GRIMA J: Structural analysis of clusterin and its subunits in ram rete testis fluid. Biochemistry (in press), 1988
11. FRITZ IB, BURDZY K, SETCHELL B, BLASCHUK O: Ram rete testis fluid contains a protein (clusterin) which influences cell-cell interactions *in vitro*. Biol Reprod 28:1173-1188, 1983
12. HON CA, GARDNER PJ, LEUSCHEN MP: Immunocytochemical staining of isolated rat Sertoli cells for anti-FSH beta. Am J Anat 167:441-450, 1983
13. JANECKI A, JAKUBOWIAK A, LUKASZYK A: Stimulatory effect of Sertoli cell secretory products on testosterone secretion by purified Leydig cells in primary culture. Mol Cell Endocrinol 42:235-243, 1985
14. LEE WM, CHENG CY, BARDIN CW, GUNSALUS GL, MUSTO NA: Measurement of a FSH responsive protein of Sertoli cell origin using an enzyme-linked immunoblot assay (ELIBA). Endocrinology 119:1914-1921, 1986
15. MATHER JP, PHILLIPS DM: Establishment of a peritubular myoid-like cell line and interaction between established testicular cell line in culture. J Ultrastruct Res 87:263-274, 1984
16. ORTH JM: The role of follicle-stimulating hormone in controlling Sertoli cell proliferation in testes of fetal rats. Endocrinology 115:1248-1255, 1984
17. ORTH J, CHRISTENSEN AK: Localization of 125_I-labeled FSH in the testis of hypophysectomized rats by autoradiography at the light and electron microscope levels. Endocrinology 101:262-278, 1977

18. PAPADOPOULUS V, KAMTCHOUING P, DROSDOWSKY MA, HOCHEREAU de REVIERS MT, CARREAU S: Adult rat Sertoli cells secrete a factor or factors which modulate Leydig cell function. J Endocrinol 114:449-458, 1987
19. PARVINEN M, NIKULA H, HUHTANIEMI I: Influence of rat seminiferous tubules on Leydig cell testosterone production *in vitro*. Mol Cell Endocrinol 37:331-336, 1984
20. SALHANICK AI, WIEBE JP: FSH receptors in isolated Sertoli cells: Changes in concentration of binding sites at the onset of sexual maturation. Life Sci 26:2281-2288, 1980
21. SELIN LK, MOGER WH: The effect of FSH on LH induced testosterone secretion in the immature hypophysectomized male rats. Endocr Res Commun 4:171-182, 1977
22. SHAHA C, CHENG CY, PHILLIPS DM, TALWAR GP, BARDIN CW: The localization of immunoreactive testibumin in the testes and epididymides of adult rats. Int J Androl (in press)
23. VAN BEURDEN WMO, ROODNAT B, de JONG FH, MULDER E, VAN DER MOLEN HJ: Hormonal regulation of LH stimulation of testosterone production in isolated Leydig cells of immature rats: The effect of hypophysectomy, FSH, and estradiol-17B. Steroids 28:847-866, 1976
24. VERHOEVEN G, CAILLEAU J: A factor in spent media from Sertoli cell-enriched cultures that stimulates steroidogenesis in Leydig cells. Mol Cell Endocrinol 40:57-68, 1985
25. VERHOEVEN G, CAILLEAU J: A Leydig cell stimulatory factor produced by human testicular tubules. Mol Cell Endocrinol 49:137-147, 1987
26. YOON DJ, GOLIMBU M, SCHINELLA R, STEIN B, SKLAR CA, DAVID R: Immunocytochemical localization of hFSH as an index of Sertoli cell function in the human testis. Acta Endocrinol (Copenh) 116:333-338, 1987

DISCUSSION

CHAIRPERSON: DO WON HAHN, Ph.D.

DR. SHAFIQ KHAN: Did you include testosterone in the Sertoli cell culture media, and did you see any influence of this steroid on Sertoli cells?

DR. CHENG: We have added both FSH and testosterone in our Sertoli cell primary cultures. The reason we did that is because studies performed by Dr. Jenny Mather in our laboratory many years ago indicated that the inclusion of these hormones would maximize the secretion of proteins by Sertoli cells.

DR. SHAFIQ KHAN: There is another factor which has been shown to be present in the interstitial fluid of the rat testis, which does not stimulate the basal testosterone production but does enhance the LH-stimulated testosterone production. Did you detect that activity in the Sertoli cell-enriched cultures too?

DR CHENG: We do not know at this stage whether this factor is present in the Sertoli cell-enriched cultures because we have not done those experiments.

DR. SHAFIQ KHAN: Why did you use 20-day-old rats for your testosterone production assay? The Leydig cell is not fully differentiated at that age.

DR. CHENG: We used 20-day-old rats for our bioassay. One of the major reasons is that the contamination with germ cells at that age is minimal.

DR. SHAFIQ KHAN: During that age, the Leydig cells in the rat testis do not produce testosterone as their major product, but 5 α-reduced products. So either you measure total androgens or you should not use rats at that age.

DR. CHENG: We did not measure testosterone. We measured androstenedione because it is the major steroid produced by Leydig cells at that age.

DR. KHAN: Androstenedione is not the major androgen produced by the rat testis, but it is the 5α-reduced androgens.

DR. SHETH: I would like to find out whether apart from studying the steroidogenic effects of this protein on Leydig cells, which you have described, have you studied its possible endocrine effects?

DR. CHENG: No, we have not, because we are just working on the purification of this protein. When we have enough material in our hand to allow us to prepare an antiserum, then we will perform physiological studies.

15

Ovarian Hyperstimulation in Monkeys: Evidence for a Gonadotropin Surge-Inhibiting Factor

ROBERT S. SCHENKEN
GARY D. HODGEN
WENDY H. ANDERSON

The concept that gonads produce a water soluble substance, termed inhibin, which specifically suppresses pituitary follicle stimulating hormone (FSH) secretion has existed for over 50 years. The recent purification and molecular characterization of inhibin in follicular fluid from several species has indeed confirmed its selectivity for regulating FSH, reportedly lacking significant effects on luteinizing hormone (LH) secretion.[5,14,19,20,22] During the past decade accumulating *in vitro* and *in vivo* data have suggested that, in addition to inhibin, follicular fluid contains another peptide that inhibits LH, and perhaps FSH, secretion under certain physiologic conditions.[3,10,12,15,16,21,25,31] In particular, porcine follicular fluid (pFF) has been shown to contain a factor(s) which inhibits the estrogen-induced LH surge and, depending on test conditions, pituitary responsiveness to gonadotropin releasing hormone (GnRH). However, purification and molecular characterization of this factor have not been accomplished.[9]

Our earliest observations on the physiological impact of this active factor involved the surprising absence of timely LH surges in monkeys receiving human menopausal gonadotropins (hMG) to induce multiple follicular maturation.[6] In more recent studies using a pure FSH preparation to induce ovarian hyperstimulation in monkeys, we again observed that, in spite of extraordinary elevation of serum estradiol (E_2), expected midcycle-like LH surges usually did not occur.[27] Simultaneously, most human *in vitro* fertilization/embryo transfer programs using clomiphene citrate with hMG or hMG alone to stimulate multiple follicular development similarly observed that endogenous LH surges were infrequent or delayed despite supraphysiologic levels of circulating E_2.[4,28]

As the data continued to merge, we suggested that primate ovaries secreted a non-steroidal factor, perhaps distinct from inhibin, that attenuated the estrogen-induced LH surge during supraphysiologic gonadotropic stimulation of the ovaries.[24,26] This entity was termed gonadotropin surge-inhibiting factor (GnSIF).[24] In this chapter we will review the studies which led us to conclude that hyperstimulated primate ovaries secrete a non-steroidal factor(s) that regulates the surge mode of pituitary LH/FSH secretion.

BLOCKADE OF THE LH SURGE DURING OVARIAN HYPERSTIMULATION

Preliminary studies in 6 monkeys demonstrated that administration of 25 or 50 I.U. of "pure" FSH (Metrodin® Serono Laboratories, Inc., Randolph, MA) on cycle days 1 to 12 resulted in significant ovarian hyperstimulation, as manifested by development of multiple bilateral follicles (Fig.1) and sustained high serum E_2 levels ($\geq$400pg/ml for 3 to 5 days).[27] In this study, only 1 monkey had a spontaneous LH surge, which occurred on day 7 of treatment and prior to maximal ovarian hyperstimulation.

Figure 1. Hyperstimulated ovaries noted at laparotomy on day 8 of FSH treatment. Both ovaries approached 4 cm in diameter and contained 15-20 large growing follicles; control ovaries were about 1.0 cm on the long axis. From Schenken RS, Hodgen GD: Follicle-stimulating hormone induced ovarian hyperstimulation in monkeys: Blockade of the luteinizing hormone surge. J Clin Endocrinol Metab 57:50, 1983. Reproduced with permission of the publisher.

Studies were then initiated to examine 5 possible explanations for the exogenous FSH-induced inhibition of the LH surge:

1. Premature or excessive elevations of progesterone since (P_4 can suppress estrogen-positive feedback for LH.
2. Multiple follicular development and associated atresia accompanying FSH therapy may have elevated serum androgen levels which, in turn, impeded estrogen-positive feedback.
3. Exogenous FSH treatments resulted in hyperprolactinemia which interfered with estrogen-positive feedback.
4. A potential negative hypothalamic-pituitary feedback by the injected FSH may have resulted in aberrations of endogenous GnRH secretion or pituitary responsiveness sufficient to deter manifestation of estrogen-positive feedback.
5. Persistent supraphysiologic FSH levels led to hypersecretion of an ovarian factor that inhibited the spontaneous LH surge.

MATERIALS AND METHODS

PRIMATE HUSBANDRY

Sexually mature cynomolgus monkeys (*Macaca fascicularis*) having regular menstrual cycles and long-term (greater than 6 months) ovariectomized rhesus monkeys (*Macaca mulatta*) were used. Housing, feeding and general husbandry practices have been described previously.[21] Body weights were 5.1 ± 1.7 kg (mean ± SE).

TREATMENT PROTOCOLS

Monkeys (N=24) were divided into study groups as described in Table 1. Blood was collected daily under ketamine anesthesia (15 mg/kg, i.m.) for 28 days in group 1 and for 11 or more days in groups 2,3,and 4. In group 1,25 or 50 I.U. FSH was administered daily in 2 divided doses for 12 days. No exogenous human chorionic gonadotropin (hCG) or LH was given and no estrogen or GnRH challenge test was performed. Frequent laparotomy/laparoscopy allowed visual assessment of ovarian follicular development in this group. FSH (25 I.U.) or normal saline (1 ml; control) was administered daily for 12 days in groups 2,3 and 4. When an estrogen challenge followed the FSH or control regimen (group 2), 50 mg/kg E_2 benzoate (E_2B) in sesame seed oil was injected at 0800 hour on day 11 of the treatment regimen and blood was collected every 8 hours for 96 hours. When a GnRH challenge followed (groups 3 and 4), 15 mg/kg GnRH was administered on day 12 of the FSH or control treatment regimen and blood was collected at -30, 0, 10, 20, 30, 60, 90, and 120 minutes. Sera were frozen at -15°C until hormone assays were performed.

Conventional radioimmunoassay (RIA) of sera were employed for LH, FSH, prolactin (PRL), E_2, P_4 and androstenedione (A), as described previously.[15] Also, serum LH was measured by a mouse testes interstitial cell bioassay.[22] The RIA employed for FSH measured both the human FSH (injected) and the monkey's endogenous FSH, whereas the RIA for LH did not cross-react with human LH or hCG.[6]

The human FSH preparation (Metrodin®) used was a gift from Serono Laboratories, Inc. Our findings, by both RIA and bioassay, confirmed that less than 1 I.U. LH was pre-

Table 1. Study design to evaluate the FSH-induced inhibition of the LH surge

Group	Status	N	FSH[a] dose (I.U./day)	Treatment regimen	Challenge test
1	Normal intact	3	25	Cycle days 1-12	None
	Normal intact	3	50	Cycle days 1-12	None
2	Ovariectomized	3	25	12 days	E_2B[c]
	Ovariectomized	3	0[b]	12 days	E_2B[c]
3	Normal intact	3	25	Cycle days 1-12	GnRH[d]
	Normal intact	3	0[b]	Cycle days 1-12	GnRH[d]
4	Ovariectomized	3	25	12 days	GnRH[d]
	Ovariectomized	3	0[b]	12 days	GnRH[d]

[a]Metrodin® (Serono Laboratories), injected i.m. in divided dose (morning/evening)
[b]Saline injections, without FSH
[c]One injection of estradiol benzoate (50 mg/kg,i.m.) at 0800 h on day 11 of FSH treatment
[d]One injection of GnRH (15 mg/kg,i.v.) at 0800 h on day 12 of FSH treatment

sent in each 75 I.U. vial. Tests for statistical significance between groups were performed using the F statistic.

RESULTS AND DISCUSSION

GROUP 1

Uniformly, injections of either 25 or 50 I.U. of FSH daily resulted in multiple follicular growth and concurrent elevations of serum E_2. This effect was somewhat less with the lower dose during the initial days of treatment. The serum gonadotropin and steroid levels in the 5 animals that failed to manifest an endogenous LH surge are presented in Figure 2. Note that no discernible changes in serum LH levels as assessed by RIA or bioassay were noted.

Assessment of daily serum P_4 levels and PRL and A levels in sera pooled every 3 days revealed no significant elevation in these hormones as compared to early follicular phase levels in primates. Thus, the blockade of the LH surge was not a result of elevations in these hormones.

GROUP 2

Treatment of ovariectomized monkeys with FSH resulted in a slight increase in serum FSH levels (25 $\pm$ 2.4, μg/ml mean $\pm$ SE) compared to levels in controls given normal saline (20 $\pm$ 1.4 μg/ml mean $\pm$ SE). No significant effects of treatment on serum LH, PRL, E_2, P_4 or A were noted. The effects of E_2B challenges on circulating gonadotropin levels at the end of the FSH or saline treatment regimens are presented in Figures 3 and 4. In

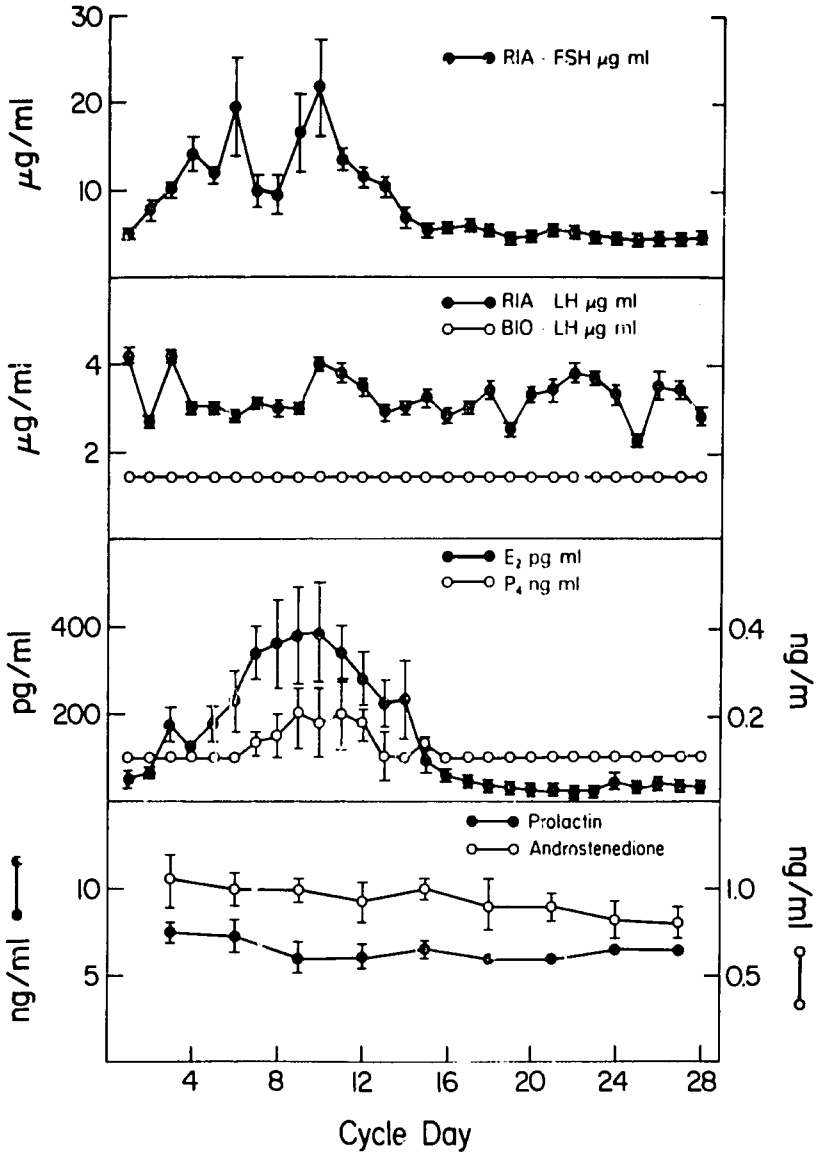


Figure 2. Composite patterns (mean ± SE) of serum LH, (RIA, radioimmunoassayable; BIO, bioassayable), FSH, PRL, E_2, P_4 and A in five intact monkeys treated with FSH (25 or 50 I.U. daily, i.m.; cycle days 1-12). Note the failure of estrogen-positive feedback for the LH. Typically, FSH doses of 12.5 I.U. or less daily did not produce ovarian hyperstimulation. From Schenken RS, Hodgen GD: Follicle-stimulating hormone induced ovarian hyperstimulation in monkeys: Blockade of the luteinizing hormone surge. J Clin Endocrinol Metab 57:50, 1983. Reproduced with permission of the publisher.

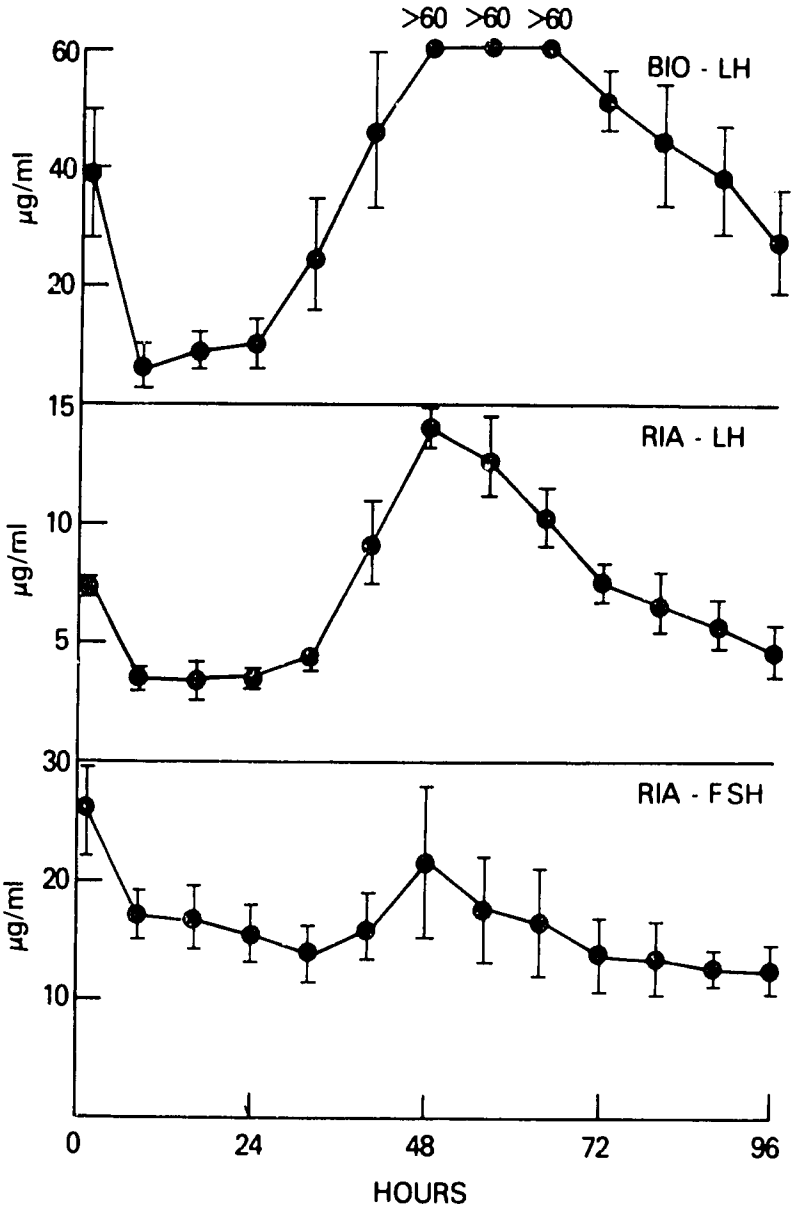


Figure 3. Retention of typical responsiveness of serum bioassayable (BIO) LH and radioimmunoassayable (RIA) LH and FSH (mean ± SE) to an estrogen challenge (estradiol benzoate [50mg/kg, i.m. at time zero]) in long-term castrate monkeys given 12 days of FSH pretreatment (25 I.U. i.m. daily). From Schenken RS, Hodgen GD: Follicle-stimulating hormone induced ovarian hyperstimulation in monkeys: Blockade of the luteinizing hormone surge. J Clin Endocrinol Metab 57:50, 1983. Reproduced with permission of the publisher.

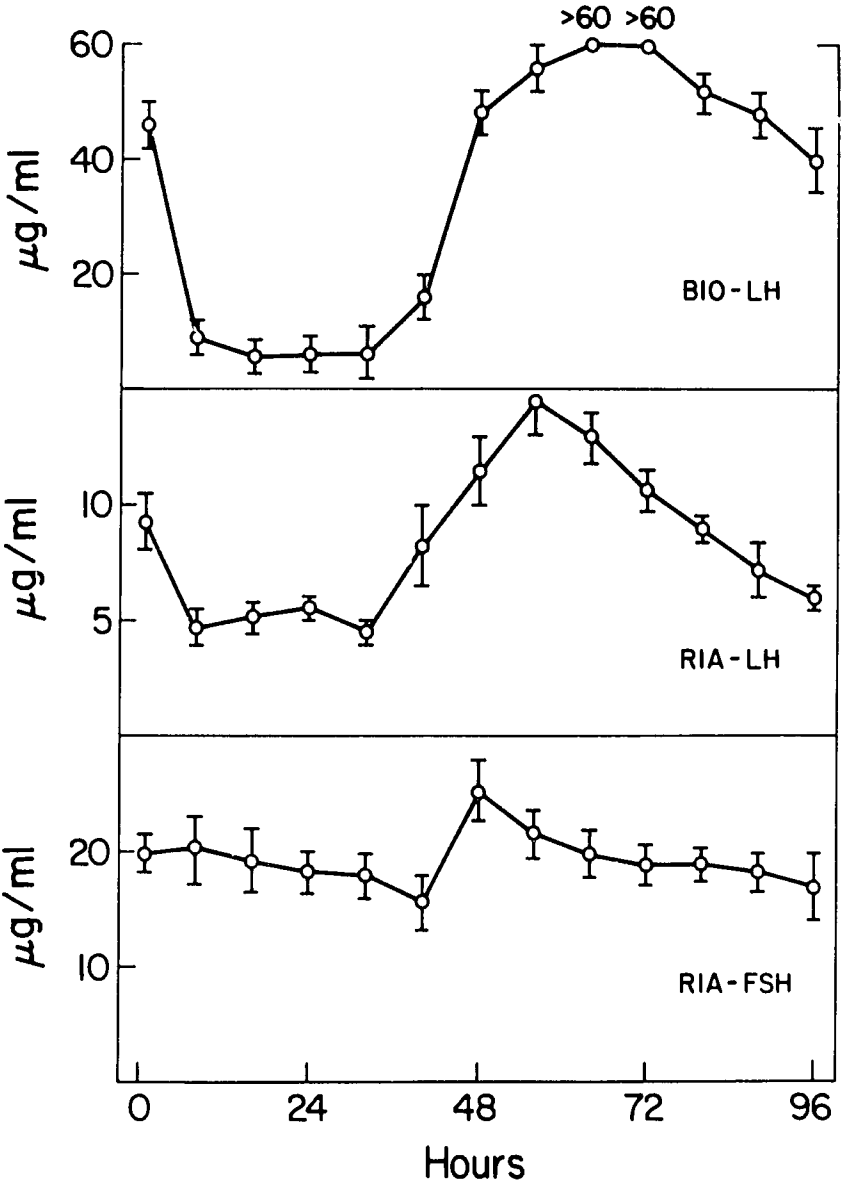


Figure 4. Typical responsiveness of serum bioassayable (BIO) LH and radioimmunoassayable (RIA) LH and FSH (mean ± SE) to an estrogen challenge (estradiol benzoate [50 mg/kg, i.m. at time zero]) in long-term castrate monkeys after 12 days of saline treatment (control 12 ml, i.m.). From Schenken RS, Hodgen GD: Follicle-stimulating hormone induced ovarian hyperstimulation in monkeys: Blockade of the luteinizing hormone surge. J Clin Endocrinol Metab 57:50, 1983. Reproduced with permission of the publisher.

the FSH—treated and control groups, E_3B resulted in similar biphasic responses with transient gonadotropin suppression followed by a surge of LH and FSH. These responses were indistinguishable between groups, eliminating the possibility that the blockage of the estrogen-induced LH surge was due to negative feedback of exogenous FSH on the hypothalamic-pituitary axis.

GROUPS 3 AND 4

Treatment of intact monkeys with 25 I.U. of FSH daily resulted in serum gonadotropin, PRL and steroid levels similar to those observed in group 1. Control monkeys in this group manifested normal follicular phase FSH (1.5 ± 0.12 μg/ml, mean ± SE) and E_2 (105 ± 14.5 pg/ml,mean ± SE) levels. Administration of GnRH to monkeys treated with saline resulted in normal 2-3-fold increases in both LH and FSH; however, the LH and FSH response to GnRH was blunted in monkeys treated with FSH (Fig. 5).

Similar to our findings in group 2, treatment of castrate monkeys with 25 I.U. of FSH daily resulted in increased serum FSH levels (28 ± 1.8 μg/ml, mean ± SE) as compared to control monkeys (19 ± 2.6 μg/ml, mean ± SE). No significant differences in serum LH, PRL, E_2, P_4 or A were noted with FSH treatments as compared to controls. GnRH challenges in group 4 resulted in acute increases in LH that were indistinguishable between FSH-treated and control monkeys (Fig. 6).

The observation that pituitary LH responsiveness to GnRH was maintained in FSH-treated castrate monkeys but not in FSH-treated intact monkeys indicates that a negative feedback of FSH on the pituitary was not responsible for the absence of an endogenous LH surge. Indeed, these findings indicate that FSH treatments impaired pituitary sensitivity to GnRH only when the ovaries were present and manifesting a hyperstimulated condition. Overall, this series of experiments provided persuasive evidence which led us to conclude that an unidentified ovarian non-steroidal factor prevented the LH surge during FSH-induced ovarian hyperstimulation.

GONADOTROPIN-INHIBITING ACTIVITY IN OVARIAN VENOUS PLASMA DURING FSH TREATMENT

Concurrent with our studies on the blockade of estrogen-induced LH surges, several groups reported that inhibin activity in follicular fluid obtained during hMG-stimulated cycles was 3 to 10 times greater than that observed in the follicular fluid from a dominant follicle during a natural cycle.[11,17] Thus, we initiated another study to determine whether FSH-stimulated ovaries secreted a non-steroidal factor(s) which was responsible for inhibiting both basal FSH (inhibin activity) and GnRH-stimulated LH secretion (GnSIF activity) by rat anterior pituitary cells in culture.

MATERIALS AND METHODS

PRIMATE HUSBANDRY

Three sexually mature female cynomolgus monkeys demonstrating menstrual cycle regularity were selected for study. Body weights were 3.2 ± 0.7 kg (mean ± SE).

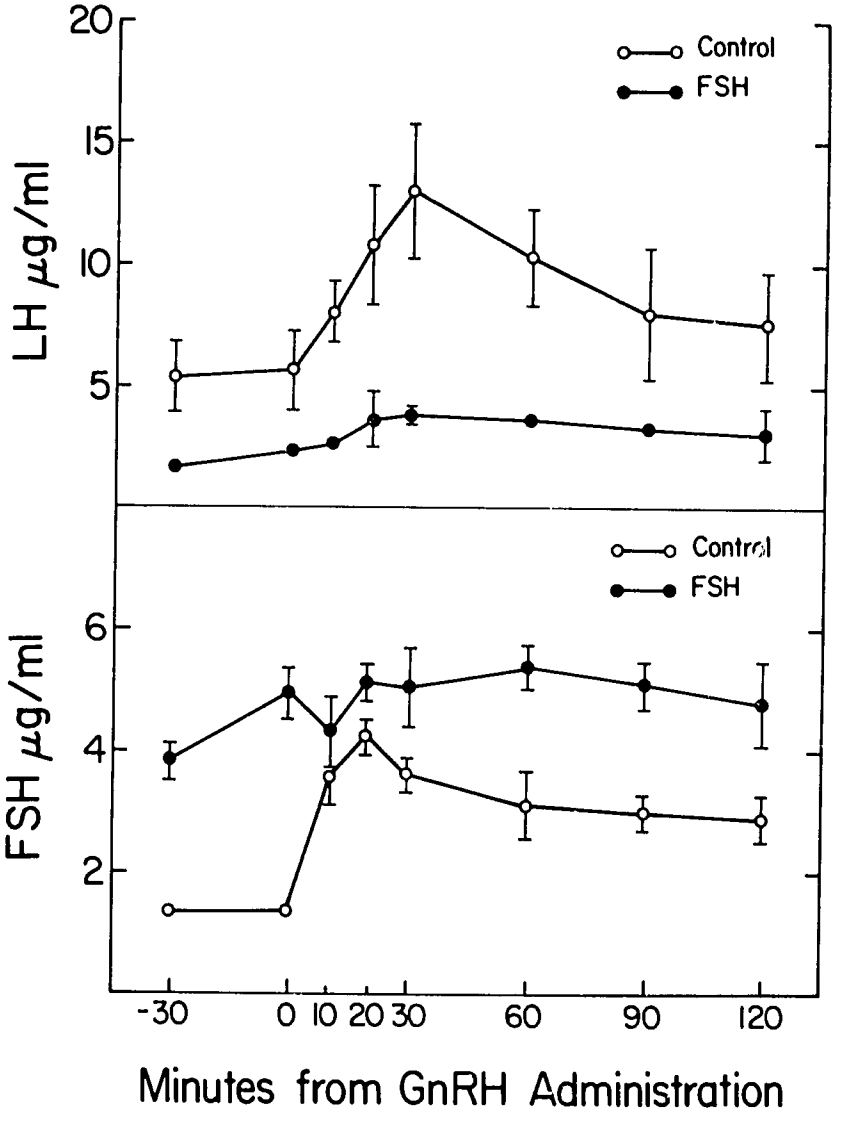


Figure 5. Serum LH and FSH response (mean ± SE) to an acute GnRH challenge (15 mg/kg, i.v. at time zero) in intact monkeys pretreated with either 25 I.U. FSH daily on cycle days 1-12 (•) or 1 ml saline daily on cycle days 1-12 (o). From Schenken RS, Hodgen GD: Follicle-stimulating hormone induced ovarian hyperstimulation in monkeys: Blockade of the luteinizing hormone surge. J Clin Endocrinol Metab 57:50, 1983. Reproduced with permission of the publisher.

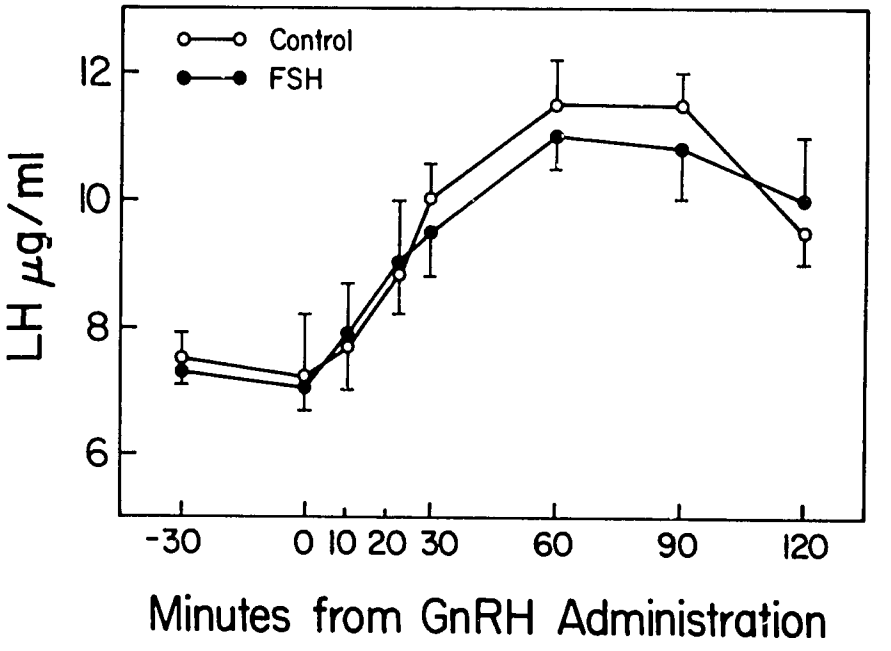


Figure 6. Serum LH levels (mean ± SE) in response to an acute GnRH challenge (15 mg/kg, i.v. at time zero) in castrate monkeys given 12 days of pretreatment with either 25 I.U. FSH daily (•) or 1 ml saline daily (o). From Schenken RS, Hodgen GD: Follicle-stimulating hormone induced ovarian hyperstimulation in monkeys: Blockade of the luteinizing hormone surge. J Clin Endocrinol Metab 57:50, 1983. Reproduced with permission of the publisher.

TREATMENT PROTOCOLS

All monkeys received 25 I.U. of FSH intramuscularly at 0800 and 1600 on cycle days 1 through 11. Laparotomy was performed on cycle day 10 under ketamine (15 mg/kg) anesthesia to visually assess ovarian follicular development and collect bilateral ovarian venous blood. Ovarian venous blood samples were collected with a 25-gauge needle inserted above the anastomosis with the uterine vein. The contralateral utero-ovarian vasculature was temporarily occluded to avoid cross-circulation. After collection of the samples, all visible follicles on the right ovary were aspirated and cauterized. On cycle day 11 laparotomy was again performed under ketamine anesthesia to collect ovarian venous blood as described above.

TREATMENT OF FOLLICULAR FLUID AND OVARIAN VENOUS BLOOD SAMPLES

Follicular fluid was pooled for individual animals and centrifuged at 800 x g for 20 minutes at 4 °C. Supernatants were stored at -20 °C. Venous blood samples were allowed to clot

at room temperature and immediately centrifuged at 1,200 x g for 20 minutes at 4 °C. Sera were stored at -20 °C.

For measurement of gonadotropin-inhibiting activity, an aliquot of each sample was charcoal-extracted to remove steroids. This was achieved by mixing 500 μl of sample with 500 μl of Dulbecco's modified Eagle's medium DMEM; (K.D. Biologicals, Inc., Lanexa, KS) and 50 ml of 10% (weight/volume) dextran-coated charcoal (Norit A, Sigma Chemical Co., St. Louis, MO). The mixture was vortexed overnight at -4 °C and centrifuged at 8,000 x g for 20 minutes. Supernatants were passed through a 0.22-mm Millipore filter (Millipore Corp, Bedford, MA). Steroid-extracted follicular fluid and ovarian venous serum (OVS) samples contained, <50 pg/ml of E_2 and , <0.3 ng/ml of P_4 as determined by RIA.

GONADOTROPIN-INHIBITING ACTIVITY ASSAY

The effects of steroid-extracted follicular fluid and (OVS) samples on basal FSH and GnRH-stimulated LH secretion by rat anterior pituitary cells in culture were determined by the method of Shander *et al.*[92] Briefly, anterior pituitary glands from Sprague-Dawley rats were collected and dispersed by 0.1% hyaluronidase and 0.35% collagenase treatment. After initial enzymatic dispersal, the mixture was centrifuged at 500 x g for 2 minutes and the pellet resuspended in HEPES-buffer solution containing 0.25% viokinase. The dispersed cells were counted using a hemocytometer and 80% of the cells were viable as determined by trypan blue dye exclusion. Viable cells were diluted to a concentration of 5 x 10^5 ml in DMEM growth medium consisting of 10% horse serum, 2.5% fetal calf serum, 0.1 mM glutamine, 1% nonessential amino acids, and penicillin-streptomycin (100 U/ml and 100 μg/ml, respectively). Cells were added to Falcon multiwell dishes (#3008, Falcon Plastics, Los Angeles, CA) in a volume of 0.5 ml growth medium/well and incubated at 37 °C in a water-saturated atmosphere of 5% CO_2:95% air. After cell attachment to the well surface (3 days) the original medium was discarded and cells were washed twice with DMEM.

DMEM containing E_2 (1 x 10^{-8}M), P_4 (1 x 10^{-7}M), and testosterone (1 x 10^{-7}M) was added to each well and 2% steroid-extracted follicular fluid or 20% steroid-extracted venous sera were added to 3 replicate wells. Twelve control wells consisted of 20% steroid-extracted long-term castrate monkey sera. The castrate serum was charcoal-extracted as described above. Steroid-extracted pFF was prepared by centrifuging crude pFF (obtained from Contraceptive Development Branch, NICHD, NIH) and mixing the supernatant with activated charcoal overnight, followed by filtration through a 0.22-μm Millipore filter. The pFF standards were added to triplicate wells in dilution of 0.005%, 0.01%, 0.02%, 0.05%, 0.1%, and 2% with DMEM. Cultures were incubated for 24 hours, at which time the medium was removed from each well and stored at -20 °C until assayed for rat FSH. Then cells were washed twice with DMEM and 500 μl DMEM with GnRH (2 x 10^{-10}M, Sigma) was added to each well. Cultures were incubated for 6 hours and the medium was removed and stored at -20 °C until assayed for rat LH.

The concentrations of E_2 and P_4 in unextracted and steroid-extracted follicular fluid and OVS samples were determined by RIA. Concentrations of rat FSH and LH in unextracted spent media were determined by RIA as described previously.

STANDARD CURVES AND DATA ANALYSIS

The coefficient of variation of each group of 3 replicate wells was calculated. If the coefficient of variation was greater than 15%, the gonadotropin assay was repeated; alternatively, 1 of 3 values was discarded. The mean basal FSH and mean GnRH-stimulated LH secretion in control wells containing castrate serum was set at 100%, and the FSH or LH secretion in each test well was expressed as percentage of control secretion of the respective gonadotropin. According to the method of Channing *et al.*, a standard curve was constructed in which a percent inhibition of basal FSH secretion of pFF standard wells was plotted versus the natural log of the nanoliters of pFF standard added.[26] Least-squares linear regression was used to construct the standard curves and linear portion of the dose response curve. The FSH dose response curve was analyzed by computer program and the inhibin activity in each unknown fluid was determined from the standard curve. Inhibin activity was expressed as units/10 μl fluid with 1 unit inhibin activity defined as that activity in 1 nanoliter of steroid-extracted pFF.

STATISTICAL ANALYSIS

The inhibition of basal FSH and GnRH-stimulated LH secretion by steroid-extracted OVS, concentrations of E_2 and P_4, and inhibin activity in sera from the right and left ovarian veins before and after aspiration of follicles were compared by paired t-test. The inhibition of basal FSH and GnRH-stimulated LH secretion by steroid-extracted OVS, pFF, monkey follicular fluid (mFF), and control castrate sera was compared by analysis of variance and Dunnett's procedure.

RESULTS AND DISCUSSION

The effects of OVS obtained on cycle days 10 and 11, 2% mFF, and 2% pFF, on 24-hour basal FSH secretion are presented in Figure 7. Right and left OVS obtained on day 10 suppressed basal FSH secretion to 48% ± 9% and 55% ± 8% (mean ± SE), respectively, of the control value. Right OVS obtained after aspiration and cautery of the right ovarian follicles also suppressed basal FSH secretion to 78% ± 13% (mean ± SE) of the control value, but the degree of FSH suppression was significantly less ($P < 0.05$) than observed with right OVS obtained on day 10. The inhibition of basal FSH secretion by OVS obtained from the left ovary with intact follicles on day 11 was significantly greater ($P < 0.05$) than observed with contralateral OVS obtained the same day. The addition of 2% mFF or pFF to pituitary well cell cultures resulted in a dramatic reduction of basal FSH secretion as compared to control ($P < 0.01$).

The inhibin activity in the steroid-extracted OVS and the steroid concentrations in unextracted follicular fluid and OVS are presented in Table 2. Right and left OVS contained measurable levels of inhibin activity and high concentrations of E_2 and P_4 prior to aspiration of follicles on the right ovary. Aspiration of the right ovarian follicles resulted in a significant decrease in inhibin activity and steroid concentration in the right OVS, whereas the inhibin activity and steroid concentration in the contralateral OVS were not significantly altered.

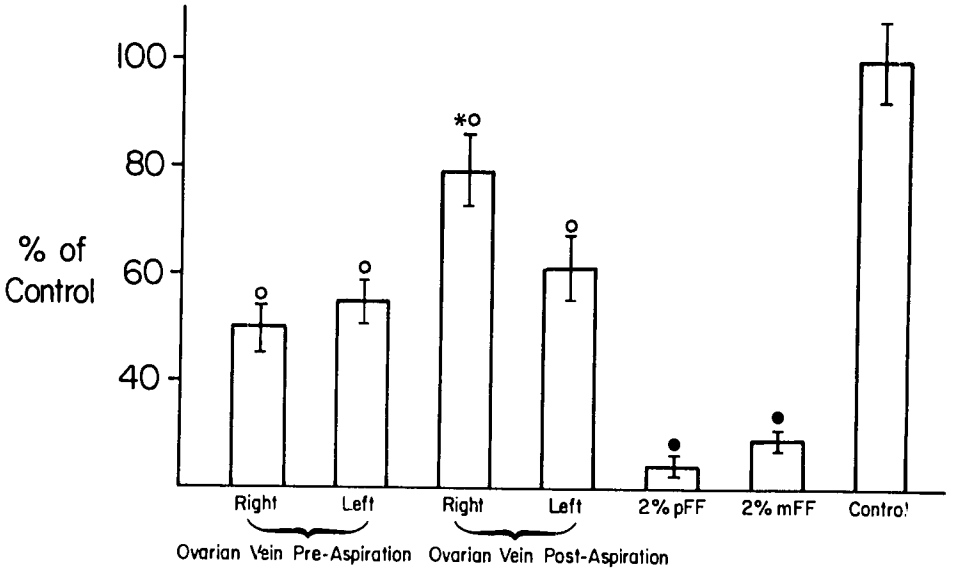


Figure 7. Effects of 20% steroid-extracted right and left OVS, before and after aspiration and cautery of right ovarian follicles; 2% steroid-extracted mFF; and 2% steroid-extracted pFF on basal 24-hour FSH secretion by rat anterior pituitary cell cultures. FSH concentrations in spent media are expressed as mean percentages of the FSH concentrations in control wells containing 20% steroid-extracted castrate monkey serum. Verticle bars represent the standard error of the mean. Asterisk (*) denotes significant difference ($P<0.05$) versus the preaspiration value for the respective vein. Circles denote significant difference (o, $P<0.05$; •, $P>0.01$) versus the control value. From Schenken RS, Anderson WH, Hodgen GD: Follicle-stimulating hormones increases ovarian vein non-steroidal factors with gonadotropin-inhibiting activity. Fertil Steril 42:785, 1984. Reproduced with permission of the publisher, The American Fertility Society.

Table 2.
Inhibin activity and steroid concentration in follicular fluid and OVS before and after aspiration of right ovarian follicles

	Before aspiration		After aspiration		
	Right	Left	Right	Left	mFF
Inhibin activity[a](U/10 ml)	13.1 ± 3.1	10.7 ± 2.6	5.1 ± 1.9[b]	9.9 ± 2.1	181.5 ± 22.5
E_2(pg/ml)a	16,167 ± 2983	14,857 ± 2778	4333 ± 1403[b]	11,436 ± 3100	>4,000,000
P_4(ng/ml)a	4.8 ± 1.1	4.3 ± 1.7	1.8 ± 0.9[b]	3.9 ± 1.3	437 ± 122

[a]Values expressed as the mean ± SE
[b]$P<0.05$ versus before aspiration value

The high levels of inhibin activity in mFF and demonstration of measurable inhibin activity in the venous effluent from hyperstimulated ovaries are supportive of a role for a non-steroidal factor(s) in the regulation of endogenous FSH secretion. These findings are also consistent with those of Lee *et al.* who demonstrated a dose-related increase in ovarian and peripheral vein inhibin activity with concurrent decreases in endogenous serum FSH concentrations after administration of exogenous gonadotropins to rats.[13]

The effects of steroid-extracted OVS obtained on cycle days 10 and 11, 2% mFF, and 2% pFF on GnRH-stimulated LH secretion by pituitary cell cultures are presented in Figure 8. Right and left OVS obtained on day 10 decreased GnRH-stimulated LH secretion to 50% ± 11% and 45% ± 16% (mean ± SE) of the control value, respectively ($P < 0.05$). Right OVS obtained subsequent to follicle aspiration and cautery also suppressed GnRH-stimulated LH secretion, but the suppression was less than observed with OVS obtained on day 10 ($P < 0.05$). OVS obtained from the left ovary on day 11 continued to suppress

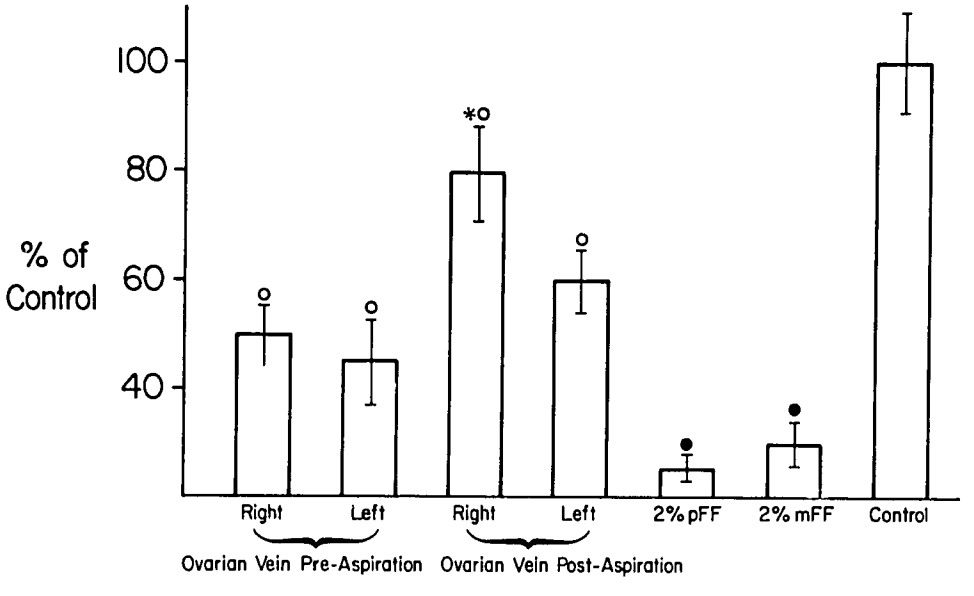


Figure 8. Effects of 20% steroid-extracted right and left OVS, before and after aspiration and cautery of right ovarian follicles; 2% steroid-extracted mFF; and 2% steroid-extracted pFF on LH-RH-stimulated 6-hour LH secretion by rat anterior pituitary cell cultures. LH concentrations in spent media are expressed as mean percentages of the LH concentration in control wells containing 20% steroid-extracted castrate monkey serum. Verticle bars represent the standard error of the mean. Asterisk (*) denotes significant difference ($P < 0.05$) versus the preaspiration value for the respective vein. Circles denote significant difference (o, $P < 0.05$; ●, $P < 0.01$ versus the control value. From Schenken RS, Anderson WH, Hodgen FD: Follicle-stimulating hormones increases ovarian vein non-steroidal factors with gonadotropin-inhibiting activity. Fertil Steril 42:785, 1984. Reproduced with permission of the publisher, The American Fertility Society.

GnRH-stimulated LH secretion to 60% ± 11% (mean ± SE) of the control value. A marked suppression of GnRH-stimulated LH secretion as compared with control values was noted with the addition of 2% mFF and pFF to pituitary cell cultures.

These findings clearly demonstrated that follicular fluid and venous effluents from hyperstimulated primate ovaries contain a nonsteroidal factor(s) with GnSIF activity. Previous studies *in vitro* have also demonstrated that pFF contains a non-steroidal factor that inhibits LH secretion by rat anterior pituitary cells in culture.[16,31] To date, *in vitro* studies have yielded conflicting results. Some have demonstrated LH suppression after pFF injections to rodents and subhuman primates,[10,21,23] whereas other investigators have shown enhanced LH secretion after administration of steroids-extracted follicular fluid.[1,8] These conflicting results are likely due to the variable follicular fluid concentrations of the non-steroidal factors which regulate LH and FSH secretion.

SUMMARY AND CONCLUSIONS

In these studies we have used the primate model to investigate the mechanism by which timely LH/FSH surges are attenuated or blocked during FSH-induced ovarian hyperstimulation. This animal model is similar to women in that LH surges are seldom elicited after hMG treatment despite overt follicular development and sustained elevations in serum E_2 levels. Our preliminary studies demonstrated that elevations in serum PRL, P_4, and A or negative hypothalamic-pituitary feedback by the injected FSH were not responsible for the observed failure of the endogenous LH surge. Our *in vitro* data demonstrated that a non-steroidal factor in the follicular fluid and venous effluent from FSH-induced hyperstimulated primate ovaries inhibited both basal FSH and GnRH-stimulated LH secretion by rat anterior pituitary cells in culture. Aspiration and cautery of the follicles on one ovary resulted in a significant decrease in ipsilateral inhibin and GnSIF activity in the ovarian venous effluent, whereas this activity in the contralateral OVS was not significantly altered. Further, the inhibin and GnSIF activity in follicular fluid was significantly greater than that observed in the OVS in this study. These findings strongly suggest that the exogenous FSH-induced ovarian hyperstimulation increased the ovarian secretion of a nonsteroidal factor(s) which was responsible for inhibiting both basal FSH secretion (inhibin activity) and the spontaneous estrogen-induced LH/FSH surge (GnSIF activity) in monkeys.

Accumulating evidence now suggests that perhaps different nonsteroidal factors are responsible for the observed inhibin and GnSIF activity in follicular fluid, although distinction between inhibin and GnSIF ultimately requires purification of GnSIF to homogeneity. The existence of a separate regulating factor(s) is supported by studies demonstrating that a peptide(s) with molecular weight greater than 10,000 daltons will specifically suppress FSH,[3,12] while a smaller follicular fluid peptide(s) having a molecular weight less than 10,000 will inhibit both LH and FSH secretion by GnRH-stimulated rat antierior pituitary cells in culture.[16,31] Another study demonstrated that pituitary refractiveness to GnRH in monkeys with hMG-induced ovarian hyperstimulated ovaries dissipates for LH release within 60 to 90 minutes after bilateral oophorectomy, whereas GnRH-induced FSH release remains subdued.[15] This suggests that a small labile molecule, which is cleared rapidly from circulaton, is responsible for the GnSIF activity. Further, Sopelak and Hodgen

reported that multiple pFF injections in monkeys led to a neutralization of pFF's suppressive effect on the E_2B-induced LH surge — possibly a result of the intrinsic antigenicity of pFF and subsequent antibody formation to GnSIF.[30]

Danforth and Hodgen have provided additional data to suggest that a peptide, distinct from inhibin, has intrinsic GnSIF activity (see following chapter). Collectively, these studies provide strong evidence that separate non-steroidal factors may regulate LH and FSH secretion in primates. Further work on the isolation and characterization of the peptide with GnSIF activity is warranted in view of its potential use as a contraceptive and as an inhibitor of spontaneous LH surges in patients undergoing ovarian stimulation.

REFERENCES

1. BRONSON FH, CHANNING CP: Suppression of serum follicle-stimulating hormone by follicular fluid in the maximally estrogenized ovariectomized mouse. Endocrinology 103:1894, 1978
2. CHANNING DP, GAGLIANO P, HOOVER DJ, TANABE K, BATTA SK, SULEWSKI J, LEBECH P: Relationship between follicular fluid inhibin-F activity and steroid content. J Clin Endocrinol Metab 52:1193, 1981
3. DE JONG FH, SHARPE RM: Evidence for inhibin-like activity in bovine follicular fluid. Nature 263:71, 1976
4. FERRARETTI AP, SPEZIALE M, FRANCESCHETTI F, MIMMI P, BOLELLI GF, FLAMIGNI C: Superovulation induction with exogenous human gonadotropins. IN LOVERRO G (ed): Human *In Vitro* Fertilization and Embryo Development, p 57. Rome, Bari Congress, Edizini International, 1984
5. FUKUDA M, MIYAMOTO K, HASEGAWA Y, NOMURA M, IGARASHI M, KANGAWA K, MATSUO H: Isolation of bovine follicular fluid inhibin of about 32 kDa. Mol Cell Endocrinol 44:55, 1986
6. GOODMAN AL, HODGEN GD: Post-partum patterns of circulating FSH, LH, prolactin, estradiol, and progesterone in non-suckling cynomolgous monkeys. Steroids 31:731, 1978
7. GOODMAN AL, NIXON WE, JOHNSON DL, HODGEN GD: Regulating of folliculogenesis in the rhesus monkey: Selection of the dominant follicle. Endocrinology 100:155, 1977
8. HERMANS WP, DEBETS MHM, VAN LEEUWEN ECM, DE JONG FH: Time-related secretion of gonadotropins after a single injection of steroid-free bovine follicular fluid in prepubertal and adult female rats. J Endocrinol 90:69, 1981
9. HODGEN GD: The dominant ovarian follicle. Fertil Steril 38:281, 1982
10. HODGEN G, CHANNING C, ANDERSON L, GAGLIANO P, TURNER L: On the regulation of FSH secretion in the primate hypothalamic-pituitary ovarian axis. In CUMMING IA, FUNDER JW, MENDELSOHN FAO (eds): Proceedings of the Sixth International Congress of Endocrinology, p 263. Amsterdam, Elsevier-North Holland, 1980
11. HOOVER DY, TANABE K, CHANNING CP: Inhibin secretion by the primate ovary. Semin Reprod Endocrinol 1:279, 1981
12. LAGACE L, LABRIE F, LORENZEN J, SCHWARTZ NB, CHANNING CP: Selective inhibitory effect of porcine follicular fluid on follicle-stimulating hormone secretion in anterior pituitary cells in culture. Clin Endocrinol (Oxf) 10:401, 1979
13. LEE VWK, McMASTER J, QUIGG H, FINDLAY J, LEVERSHA L: Ovarian and peripheral blood inhibin concentrations increase with gonadotropin treatment in immature rats. Endocrinology 108:2403, 1981
14. LING N, YING S-Y, UENO N, ESCH F, DENOROY L, GUILLEMIN R: Isolation and partial characterization of a M_r 32,000 protein with inhibin activity from porcine follicular fluid. Proc of Natl Acad Sci 82:7217, 1985
15. LITTMAN BA, HODGEN GD: Human menopausal gonadotropin stimulation in monkeys: Blockade of the luteinizing hormone surge by a highly transient ovarian factor. Fertil Steril 41:440, 1984
16. LORENZEN JR, CHANNING CP, SCHWARTZ NB: Partial characterization of FSH suppressing activity (folliculostatin) in porcine follicular fluid using the metestrous rat as an *in vivo* bioassay model. Biol Reprod 19:635, 1978

17. MARRS RP, LOBO R, CAMPEAU JD, NAKAMURA RM, BROWN J, UJITA EL, DIZEREGA GS: Correlation of human follicular fluid inhibin activity with spontaneous and induced follicle maturation. J. Clin Endocrinol Metab 58:704, 1984
18. MARUT EL, WILLIAMS RF, COWAN BD, LYNCH A, LERNER SP, HODGEN GD: Pulsatile pituitary gonadotropin secretion during maturation of the dominant follicle in monkeys: Estrogen positive feedback enhances the biological activity of LH. Endocrinology 109:2270, 1981
19. MASON AJ, HAYFLICK JS, LING N, ESCH F, UENO N, YING S-Y, GUILLEMIN R, NIALL H, SEEBURG PH: Complementary DNA sequences of ovarian follicular fluid inhibin show precursor structure and homology with transforming growth factor-b. Nature 318:659, 1985
20. MASON AJ, NIALL HD, SEEBURG PH: Structure of two human ovarian inhibins. Biochem Biophys Res Commun 135:957, 1986
21. RETTORI V, SILER-KHODR TM, PAUERSTEIN CJ, SMITH CG, ASCH RH: Effects of porcine follicular fluid on gonadotropin concentration in the rhesus monkey. J Clin Endocrinol Metab 54:500, 1982
22. ROBERTSON DM, FOULDS LM, LEVERSHA, MORGAN FJ, HEARN MTW, BURGER HG, WETTENHALL REM, DE KRESTER DM: Isolation of inhibin from bovine follicular fluid. Biochem Biophys Res Commun 126:220, 1985
23. RUSH ME, ASHIRU OA, LIPNER H, WILLIAMS AT, McRAE C, BLAKE CA: The actions of porcine follicular fluid and estradiol on periovulatory secretion of gonadotropin hormones in rats. Endocrinology 108:2316, 1981
24. SCHENKEN RS, ANDERSON WH, HODGEN GD: Follicle-stimulating hormone increases ovarian vein nonsteroidal factors with gonadotropin-inhibiting activity. Fertil Steril 42:785, 1984
25. SCHENKEN RS, ASCH RH, ANDERSON WH: Effect of porcine follicular fluid on the post-castration secretion of gonadotropinsion rhesus monkeys. J Clin Endocrinol Metab 59:436, 1984
26. SCHENKEN RS, HODGEN GD: Follicle-stimulating hormone induced ovarian hyperstimulation in monkeys: Blockade of the luteinizing hormone surge. J Clin Endocrinol Metab 57:50, 1983
27. SCHENKEN RS, HODGEN GD: FSH ovarian hyperstimulation in monkeys. 29th Annual Meeting of the Society for Gynecologic Investigation, Dallas, TX, Abstract No. 282, 1982
28. SCHOEMAKER J, WENTZ AC, JONES GS, DUBIN NH, SAPP KC: Stimulation of follicular growth with "pure" FSH in patients with anovulation and elevated LH levels. Obstet Gynecol 51:270, 1978
29. SHANDER D, ANDERSON LD, BARRACLOUGH CA, CHANNING CP: Interactions of porcine follicular fluid with ovarian steroids and luteinizing hormone-releasing hormone on the secretion of luteinizing hormone and follicle-stimulating hormone by cultured pituitary cells. Endocrinology 106:237, 1980
30. SOPELAK VM, HODGEN GD: Blockade of the estrogen-induced luteinizing hormone surge in monkeys: A non-steroidal, antigenic factor in porcine follicular fluid. Fertil Steril 41:108, 1984
31. YING S, LING N, BOHLEN P, GUILLEMIN R: Gonadostatins and gonadocrinin: Peptides from the gonads regulating the secretion of gonadotropins. In JAGIELLO G, VOGEL HJ (eds): Bioregulators of Reproduction, p 389. New York, Academic Press, 1981

DISCUSSION

CHAIRPERSON: JEFFREY M. SPIELER

DR. GOODMAN: If I understand what you said correctly, the monkeys with intact ovaries that were treated with FSH, and had hyperstimulation of the ovaries, responded appropriately to GNRH. In other words, there is no difference between the animals that did not receive FSH and the animals that did receive FSH; is that correct?

DR. SCHENKEN: The intact, normally-cycling primates that were treated with FSH had a blunted LH response to GNRH.

DR. WINTERS: There seems to be a great deal of steroids in the ovarian venous plasma which also changed after you aspirated the dominant follicle. Why is this not a steroid effect?

DR. SCHENKEN: I do not think it is a steroid effect because the serum samples that we used for the *in vitro* bioassay were charcoal extracted and did not contain significant levels of steroids. In addition, in the bioassay we used a concentration of estradiol, progesterone and testosterone which was several-fold higher than the levels that we obtained after the charcoal extraction. So, I do not think that the steroids were causing the observed effect.

DR. RAJ: Did you give Pergonal, which is a FSH/LH preparation, to the monkeys to induce hyperstimulation, or did you just use FSH?

DR. SCHENKEN: All of these studies were conducted with the pure FSH that was provided by Serono. Subsequently, there were studies conducted with Pergonal that showed essentially similar results.

DR. SHETH: Can you ascribe your results to FSH only or is there a joint contribution by both FSH and LH?

DR. SCHENKEN: I think that is a very interesting question. Obviously, with the pure FSH preparation we observed this effect. We have not looked at LH or hCG administration alone, and we have not quantified the effect to determine whether or not LH is additive.

DR. BIALY: These animals were driven with a lot of FSH. What do we have under normal circumstances? Is there any evidence for the presence of this factor?

DR. SCHENKEN: Yes, I think there are several lines of evidence. You are absolutely correct that we have administered a pharmacological dose of FSH to these primates. The dose is equivalent to giving humans 400 to 600 I.U. of Pergonal daily. However, as I mentioned in the introduction, in IVF programs women undergoing ovarian hyperstimulation frequently, but not uniformly, have gonadotropin surges or LH surges that are, in fact, blocked or attentuated. We have not yet had the opportunity to look at the ovarian venous serum from women undergoing hyperstimulation; however, in the studies by Burger, peripheral inhibin levels are elevated, and they increase to a much greater degree during hyperstimulated cycles than they do during natural cycles.

So, I think it is entirely possible that this factor, be it inhibin or some related factor, is responsible for the observed effect in these hyperstimulated cycles. Clearly, further confirmation of this is necessary.

MR. SPIELER: How do you, then, explain the incidence of premature LH rises in women being hyperstimulated, particularly in IVF programs?

DR. SCHENKEN: There are a couple of possible explanations. We also observed in the primate model that the blockade or attenuation of the LH surge is not seen in every case.

I presented data showing that one of the six monkeys treated had a spontaneous LH surge, and this LH surge occurred very early during the stimulation, approximately on day seven. We certainly see this in women undergoing ovarian hyperstimulation, and it could be due to a number of factors. It could simply be related to the concentration of GnSIF, or inhibin, that is being secreted, and there is some evidence to suggest that this is the case. Nader and colleagues in Houston compared a group of women who had spontaneous LH surges during ovarian hyperstimulation to another group of women that did not have spontaneous LH surges (Fertil. Steril. 45:75, 1986). They found that women who did not have spontaneous LH surges consistently had a greater number of follicles that exceeded, I believe, 15 mm in diameter. Estradiol levels were similar. This suggests that these multiple micro-follicles were secreting greater quantities of inhibin or GnSIF.

DR. KHAN: I would like to know if you ever tried unextracted plasma, since charcoal treatment of the follicular fluid may remove these factors. I am asking this question because some of our studies have shown that if you charcoal extract the follicular fluid, you actually also remove a lot of biologically active proteins.

DR. SCHENKEN: That is a very good point. However, we have not studied non-charcoal extracted plasma.

DR. NEKOLA: Has anybody found this factor in a multiparous animal, such as a pig, where it might be easier to demonstrate a dose response after removing a certain number of follicles?

DR. SCHENKEN: No, not that I am aware of.

16

The Regulation of Pituitary Gonadotropin Secretion by Gonadotropin Surge-Inhibiting Factor (GnSIF) and Inhibin

DOUGLAS R. DANFORTH
GARY D. HODGEN

The regulation of pituitary gonadotropin secretion involves a complex coordination of ovarian (or testicular) steroids, pulasatile GnRH from the hypothalamus, and non-steroidal gonadal factors. The effects of gonadal steroids, and their interaction with GnRH in the regulation of LH and FSH secretion has been thoroughly examined.[6,8,12,13,19] In contrast, the role of nonsteroidal factors from the gonads remains relatively poorly understood. Inhibin is by far the best characterized of these follicular fluid hormones, yet its molecular structure and amino acid acid sequence have only been known for three years.[17,18] In addition, it is now becoming evident that there are several molecular members of the inhibin family with markedly different effects on pituitary gonadotropin secretion.[15,27] The recent elucidation of the molecular structure of inhibin, and the expanding interest in other non-steroidal gonadal factors will surely help unravel the intracies of the physiological control of gonadotropin secretion.

It was almost a decade ago when it was first recognized that administration of exogenous gonadotropins to cynomolgus monkeys resulted in persistently elevated levels of estradiol, however no gonadotropin surges occurred.[10] Subsequently, several investigators have observed that administration of hMG to normally cycling monkeys or women for ovulation induction rarely culminates in gonadotropin surges; ovulation is blocked unless exogenous hCG is administered.[11,7,9] In 1984, the term Gonadotropin Surge-Inhibiting Factor (GnSIF) was coined to represent a nonsteroidal factor which blocked the surge mode of LH and FSH secretion induced by either a bolus of estradiol or GnRH.[26] In the ensuing years, many similar observations *in vivo*[16,21,22,23] have strengthened our understanding of this putative substance, and revealed the need for an assay system to measure GnSIF activity in biological fluids and during a purification procedure.

In what follows, we describe the development of an *in vitro* bioassay for GnSIF activity in follicular fluid, and discuss the evidence that GnSIF and inhibin may be distinct molecular entities, with different physico-chemical characteristics, and differential effects on pituitary gonadotropin secretion.

MATERIALS AND METHODS

The detailed methodology for pituitary cell culture has been previously reported.[4] Briefly, we removed anterior pituitaries from young adult female Sprague-Dawley rats and dispersed the tissue for 1h at 37°C using Earle's Balanced Salts solution, pH 7.4 containing 0.4% collagenase, 0.1% hyaluronidase, 0.1% DNAse, 3.0% bovim serum albumin (BSA), 2.5 mM Hepes, 10 mM dextrose and 100 units/ml Penicillin/100 μg/ml Streptomycin. After dispersion, the cells were washed and counted (using Trypan blue exclusion), and suspended in M199 tissue culture media supplemented with Penicillin/Streptomycin, 10% horse serum, and 2.5% fetal bovine serum.

FOLLICULAR FLUID

Porcine follicular fluid (pFF) was generously provided by the Contraceptive Development Branch, Center for Population Research, NICHD. One liter of pFF was extracted twice with dextran-coated charcoal, filter sterilized (0.22 μM filters), and stored frozen (-20°C) prior to use in bioassay.[4] Estradiol and progesterone levels were reduced by this charcoal extraction procedure to <20 pg/ml and <0.1 ng/ml, respectively. All experiments were performed with charcoal extracted pFF, hereafter referred to simply as pFF.

Human follicular fluid (hFF) was collected from patients undergoing hMG stimulation for *in vitro* fertilization. Patients received 150-225 IU hMG (Pergonal, Serono) on days 3 and 4, followed by 150 IU hMG/day until estradiol levels reached 725-1300 pg/ml. On approximately day 11, 34.5 hours after 10,000 IU hCG was administered im, all visible follicles were aspirated at laparoscopy. Follicular fluid volumes ranged from 1.5 to 4.0 ml and were diluted with 4.0 ml PBS, charcoal extracted, and frozen prior to assay.

HEPARIN SEPHAROSE AFFINITY CHROMATOGRAPHY

Five ml pFF was applied on a 2.6 x 10 cm column of heparin-Sepharose equilibrated with 10 mM Tris-HCl buffer at 25°C, pH 7.8 containing 100 mM NaCl. The follicular fluid was allowed to absorb into the matrix for 15 minutes, and the the non-bound proteins were eluted at 1 ml/min with buffer until absorbance (280 nM) returned to baseline. The proteins bound to the heparin-Sepharose were eluted with 10 mM Tris-HCl containing 2.0 M NaCl. Heparin bound and non-bound proteins were concentrated to original volume on a Centriprep 10 concentrator at 1500 x g and assayed for GnSIF and inhibin activity.

RIA's FOR rFSH AND rLH AND PROTEIN ESTIMATION

Assays for rLH and rFSH were performed using materials provided by the National Hormone and Pituitary Program of the NIADDKD, NIH. Sensitivities of the radioimmunoassays were 0.39 and 3.1 ng/ml, respectively. Protein determinations were performed according to the methods of Bradford[2] using BSA as a standard. The total protein concentration in "crude" pFF was 90 mg/ml. Charcoal extraction of pFF reduced the protein concentration to 62 mg/ml.

STATISTICAL ANALYSES

Mean LH and FSH levels following exposure to pFF and inhibin were analyzed by two-way analysis of variance. After demonstration of significance ($p < 0.05$) with an F-test, comparisons were made between pairs of means with a least significant difference test.[25] Student's t-tests were used to compare GnSIF and inhibin activity in heparin-Sepharose bound and nonbound fractions.

RESULTS

ASSAY OF GnSIF

One of the major problems in identifying and characterizing gonadal hormones which affect pituitary gonadotropin secretion is the lack of a standardized and carefully characterized bioassay system. As such, we spent considerable effort to maximize the characteristics of our pitiutary cell bioassay for GnSIF. We carefully examined the effects of different dispersion methods, plating time, cell concentration, and GnRH dose and stability. In summary, maximal LH production from pituitary cells was maintained following dispersion with collagenase (see Methods), plating 500,000 cells/well for at least 48 hours, and incubating with a dose of 10^{-8}M GnRH for 4 hours.

Next we examined the effects of pFF on GnRH-stimulated LH secretion. Cultures were incubated with 0.01-100 μl pFF for 0-72 hours prior to exposure to GnRH (10^{-8}M) for 4 hours (Fig. 1). Without prior incubation with pFF (0h pre-exposure), GnRH-stimulated LH secretion was unchanged except at very high doses of follicular fluid (100 μl). Longer exposure of pituitary cells to pFF (24-72 hours) prior to GnRH exposure resulted in greater inhibition of LH secretion and greater sensitivity of the GnSIF assay; GnSIF activity was detected in as little as 0.01 μl pFF following 48-72 hours incubation. Maximal LH secretion was beginning to decline following 72 hours preincubation, therefore 48 hours exposure was chosen for all subsequent experiments. Figure 2 illustrates the effects of pFF on basal and GnRH-stimulated LH and FSH secretion by pituitary cells. The cultures were incubated with pFF for 48 hours prior to GnRH stimulation. The presence of 0.01-100 μl pFF inhibited basal FSH, and GnRH-stimulated LH and FSH secretion in a dose-dependent manner. Basal LH production was unaffected. Figure 3 depicts the results of 4 separate experiments on the effects of pFF on GnRH-stimulated LH secretion, our GnSIF "standard curve". We have defined one unit of GnSIF activity as the amount required to inhibit 50% of the GnRH-stimulated LH secretion, and this is contained in approximately 1 μl pFF (62 μg total protein). Linear regression analysis up to the 10 μl dose revealed a straight line with a correlation coefficient (r) of 0.98, and a slope of -16.6. The interassay CV, intraassay CV, and the index of precision for the GnSIF assay were 14.7%, 8.4%, and 0.0063%, respectively.

INHIBIN ACTIVITY

That follicular fluids from several species are rich sources of inhibin activity is well established. The classical action of inhibin is the suppression of basal FSH release with

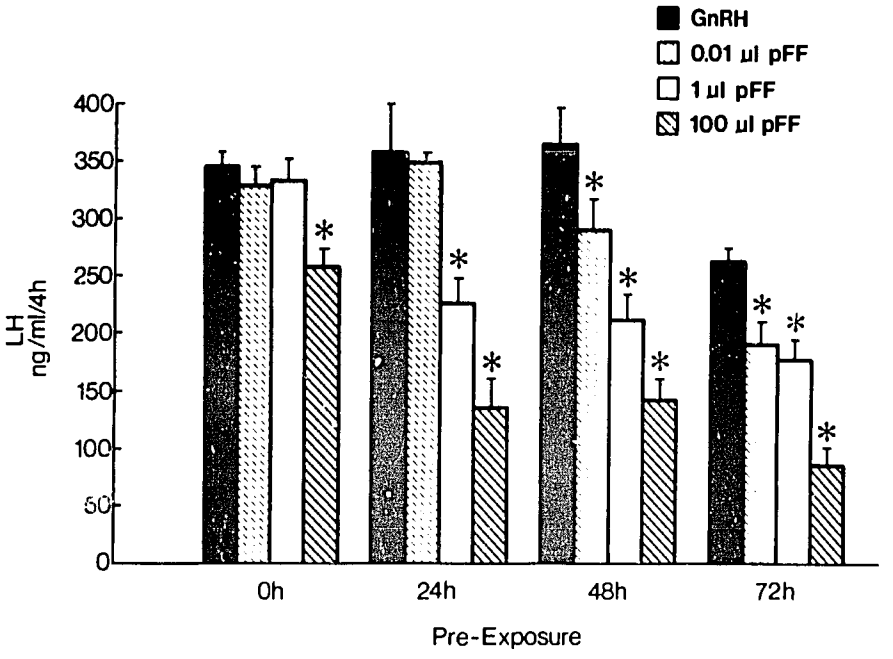


Figure 1. Inhibition of GnRH-stimulated LH release from cultured anterior pituitary cells by pFF. Following 48 hour plating, cultures (500,000 cells/well) were incubated with 0.01-100 μl pFF for 0 hour, 24 hours, 48 hours, or 72 hours, washed twice, and then incubated with 1 x 10^{-8}M GnRH for 4 hours. Each bar represents the mean $\pm$ SE of four observations. Asterisks denote significant differences from control levels.

no effect on LH secretion. We examined this inhibin activity in our pFF preparation (Fig. 4). Pitiutary cells were incubated for 48 hours in the presence of increasing concentrations of pFF, and the amount of basal FSH and LH release was measured. The presence of 0.01-100 μl pFF inhibited basal FSH release in a dose-dependent manner, with no effect on basal LH secretion. However, several investigators, using raw or partially purified follicular fluid, have reported that inhibin will also block GnRH-stimulated LH and FSH secretion as well as basal FSH release.[1,5,24] However, it is impossible from these data to determine whether the effects were due to a single substance or separate molecules in folllicular fluid. More recent studies using pure preparations of porcine and bovine inhibin reveal that inhibin does indeed block GnRH-stimulated LH and FSH secretion in a dose-dependent manner *in vitro,* although much higher concentrations (10-100x) are required to observe this effect[3,20] (these proceedings). Nevertheless, several *in vivo* observations suggest that GnSIF activity and classical inhibin activity can be differentiated, suggesting that there may be more than one substance in follicular fluid which affects GnRH-stimulated gonadotropin secretion. The differences between the GnSIF assay and the inhibin assay are outlined below.

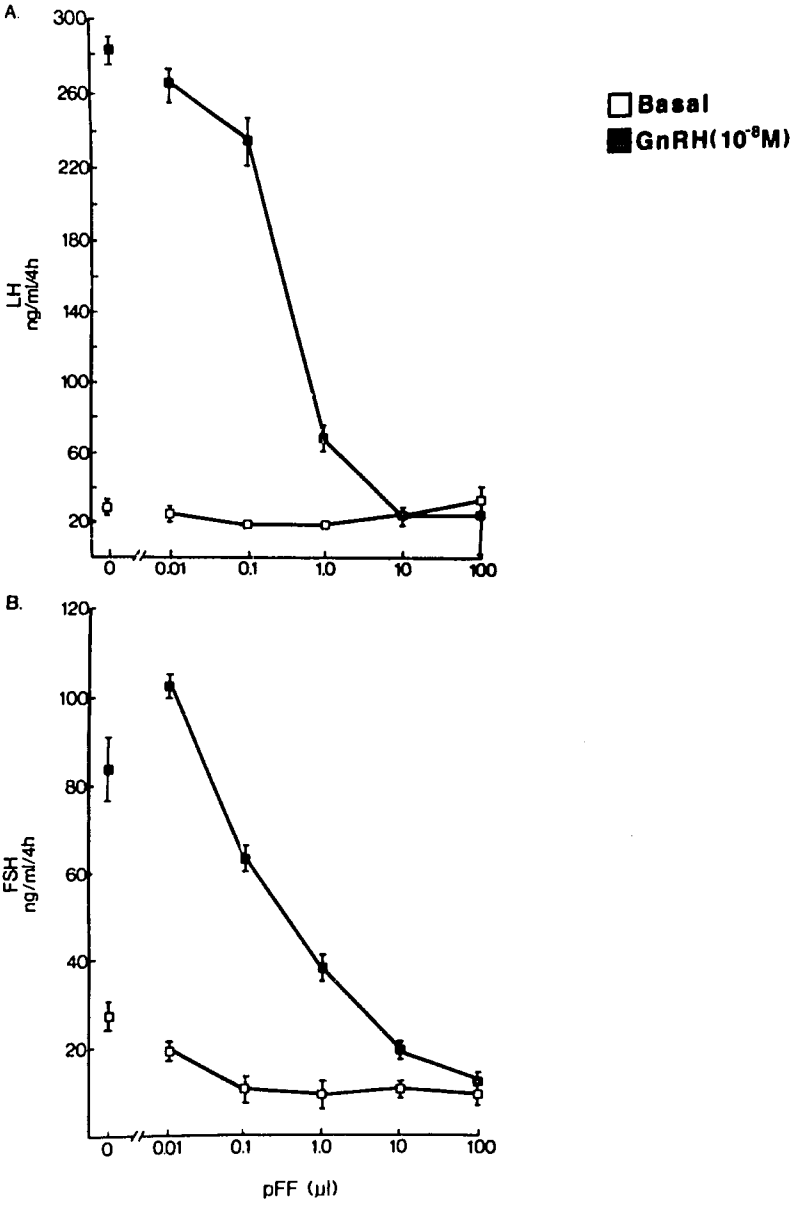


Figure 2. The effect of pFF on basal (□) and GnRH-stimulated (■) LH and FSH release by cultured anterior pituitary cells. After plating, pituitary cells were incubated with 0.01-100 μl pFF for 48 hours, washed twice, and then incubated in the presence or absence of GnRH (1 x 10^{-8}M) and the appropriate amount of pFF for 4 hours. Each point represents the mean $\pm$ SE of 4 observations.

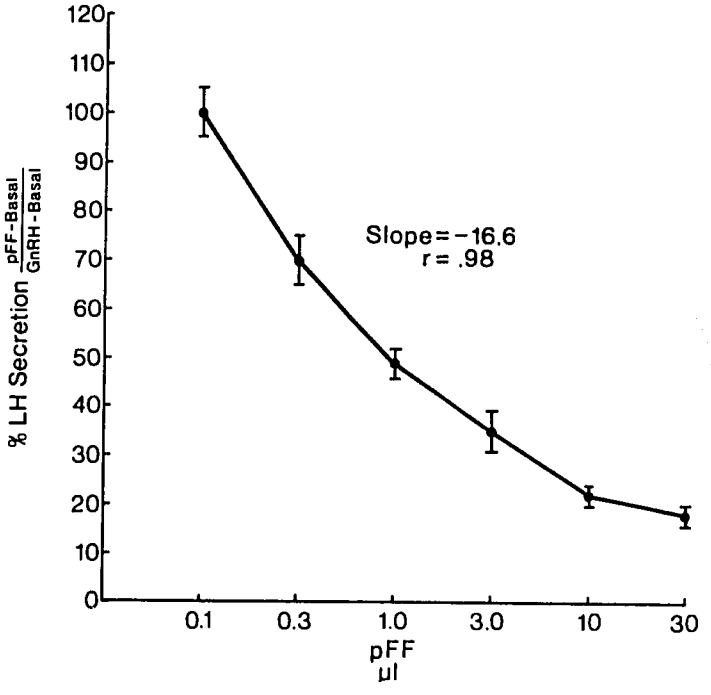


Figure 3. The effect of pFF on GnRH-stimulated LH secretion. Pituitary cells were incubated with 0.1-30 µl pFF for 48 hours, washed twice, and then incubated with 1 x 10^{-8}M GnRH and pFF for 4 hours. LH production is expressed as a percentage of the control value (GnRH only, no pFF) and basal LH has been subtracted out. Each point represents the mean of four separate experiments with four replicates each.

INHIBIN ASSAY	GnSIF ASSAY
- plate cells for 48 hours	- plate cells for 48 hours
- wash, add test substances	- wash, add test substances
- incubate 48 hours, collect media	- incubate 48 hours
- ng FSH/ml/48 hours	- wash, add test substances + GnRH
	- incubate 4 hours, collect media
	- ng LH/ml/4 hours

THE SEPARATION OF GnSIF ACTIVITY FROM INHIBIN

Several different methodologies have been employed to purify inhibin from follicular fluid. One of the most successful is the use of heparin-Sepharose chromatography.[14] Inhibin is among a minor proportion of proteins in follicular fluid which bind to heparin, therefore

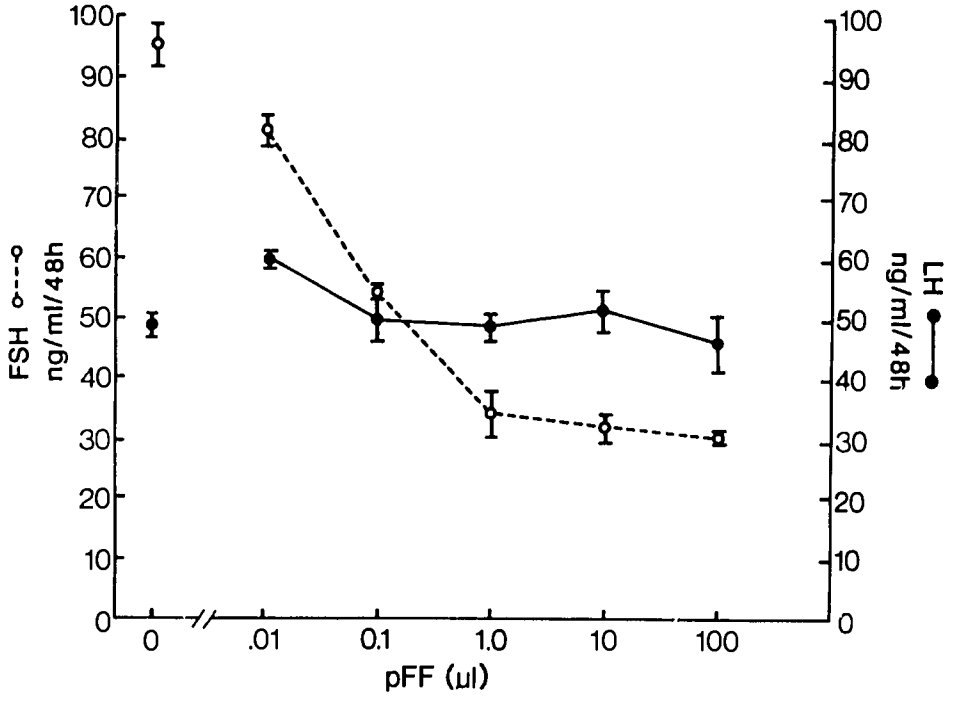


Figure 4. Inhibin activity in porcine follicular fluid. Pituitary cells were incubated for 48 hours with pFF and the media was removed and assayed for FSH and LH. Each point represents the mean ± SE of four observations.

heparin affinity chromatography is a very useful step in the early stages of inhibin purification. We examined the inhibin and GnSIF activity in pFF after passing the follicular fluid through a heparin-Sepharose column (Table 1). As expected, inhibin activity was recovered among those proteins which bound to heparin. However, at the dose tested, GnSIF activity was exclusively located in the non-bound fraction. Dose-response experiments on the bound and non-bound fractions of pFF following heparin-Sepharose chromatography are depicted in Figure 5. Again, GnSIF activity was almost exclusively located in the non-bound fraction; a dose-dependent inhibition of GnRH-stimulated LH secretion was observed with no effect on basal FSH. Conversely, dose-dependent inhibition of basal FSH secretion (inhibin activity) was almost exclusively recovered in the bound fraction; inhibition of GnRH-stimulated LH secretion was only observed at elevated doses (10 and 30 µl, data not shown). Whereas heparin-Sepharose chromatography does not result in significant purification of GnSIF (90-95% of the total proteins remain in the non-bound fraction), it does provide a useful means for removing the confounding influence of inhibin in the GnSIF assay.

As noted earlier, patients undergoing gonadotropin treatment for ovarian stimulation rarely exhibit LH surges, suggesting that preovulatory follicular fluids might be rich in GnSIF and/or inhibin. We examined inhibin and GnSIF activity in individual follicular

fluids obtained from women undergoing hMG/hCG therapy for ovulation induction (Table 2). Inhibin activity was exclusively recovered from follicles containing mature granulosa cells (morphological evaluation at aspiration). In contrast, GnSIF activity was found in follicles containing either mature or immature granulosa cells. Importantly, some follicular fluid samples contained both GnSIF and inhibin, some contained only inhibin, others contained only GnSIF, and one sample contained neither. Figure 6 compares the mean GnSIF and inhibin levels in follicles containing mature vs immature granulosa cells. Whereas inhibin levels were significantly elevated in follicular fluids which contained mature versus immature granulosa cells, GnSIF activity was similar in both groups. These data again

TABLE 1

SEPARATION OF GnSIF AND INHIBIN ACTIVITY BY CHROMATOGRAPHY OF pFF ON HEPARIN-SEPHAROSE

	Inhibin Activity Basal FSH ng/ml/48 h (% Control[a])	GnSIF Activity GnRH-stimulated LH ng/ml/4 h (% Control[b])
Unbound	234 ± 4 (107%)	58 ± 6 (49%)*
Bound	70 ± 2 (32%)*	128 ± 9 (108%)

Values are the mean ± SEM, n = 4
[a]Control = 219 ± 2 ng FSH/ml/48 h
[b]Control = 118 ± 4 ng LH/ml/4 h
*Significantly different from control $p < 0.05$.

suggest that the expression of GnSIF activity, that is the selective supression of GnRH-stimulated LH secretion with no effect on basal gonadotropin production, can be separated from inhibin, both *in vitro* and *in vivo*.

CONCLUSION

In conclusion, we have developed a reliable and sensitive *in vitro* bioassay for the detection of GnSIF activity in follicular fluid. We have significant evidence that GnSIF and inhibin are probably distinct molecular entities, with different physicochemical characteristics, and differential effects on pituitary gonadotropin secretion.

However, until GnSIF is isolated and purified to homogeniety, and its exact molecular structure determined, we must also consider the possibility that GnSIF activity is simply an expression of one of the several molecular members of the inhibin family, if not native inhibin itself.

The importance of GnSIF in the physiological regulation of gonadotropin secretion by the pituitary remains to be determined. Nevertheless, gonadotropin regulation by ovarian "hormones", such as inhibin and possibly GnSIF, may play an important role in formulating a contraceptive strategy during the next decade.

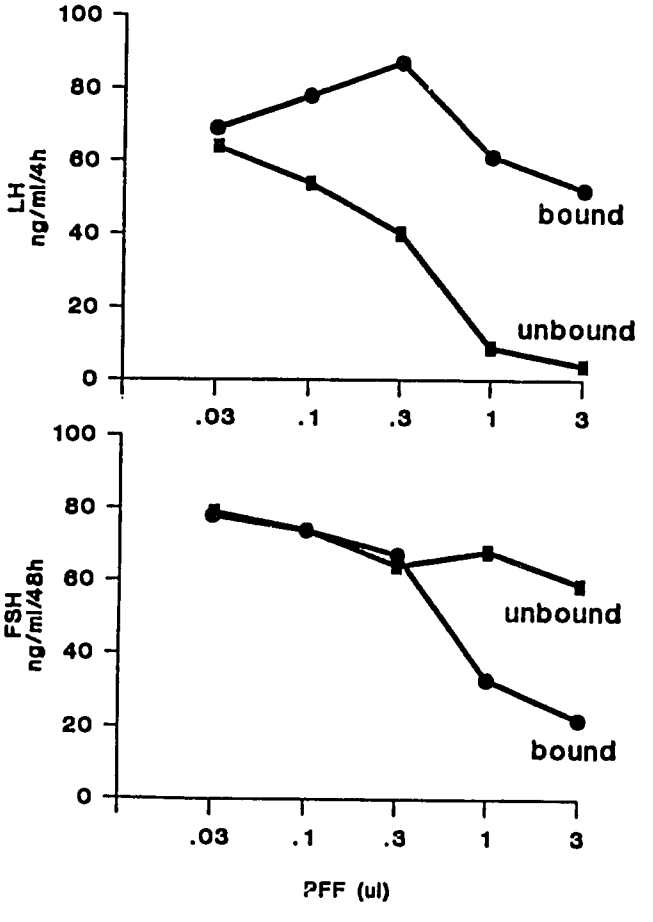


Figure 5. GnSIF activity (top) and inhibin activity (bottom) in the bound and non-bound fractions of pFF after heparin-Sepharose chromatography. Pituitary cells were incubated with increasing doses of the bound and non-bound fractions for 48 hours and the media was removed (inhibin assay, top). The cells were then washed twice and incubated with GnRH (1 x 10^{-8}M) and fractions for 4 hours (GnSIF assay bottom. Each point represents the mean $\pm$ SE of four observations.

Table 2. Inhibin vs GnSIF Activity in Human Follicular Fluid

Follicle #	Granulosa Cell Status	GnSIF Activity GnRH-Stimulated LH ng/ml/4h[a]	Inhibin Activity Basal FSH ng/ml/48h[b]
S-6	Mature	179 +/- 3	* 49 +/- 3
C-1	Mature	* 66 +/- 2	* 36 +/- 1
C-9	Mature	* 61 +/- 10	* 34 +/- 1
C-15	Immature	* 81 +/- 3	115 +/- 12
S-8	Immature	194 +/- 29	109 +/- 4
S-9	Immature	* 87 +/- 7	118 +/- 11
S-10	Immature	* 86 +/- 9	95 +/- 6

Values are the Mean +/- SEM, n=3.
[a] Control (GnRH 10^{-8} M) = 140 +/- 32 ng LH/ml/4h
[b] Control (Basal) = 119 +/- 12 ng FSH/ml/48h
* Significantly different from control ($p<0.05$)

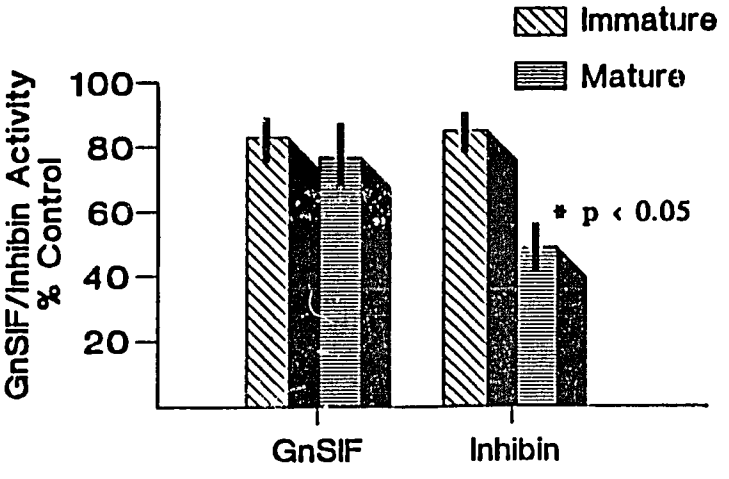


Figure 6. Correlation of inhibin and GnsIF activity with morphological status of the granulosa cells from human follicular fluid aspirates following hMG/hCG treatment. Each bar represents the mean ± SE of at least 4 follicular fluid samples assayed in triplicate.

ACKNOWLEDGMENTS

The authors would like to thank Lynn Sharpe, Susan Poland, Wendie Kaiafsky, and Kim Golightly for their expert technical assistance. We are grateful to Patricia Reese for her help in the preparation of this manuscript.

REFERENCES

1. BAKER HWG, EDDIE LW, HIGGINSON RE, HUDSON B, KEOGH EJ, NIALL HD: Assay of inhibin. In FRANCHIMONT P, CHANNING CP (eds): Intragonadal Regulation of Reproduction. London, New York, Toronto, Sydney, San Francisco: Academic Press, p 193, 1981
2. BRADFORD MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248, 1976
3. BURGER HG: Inhibin—regulation and mechanism of action. In HODGEN GD, ROSENWAKS Z, SPIELER JM (eds): Nonsteroidal Gonadal Factors: Physiological Roles and Possibilities in Contraceptive Development. Proceedings of the CONRAD First International Workshop, Norfolk, Chp 10, 1988
4. DANFORTH DR, SINOSICH MJ, ANDERSON TL, CHENG CY, BARDIN CW, HODGEN GD: Identification of Gonadotropin Surge Inhibiting Factor (GnSIF) in follicular fluid and its differentiation from inhibin. Biol Reprod 37(5):1075, 1987
5. DE JONG FH, SMITH SD, VAN DER MOLEN HJ: Bioassay of inhibin-like activity using pituitary cells *in vitro*. J Endocrinol 80:91, 1979
6. EVERETT JW: Central neural control of reproductive functions of the adenohypophysis. Physiol Rev 44:373, 1964
7. FERRARETTI AP, GARCIA JE, ACOSTA AA, JONES GS: Serum luteinizing hormone during ovulation induction with human menopausal gonadotropin for *in vitro* fertilization in normally menstruating women. Fertil Steril 40:742, 1983
8. FRAWLEY LS, NEILL JD: Biphasic effects of estrogen on gonadotropin-releasing hormone induced luteinizing hormone release in monolayer cultures of rat and monkey pituitary cells. Endocrinology 114:659, 1984
9. GARCIA JE, JONES GS, ACOSTA AA, WRIGHT G JR: Human menopausal gonadotropin/human chorionic gonadotropin follicular maturation for oocyte aspiration: Phase I, 1981. Fertil Steril 39:167, 1983
10. GOODMAN AL, HODGEN GD: Postpartum patterns of circulating FSH, LH, prolactin, estradiol, and progesterone in nonsuckling cynomolgus monkeys. Steroids 31:731, 1978
11. HODGEN GD: The dominant ovarian follicle. Fert Steril 38:281, 1982
12. KAMEL F, KREY LC: Gonadal steroid modulation of LHRH-stimulated LH secretion by pituitary cell cultures. Molecular and Cellular Endocrinology 26:151, 1982
13. KNOBIL E: The neuroendocrine control of the menstrual cycle. In GREEP RO (ed): Recent Progress in Hormone Research, Vol. 36, p 53, Academic Press, New York, 1980
14. LING N, YING S, UENO N, ESCH F, DENOROY L, GUILLEMIN R: Isolation and partial characterization of a M_r 32,000 protein with inhibin activity from porcine follicular fluid. Proc Natl Acad Sci USA 82:7217, 1985
15. LING N, YING S, UENO N, SHIMASAKI S, ESCH F, HOTTA M, GUILLEMIN R: Pituitary FSH is released by a heterodimer of the β-subunits from the two forms of inhibin. Nature 321:779, 1986
16. LITTMAN BA, HODGEN GD: Human menopausal gonadotropin stimulation in monkeys: blockade of the luteinizing hormone surge by a highly transient ovarian factor. Fertil Steril 41:440, 1984
17. MASON AJ, HAYFLICK JS, LING N, ESCH F, UENO N, YING S, GUILLEMIN R, NIALL H, SEEBURG PH: Complementary DNA sequences of ovarian follicular fluid inhibin show precursor structure and homology with transforming growth factor-β. Nature 318:659, 1985
18. MAYO KE, CERELLI GM, SPIESS J, RIVIER J, ROSENFELD MG, EVANS RM, VALE W: Inhibin A-subunit cDNAs from porcine ovary and human placenta. Proc Natl Acad Sci USA 83:5849, 1986
19. MOLL GW, ROSENFIELD RL: Direct inhibitory effects of estradiol on pituitary luteinizing hormone responsiveness to luteinizing hormone releasing hormone is specific and of rapid onset. Biol Reprod 30:59, 1984

20. MIYAMOTO K: Structure and Function of inhibin and its related peptides. In HODGEN GD, ROSENWAKS Z, SPIELER JM (eds): Nonsteroidal Gonadal Factors: Physiological Roles and Possibilities in Contraceptive Development, Proceedings of the CONRAD First International Workshop, Norfolk, chp 5, 1988
21. SCHENKEN RS, HODGEN GD: Follicle-stimulating hormone induced ovarian hyperstimulation in monkeys: blockade of the luteinizing hormone surge. J Clin Endocrinol Metab 57:50, 1983
22. SCHENKEN RS, ANDERSON WH, HODGEN GD: Follicle-stimulating hormone increases ovarian vein nonsteroidal factors with gonadotropin-inhibiting activity. Fertil Steril 42:785, 1984
23. SCHENKEN RS, HODGEN GD: Follicle-stimulating hormone blocks estrogen-positive feedback during the early follicular phase in monkeys. Fertil Steril 45:556, 1986
24. SHANDER D, ANDERSON LD, BARRACLOUGH CA, CHANNING CP: Interactions of porcine follicular fluid with ovarian steroids and luteinizing hormone-releasing hormone on the secretion of luteinizing hormone and follicle-stimulating hormone by cultured pituitary cells. Endocrinology 106:237, 1980
25. SNEDCOR GW, COCHRAN WG: Statistical Methods, ed 6. Ames: Iowa State University Press, 1967
26. SOPELAK VM, HODGEN GD: Blockade of the estrogen-induced luteinizing hormone surge in monkeys: a nonsteroidal, antigenic factor in porcine follicular fluid. Fertil Steril 41:108, 1984
27. VALE W, RIVIER J, VAUGHAN J, MCCLINTOCK R, CORRIGAN A, WOO W, KARR D, SPIESS J: Purification and characterization of an FSH releasing protein from porcine ovarian follicular fluid. Nature 321:776, 1986

DISCUSSION

CHAIRPERSON: JEFFREY M. SPIELER

DR. KARTEN: I would like to make a couple of comments regarding the material that you were using as an inhibin standard.

At that time, NIH had contracted with The Salk Institute to prepare inhibin material. Now, we assumed it was inhibin, but I think it was labeled as inhibin-like. It has a molecular weight of sixty thousand. It was fairly easy to actually make, isolate and prepare. The purification factor was approximately tenfold.

However, more recently, after Dr. Ling had determined the structure of follistatin, we were a little concerned as to whether that original material was inhibin. And the only information I can report now is that the material that you received as an inhibin standard, which is different from the Ward material, does not cross-react with inhibin. We do not know if it is follistatin, but we do know that it does not cross-react with inhibin, and its sequence is currently under investigation.

DR. DANFORTH: Are you talking about the 119-6-4 preparation?

DR. KARTEN: Yes.

DR. PETRAGLIA: Did you try to immunoneutralize the *in vitro* GnSIF activity with an inhibin antiserum?

DR. DANFORTH: Yes and no. We have generated several polyclonal antisera against the first 30 amino acids of the alpha chain of human inhibin. All of the antisera that we have tested cross-react very poorly with porcine inhibin. One of the antisera cross-reacts very well with human inhibin and will neutralize human inhibin activity *in vitro*. We have not tested whether this antisera will block GnRH-stimulated LH secretion as well. We certainly plan to try this.

DR. KHAN: Did you do other pituitary hormones in the GnSIF assay to see whether it was a specific response?

DR. DANFORTH: We sporadically measure FSH, but other than that, we have not checked. And, uniformly, GnRH-stimulated FSH secretion is suppressed as well, although often not to the same degree as GnRH-stimulated LH secretion.

DR. KHAN: That is what concerns me, because the 100 μl dose of follicular fluid you use could have a nonspecific effect because of its very high protein content.

DR. DANFORTH: I agree. In fact, after the preliminary investigations, we never added more than 30 μl of follicular fluid and rarely more than 10 μl.

DR. KHAN: What is the approximate molecular weight of the GnSIF?

DR. DANFORTH: Greater than ten thousand. That is all I can tell you.

We spent a lot of time initially trying to purify this molecule before we knew about the effect of heparin, and we made a lot of progress. Our plans now are to use the heparin nonbound material as our starting material for subsequent purification steps.

DR. FAIMAN: I have some difficulty with the terminology in terms of the GnSIF. The presumption here is that you are really talking about, as you have alluded to, LH release following a GnRH challenge *in vitro.* How does this relate to the *in vivo* surge mechanism? Is it, indeed, gonadotropin releasing hormone-dependent?

DR. DANFORTH: I inherited the terminology, although I think it was appropriate at the time. All of our preliminary data suggesting that this factor exists came from *in vivo* experiments on the inhibition of the gonadotropin surge. Whether or not the material in the nonbound heparin fraction is a gonadotropin surge inhibitor or, more generically, a GnRH-induced LH secretion inhibitor, I do not know at this point.

DR. GOODMAN: Since you have access to human granulosa cells, have you looked at whether media condition by human granulosa cells is active in your assay in the same kind of way that the follicular fluid is? And, since you have an antiserum against human inhibin, can you immunoneutralize the inhibin out of the human granulosa cell conditioned medium and perhaps see an activity remaining that would be an LH suppressor?

DR. DANFORTH: I have not done any work with the granulosa cells from the IVF program. We are, as I said before, going to try the neutralization experiments with our antiserum against the alpha chain of human inhibin.

DR. TSAFRIRI: I wish to push a little bit further than the question raised by Dr. Faiman. In view of the selective preponderance of the GnSIF in early follicular development, would you speculate, then, on a physiological action of this factor?

DR. DANFORTH: We have constructed a hypothetical profile of inhibin activity and GnSIF activity. And this is based on stimulated cycles for IVF, but I think the same pattern may hold true in normal cycles. In the follicular phase, estradiol rises and inhibin rises, maybe not in exactly the same pattern. But at the time of hCG stimulation and follicle aspiration, both estradiol and inhibin decrease, probably more than I have shown in the slides.

Regardless of whether GnSIF peaks early, which agrees with the data from Dr. Schenken, or actually peaks a little bit later, it is clear that most of the GnSIF activity is gone by the time of ovulation or follicle aspiration. I would speculate, although I have absolutely no data for this, that if GnSIF has a physiological role, it would be in maintaining the ovulatory quota and blocking ovulation from occurring at an inappropriate time. GnSIF levels may be higher earlier in the follicular phase; therefore, no ovulation and no LH surges occur. Once GnSIF has decreased to an appropriate level, it may be one of the factors involved in allowing ovulation to occur.

DR. RAJ: The estradiol levels as they occurred in one ovulation in your slide did not take off and remain elevated. From the data of Dr. Hasegawa and his group it is during the descending slope of this estradiol surge that the inhibin drops off. So, I was just wondering whether the GnSIF activity is in some way affecting the estradiol surge in your stimulated cycles. What is the relationship between the level of GnSIF and the high levels of estradiol that you see in ovarian hyperstimulation to the inhibition of the surge of gonadotropin?

DR. DANFORTH: That is a very good point. I have no idea at this time. I think many of the studies on raw follicular fluid can be confounded by the presence of inhibin. Until we separate the inhibin and GnSIF, and look at their effects independently, I think the administration of raw follicular fluid does not give us a clear answer on the relationship between the ovarian steroids to the gonadotropins and to the nonsteroidal factors in follicular fluid.

DR. WILLIAMS: I would like to make a comment directed more at one of the latter questions in Dr. Schenken's presentation about other species. If we think back to the literature of the 1960s when embryo transfer was developing in domestic animals, in this body of literature, in pigs, sheep, and cows, with hyperstimulation, ovulation did not occur, and it became necessary to administer LH or hCG to induce ovulation. And then as preimplantation embryo culture evolved in the late 1960s and Robert Foote and Ralph Mower published methodology papers on how to hyperstimulate rabbits and to do preimplantation culture of rabbit embryos, they also reported that ovulation did not occur when they hyperstimulated a rabbit. And that is most interesting, because these rabbits were being mated and, of course, the rabbit is a reflex ovulator. The fact is that you still had to administer LH to rabbits even after mating to get ovulation when they were hyperstimulated.

17

Experience with Follicle Regulatory Protein as an Inhibitor of Folliculogenesis and Spermatogenesis *In Vivo*

GERE S. diZEREGA
KATSUHIKO FUIMORI
SHARON A. TONETTA
SREEMATHI CHARI
FLORENCE LEDWITZ-RIGBY
BRIAN RIGBY
KATHLEEN E. RODGERS

Using inhibition of aromatase activity in granulosa cells as the reference bioassay, we identified a protein, referred to as follicle regulatory protein (FRP), which was partially purified from human (Chari, unpublished data) and porcine[13,19] follicular fluid. These follicular proteins have similar molecular weights (15,000 kDa) and isoelectric points (pH 4.5). When FRP was tested *in vitro* for inhibin activity (Channing, Wehrenberg and Campeau, personal communications) or injected into female (Fujimori, unpublished results) and male rats,[23,24] no reduction in FSH levels was detected. In addition, FRP demonstrates biochemical and biological properties different from those of growth factors which are known to influence ovarian steroidogenesis, for example, IGF-I, IGF-II, EGF, TGFα, and TGFβ. This chapter will summarize data from studies evaluating both cellular and whole animal responses to FRP. It is important to remember that in none of these studies was homogeneous FRP used, thus effects from contaminants cannot be excluded. The focus of this review will be to outline the potential utility for FRP as a male and female contraceptive.

OVARIAN STUDIES

STEROIDOGENESIS

FRP inhibits aromatase activity in porcine granulosa cells from medium and small-sized follicles.[15] However, FRP does not appear to have an effect on the secretion of estrogen by cells from large follicles. Increases in intrafollicular levels of androgens enhance the sensitivity of FSH-responsive steroidogenic enzymes in granulosa cells to FRP (Ledwitz-Rigby and Rigby, unpublished observation). The demonstration that FRP inhibits aromatase

activity primarily in granulosa cells of less mature follicles supports the hypothesis that FRP may facilitate follicle selection and suggests a role for FRP in atresia.[8] Once the follicle begins to grow, its ability to aromatize its own androgens reduces the sensitivity of the growing follicle to FRP and the likelihood of it becoming atretic. The effects of FRP on steroidogenesis in granulosa cells are reversible unless aromatase activity is inhibited by more than 85-90%.[21] This may correlate with a feature of the preovulatory follicle, namely, that if the dominant ovarian structure (follicle or corpus luteum) is destroyed, no other follicle can soon take its place. Thus, FRP may irreversibly suppress the ability of non-ovulatory follicles to produce estrogen.

FRP induces a biphasic effect on the secretion of progesterone by granulosa cells from sows[1] and rats.[21] At low doses, FRP stimulates the secretion of progesterone while, at higher doses, it is inhibitory. When FRP is added to hMG-treated human and porcine granulosa cells, there is a dose-dependent decrease in the concentrations of progesterone in culture media and cytosol as well as microsomal 3β-hydroxysteroid dehydrogenase activity (3β-HSD)[1-3] FRP-enriched fractions of follicular fluid stimulate basal levels of progesterone by porcine granulosa cells from small and medium-sized follicles, but not by granulosa cells from large follicles. As estrogen and androgen inhibit 3β-HSD activity in granulosa cells, the apparent biphasic effects of FRP on the secretion of progesterone by granulosa cells may be indirectly mediated by alterations in aromatase activity. Testosterone appears to sensitize granulosa cells to FRP-related alterations in the secretion of progesterone (Ledwitz-Rigby and Rigby, unpublished observation). It is unlikely that the effects of FRP on granulosa secretion of progesterone are due to toxicity of FRP, since FSH in high concentration is able to overcome the FRP-induced inhibition of progesterone secretion.[21]

Taken together with the findings that FRP modulates other FSH-responsive activities in granulosa cells, for example, LH/hCG receptor induction,[18] cAMP formation[25] and 3β-HSD activity,[3] these data indicate that recruitment and selection of the preovulatory follicle may occur as a result of early follicular exposure to FSH and a reduction in sensitivity to and/or production of FRP by the follicle. These results demonstrate that the effects of FRP on ovarian steroidogenesis are complex and depend on: 1) the concentration of gonadotropins, especially FSH; 2) the concentration of FRP; and 3) the availability of substrate.

MENSTRUAL CYCLE

Levels of FRP rise beginning in the mid-follicular phase to reach their zenith by the midluteal phase,[14] a pattern which correlates with the decrease in the number of healthy follicles during the periovulatory and midluteal phases of the menstrual cycle.[4,17] FRP is secreted by granulosa cells from small and medium-sized porcine follicles, but not by thecal cells, as determined by both biological[20,22] and immunological assays. Enlargement of the porcine follicle to preovulatory size (8-10 mm in diameter) leads to a marked reduction in granulosa secretion of FRP.[22] Using immunohistochemical techniques, granulosa cells in medium and small-sized follicles in the non-luteal porcine ovary were found to bind antibodies to FRP; whereas, no staining of the theca was seen.[12] Localization of anti-FRP was limited to the perifollicular components outside of the basal lamina in both large-viable and atretic follicles. In porcine ovaries obtained during the luteal phase, staining

was restricted to the large cells of the corpus luteum which are thought to be the successors of granulosa cells from the preceding preovulatory follicle. Recently, Tonetta and Rodgers confirmed that single cell suspensions of porcine luteal cells secrete immunoreactive FRP *in vitro* (unpublished observation). Luteal cells may therefore be a principal source of FRP.

The nadir of FRP levels occur during the early follicular phase, a time which is characterized by the largest number of nonatretic follicles during the menstrual cycle.[4] In the luteal phase nonatretic follicles are not likely to be a predominant source of FRP since their number decreases during this time. This temporal correlation between rising levels of FRP and atretic follicles provides a new focus for further studies of FRP secretion. The small number of healthy follicles available for recruitment by the rise in serum levels of FSH during the late luteal-early follicular phase may be determined by the numbers of follicles which survive the early-midluteal phase. Thus, FRP produced in the periovulatory interval and subsequent luteal phase may play a role in the reduction of the follicular population to a number consistent with the ovulatory quota.

ANOVULATION

A group of anovulatory women was recently found to have elevated levels of immunoreactive FRP in their peripheral serum.[16] A differential ovarian response to clomiphene citrate by these anovulatory patients was observed on days 11-12 of the menstrual cycle. The appearance of abnormalities of follicular recruitment during the intercycle interval was demonstrated by reduced levels of estradiol in serum and concomitant elevations in serum levels of FRP 3-5 days after the onset of the last menstrual period. From these limited data, it is tempting to speculate that anovulation in these patients may result from an overproduction of FRP, presumably by the granulosa cells, and subsequent reduction in follicular responsivity to stimulation by gonadotropins. Additional exposure to gonadotropins was not successful in overcoming this putative FRP-induced block in follicular maturation. Levels of FRP were elevated in other anovulatory patients 11-12 and 22-23 days after the onset of the last menstrual period even after receiving clomiphene citrate therapy on days 3-7 of the cycle. A separate group of anovulatory patients was identified by both relatively low levels of estradiol and FRP in serum determined on cycle days 3-5.[16] Follicles in these patients may be understimulated by gonadotropins. Anovulatory patients who responded to clominhene citrate therapy by increasing their peripheral levels of estradiol on days 11-12 of the menstrual cycle and presumably ovulated, as evidenced by elevated serum levels of progesterone on days 22-23, did not have elevated levels of FRP during these intervals. Thus, inappropriate exposure to elevated levels of FRP could limit the ability of follicles to respond to FSH resulting in suppressed follicular maturation.

FRP TREATMENT: INHIBITION OF OVULATION

Female Sprague-Dawley rats (23 days old) received once daily intraperitoneal injections of a partially purified preparation of FRP beginning either at estrus, proestrus, or metestrus. After 7 days of treatment, all rats manifested leukocytic vaginal smears. Although treatment with FRP was discontinued on treatment day 12 (after 5 consistent days of diestrus smears), all rats remained in diestrus for at least another 10 days. At this time, animals

were sacrificed, ovaries removed for histological evaluation, and trunk blood collected for determination of hormone levels by standard radioimmunoassays. All of the rats had elevations in serum concentrations of progesterone and most of the rats manifested decreased estradiol levels. Histological evaluation of the ovaries from these rats demonstrated granulosa hyperplasia, thecal and stromal hypertrophy, and no evidence of ovulation (Fig. 1).

An FRP-enriched fraction of follicular fluid which did not contain inhibin activity was administered to normally cycling rhesus monkeys in daily intramuscular injections.[9] Normal follicular development was disrupted in all monkeys. Three monkeys treated with FRP had menstrual cycles characterized by long follicular phases, low estradiol values, and subnormal luteal function; two of the monkeys treated with FRP had anovulatory menstrual cycles. The association of abnormally long follicular phases and reduced serum concentrations of estradiol with treatment suggests that FRP has a direct ovarian action which disrupts the normal process of folliculogenesis, resulting in diminished production of steroids by the corpus luteum (luteal insufficiency).[7] In its most complete form, the disruption of folliculogenesis by FRP produced anovulation.

Figure 1. Representative ovary from a 23-day old Sprague-Dawley rat treated with partially purified FRP prepared from porcine follicular fluid. Follicles contained granulosa hyperplasia, thecal and stromal hypertrophy and there was no evidence of ovulation (original magnification 100 x).

We assessed the effects of partially purified FRP on folliculogenesis in intact and hemicastrated guinea pigs.[11] The first day of vaginal opening (either partial or full) in the presence of nucleated and/or cornified cells in the vaginal lavage was designated day 0 of the estrous cycle. All estrous cycles included in this study were immediately preceded by at least two consecutive, regular cycles. The guinea pigs received intraperitoneal injections of FRP or vehicle every morning from day 8 of the estrous cycle until they were sacrificed. The morphology of ovaries after 3 days (days 8-10 of the estrous cycle) or 5 days (days 8-12 of the estrous cycle) of FRP administration demonstrated a significant increase in the percentage as well as the actual number of atretic follicles. Since atretic follicles predominantly emanate from the population of follicles >400 μm in diameter, morphometric quantitation of atretic follicles was performed on those large follicles. The size distribution of follicles on day 10 of the estrous cycle (after 3 days of FRP therapy) showed an increase in the number of atretic follicles 500-800 μm in diameter. If FRP suppressed aromatization in follicles of all size categories, an increase in the number of atretic follicles would have occurred in each size category. However, an increase in the number of FRP-induced atretic follicles was observed only in the group of follicles 500-800 μm in diameter, the preferential size range for atresia in the guinea pig. Thus, exogenous FRP did not significantly inhibit follicular growth per se, but rather facilitated or amplified ongoing atresia.

ANTI-FRP TREATMENT

To determine if treatment with anti-FRP affected ovarian function, 23 day old Sprague-Dawley rats with regular 4 day estrous cycles were treated beginning on day 1 of the estrous cycle with rabbit antisera prepared against porcine FRP. Treatment was continued for 4 days (one estrous cycle) or 8 days (N=5, 4 respectively). A control group of rats was treated with non-specific antisera (daily, IP) for 4 (N=5) or 8 (N=4) consecutive days. Thereafter, the rats were sacrificed by decapitation, ovaries removed for quantitative histology, and trunk blood collected for quantitation of estradiol levels by standard RIA. The mean levels of estradiol in serum from both the 4 and 8 day treatment group were significantly greater than the control ($P<.05$, $p<.01$ respectively, Fig. 2). Figure 3 displays the distribution of viable follicles from these rats determined by standard morphometric analysis. There was a significant increase in the number of large (>500 μm) follicles in the antisera-treated rats ($p=0.05$; Mann-Whitney U).

PERSPECTIVE

These studies from a variety of species suggest that exogenous FRP inhibits follicular growth by induction of atresia which becomes morphologically evident at a specific follicular size which is species dependent. For the guinea pig, this follicular size is consistent with the follicular diameter at which generalized atresia typically occurs in the normal estrous cycle. Since this amplification of atresia can be prevented, in part, in the rat by administration of anti-FRP, these findings suggest that FRP may function as a natural atretogen. In this context, recruitment and selection of the preovulatory follicle may occur as a result of an FRP-mediated reduction in the number of healthy follicles during the follicular phase,

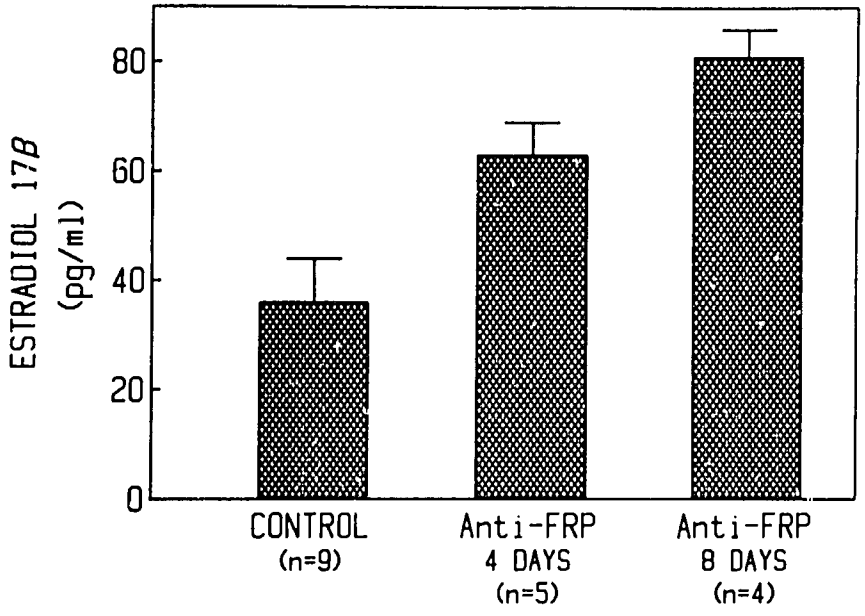


Figure 2. Stimulation of serum estradiol levels in 23-day old Sprague-Dawley female rats treated with rabbit antisera prepared against porcine FRP for 4 or 8 days beginning on cycle day 1. Estradiol concentrations were significantly greater in antisera treated rats compared to rats treated with nonspecific antisera.

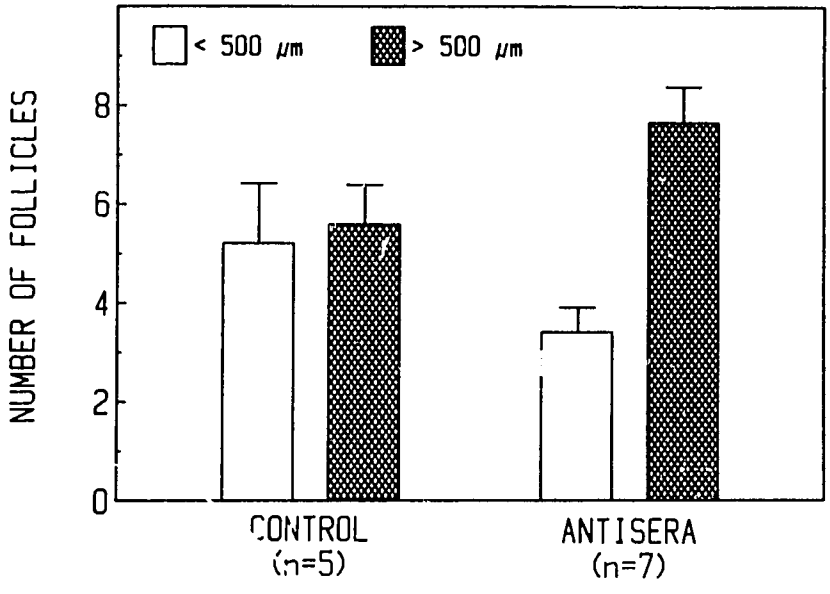


Figure 3. Morphometric analysis of antral follicles without evidence of atresia in ovaries from 23-day old SpragueDawley rats treated 4 or 8 days with rabbit antisera prepared against porcine FRP or nonspecific rabbit antisera. Rats treated with anti-FRP had more viable follicles >500μm compared to rats treated with nonspecific antisera (mean ± SEM).

followed by an FSH-mediated stimulation of the remaining viable follicle(s) in the subsequent luteal phase. Within the follicle, FRP may mediate a dose-response set point for FSH, which increasing exposure of the follicles to FSH can override, allowing for the recruitment of ovulatory follicles. When intrafollicular levels of FRP are low, multiple follicles are recruited. However, when intrafollicular levels of FRP are high, ovarian dysfunction (*i.e.* anovulation) may ensue. High levels of intrafollicular FSH can reduce the action of FRP in granulosa cells which may, in turn, allow for enhanced granulosa aromatization and the production of estrogen. If FSH is unable to overcome the intrafollicular effects of FRP, the follicle becomes atretic.

TESTICULAR STUDIES

INHIBITION OF SPERMATOGENESIS

We evaluated the effects of a partially purified preparation of FRP derived from porcine follicular fluid on testicular histology in mongrel dogs.[10] Partially purified preparations of FRP were administered intramuscularly once each day. Morphometric quantitation of these testes revealed a reduction in spermatogonial and spermatid forms, suggesting an alteration in seminiferous epithelial function. Accordingly, we performed time and dose-response studies to assess the effects of partially purified FRP on the function of rat seminiferous epithelium. After 75[24] or 30[23] days of intraperitoneal injections, there was a significant decrease in sperm head counts in 45 day old Sprague-Dawley rats treated with FRP when compared to vehicle-treated controls (Fig. 4). Although a reduction in sperm head counts can result from decondensation of nuclei, there were no gross alterations in elongated spermatid nuclei in the rats treated with FRP. Rather, the reductions in the number of sperm head nuclei by FRP may result from a decrease in progenitor cells. This was suggested by the formation of ring chromatin in the round spermatids.[23] After 60 days of treatment, an insufficient number of round spermatids were present even to identify a discrete Stage VII. The presence of ring chromatin in the round spermatids may result from either direct toxicity or deficient hormonal stimulation. That these effects, including the formation of ring chromatin, were essentially reversible within 6 weeks of discontinuing FRP therapy (Tsutsumi and diZerega, unpublished observation) suggest an involvement of the Sertoli-germ cell paracrine system.

Spent media from cultures of rat Sertoli cells was found to contain enhanced levels of immunoreactive FRP (Skinner and Rogers, unpublished observation). Accordingly, we tested the effect of FRP on FSH-dependent secretion of proteins by Sertoli cells.[23] In the presence of FRP, a significant increase in secretion of transferring by rat Sertoli cells treated *in vitro* with and without FSH was noted after 48 hours of culture (2 and 1.5-fold, respectively). Secretion of androgen binding protein (ABP) after administration of FSH also increased from control levels. The addition of FRP stimulated a further rise in concentrations of ABP with and without FSH (2.5 and 2-fold, respectively). Thus, FRP enhances the secretion of these proteins by Sertoli cells in response to FSH treatment *in vitro*.

Seminiferous tubules microdissected from rats treated with partially purified FRP were markedly more translucent at stage VII of the seminiferous epithelial cycle than those from

vehicle-treated control rats after 45 and 70 days of treatment.[23] The translucent seminiferous tubules in rats treated with FRP appeared to result from decreased mature sperm forms, consistent with the decreased sperm head counts. Squash preparations of seminiferous epithelial segments contained many abnormal spermatids in FRP-treated rats, which were not found in vehicle-treated controls. This suggests failure in condensation of the spermatid nuclei arrangement on the top of the epithelium, or other failures in spermatid development. That the percentage of dark segment lengths decreased at the same time as the sperm head count fell may be related to the overall reduction in the number of sperma-

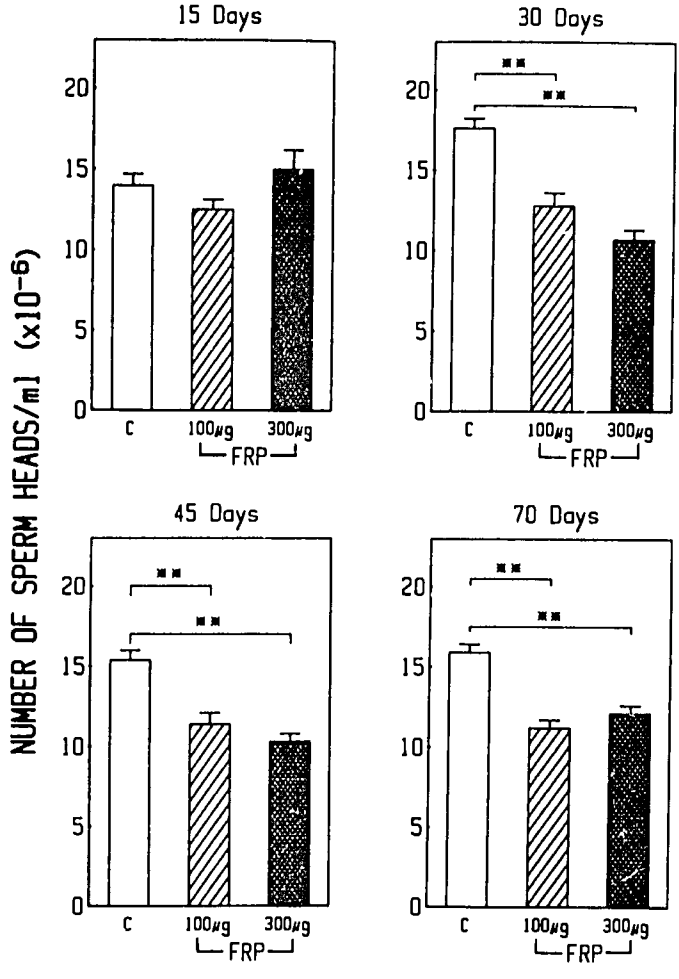


Figure 4. Disruption of seminiferous epithelial function in the rat by partially purified FRP. Sperm head counts (mean ± SEM) were performed after injection of rats with vehicle (C) or follicle regulatory protein (FRP: 100 µg or 300 µg) for 15, 30, 45, and 70 days. Both the 100 and 300-µg FRP-treated groups contained significantly decreased sperm head counts at 30 ($p<0.01$), 45, and 70 days ($p<0.01$) of treatment.

tids in tubules as FRP therapy continued. The reduction of elongated spermatids after FRP administration may result from either degeneration of these cells or reduction of more immature forms. Disruption in spermatogonial maturation in dogs treated with a crude preparation of FRP supports the latter possibility.[14]

FERTILITY TESTS

Fertility was assessed by cohabitating 5 female rats with a single male for the last 10 days of FRP treatment.[24] Each male rat previously received daily intraperitoneal injections of FRP for 45 days which was continued during the period of fertility testing. Controls were treated in the same manner with daily injections of vehicle. Thereafter, the females were separated from the males and subsequently evaluated for implantation sites in their uterine horns. The percentage of female rats that conceived when cohabitated with vehicle-treated controls was 44% to 68% during the study interval (Fig. 5). Fertility rates for male rats treated with a partially purified preparation of FRP for 45 and 60 days were significantly lower than those of controls.

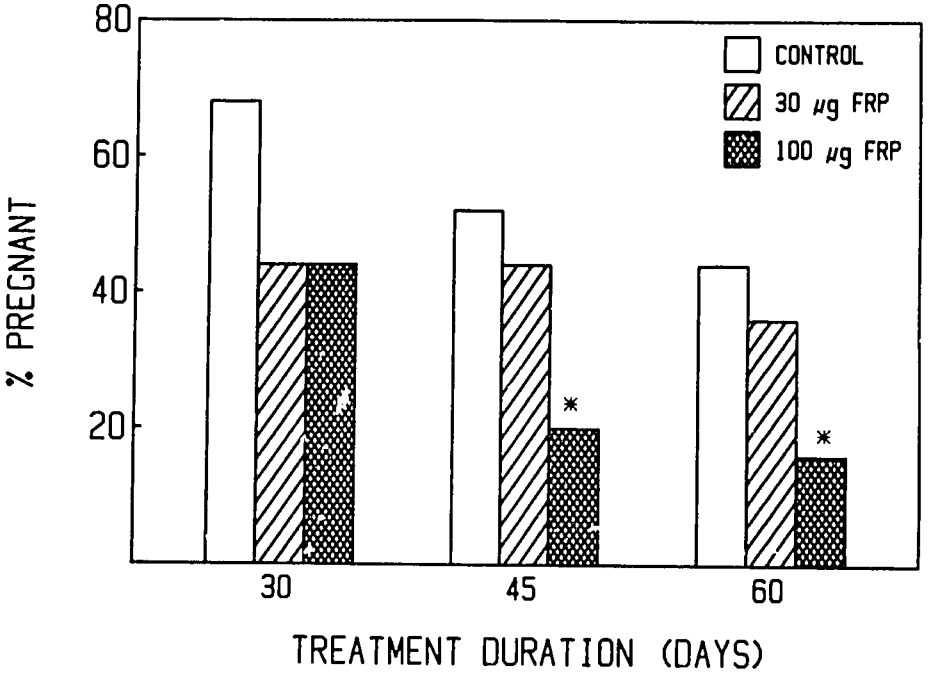


Figure 5. Reduction of fertility in the male rat by systemic treatment with partially purified FRP prepared from porcine follicular fluid. Percentage of pregnant rats after 10-days cohabitation with males treated with vehicle (C) or FRP for 30 to 60 days. Study design was five females per male; five males per treatment group. Females cohabitated with males treated with FRP, 100 μg for 45 and 60 days had significantly fewer pregnancies than controls. *$p < 0.05$; data are expressed as percent of total number of females.

PERSPECTIVE

Speculation of potential mechanisms for action of FRP in the testis is less clear than for the ovary. However, it is apparent that autocrine/paracrine regulation of spermatogenesis by mediators such as FRP provide a special opportunity to reduce the production of mature germ cells in the presence of normal levels of steroid hormones in the peripheral circulation. However, the duration of treatment required to sufficiently inhibit spermatogenesis to allow for azoospermia remains problematic. Although the limited dose-response studies performed with FRP *in vivo* clearly indicate a reduction in male fertility, evidence for reversible sterility has not been obtained. Whether higher doses of FRP and/or more prolonged treatment cycles will result in a useful male contraceptive awaits further study.

REFERENCES

1. BATTIN DA, diZEREGA GS: Effects of follicular fluid protein(s) on gonadotropin modulated secretion of progesterone in porcine granulosa cells. Am J Obstet Gynecol 60:1116, 1985
2. BATTIN DA, diZEREGA GS: Effect of hMG and follicle regulatory protein on 3β-ol-dehydrogenase activity in human granulosa cells. J Clin Endocrinol Metab 153:432, 1985
3. CHICZ R, CAMPEAU JD, NAKAMURA RM, diZEREGA GS: Follicular regulatory protein noncompetitively inhibits human placental microsomal aromatase activity. J Steroid Biochem 23:663, 1985
4. CHIKAZAWA K, ARAKI S, TAMADA I: Morphological and endocrinological studies on follicular development during the human menstrual cycle. J Clin Endocrinol Metab 62:305, 1986
5. diZEREGA GS, CAMPEAU JD, UJITA EL, KLING OR, MARRS RP, LOBO RA, NAKAMURA RM: Possible role for a follicular fluid protein in the intraovarian regulation of folliculogenesis. Sem Reprod Endocrinol 11:309-322, 1983
6. diZEREGA GS, MARRS RP, CAMPEAU JD, NAKAMURA RM, KLING OR: Human granulosa cell secretion of proteins(s) which suppress follicular response to gonadotropins. J Clin Endocrinol Metab 56:147, 1983
7. diZEREGA GS, ROSS GT: Regulation of follicle growth. In TAYMOR MC, NELSON J (eds): Progress in Gynecology, Volume 7, p 21. New York, Grune and Stratton, 1983
8. diZEREGA GS, TONETTA SA, WESTHOF G: Role of follicle regulatory protein in follicle selection. In HAZELTINE F, FIRST N (eds): Molecular Control of Meiosis. New York, Alan Liss Publications, 1988
9. diZEREGA GS, WILKS JF: Inhibition of the primate ovarian cycle by a porcine follicular fluid protein. Fertil Steril 41:635, 1984
10. FUJIMORI K, MONTZ FJ, NAKAMURA RM, diZEREGA GS: Inhibition of canine spermatogonial mitosis with follicle regulatory protein. Arc Androl 16:25, 1986
11. FUJIMORE K, NAKAMURA RM, TONETTA SA, diZEREGA GS: Cessation of transition phase follicle growth in the guinea pig by follicle regulatory protein. Biol Reprod, 37:812, 1987
12. FUJIMORI K, RODGERS KE, NAKAMURA RM, diZEREGA GS: Ovarian distribution of follicle regulatory protein assessed by immunohistochemistry. J. Histochem Cytochem 36:589, 1988
13. HOLMBERG EA, CAMPEAU JD, ONO T, diZEREGA GS: Comparison of isoelectric focusing in sephadex vs immobilized flat beds for the preparative purification of follicular fluid. Prep Biochem 16:275, 1986
14. KATT E, YANAGIHARA DI, HOLST P, MISHELL DR, Jr., TONETTA SA, RODGERS KE, WESTHOF G, HORNSTEIN J, DeVINNA R, LaCARRA M, GREENWALT D, diZEREGA GS: Characterization of follicle regulatory protein in urine during the normal menstrual cycle. J. Clin Endocrinol Metab 66:1213, 1988
15. KLING OR, ROCHE PC, CAMPEAU JD, diZEREGA GS: Identification of protein(s) in porcine follicular fluid which suppress follicular response to gonadotropins. Biol Reprod 30:564, 1984
16. LEW MW, KATT EL, RODGERS KE, diZEREGA GS: Alteration of follicle regulatory protein levels in human reproductive disorders and anovulation. Obstet Gynecol 70:157, 1987
17. McNATTY KP: Ovarian follicular development from the onset of luteal regression in humans and sheep. In ROLLAND R, VAN HALL EV, HILLIER SG, McNATTY KP, SCHOEMAKER J (eds): Fourth Reinier De Graff Symposium, Amsterdam: p 1, Elsiever, 1982

18. MONTZ FJ, UJITA EL, CAMPEAU JD, diZEREGA GS: Inhibition of luteinizing hormone/human chorionic gonadotropin binding to porcine granulosa cells by a follicular fluid protein. Am J Obstet Gynecol 148:436, 1984
19. ONO T, CAMPEAU JD, HOLMBERG EA, NAKAMURA RM, UJITA EL, DEVEREAUX DL, TONETTA SA, DeVINNA R, UGALDE M, diZEREGA GS: Biochemical and physiological characterization of follicle regulatory protein: A paracrine regulator of folliculogenesis. Am J Obstet Gynecol 154:709, 1986
20. RODGERS K, TONETTA SA, diZEREGA GS: Development of an enzymelinked immunoassay to measure follicle regulatory protein. 1988 Alan Liss, Albertson, B. and Hazeltine F., (in press)
21. SCHREIBER JR, diZEREGA GS: Porcine follicular fluid protein(s) inhibit rat ovary granulosa cell steroidogenesis. Am J Obstet Gynecol 155:128, 1986
22. TONETTA SA, YANAGIHARA KL, DeVINNA RS, diZEREGA GS: Secretion of follicle regulatory protein by porcine granulosa cells. Biol Reprod 38:1001, 1988
23. TSUTSUMI I, FUJIMORI K, ONO T, NAKAMURA RM, diZEREGA GS: Inhibition of spermatogenesis in the rat with follicle regulatory protein. Biol Reprod 36:451, 1987
24. TSUTSUMI I, TOPPARI J, CAMPEAU JD, diZEREGA GS: Reduction of fertility in the male rat by systemic treatment with follicle regulatory protein. Fertil Steril 47:689, 1987
25. UJITA EL, CAMPEAU JD, diZEREGA GS: Inhibition of porcine granulosa cell adenylate cyclase activity by an ovarian protein. Expl Clin Endocrinol 89:153, 1987

DISCUSSION

CHAIRPERSON: JEFFREY M. SPIELER

DR. KEEPING: Is there any evidence for FRP production in the testis or Sertoli cells?

DR. DIZEREGA: Yes, there is. Dr. Jorma Toppari, who has been in our lab for the last year or so, has looked carefully at FRP in the testis using immunohistochemical techniques. In the human testis we find immunolocalization with the Sertoli cells without germ cell nor interstitial cell uptake.

In terms of Sertoli cell secretion, we can measure FRP secretion in the spent Sertoli culture media, not in Leydig cell culture media. And, once again, in response to FSH there is no change in immunoreactive FRP levels.

A number of years ago we published some papers using a much less purified preparation of FRP and found that we could readily reduce the sperm head count in rats treated with these impure preparations, and that we could reduce pregnancy rates with cohabited females with administration of a partially purified preparation of FRP to the males.

DR. CORBIN: Did you see any blockade of ovulation in rats treated with FRP that had disrupted cycles? Did you look for eggs?

DR. DIZEREGA: In our studies we did not flush out the tubes nor look for eggs. We have, however, collaborated with Keith Demerest of Ortho Pharmaceutical Company who reproduced these studies. He did flush out the fallopian tubes and found that in animals with disruption of the estrus cycle, as shown by changes in vaginal cytology, that there were no eggs in the uterine horns.

DR. CORBIN: Were there any associated hormonal changes? Were any mating studies done on these animals to see if they could become pregnant? Did you, or did somebody else, try to block the LH surge with FRP?

DR. DIZEREGA: There were no attempts to block the LH surge. However, we did measure serum steroid levels in FRP treated rats. The estradiol levels were suppressed and androgens were questionable; some were slightly elevated and some were slightly suppressed. The only unusual finding was that progesterone was elevated in virtually all of the FRP treated rats. Although there was luteinization of many of the ovaries, there were no corpora lutea to account for the progesterone elevation.

DR. CORBIN: One other question regarding the relationship between cancer and FRP. Did you say that patients with ovarian cancer have high levels of FRP in their serum?

DR. DIZEREGA: Thirty-five percent of the patients that we studied — we have studied a little over five hundred now — with serous adenocarcinomas, mucinous adenocarcinomas, or granulosa cell tumors do have elevated levels of FRP in their serum.

DR. CORBIN: On the other hand, when you gave FRP to the nude mice that have tumors growing in them, you got the opposite effect. In the human, it looked like there was some relationship between the FRP and the generation of the cancer.

DR. DIZEREGA: I think that is a very important point to revisit. In the tumor patients, be they male or female, when we find immunoreactive FRP at these very high levels, we have never found it to be biologically active. And, as I indicated on that Western blot, the positioning of the immunoactivity is not at 15,000 kDa; it is at a much higher molecular weight position. We do not know if this is due to post-translational disruption. But quite clearly the immunoreactive molecule is biologically inactive. In those tumors, when we add FRP either *in vitro* or *in vivo* in the nude mice, we find disruption of tumor growth.

DR. GOODMAN: I was intrigued about the finding of ovarian hyperstimulation in the passive immunization studies in the rat. The estradiol levels seem to be very high, and one would suspect, then, that the gonadotropins would be low. If so, why are those ovaries so big? Do you have any hormone data from these studies?

Also, if FRP is in circulation, and if FRP is an aromatase inhibitor, what impact does that have on using a FSH bioassay that measures aromatase? And, is FRP an intra-ovarian hormone in the sense that follicles are talking to each other through FRP, and FRP in circulation is simply an epi phenomenon of the message leading out into the circulation?

DR. DIZEREGA: If FRP acts as an intragonadal inhibitor of aromatase activity, then the increased serum estradiol levels from passive immunization would be expected. In terms of gene leakage, we have found immunoactive material from colon, breast, and large, as well as oat cell, cancers of the lung. In testicular material, there is really no reason why you would expect the elevated immunoreactive FRP levels in serum from seminoma patients. What the role is, if any, at extragonadal sites, I do not know. I do know that in func-

tional castrates, or in postcastrate men and women, as well as postmenopausal women, typically, we do not find immunoactive material in either serum or urine. So, I really do not postulate an extragonadal mode of action.

In terms of the peripheral effects of FRP, we do think they are important. The amount of FRP that Dr. Gregor Westhof got on a per cell basis from perifused whole follicles is far more than we get from granulosa cell culture media. And, I wonder if that would be the case with inhibin as well, but I have not seen any data on that.

DR. SAIRAM: Is the FRP confined to the gonad? When you castrate the animal, does it appear in circulation?

DR. DIZEREGA: The answer to the first question is yes; the second, no.

DR. SAIRAM: Does FRP affect postcyclic AMP events? And, do you see that effect in other tissues as well, like other steroid producing tissues such as the adrenal gland?

DR. DIZEREGA: We have not studied placental, adrenal, nor adipose tissue. In terms of postcyclase events, we have submitted an abstract to the Endocrine Society that addresses this issue. That data, hopefully, will be presented at the 70th Annual Meeting of the Endocrine Society, June 1988, in New Orleans.

DR. MASON: Is the amino acid sequence you presented contained in the larger forms that you see in tumors?

DR. DIZEREGA: I wish I knew, but I have no information at this time. We have not done any sequencing at all on those large forms; we have just recovered them and stored them.

The only thing I can say about those large forms is that they are not due to aggregation. Native FRP readily aggregates, and that is why it took us so long to develop the N-terminal sequence. We are currently working on cloning the FRP gene.

DR. CROWLEY: My question stems from an interesting observation of which you might be aware. A lot of the older literature about patients with ovarian cancer reported a variable degree of estrogenization of the vaginal epithelium. It was always an anomalous thing. One of the curious features is that not all ovarian cancer patients have it. It struck me, as I was looking at your data, that you potentially had a reason why some did not have it, and that is because they are making an aromatase inhibitor. Have you looked at that in terms of clinical expression and levels of FRP in some of these ovarian cancer patients?

DR. DIZEREGA: It turns out that although it would be nice if there were, there really is no correlation. It is great to put things in little neat packages, but it does not work this time. The immunoreactive material is biologically inactive. In other words, if we recovered the material from our immunoaffinity chromatography and you put it in the granulosa cell aromatase assays, there would be no inhibition. So, we would not necessarily presuppose there to be a cause-and-effect relationship there.

I think another aspect of physiology that this type of data will begin to readdress is the full role of prolactin in gonadal function. There is a lot of data suggesting that prolactin added to granulosa cell systems reduces estrogen production and aromatase activity. And, perhaps prolactin will become an important indirect regulator of intra-gonadal function. At least we are paying a lot more attention to it now.

DR. HASELTINE: As usual, the last slide presented leads to a lot of questions. In response to further questioning, you mentioned that other tumors, from other sites, produce FRP. Have you done any nude mouse studies similar to the one you already reported?

DR. DIZEREGA: Yes, we have, and it is quite exciting. The ovarian tumors are actually some of the more relatively resistant tumors to FRP therapy, and we are getting dose response characteristics down in the nanogram levels, well below those of other biologic modifiers in terms of antineoplastic therapy. The tumor that we have found to be the most sensitive is the seminoma. Many breast cancers are also quite sensitive. The ovarian cancers are next, and on down the list. We also have quite a few tumors which do not respond to FRP therapy. For instance, endometrial adenocarcinoma of the uterus is negative, melanomas are negative, and so forth, so there is tissue specificity.

18

Anti-FSH Antibody as a Probe in Determining the Role of FSH in the Maintenance of Gonadal Function in the Primate

RAGHUVEER N. MOUDGAL
NEELAKANTA RAVINDRANATH
ARAVINDAN GNANAKAN
RAJAN R. DIGHE
GUNDLUPET S. MURTHY

We wish to dedicate this paper to the memory of the Late ProfessorM.R.N. Prasad, Emeritus Scientist at the Department of Biochemistry, Indian Institute of Science, Bangalore, India.

Earlier studies from this laboratory have shown that well characterized gonadotropin antibodies can be used effectively in understanding the role of LH in regulating luteal function in both the rodent [6,9] and the primate[8,11] and that of FSH in regulating the follicular maturation process in the rodent[19,21] and testicular function in the primate.[10,17] These antibodies have also added to our knowledge on gonadotropin-receptor interaction.[12,13] FSH antibodies are currently being used in our laboratory to further our understanding of FSH regulation of follicular maturation and luteal function as well as the role of FSH in the regulation of testicular function in the primate. The results of some of these investigations are presented here.

METHODS

PRODUCTION AND CHARACTERIZATION OF FSH ANTIBODY

Both passive and active immunization approaches have been used in the current study. In the passive immunization study antiserum raised in an adult male monkey to oFSH (antiserum #603) has been used. This antiserum neutralizes the biological activity of both ovine and primate FSH as determined in the receptor FSH binding inhibition test (Fig. 1). The binding affinity and capacity of antiserum #603 to hFSH was 0.3 $\times 10^{10}$ M^{-1} and 43.8 μg/ml, respectively. As little as 25 μl of this antiserum is adequate to neutralize endogenous FSH over a 8 to 12 hour period when given to cycling female monkeys (Fig. 2). This antiserum has no effect on bioactivity of primate LH.

For active immunization, a purified preparation of oFSH was passed through an immunoaffinity column of LH antibody to remove any LH contamination. Immunization of adult male monkeys with this preparation did not lead to any diminution of testosterone levels, anindex of the absence of LH antibodies. Antiserum thus raised was capable of bioneutralizing human and monkey FSH; a binding affinity (ka) of 5-15 x 10^9 M^{-1} was determined, using oFSH as the ligand.

ANIMAL MODEL

The non-human primate used in these studies is the South Indian bonnet monkey (*Macaca radiata*). The adult females exhibit a regular 28 ± 4.2 day cycle, nine months of the year (July-March), whereas the adult males are non-seasonal and continue to produce sperm throughout the year. The endocrine profiles of these monkeys as well as details of husbandry conditions have been described elsewhere.[16,18] All animals used in this study, unless otherwise stated, were proven fertile monkeys.

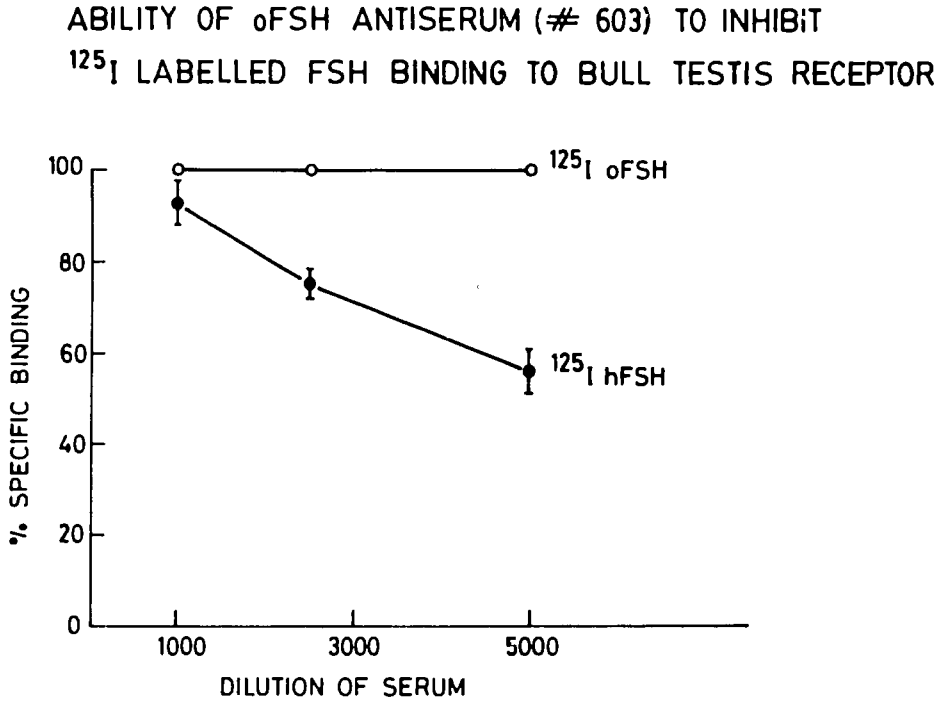


Figure 1: Demonstration of cross-reactivity of human FSH with oFSH antiserum.

A bull testis FSH preparation was incubated in the presence of different dilutions of oFSH antiserum (#603) with either ^{125}I labelled oFSH or hFSH for 1 hour at 37 °C. Non-specific binding was determined by carrying out the incubation in the presence of excess unlabelled hormone.

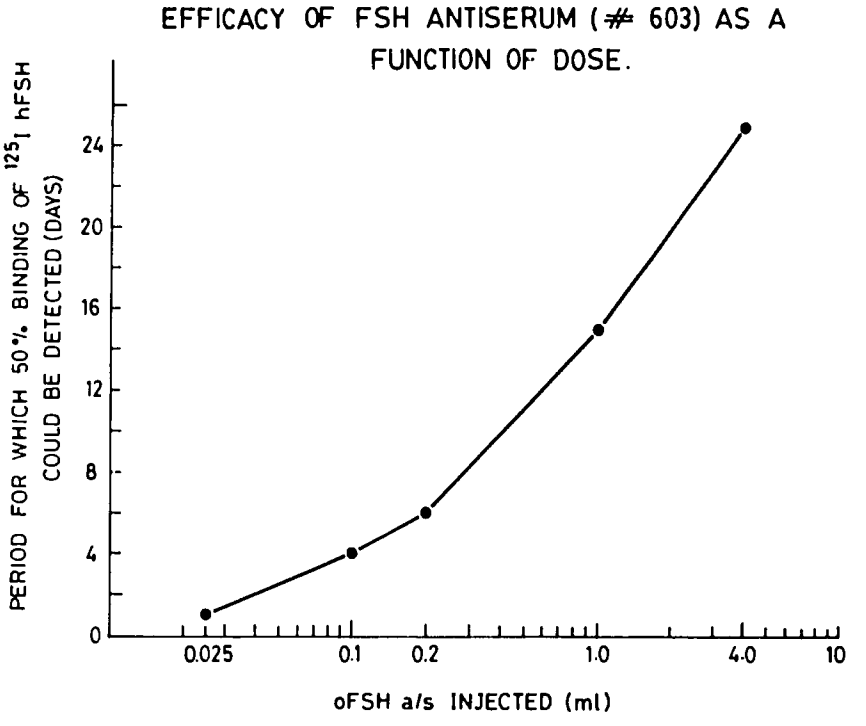


Figure 2: Efficacy of oFSH antiserum (#603) to neutralize endogenous monkey FSH-a function of the dose injected.

Female monkeys on day 1 of cycle received different volumes of the antiserum injected intraperitoneally. Blood samples removed at specific periods were processed for ^{125}I hFSH binding to different dilutions of sera. For comparative purposes 50% binding data is used.

RESULTS AND DISCUSSION

EFFECT ON OVARIAN FUNCTION OF PASSIVE IMMUNONEUTRALIZATION OF FSH DURING VARIOUS PHASES OF THE REPRODUCTIVE CYCLE

Using serum estrogen (E_2) levels as an index of follicular activity, it has been observed that in the cycling bonnet monkey the follicular maturation process is almost complete with estrogen levels peaking by day 9-10 of the cycle. While injection of 25 μl of FSH antiserum (antiserum #603) on day 8 had a minimal inhibitory effect (25%) on day 9 estrogen levels, the same dose given on days 7, 6, or 5 resulted in differential effects which are summarized in Table 1. Of the 4 days tested, the day 7 follicles seem most vulnerable to the lack of FSH. The estrogen surge in this group occurs on day 16, indicating the resurgence of a new set of follicles shortly after the effect of the antiserum has worn off. The day 5 and 6 follicles seem to override the effect of the FSH antiserum administered on these days, the E_2 surge occurring in the day 5 group on day 11 instead of day 9, while the day 6 group

Table 1

EFFECT OF NEUTRALIZING FSH ON DIFFERENT DAYS UPON FOLLICULAR FUNCTION

Day of Cycle	n	Dose of a/s (μl)	Effect on E_2 Level on Day 9	Day on Which next E_2 Surge Occurred	Luteal Length/ Cycle Length (Days)
5	4	25	NIL	11	15.0 ± 0.8/27.0 ± 1.4
6	3	25	NIL	14	14.0 ± 2.3/27.3 ± 2.3
7	9	25	reduced by 80%	16	13.1 ± 2.2/28.2 ± 2.6
8	4	25	reduced by 25%	—	20.2 ± 2.6/30.2 ± 2.2
9	3	50	NIL	—	19.8 ± 2.1/29.2 ± 2.8

1. Normal Midcycle E_2 Surge in Bonnets occurs on day 9/10 and the E_2 Levels are 610 ± 180 pg/ml.

2. Normal Length of Luteal Phase from E_2 Peak on day 9 or 10 to the appearance of next Menses range from 18-22 days (Mean ± SD: 20.0 ± 1.6)

exhibits 2 estrogen surges — one on day 9 and the other on day 14 of the cycle. In the day 6 group the dominant follicle, though it appears to override the effects of the antibody, still does not seem capable of meeting the ardors of ovulation/luteinization with the result that it either undergoes atresia or luteinization with an entrapped ovum. The increase in progesterone levels seen between days 9 and 14 in this group suggests the latter possibility. The net result of such treatment is the development of a new dominant follicle which is responsible for the E_2 surge seen on day 14 of the cycle. Laparoscopic examination on day 14 confirmed the existence of a new follicle on the contralateral ovary.

As far as we are aware, the present study provides the first clear evidence that follicular dominance is achieved on day 7 or 8 of the cycle, depending on whether maximal sensitivity to FSH (day 7) or independence from FSH support (day 8) is considered the mark of dominance. It remains to be seen what cellular events FSH controls in the day 7 follicle.

The partial neutralization of endogenous FSH during the early phases of follicular maturation seems to influence the quality of granulosa cells produced, particularly in terms of their future ability to luteinize and produce normal levels of progesterone. The experiment described below made this conclusion evident. Among the new set of follicles initiated to maturation following antiserum injection on day 7, one becomes dominant and produces normal levels of estrogen, showing a surge of E_2 9 to 10 days later (on day 16 of the cycle). The follicle luteinizes normally following ovulation but the newly formed corpus luteum fails to sustain function for the normal duration. One of the best ways of finding out if the corpus luteum is functioning normally is to ascertain if it can support the establishment of pregnancy. A group of proven fertile female monkeys (n=6), caged for the duration of the experiment with individual fertile males, were injected on day 7 of 3 consecutive cycles, with 25 μl of antiserum per monkey. The antiserum injection had no

effect on cycle length, nor on estrogen and progesterone levels, except for the inhibition of estrogen (75%) on day 9. The luteal phase lengths, however, were markedly reduced (C:18.2 ± 1.5 vs Expt: 10.8 ± 1.9 days) and no pregnancies occurred during the treatment period consisting of 18-cycle exposures. However, with the stoppage of the antiserum injection, 5 out of 6 monkeys became pregnant within 3 post-treatment cycles, providing a pregnancy index of 83%, which corresponds to colony norms. It is thus clear that luteal tissue of monkeys treated with FSH antiserum on day 7 of the cycle is in some ways defective; incapable of being rescued as shown by the inability of the monkeys to conceive. It should be added here that FSH antiserum injection throughout the luteal phase had no effect on luteal function as judged by progesterone levels or luteal phase length. Interestingly, neutralization of FSH during this period had no effect on the follicular maturation process in the following cycle. The above experiment could perhaps provide a focal point of attack for developing a contraceptive capable of blocking or partially neutralizing endogenous FSH on day 7 of the cycle, a crucial juncture in the follicular maturation process of the primate. Interestingly, preliminary studies have indicated that injection of inhibin (charcoal treated pFF) to bonnet monkeys on day 6/7 of the cycle also leads to a luteal phase defect, but the mechanism by which this is brought about appears to be different.[15]

EFFECT OF PASSIVE AND ACTIVE IMMUNONEUTRALIZATION OF FSH ON TESTICULAR FUNCTION OF ADULT MALE MONKEYS

We reported earlier that chronic neutralization of endogenous FSH in adult male monkeys for a period of 200 days leads to testicular dysfunction. Testicular function was assessed in several ways. In addition to showing significant reduction in the rate of ^{3}H thymidine incorporation into testicular DNA *in vitro* and testicular hyaluronidase activity, both used here as indices of tubular functionality,[20] the animals exhibited marked reduction in the quality and quantity of sperm voided during electroejaculation (Fig. 3). However, these animals did not show any change in serum testosterone levels, and proved fertile in controlled mating experiments.

The above study prompted us to determine if oFSH can be used as a contraceptive vaccine in the male primate. Specifically, we would like to discuss two aspects of this study. The first refers to the use of Flow Cytometric analysis [FCM] to assess the effect of immunization on the germ cell population in testes and, secondly, determine how FSH immunization induced oligospermia leads to infertility. In recent years, FCM is gaining acceptance as a method for quantitating the germ cell population in testicular biopsies of human[2,3] and experimental animals.[1]

A group of five adult proven fertile male bonnet monkeys 6 to 7 years of age were immunized with oFSH given adsorbed on Alugel as per protocol standardized earlier in this laboratory.[14] Testicular biopsy samples (100 mgs of tissue/monkey) were removed a week prior to the start of immunization, and on day 85 of immunization using every precaution to minimize permanent damage to the testicular architecture. While one portion of the biopsy material was used after due processing for determining Johnson's biopsy score according to standardized procedures,[5] the other portion was used for Flow cytometric analysis (FCM) using a PHYWE ICP 22 Flow cytometer.

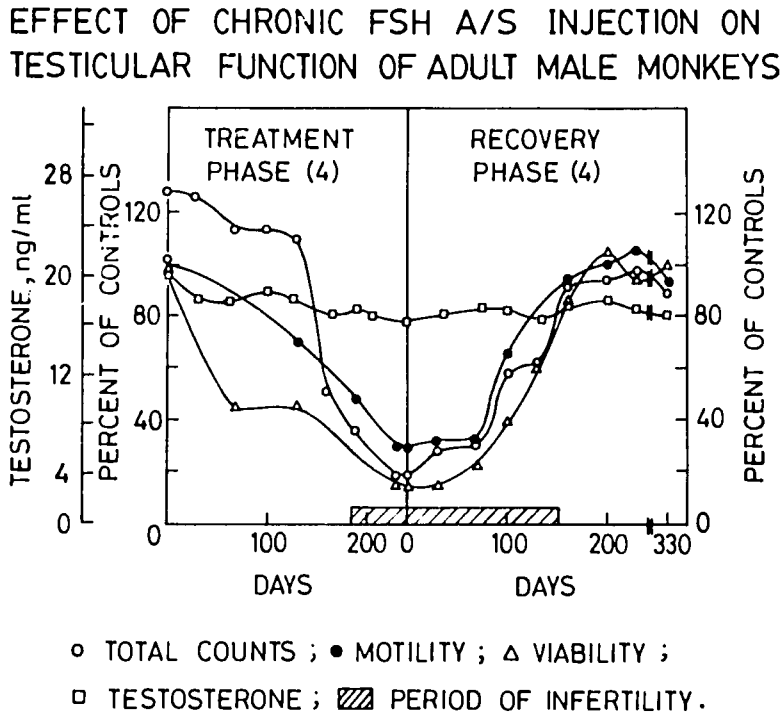


Figure 3: Effect of chronic FSH neutralization on testicular function of adult male bonnet monkeys.

Two ml of a well characterized oFSH antiserum raised in donor monkeys was injected alternate days for 200 days to proven fertile adult male bonnet monkeys. Fertility was tested by caging each of the males (n=4) with a cycling female from days 9-14 of cycle. Each male was exposed to four females. Infertility was established by about 150 days of treatment and for restitution of fertility it took 150-200 days post-treatment. Passive immunization had no effect on libido.

The tissue sample for FCM was processed by mincing the tissue into fine pieces and incubating in a DMEM medium containing of 0.10% Trypsin and 17 μg/ml DNAse for 30 minutes at 37°C.[7] The germ cells released by this procedure were filtered to remove the debris, fixed in 70% ethanol and stained with DAPI (diaminido phenyl indole) according to standardized procedure.[4] The results clearly show that immunization with FSH does affect the spermatogenic process (Table 2). The block at the tetraploid stage (4C- from leptotene to diplotene stage) appears most striking. There is significant reduction in both haploid (1C-spermatids and spermatozoa) and diploid cells (2C-spermatogonia and secondary spermatocyte) population. Interestingly, monkeys having a higher FSH antibody titer showed a more pronounced effect on their FCM pattern compared to those having a lesser titer.

Active immunization of monkeys with oFSH for extended periods (beyond 150 days) brings about a marked reduction in sperm counts (over 70%). These animals in controll-

ed mating experiments proved infertile. It is generally held that for a male contraceptive to succeed it has to bring about azoospermia. Since this assumption is at variance with what has been observed in FSH immunization experiments, we attempted to examine the quality of sperm voided by FSH immunized monkeys. It is evident from Table 3 that the lack of FSH affects markedly the quality of sperm voided. Particular attention is drawn to the marked reduction in gel penetrability and sperm acrosin levels. While penetrability or motility is necessary for translocation of sperm from the site of deposition to the fallopian tube, the enzymes hyaluronidase and acrosin are required for the dispersion of the cumulus and penetration of the zona respectively. We believe it is a summation of effects — reduction in sperm counts, viability, forward motility and acrosomal enzyme activity — that contributes to the setting in of infertility. For this to be achieved, there should be a near total neutralization of FSH for continuous periods.

TABLE 2

JOHNSON'S BIOPSY SCORES AND FLOW CYTOMETRIC ANALYSIS OF DNA DISTRIBUTIONS IN GERM CELLS OF CONTROL AND IMMUNIZED MONKEYS

Attributes	n	Biopsy Score	% Distribution of Germ Cells HC	1C	2C	4C
Controls	(5)	8.1 ± 0.3	21.9 ± 3.7	57.8 ± 2.9	13.8 ± 1.2	6.0 ± 1.8
Immunized (90 days)	(5)	3.8 ± 1.1	74.5 ± 7.3	20.8 ± 6.1	4.8 ± 1.9	0
% Change		53	240	64	65	100

Values are mean = SD

See Text for Details of Methodology.

HC = Hypohaploid; 1C = Haploid; 2C = Diploid and 4C = Tetraploid Table 3

CONCLUSION

We have tried to highlight here how an FSH antibody can be used to probe FSH regulation of the gonadal function. This approach, in addition to furthering our knowledge and basic understanding of FSH action, provides an opportunity to assess exactly where we might interfere with FSH action to achieve control of fertility. Considering the differential requirement for FSH by the female and the male, it is perhaps feasible to develop inhibin as a female contraceptive and a FSH vaccine as a male contraceptive.

TABLE 3

ASSESSMENT OF QUALITY OF SPERMATOZOA VOIDED BY MONKEYS IMMUNIZED WITH oFSH

	Control	Experimental	% Change	P Value
1. Sperm Counts (Millions/EJA)	361.5 ± 44.5	135.6 ± 26.5	62.5	<0.01
2. Viability	75.6 ± 2.5	49.8 ± 4.0	34.13	<0.01
3. Motility % (Overall)	57.0 ± 6.2	21.0 ± 3.1	63.17	<0.01
4. Gel Penetrability				
Max. Distance (mm)	32.3 ± 3.7	14.4 ± 3.1	55.42	<0.01
Density at Max.[1] Distance (Number of Sperms	5.3 ± 2.0	4.5 ± 1.0	15.1	NS AT 5% Level
5. Acrosin[2] (mIU/10 Sperms)	15.57 ± 1.03	3.68 ± 0.73	74	<0.01
6. Hyaluronidase (ug. NAGA/ 10 sperms/h)	15.25 ± 1.07	10.12 ± 1.61	34	NS AT 5% Level
7. Fertility status[3]	15/26	0/50	—	Significant at 5% Level

Values are Mean ± SEM. The Value of n Varied from 8-14.

[1]Sperm Density in Controls at 15 mm Distance (53.8 ± 0.7) is Significantly Different From That Seen in Experimentals at 15.0 mm Distance

[2]ImIU = Amount of Acrosin causing the Hydrolysis of 1 Nanomole of Benzoyl Arginine Ethyl Ester in 1 MTS at Room Temperature.

[3]Fertility Status: No. Pregnant/No. Mated. Males of Each Group Tested ≤ 10.

ACKNOWLEDGMENT

This research was aided by grants from the Indian Council of Medical Research; the Department of Biotechnology, the Government of India, New Delhi; and the World Health Organization, Geneva. The assistance rendered by Flora Martin, Srilatha, Ramesh, Sudarshan, Krishnamurthy, and Shambhavi is gratefully acknowledged. We thank Dr. Joshi of the Biophysicals Group of NIMHAMS, Bangalore, for allowing us to use the FCM and to Dr. Kamala Gopalakrishnan of the Institute of Research in Reproduction, Bombay, for providing the Johnsons Biopsy score of monkey testes.

REFERENCES

1. CLAUSEN OPF, PURVIS K, HANSSON V: Micro flow fluorimetric DNA measurements of isolated testicular cells from the rat. Ann Biol Anim Bioch Biophys 18:541, 1978
2. CLAUSEN OPF, ABYHOLM T: DNA flow cytometry of germ cells in the investigation of male infertility. Fertil Steril 34:369, 1980
3. EVENSON PD, MELAMED RM: Rapid analysis of normal and abnormal cell types in human semen and testis biopsies by flow cytometry. J Histochem Cytochem 31:248, 1983
4. JAIN VK, OTTO FJ, GOPINATH PM: Application of flow cytometry in genetic research. In MARIMUTHU KM, GOPINATH PM (eds): Advances in Biosciences Vol. 56, Pergamon Press, London, 1986
5. JOHNSON SG: Testicular biopsy score count. A method for registration of spermiogenesis in human testis: Normal values andresults in 335 hypogonadal males. Hormones 1:2, 1970
6. MADHWARAJ HG, MOUDGAL NR: Hormonal control of gestation in the intact rat. Endocrinology 86:874, 1970
7. MEISTRICH ML, LONGTIN J, BROCK WA, GRIMES SR, Jr., MACE ML: Purification of rat spermatogenic cells and preliminary biochemical analysis of these cells. Biol Reprod 25:1065, 1981
8. MOUDGAL NR, MACDONALD GJ, GREEP RO: Role of endogenous primate LH in maintaining corpus luteum of the monkey. J Clin Endocrinol Metab 35:113, 1972
9. MOUDGAL NR: The luteotropic action of luteinizing hormone (LH). In SEGAL, CROZIER, CORFMAN, CONDLIFEE, THOMAS CC (eds): The Regulation of Mammalian Reproduction. CHARLES C. THOMAS, Illinois, 428, 1973
10. MOUDGAL NR: A need for FSH in maintaining fertility of adult male sub-human primates. Arch Androl 7:117, 1981
11. MOUDGAL NR: Corpus luteum of the non-human primate. In CORRELIUSCE, SIMPSON CF, HENDRIX AG (eds): Advances in Veterinary Science and Comparative Medicine. Academic Press, New York 28:343, 1984
12. MOUDGAL NR, DIGHE PR: An immunological approach to study gonadotropin action at the cellular and molecular level. Bioscience Reports 5:943, 1985
13. MOUDGAL NR, SAIRAM MR, DIGHE RR: Interaction of follicle stimulating hormone with its receptor and antibody. Mol Cell Endocrinol (in press)
14. MOUDGAL NR, MURTHY GS, RAVINDRANATH N, RAO AJ, PRASAD MRN: Development of contraceptive vaccine for use by the human male results of feasibility study carried out in adult male bonnet monkey (*Macaca radiata*). Contraceptives for The Nineties. Inaugural Symposium, National Institute of Immunology, New Delhi (in press)
15. MOUDGAL NR, DIGHE RR, SRINATH BR, RAVINDRANATH N, KURKALLI BS: Assessment of contraceptive potential of inhibin using non-human primate models. In BURGER HG, deKRETSER DM, FINDLAY JK, IGARASHIM (eds): Inhibin and Nonsteroidal Regulation of Follicular Stimulating Hormone Secretion. Serono Symposium Series, Raven Press, 42:289, 1987
16. MUKKU VR, PRAHALADA S, MOUDGAL NR: Effect of constant light on nycthemeral variations in serum testosterone in male *Macaca radiata*. Nature 260:778, 1976
17. MURTY GSRC, SHEELA RANI CS, MOUDGAL NR, PRASAD MRN: Effect of passive immunization with specific antiserum to FSH on the spermatogenic process and fertility of adult male bonnet monkeys *Macaca radiata*. J Reprod Fertil (Suppl) 26:37, 1979
18. RAVINDRANATH N, MOUDGAL NR: Use of tamoxifen, an anti estrogen in establishing a need for estrogen in early pregnancy in the bonnet monkey. J Reprod Fertil 81:327, 1987
19. SHEELA RANI CS, MOUDGAL NR: Role of proestrus surge of gonadotropins in the initiation of follicular maturation in the cyclic hamster: A study using antisera to follicle stimulating hormone and luteinizing hormone. Endocrinology 101:1484, 1977
20. SHEELA RANI CS, MURTY GSRC, MOUDGAL NR: Effect of chronic neutralization of endogenous FSH on testicular function in the adult male bonnet monkey assessment using biochemical parameters. Int J Andrology, 1:489, 1978
21. SHEELA RANI CS, MOUDGAL NR: Advances in immunology ofgonadotropins. In CHI LI (ed): Hormonal Proteins and Peptides Academic Press. New York, 11:135, 1983

DISCUSSION

CHAIRPERSON: JEFFREY M. SPIELER

DR. BURGER: I think that the data you presented on the work of your group in the area of the dependence of folliculogenesis and spermatogenesis on FSH is really striking. One of the really controversial questions to me concerning the control of spermatogenesis, is whether FSH is really essential and the interaction between FSH and testosterone.

Have you had the chance to look at any of your immunized monkeys given hCG, particularly during the period that spermatogenesis has been suppressed with FSH immunization, to see whether, in the presence of FSH antibody, it is possible to reinitiate spermatogenesis as was done in Dr. Bremner's department in an FSH-suppressed individual?

DR. MOUDGAL: No, we have not done that specific experiment, though it may be worthwhile. We have, instead, done another experiment testing the effect on testicular function of testosterone supplementation in monkeys whose FSH and LH were suppressed by about 80 and 60%, respectively, by the continuous infusion of Buserelin via Alzet pumps for hours on end. Bonnet monkeys exhibit basal levels of testosterone during morning hours and surge levels during the night. Buserelin infusion blocked surge testosterone levels, but not basal levels. Such animals exhibited azoospermia and also a marked shrinkage in testes volume as well as semen volume. In addition to restoration of normal testosterone levels in these monkeys by introducing a testosterone implant, some of these animals received an additional injection of testosterone (1 mg per monkey) in the evenings. This led to restoration of normal ejaculatory response and semen volume, but sperm counts continued to be zero.

DR. SEGAL: I think that the work you have presented here in the female, and your work on anti-FSH in the male, is extremely interesting and should be of considerable interest to this group with their particular concern for intragonadal regulation and substances that may have a role in FSH regulation. If these substances are ever to play a role in fertility regulation by interfering with FSH production or activity, a model showing the effectiveness of FSH immunization is extremely important. I think it is difficult to understand how the effect that you found is brought about with such a small volume of antibody. That is the first thing. Secondly, I think that you used the total FSH molecule as the antigen. Did you have any problems with LH or FSH cross-reaction?

DR. MOUDGAL: The dose of 25 μl of antiserum was arrived at by undertaking a pilot study wherein monkeys received different volumes of antiserum (from 10 μl to 1,000 μl) and their sera at different times thereafter was analyzed for antibody capable of binding ^{125}I-hFSH. In the experiment 25 μl of antiserum was capable of neutralizing FSH for a minimum period of 12-24 hours. The antiserum used, incidentally, was raised in donor monkeys to oFSH and, as such, there was no problem of serum incompatibility. Besides this, the specificity of the effect was established by the fact that the antiserum proved to be effective only when given at times when the follicle is sensitive to FSH lack and not otherwise. For example, the effect produced by the day 7 injection was dramatic compared

to a minimal effect on day 8 and no effect on day 9. Similarly, the follicle on day 5 versus 6 of the cycle appeared less sensitive to FSH lack compared to that of day 7.

In response to the second question, this FSH antiserum does not affect either testosterone or thyroxine levels, good indices of lack of LH and FSH antibodies.

DR. ALEXANDER: Would you care to speculate on the polyclonality of the immune response which may cause some monkeys to develop more neutralizing antibodies and some might develop more antibodies that were specific for the receptor, and how that could affect the response in both females and males?

DR. MOUDGAL: If you are asking whether there is a difference in the type of antibodies produced, receptor blocking versus plain binding depending on the immunogen used, we do find that the antibodies raised to intact oFSH are far superior to those raised to the oFSH β subunit. Both antibodies were raised in monkeys. While both the antibodies bind the hormone with equal affinity, their behavior on a particulate FSH receptor or FSH responsive cell is introduced into the system. The antibody to intact oFSH is capable of effectively competing with the receptor in binding and blocking response to FSH. In contrast, the antibody to oFSH-β turned out to be a poor competitor or blocker of response to FSH. This work is currently being published.

DR. KOJI YOSHINAGA: Since treatment with anti-FSH seems to affect cyclicity, how do you determine Day 7 of the cycle for the purpose of treatment with antiserum?

DR. MOUDGAL: By giving antiserum on Day 7 of the cycle, we believe the active growth phase of the dominant follicle is arrested. If one looks at the estrogen profile in the normal monkey during this period, the increase in estrogen levels starts from Day 7, peaking on Day 9 or 10 of the cycle. The follicle appears most sensitive to the absence of FSH by Day 7 and, unlike that of Day 5 or 6, is unable to regain function after 24 hours. The new follicle that comes up appears to grow normally as judged by a normal estrogen surge on Day 16 (9 days post-antisera treatment) followed by ovulation and corpus luteum formation as seen by normal progesterone levels. However, the normality is only apparent as the corpus luteum cannot be rescued from undergoing cyclic demise. We preferred to test the rescue process by undertaking mating experiments rather than giving exogenous hCG. The effect of the antiserum is confined to only one cycle and the next cycle is normal, and, as such, an effective dose of antiserum can be administered in consecutive cycles without interfering with the normal cycle length. These experiments clearly show that follicles developed under partial FSH lack have granulosa cells which are luteinized and developed into defective luteal cells incapable of being rescued.

19

Follicular Regulation of Oocyte Maturation: Role of Oocyte Maturation Inhibitor

ALEXANDER TSAFRIRI

Meiotic maturation in mammalian oocytes is initiated during prenatal life or shortly after birth, but the process is arrested (first meiotic arrest) at the diplotene of the prophase of first meiotic division, usually referred to as the dictyate stage. The meiotic arrest is maintained throughout oocyte and follicular development to the Graafian follicle stage. *In vivo* the meiotic process is resumed only in fully grown oocytes in follicles responding to the preovulatory surge of gonadotropins[1] or in follicles undergoing atresia.[24] At ovulation, in most mammalian species, a secondary oocyte arrested at the metaphase of the second meiotic division (second meiotic arrest) is released. The second meiotic division is completed only after fertilization or a parthenogenetic stimulus by abstriction of the second polar body. We shall use the term "oocyte maturation" to denote the preovulatory resumption of the meiotic process including chromatin condensation, germinal vesicle breakdown (GVB), completion of the first meiotic division, abstriction of the first polar body and formation of the second meiotic spindle. Since GVB is the first change readily observed in living oocytes of many species, this is taken in many studies as the criterion for resumption of the meiotic process. While the presence of an intact germinal vesicle is a valid criterion for the maintenance of meiotic arrest, only the abstriction of the polar body and presence of second meiotic spindle confirm the completion of oocyte maturation.

RESUMPTION OF MEIOSIS: MODELS

The study of the regulation of oocyte maturation reflects a common problem inherent in biological research. The process is complex, involving a multitude of regulatory mechanisms. Therefore, simpler substitute "model systems" which are more accessible to investigation were established. It is assumed that these models retain some resemblance to the real biological system. In choosing any model system, however, we should be aware that we sacrifice some of the features of the real object of our study, such as the manifold intercellular interactions and homeostatic mechanisms that operate *in vivo*. As we progress from organ to cell cultures and cell-free systems, we may gain greater reduction for our analytical approach, but the relevance of our findings to the physiological situation is at times entirely lost. It also happens that the investigator so falls in love with his model that he becomes oblivious to the original objectives of his study. In the following we shall

describe some of the *in vitro* models that have been exploited to gain an understanding of the control of oocyte maturation with special emphasis on follicular regulation and assay of oocyte maturation inhibitor (OMI) activity.These models differ in complexity and in some respect yield seemingly contradictory results. Thus, mammalian oocytes dislodged from their follicles will resume meiosis spontaneously in hormone-free media, even if collected before the onset of the endogenous LH-surge, whereas oocytes explanted within their intact follicles, at the same stage of development, fail to do so.[22,29] Such follicle-enclosed oocytes, however, can be induced to undergo maturation by addition of the appropriate hormone(s) to the culture medium[27] closely resembling the requirements prevailing *in vivo*. Being a multicompartmental model it does not allow us to draw unequivocal conclusions regarding the primary site(s) of gonadotropin action in inducing oocyte maturation and the elucidation of the cascade of events involved in this response.

The contrasting behavior of the isolated oocyte and of follicle-enclosed oocytes led to the suggestion that maturation of the mammalian oocyte is normally prevented by an inhibitory action exerted by follicular cells. In order to test this hypothesis a third model was elaborated: co-culture of isolated ooyctes with various follicular components.[9,25,22,29] Studies employing this model corroborated the concept of follicular inhibitory action and led to partial characterization and purification of OMI as detailed by Dr. S.H. Pomerantz (Chapter 20). It should be remembered, however, that the coculture of oocytes with follicular components can hardly restore the precise follicular interactions between the ovum and its micromilieu. Furthermore, OMI purified from the follicular fluid may not necessarily be the native molecule regulating meiosis upon direct transfer from follicle cells to the ovum through the gap junctions connecting these two follicular compartments. At most, we can hope for an approximation and the gaining of some insight into the paracrine mechanisms participating in follicular control of meiosis *in situ*.

FOLLICULAR CONTROL OF OOCYTE MATURATION

The spontaneous maturation of isolated oocytes in culture led Pincus and Enzmann[19] to suggest that follicle cells in mammals "supply to the ovum a substance or substances which directly inhibit nuclear maturation." This suggestion was supported by the inhibition of the resumption of meiosis of porcine oocytes co-cultured with segments of follicle wall, but not with its theca layer[9,25] or ovarian bursa.[16] Likewise, meiosis was inhibited in porcine oocytes transferred to host follicles and cultured in hormone-free medium[8,17] and maturation of rat oocytes injected into porcine follicles was markedly delayed.[8] Further, co-culture of porcine oocytes with porcine granulosa cells inhibited the spontaneous resumption of meiosis.[20,25] The inhibitory effect of granulosa cells depended upon their concentration in culture. Granulosa cells collected from small follicles inhibited meiosis more effectively than cells from medium or large follicles.[2,25] Similarly, when rat oocytes were added to rat granulosa cells previously cultured for 24 or 48 hours, resumption of meiosis was suppressed and the degree of inhibition depended upon the number of granulosa cells in the culture. This inhibitory effect of rat granulosa cells was reversed by the addition of LH to the co-cultures.[22,26] Nuclear maturation of rabbit oocytes was significantly delayed when cultured with granulosa cells.[18]

Extracts of granulosa cells, as well as granulosa cell-conditioned medium, *i.e.* a medium in which granulosa cells were cultured previously, inhibited the resumption of meiosis.[2,20,22] Collectively these results suggest that within the follicle meiosis is arrested by an oocyte maturation inhibitor (OMI) produced by granulosa cells. It was found that addition of FSH or prolactin to suspension cultures of porcine granulosa cells increases OMI activity in the medium, whereas testosterone or dihydrotestosterone reduces it.[5]

Follicular fluid (FFI) from ovaries of rabbit, ovine, bovine, porcine, hamster and the human exerted an inhibitory effect upon the spontaneous maturation of isolated oocytes.[3,10,13,15,25,26] This inhibitory effect is not species-specific; thus human FFI inhibited the maturation of porcine[11] and rat oocytes,[6] bovine FFI inhibited hamster oocytes,[10] and porcine FFI inhibited the maturation of rat oocytes.[28] The OMI activity in porcine FFI appears to decline in the course of follicular growth. This was demonstrated by assaying FFI freshly collected from small, medium and large follicles[21] or fluid collected from pigs on specified days of their reproductive cycle.[30] Similarly, in FFI collected from women participating in an *in vitro* fertilization-embryo transfer program, the OMI activity was significantly lower in follicles yielding mature and fertilizable oocytes as compared to follicles yielding immature or atretic oocytes.[4,12]

OMI from porcine follicular fluid inhibited the resumption of meiosis by oocytes cultured within their intact cumuli, but it did not interfere with the maturation of denuded oocytes of the pig[14] or rat.[23] By contrast, porcine FFI attenuated maturation of mouse denuded oocytes.[7] Nevertheless, this study also showed that the suppression of the resumption of meiosis in mouse denuded oocytes was consistently less than in cumulus-enclosed oocytes.

OMI AS A PHYSIOLOGICAL REGULATOR OF RESUMPTION OF MEIOSIS

In order to serve as a physiological regulator of meiosis, a substance has to fulfil the following criteria: 1) it should be present in a compartment relevant to regulation of meiosis at the proper timing; 2) its action should be reversible with no adverse effects on further stages of ovum maturation, fertilization and embryonic development; and 3) the physiological trigger of resumption of meiosis, gonadotropin, should be able to either supersede its inhibitory action or cause its degradation or removal from the relevant compartment. The inhibitory action of porcine FFI and of its partially purified fractions could be reversed by either transferring the oocytes after an initial 24 hour incubation with the inhibitor to a medium without OMI[11,14,21] or by the addition of an antibody prepared against the low molecular weight fraction of porcine FFI.[22] Thus, the inhibition of meiosis by OMI from porcine FFI does not appear to be due to a toxic and irreversible action.

In vivo or in follicle-enclosed oocytes *in vitro* resumption of meiosis is induced by LH. Hence the ability of LH (or other gonadotropins) to overcome the inhibition of meiosis by FFI or by coculture with granulosa cells lends support for the physiological role of OMI in the regulation of meiosis. It was found that the addition of LH to cultures of porcine oocytes with porcine FFI or its purified fractions overcame their inhibition of meiosis.[25] Also, the inhibitory action of bovine or hamster FFI,[10] of porcine FFI, rat granulosa cell conditioned medium as well as coculture with rat granulosa cells[22,26] were all alleviated by the addition of LH, closely resembling the regulation *in vivo* of oocyte maturation.

Follicular OMI activity was demonstrated by several laboratories and by a variety of approaches. Some investigators, however, were unable to confirm the presence of OMI activity in various follicular preparations. These varying findings may be related, at least in part, to the low OMI activity of follicular fluid and of granulosa cell cultures; the finding that fluid and granulosa cells from large follicles are devoid of inhibitory activity; the relative instability of OMI and to differences in the methods of oocyte collection and culture.

Collectively, the studies reviewed above clearly support the role of local paracrine mechanisms in regulation of oocyte maturation. Follicular OMI fulfills several criteria of a physiological regulator of meiosis.

REFERENCES

1. AYALON D, TSAFRIRI A, LINDNER HR, CORDOVA T, HARELL A: Serum gonadotropin levels in proestrous rats in relation to the resumption of meiosis by the oocytes. J Reprod Fertil 31:51, 1972
2. CENTOLA GM, ANDERSON LD, CHANNING CP: Oocyte maturation inhibitor (OMI) activity in porcine granulosa cells. Gamete Res 4:451, 1981
3. CHANG MC:The maturation of rabbit oocytes in culture and their maturation, activation, fertilization and subsequent development in fallopian tube. J. Exp Zool 128:378, 1955
4. CHANNING CP, ANDERSON LD, HOOVER DJ, KOLENA J, OSTEEN KG, POMERANTZ SH, TANABE K: The role of non-steroidal regulators in control of oocyte and follicular maturation. Recent Prog Horm Res 8:331, 1982
5. CHANNING CP, LIU CO, JONES GS, JONES H: Decline in follicular oocyte maturation inhibitor coincident with maturation and achievement of fertilizability of oocytes recovered at midcycle of gonadotropin-treated women. Proc Natl Acad Sci USA 80:4184, 1983
6. CHARI S, HILLENSJO T, MAGNUSSON C, STURM G, DAUME E: *In vitro* inhibition of rat oocyte meiosis by human follicular fluid fractions. Arch Gynecol 233:155, 1983
7. DOWNS SM, EPPIG JJ: Cyclic adenosine monophosphate and ovarian follicular fluid act synergistically to inhibit mouse oocyte maturation. Endocrinology 114:418, 1984
8. FLEMING AD, KUEHL TJ, ARMSTRONG DT: Maturation of pig and rat oocytes transplanted into surrogate pig follicles *in vitro*. Gamete Res 11:107, 1985
9. FOOTE WD, THIBAULT C: Recherches experimentales sur la maturation *in vitro* des ovocytes de truie et de veau. Ann Biol Anim Bioch Biophys 9:329, 1969
10. GWATKIN RBL, ANDERSEN OF: Hamster oocyte maturation *in vitro* inhibition by follicular components. Life Sci 19:527, 1976
11. HILLENSJO T, BATTA SK, SCHWARTZ-KRIPNER A, WENTZ AC, SULEWSKI J, CHANNING CP: Inhibitory effect of human follicular fluid upon the maturation of porcine oocytes in culture. J Clin Endocr Metab 47:1332, 1978
12. HILLENSJO T, BRANNSTROM M, CHARI S, DAUME E, MAGNUSSON C, NILSSON L, SJOGREN A, TORNEL J: Oocyte maturation as regulated by follicular factors. Ann NY Acad Sci 442:73, 1985
13. HILLENSJO T, CHANNING CP, POMERANTZ SH, SCHWARTZ-KRIPNER A: Intrafollicular control of oocyte maturation in the pig. *In Vitro* 15:32, 1979
14. HILLENSJO T, KRIPNER AS, POMERANTZ SH, CHANNING CP: Action of porcine follicular fluid oocyte maturation *in vitro:* Possible role of the cumulus cells. In CHANNING CP, MARSH JM, SADLER WA (eds): Ovarian Follicular and Corpus Luteum Function, p 282, New York, Plenum Press, 1979
15. JAGIELLO G, GRAFFEO J, DUCAYEN M, PROSSER R: Further studies of inhibitors of *in vitro* mammalian oocyte maturation. Fertil Steril 28:476, 1977
16. LEIBFRIED L, FIRST NL: Follicular control of meiosis in the porcine oocyte. Biol Reprod 23:705, 1980
17. MEINECKE B, MEINECKE-TILLMAN S: Induction and inhibition of meiotic maturation of follicle-enclosed porcine oocytes in vivo. Theriogenology 216:205, 1981
18. MOTLIK J, FULKA J: Fertilization of rabbit oocyte cocultured with granulosa cells. J Reprod Fertil 63:425, 1981

19. PINCUS G, ENZMANN EV: The comparative behaviour of mammalian eggs *in vivo* and *in vitro*. J Exp Med 62:655, 1935
20. SATO E, ISHIBASHI T: Meiotic arresting action of the substance obtained from cell surface of porcine ovarian granulosa cells. Jap J Reprod Fertil 68:335, 1933
21. STONE SL, POMERANTZ SH, SCHWARTZ-KRIPNER A, CHANNING CP: Inhibitor of oocyte maturation from porcine follicular fluid: Further purification and evidence for reversible action. Biol Reprod 19:585, 1978
22. TSAFRIRI A: Oocyte maturation in mammals. In JONES RE (ed): The Vertebrate Ovary, p 409, New York, Plenum Press, 1979
23. TSAFRIRI A, BAR-AMI S: Oocyte maturation inhibitor: A 1981 perspective. In CP CHANNING, SJ SEGAL (eds): Intraovarian Control Mechanisms, p 145, Plenum, New York, 1982
24. TSAFRIRI A, BRAW R: Experimental approaches to atresia in mammals. Oxf Rev of Reprod Biol 6:226, 1984
25. TSAFRIRI A, CHANNING CP: Inhibitory influence of granulosa cells and follicular fluid upon porcine oocyte meiosis *in vitro*. Endocrinology 96:922, 1975
26. TSAFRIRI A, CHANNING CP, POMERANTZ SH, LINDNER HR: Inhibition of maturation of isolated rat oocytes by porcine follicular fluid. J Endocrinol 75:285, 1977
27. TSAFRIRI A, LINDNER HR, ZOR U, LAMPRECHT SA: *In vitro* induction of meiotic division in follicle-enclosed rat oocytes by LH, cyclic AMP and prostaglandin E_2. J Reprod Fertil 31:39, 1972
28. TSAFRIRI A, POMERANTZ SH, CHANNING CP: Follicular control of oocyte maturation. In: CROSIGNANI PG, MISHELL DR (eds): Ovulation in the Human, p 31, New York, Academic Press, 1976
29. TSAFRIRI A, REICH R, ABISOGUN AO: The ovarian egg and ovulation. In LAMMING GE (ed): Marshalls Physiology of Reproduction, Vol III, London, Churchill Livingstone, (in press)
30. VAN DE WIEL DFM, BAR-AMI S, TSAFRIRI A, DE JONG FH: Oocyte maturation inhibitor, inhibin, and steroid concentrations in porcine follicular fluid at various stages of the oestrous cycle. J Reprod Fertil 68:247, 1983

DISCUSSION

CHAIRPERSON: MILAN R. HENZL, M.D., Ph.D.

DR. NEKOLA: I have a question concerning the disappearance of OMI or the decrease of its concentration in the preovulatory follicles. Would you care to comment on what is keeping the oocyte arrested if the concentration of OMI is going down?

DR. TSAFRIRI: It should be emphasized that OMI is not the sole regulator of resumption of meiosis. Thus, it is clear that high oocyte levels of cyclic AMP are inhibitory. As mentioned during my presentation, OMI and dibutyryl cAMP act synergistically to inhibit maturation *in vitro*. Similar mechanisms may operate also *in vivo*. The concerted action of OMI, cAMP and probably other, yet unidentified factors, suppresses meiosis in antral follicles. We have, indeed, observed that the OMI activity in porcine follicular fluid is lower in large as compared to small follicles. But this does not say that OMI activity in large follicles is not sufficient to maintain meiotic arrest *in vivo* prior to the LH surge. Nevertheless, this does suggest a mechanism which facilitates the meiotic response to LH stimulation. The fact that the present studies on the control of resumption of meiosis in mammals deal with inhibitory factors should not lead us to the conclusion that positive stimuli, or regulators, are absent in mammalian follicles, and I am saying that in spite of my failure to demonstrate such positive activity. In summary, the answer to your question is that the regulation of meiosis is complex, involving several factors and follicular sites, which cannot be explained entirely by one regulator such as OMI.

20

Oocyte Maturation Inhibitor: Several Candidates for the Title

SEYMOUR H. POMERANTZ

This chapter offers a brief review of candidates for the title of oocyte maturation inhibitor and the evidence that is available to support each one. In these proceedings Dr. Alexander Tsafriri has reviewed the general role of an oocyte maturation inhibitor as a regulator of oocyte maturation. Here I focus on specific compounds and build on the reviews by Eppig and Downs,[21] Tsafriri and Pomerantz,[54] and Dekel[9] that have appeared in the past four years. No attempt has been made to include all possible citations.

CYCLIC AMP

Evidence that a derivative of cyclic AMP inhibits the spontaneous maturation of mammalian oocytes was first brought by Cho *et al.* in 1974.[7] Since that time several lines of evidence converge to support the idea that intra-oocyte levels of cyclic AMP help regulate oocyte maturation.

(A) Derivatives of cyclic AMP as well as phosphodiesterase inhibitors such as 3-isobutyl-1-methyl xanthine (IBMX) inhibit the spontaneous maturation of oocytes.[10,31,61] Gonadotropins reverse the inhibitory action of IBMX and dibutyryl cyclic AMP.[10] Recently Aberdam *et al.*[1] showed that adenylate cyclase of *Bordetella pertussis*, which invaded the denuded rat oocyte, generated cyclic AMP within the oocyte and maintained meiotic arrest. Cyclic AMP within the oocyte is also elevated by stimulation of endogenous adenylate cyclase. Several reports show that cholera toxin,[11] which stimulates adenylate cyclase by binding the GS protein, and forskolin, which activates the enzyme catalytic subunit,[12,39] inhibit the spontaneous maturation of cumulus-enclosed, but not denuded, rat oocytes. There are other investigators, however, who report that forskolin did increase the concentration of cyclic AMP of denuded rat or mouse oocytes and inhibited meiotic maturation.[19,20,50,58] As Dekel points out[9] the explanation for these opposite findings may lie in the fact that some preparations of denuded oocytes probably contain large numbers of projections of cumulus cells embedded in zona pellucida. These remnants may contain adenylate cyclase and respond to forskolin.

(B) Dekel and Beers hypothesized[10,11] that cyclic AMP from cumulus cells is transferred to the oocyte by intercellular coupling and that cyclic AMP plays a vital role in regulating oocyte maturation. This hypothesis has now been amply supported by the work of three laboratories. Racowsky[39] showed that incubation of cumulus-enclosed rat oocytes with

The author's research reported here was supported in part by NIH Grant HD-14696.

forskolin increased the cyclic AMP content of oocytes and cumulus and there was a significant positive correlation between the amount of cyclic AMP within the cumulus and that in the enclosed oocyte. Racowsky further showed that a gradual increase in germinal vesicle breakdown was accompanied by a fall in intra-oocyte cyclic AMP while the cumulus cell content of cyclic AMP remained constant. Similar results were obtained by Dekel with rat oocytes,[9] Bornslaeger and Schultz with mouse oocytes,[3] and Racowsky with pig[40] and hamster[41] oocytes. The earlier report by Schultz *et al.*[50] that cyclic AMP did not equilibrate between cumulus cells and associated oocyte was probably the result of using incubation conditions for the complexes which did not completely inhibit the oocyte phosphodiesterase, thus allowing the transferred cyclic AMP to be hydrolyzed.

(C) Dekel[9] has observed with rat oocyte-cumulus complexes that the coupling between oocyte and cumulus cells, as measured by transport of [^{3}H]uridine, is broken before resumption of meiosis. These results differ from an earlier report by Dekel *et al.*[13] in which electrical coupling was used to measure the oocyte-cumulus connection. Dekel[9] feels that the chemical coupling measurements are better able to show the changes in intercellular communication which accompany hormonal stimulus and which occur before complete uncoupling. In support, Racowsky and Satterlie[43] showed, with hamster oocyte-cumulus complexes, that biphasic changes in ionic coupling precede and accompany meiotic maturation while there is a steady decrease in the transfer of [^{3}H]uridine from cumulus to oocyte. However, the temporal relation found by Dekel between the breaking of oocyte-cumulus coupling and meiosis is not supported by results with hamster,[43] sheep,[34] and mouse[20,45] oocytes. These latter investigators show reinitiation of maturation in advance of the reduction in oocyte-cumulus cell coupling. These different observations could be the result of species differences.

(D) Schultz *et al.*[49] and Vivirelli *et al.*[59] demonstrated that a decrease in cyclic AMP within the mouse oocyte occurs shortly before germinal vesicle breakdown and Dekel[9] reported similar unpublished results for rat oocytes. This means that reduction of the concentration of cyclic AMP within the oocyte is an early event in the sequence of reactions leading to germinal vesicle breakdown.

(E) In summary, elevated levels of cyclic AMP within the oocyte prevent the reinitiation of oocyte maturation and reductions of those levels are correlated with resumption of meiosis. Reduction of intra-oocyte cyclic AMP is an early event in the process of meiosis. In several species cyclic AMP from cumulus cells can be transferred to the oocyte and interruption of oocyte-cumulus communication may precede meiosis.

STEROIDS

Several investigators[8,23,38,44] have reported that various steroids augment the inhibition of oocyte maturation brought about by dibutyryl cyclic AMP or agents which increase the intracellular concentration of cyclic AMP. Steroids are ineffective on their own. Testosterone[38,44] progesterone,[23] and estradiol[23] were effective. Daniel *et al.*[8] showed that 19-norandrostenedione, a steroid found at high concentration in pig follicular fluid (2 x 10^{-6}M),[28] was also effective in augmenting inhibitory effect of cycle AMP. There is some disagreement as to whether intact cumulus cell-oocyte complexes are required. Eppig *et al.*[23] reported that testosterone, progesterone, and estradiol are effective in augmenting

inhibition of maturation of denuded oocytes in the presence of dibutyryl cyclic AMP or forskolin, but Daniel *et al.*[8] found that 19-norandrostenedione was ineffective in a denuded oocyte system containing dibutyryl cyclic AMP. There is some ambiguity also with dihydrotestosterone: Eppig *et al.*[23] found it to be inactive with the cumulus-oocyte complex but it was active in the denuded system, but only in the presence of dibutyryl cyclic AMP, not forskolin. I agree with the conclusion reached earlier by Eppig and Downs[21] that a physiological role for steroids in the maintenance of meiotic arrest has not yet been demonstrated. The steroids are effective only when an inhibitory intracellular concentration of cyclic AMP has already been reached.

PURINES

Downs *et al.*[17] identified hypoxanthine as one of at least two inhibitors of mouse oocyte maturation in a low molecular weight fraction of pig follicular fluid. Hypoxanthine accounted for most of the activity. In that report several other purines — adenine, guanine, xanthine, adenosine, guanosine, and inosine—also had activity at 0.5 mM or 1.0 mM concentration when tested in a system of cumulus-enclosed mouse oocytes and dibutyryl cyclic AMP. Except for guanosine—the most active compound — none were active alone. Hypoxanthine was shown[22] to be effective in denuded oocytes if the concentration was raised to 4 to 5 μM and hypoxanthine and adenosine were identified in mouse follicular fluid at concentrations of 2-4 mM and 0.35-0.70 mM, respectively. In this report[22] adenosine and inosine were each synergistic with hypoxanthine. Salustri *et al.*[46] showed that adenosine action is mediated by reaction with cell surface receptors.

In a rat system[2] guanosine (50 μM) and hypoxanthine (2 μM) alone decreased oocyte maturation and each greatly potentiated the inhibitory effect of FSH, particularly guanosine. Adenosine had no effect on its own but potentiated the action of FSH.[2,33,46] Miller and Behrman[33] however, report no potentiation of FSH action in the rat oocyte system by adenine or inosine but show potentiation by ATP, ADP, or AMP. Preston *et al.*[37] showed that two calcium ionophores, in a dose-dependent manner, potentiated oocyte maturation in the presence of FSH alone or with FSH and adenosine, and also inhibited accumulation of cyclic AMP. The ionophores had no effect when maturation was inhibited by dibutyryl cyclic AMP. Adenosine reversed ionophore-dependent inhibition of cyclic AMP accumulation and induction of oocyte maturation in the presence of FSH. Adenosine, ionophores, or FSH had no effect on denuded rat oocytes. The authors concluded that calcium mediates a potentiation of oocyte maturation by inhibiting FSH-dependent accumulation of cyclic AMP in cumulus cells and that adenosine antagonizes this effect of calcium.

The mechanism of action by purines has been studied in the mouse oocyte system by Eppig and colleagues. Downs *et al.*[16] showed that cumulus-enclosed oocytes take up much more hypoxanthine or adenosine than do denuded oocytes. Both compounds were completely metabolized *in vitro*: most of the hypoxanthine was metabolized to uric acid and most of the adenosine to ADP; a small amount of each was converted to inosine. The following reactions probably account for these findings:

$$\text{Hypoxanthine} \xrightarrow[\text{oxidase}]{\text{xanthine}} \text{Xanthine} \xrightarrow[\text{oxidase}]{\text{xanthine}} \text{Uric acid(l)}$$

$$\text{Adenosine} \xrightarrow[\text{phosphorylase}]{\text{nucleoside}} \text{Adenine} \quad (2)$$

$$\text{Adenine} + \text{5-phosphoribosyl-1-pyrophosphate (PRPP)} \xrightarrow[\text{phosphoribosyl transferase}]{\text{adenine}} \text{AMP} \quad (3)$$

$$\text{AMP} + \text{ATP} \xrightarrow[\text{kinase}]{\text{adenylate}} \text{2 ADP} \quad (4)$$

$$\text{Adenosine} \xrightarrow[\text{deaminase}]{\text{adenosine}} \text{Inosine} \quad (5)$$

There are other reactions which can take place with hypoxanthine and adenosine which lead to inosine monophosphate (IMP) and the latter compound can be converted to xanthylic acid (XMP). XMP is the source for guanylic acid (GMP), guanosine, and guanine:

$$\text{Hypoxanthine} + \text{PRPP} \rightarrow \text{IMP} \quad (6)$$

$$\text{AMP} \xrightarrow[\text{deaminase}]{\text{adenylate}} \text{IMP} \quad (7)$$

$$\text{IMP} + \text{NAD} \xrightarrow[\text{dehydrogenase}]{\text{IMP}} \text{XMP} \quad (8)$$

$$\text{XMP} \rightarrow \text{GMP} \rightarrow \text{Guanosine} \rightarrow \text{Guanine} \quad (9)$$

Downs *et al.*[16] employed inhibitors of IMP dehydrogenase in the cumulus-enclosed oocyte system and observed that one of these inhibitors, mycophenolic acid, reversed, in a dose-dependent manner, the inhibition of meiosis brought about by hypoxanthine or hypoxanthine plus adenosine, but it did not reverse the inhibition by guanosine. Mycophenolic acid had no effect on the uptake of hypoxanthine by cumulus-enclosed oocytes. The conclusion reached was that xanthyl or guanyl compounds or both, produced by the oocyte-cumulus complex, play an important role in maintenance of meiotic arrest. This would account for the observation that guanosine was the most active purine in the mouse system[17].

Downs and Eppig[15] injected immature mice with inhibitors of purine synthesis to obtain further evidence on the role of guanyl and/or xanthyl compounds in maintaining meiotic arrest *in vivo*. Cumulus-enclosed oocytes were isolated from ovaries at various times after treatment with mycophenolic acid and bredinin, inhibitors of IMP dehydrogenase; azaserine, an inhibitor of de novo purine synthesis; and sodium hadacidin and dl-alanosine, inhibitors of adenylosuccinate synthetase. Inhibition of IMP dehydrogenase and of de novo purine synthesis each induced maturation in a dose-dependent manner. Furthermore, the actions appeared to be direct and not as a result of stimulation of gonadotropin release or of atresia. However, the inhibitors of adenylosuccinate synthetase had no effect on maturation. The authors conclude that guanyl and/or xanthyl compounds are essential for the maintenance of meiotic arrest *in vivo*.

The interpretation is clouded however by the following observations:

(a) There was no showing that the compounds actually did inhibit the appropriate purine pathways in the ovary *in vivo* at the doses used.

(b) Since both salvage and de novo pathways are operating would one expect acute inhibition of one pathway to be so disruptive?

I judge that the data gathered so far on a role for purines in maintaining meiotic arrest in the mouse and rat are quite persuasive, but that we must await additional data on the biochemical and physiological mechanism, and on results in other species. So far, we have found no effect of hypoxanthine at 0.5 mM in either pig or toad oocyte assays. In addition our OMI activity chromatographs on reverse phase HPLC in a different position from authentic hypoxanthine.

OOCYTE MATURATION INHIBITOR (OMI) IN PIG FOLLICULAR FLUID

The work that I will describe from my own laboratory is a direct outgrowth of work reported by Foote and Thibault[25] and later by Tsafriri and Channing[52] who showed that granulosa cells inhibited meiotic maturation of pig oocytes. Tsafriri and Channing[52] found in addition that pig follicular fluid (FFI) inhibited meiotic maturation of pig oocytes. The FFI inhibitor is not species specific since pig FFI inhibited the maturation of rat[56] and mouse (Channing and Tsafriri, unpublished[14,17]) oocytes; bovine FFI affected hamster oocytes;[26] and human FFI inhibited pig[27], rat[6], and toad[4] oocytes. Although there are reports[24,29,42,47] that FFI or a PM-10 filtrate (<10,000 molecular weight fraction) of FFI did not inhibit oocyte maturation, these observations are explained at least in part by the low activity of some batches of FFI, the likelihood that OMI in FFI is sometimes masked by substances which stimulate oocyte maturation[5,36] and the use of small pools of FFI.[27] However, some of these authors found that co-culture of granulosa cells with oocytes inhibited meiosis[47] whereas others[29] did not. The latter group[29] did find that co-culture with follicle hemisection prevented meiosis.[30]

The action of OMI was reversed by addition of LH to the culture medium[56] or by replacement with control medium.[51]

ASSAY OF OMI

Cultures of cumulus-enclosed pig oocytes plus the putative inhibitor alone have served as the primary system for OMI.[51,52,55] Tsafriri *et al.*[56] showed that rat oocyte cultures

could be used to follow the purification.

Mouse oocytes have been used[14,17] to test FFI and crude fractions of FFI for OMI. However, the system employed—cumulus-enclosed oocytes together with something which increases intracellular cyclic AMP or a derivative of cyclic AMP — is different from the conceptually simpler system of cumulus-enclosed oocytes used by us. However, one can enhance the activity of OMI in the rat system by adding dibutyryl cyclic AMP to the system.[54]

XENOPUS OOCYTES

Inhibitory effects of human follicular fluid on toad[4] and frog[48] oocytes have been demonstrated. We[35] have now developed an assay for OMI using oocytes from *Xenopus laevis*. This latter assay is easier to conduct than the assay with pig oocytes and gives comparable results after a certain stage of purification.

The process of meiotic maturation of *Xenopus* oocytes appears to differ from that of mammalian oocytes. For example, *Xenopus* oocytes require progesterone exposure *in vitro* systems, whereas mammalian oocytes mature spontaneously in culture when they are removed from the follicle. However, there are some similarities. A principal one is that in both mammalian[49] and *Xenopus*[32] oocyte systems there is an early fall in cyclic AMP within the oocyte.

As we developed the assay we learned that oocytes from different toads are quite variable. In order to get 60% or more of the eggs to mature we have found it useful to inject toads with 12 IU pregnant mares serum gonadotropin (PMSG) from 4 to 10 days before they are used. The animals are anesthetized in an ice-water slurry and an ovary is cut off and placed in a 0.01 M Hepes-Ringer solution, pH 7.8.[60] The eggs are harvested by gently scraping the ovary. Stage VI oocytes[18] are selected and counted. Usually 600-1000 oocytes can be obtained from one ovary. Hepes-Ringer, OMI standard, and fractions to be tested for activity are each placed in groups of 2 or 3 culture wells in the amount of 1 ml per well. Oocytes (15 to 20) are added to each well and after 1 hour progesterone (8 mM final concentration; 2.5 μmole/ml) is added. The incubation is continued at room temperature until there is maximum maturation in the control (5 to 8 hour). The oocytes are scored as mature (characteristic white spot on the animal pole), immature, or mottled (oocytes which have streaked areas in the pigmented hemisphere which make it difficult to determine whether maturation has occurred). The percent maturation is calculated from the number of mature oocytes divided by the total number of oocytes. The data in Figure 1[35] shows one of many trials in which 3 toads were each injected with 12 IU of PMSG and portions of their ovaries were removed on different days after treatment. Figure 1 shows inhibition of oocyte maturation achieved with various dilutions of an OMI standard on 2,3,4 and 7 days, respectively, after treatment with PMSG. Better inhibition was observed by 4 days past PMSG and this response was maintained for at least 7 days. Dose responses were usually seen. The effect of 12 IU of PMSG held up for about 2 weeks and disappeared by 23 days after injection.[35] In other experiments[35] reversibility of inhibition was found when OMI medium was replaced by control medium after 6.5 hour of OMI exposure.

INITIAL PURIFICATION AND CHARACTERIZATION OF OMI

Tsafriri *et al.*[55] reported that OMI passed through an Amicon PM-10 filter. Chromatography of the concentrated filtrate on Sephadex G-25[52,55] revealed that OMI had a molecular weight of <2000. FFI lost its activity when it was treated with trypsin but retained its activity on repeated freezing and thawing, treatment with charcoal, and when heated to 60°C for 20 minutes.[55] In some unpublished experiments we have found no loss of activity after heating a partially purified fraction to 100°C for 15 minutes. In further experiments we obtained conflicting results after treatment with trypsin or with a bacterial protease. In one experiment we destroyed the activity of a Biogel P_2 fraction (unpublished experiments of Pomerantz and Bilello) with either the bacterial protease or with trypsin, but in a later experiment with a more highly purified HPLC fraction (see next section), neither trypsin nor the bacterial protease destroyed the activity. We have no explanation as yet for these contradictory findings.

In early experiments we tried ion exchange chromatography on CM-Sephadex and DEAE-Sephadex.[36] These experiments showed that OMI is an acidic compound since it was bound to DEAE-but not CM-Sephadex.

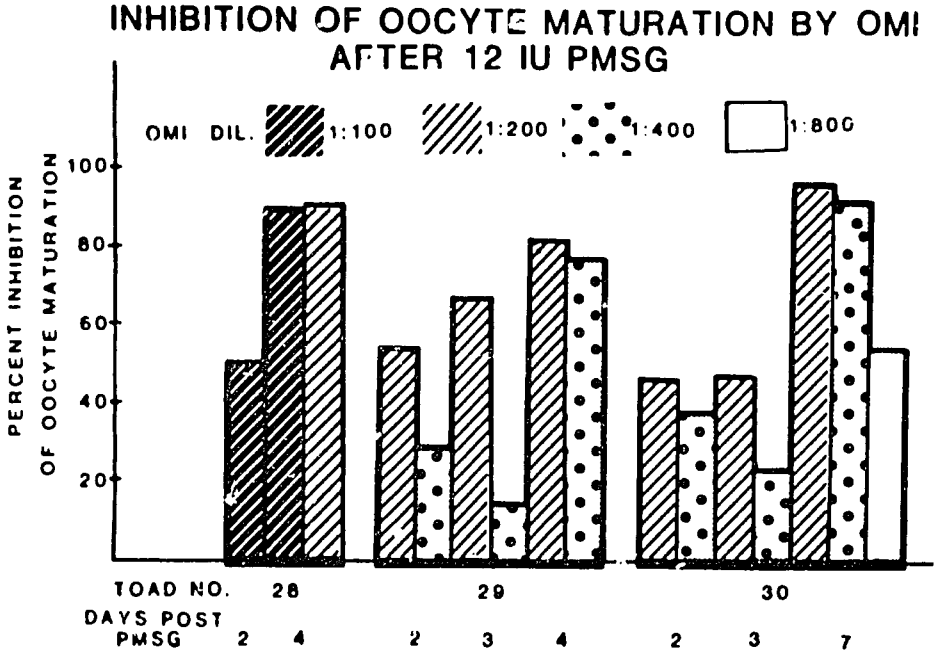


Figure 1. Inhibition of oocyte maturation by OMI after injection of toads with 12 IU of PMSG[35]. Stage VI oocytes were exposed for 1 hour to QAE-Sephadex Fraction A OMI dissolved in Ringer solution at dilutions of (1:100), (1:200), (1:400), or (1:800). Then progesterone was added at a final concentration of 8 mM and remained for 6 hours until the eggs were scored. The number of oocytes in each group (from left to right in the figure): Toad 28: 45, 47, 50; Toad 29: 48, 47, 44, 49, 45, 43; Toad 30: 45, 50, 45, 50, 47, 52, 49.

We conclude from these results that OMI is a low molecular weight acidic compound, probably not a steroid, and possibly containing some peptide linkages.

CURRENT OMI PURIFICATION PROCEDURES

The procedure exploits the acidic nature of OMI. The Amicon PM-10 filtrate is diluted with an equal volume of water and passed onto a column of QAE-Sephadex. This strongly basic ion exchange resin binds OMI and it is eluted with a steep gradient of NH_4HCO_3. In Figure 2[53] (upper panel) I show the results of one of six runs made on QAE-Sephadex. There were two major areas of OMI activity which were collected as fractions A and B according to the pig oocyte assay. The lower panel shows the results of a blank run using

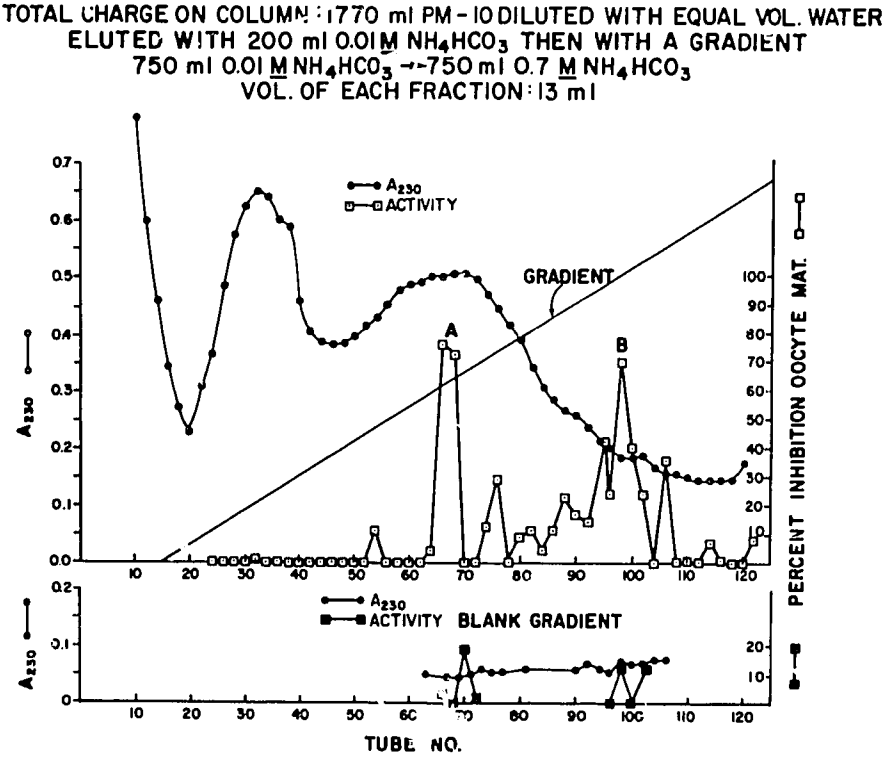


Figure 2. Chromatography of PM-10 filtrate on QAE-Sephadex (5 x 4.5 cm) suspended in 0.01 M ammonium bicarbonate.[53] 1770 ml of PM-10 filtrate (1.5 units/ml; 0.18 units/mg) was diluted with an equal volume of water and passed onto the column. The column was eluted with 200 ml of 0.01 M ammonium bicarbonate and then with a linear gradient formed by passing 750 ml of 0.7 M ammonium bicarbonate into 750 ml of 0.01 M ammonium bicarbonate. Active fractions were pooled to form fractions A^{65-69} and B^{95-102}. The lower panel shows the result of a BLANK RUN conducted in the same way as the gradient after the resin was washed with 1.0 M ammonium bicarbonate and re-equilibrated with 0.01 M ammonium bicarbonate.

the same resin after cleaning and re-equilibration with the same buffer. The results showed that no inhibitory activity eluted from the blank resin and that only a small amount of material absorbed at 230 nm.

Fractions A and B were chromatographed separately on a reverse phase HPLC column. The data for fraction A is shown in Figure 3.[53] The OMI activity was eluted in fractions (24+25) and (26+27). These fractions were combined and rechromatographed and the results are shown in Figure 4. The OMI activity was entirely in fraction 26. The data for HPLC of fraction B are shown in Figure 5. Only fraction (26+27) had significant OMI activity. The activity in tube 15 fell short of statistical significance ($p < 0.2$) but other runs have shown significant activity in this region. The apparent activity in 30-34 was not significant and in other runs we did not find activity in this region. The total recovery of activity from the QAE-Sephadex column was 72%, equally divided between fractions A and B. The recovery of activity from fraction A after the two HPLC runs was only 50% and that of fraction B was 53%. Fraction A was purified 15,000-fold over the PM-10 fraction, equivalent to about 150,000-fold over EFI. Since fractions A and B give similar reverse phase HPLC profiles we are still uncertain whether they are indeed different from each other.

We have compared the results of mammalian and *Xenopus* oocyte assays on fractions obtained from QAE-Sephadex and reverse phase HPLC in order to determine whether toad oocyte assays could be used in place of pig oocyte assays to follow the purification of OMI. The results (not shown) for QAE-Sephadex showed that while the toad and pig assays both showed inhibition for two fractions, there were several fractions which were positive by the toad assay but negative by the pig assay. However, as shown in Figure 6, the results of the pig and toad oocyte assays coincided exactly for fractions eluted from reverse phase HPLC. Similarly, we did some preliminary trials of chromatography of a reverse phase HPLC fraction on an anion exchange HPLC column. The results (not shown) showed exact coincidence between pig and toad oocyte assays. As a result of these studies we are using the toad oocyte assay to follow OMI purification from the reverse phase HPLC step onwards.

POLYCLONAL ANTIBODIES TO OMI

Tsafriri *et al.*[57] were successful in raising antibodies in rabbits against a PM-10 fraction of OMI which blocked the action of OMI in a rat oocyte system. More recently we have generated polyclonal antibodies in three rats by injecting the animals with a QAE-Sephadex fraction linked to bovine serum albumin (unpublished experiments of S.H. Pomerantz, F. Kohen, and A. Tsafriri, 1986). All three antisera were effective in blocking OMI activity in the rat and pig oocyte assays, while only one blocked OMI in the toad assay. The results of the test using rat oocytes are shown in Figure 7. In this and in the other tests we observed that normal rat serum alone appeared to have some neutralizing capacity even though it did not reach the 95% level of significance. We hope to be able to use antibodies to pure OMI in later studies of its mechanism of action.

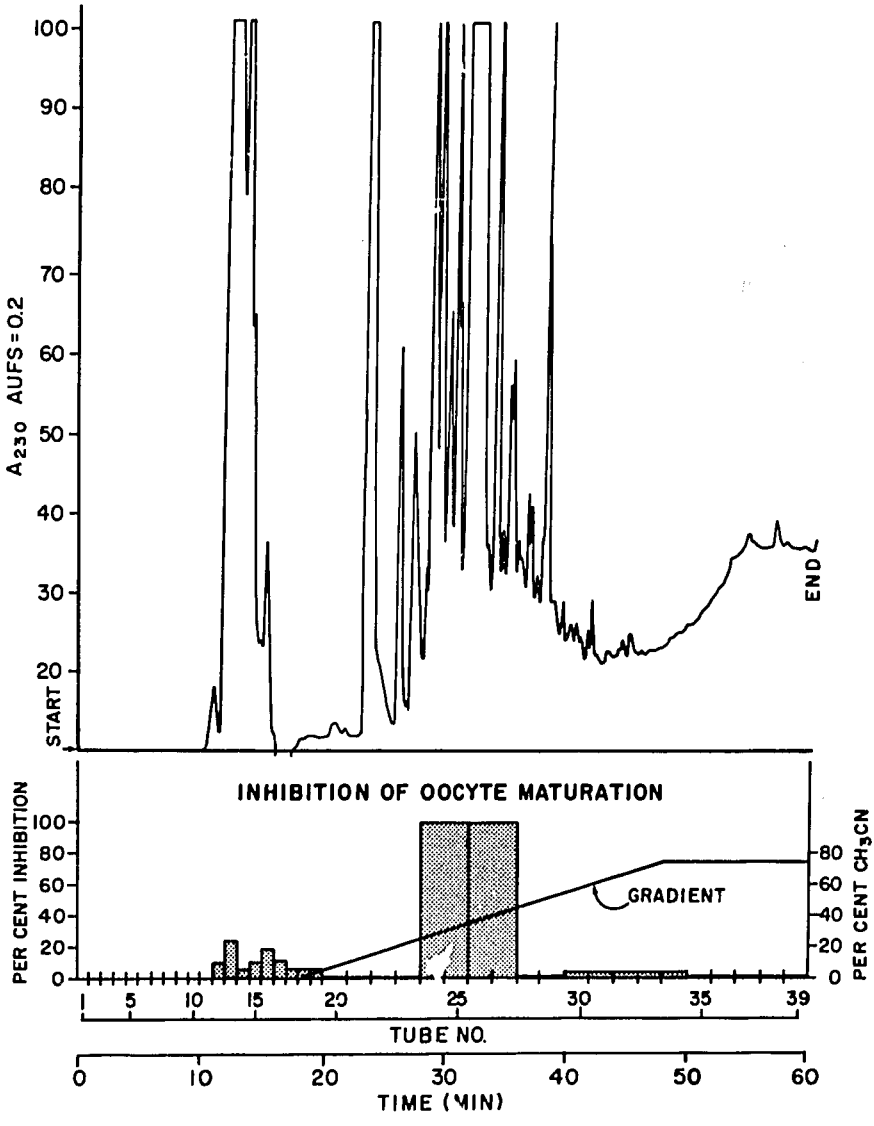


Figure 3. HPLC of fraction A of Figure 2.[53] Fraction A (1.73 mg; 750 units), acidified to 0.1% trifluoroacetic acid (TFA), was chromatographed in two portions on a Whatman Magnum 9 reverse phase column. Buffer A was 0.1% TFA in water and B, 0.1% TFA in acetonitrile. The column was eluted with Buffer A for 18 minutes, then with a linear gradient with Buffer B for 30 min so that at the end of 48 min the final mixture was 25% A: 75% B. This last was held for a further 12 minutes to complete the run at 60 minutes During the first 18 minutes the elution rate was 1 ml/min and 1 ml fractions were collected; the rate was then changed to 2 ml/min and fractions of 4 ml were collected. The dashed bars show areas of significant inhibition of oocyte maturation. The length of each bar shows the tubes pooled for the assay. Only the height of each bar is indicative of activity.

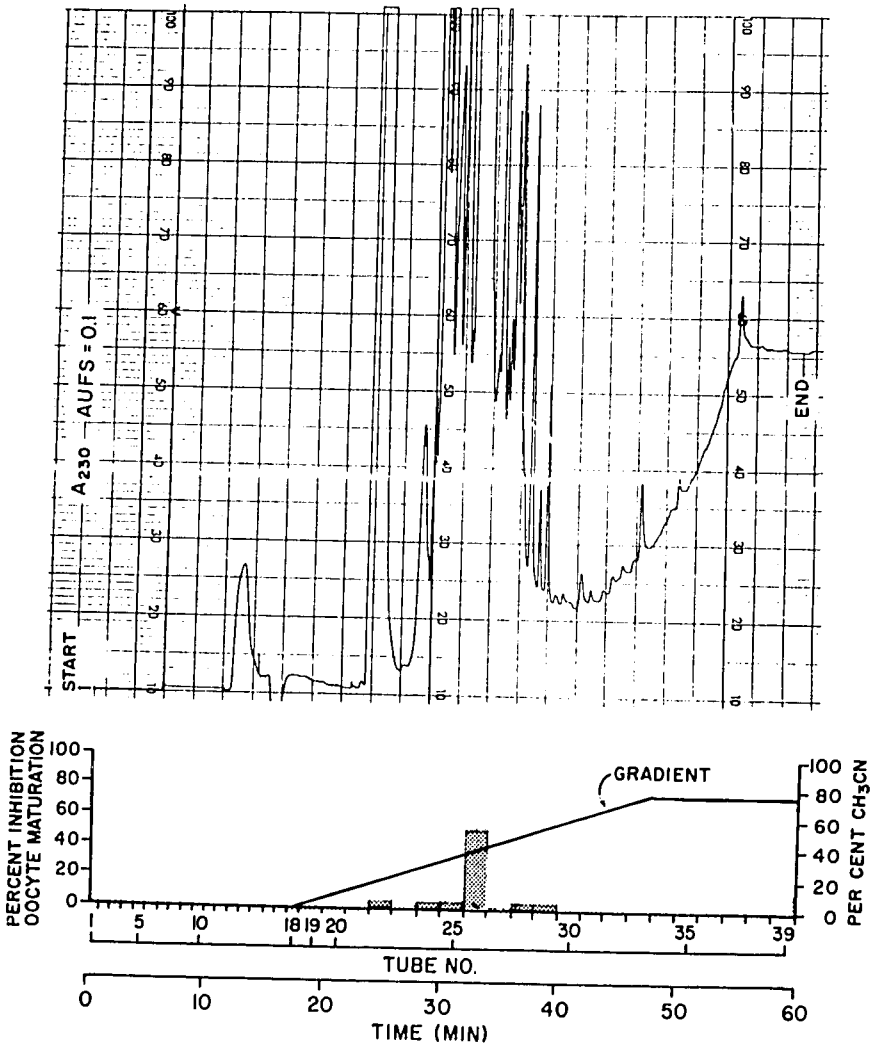


Figure 4. Chromatography of (24 + 25) + (26 + 27) of Figure 3 on HPLC. The active samples were mixed (0.487 mg/ about 750 units), acidified to 0.1% TFA, and chromatographed in three portions on a Whatman Magnum 9 reverse phase column as described in the legend to Figure 3. Significant inhibition of oocyte maturation was measured only in #26.

CONCLUSION

It appears that several compounds probably play roles in the maintenance of meiotic arrest and there is the strong possibility that there may be some species variation in the

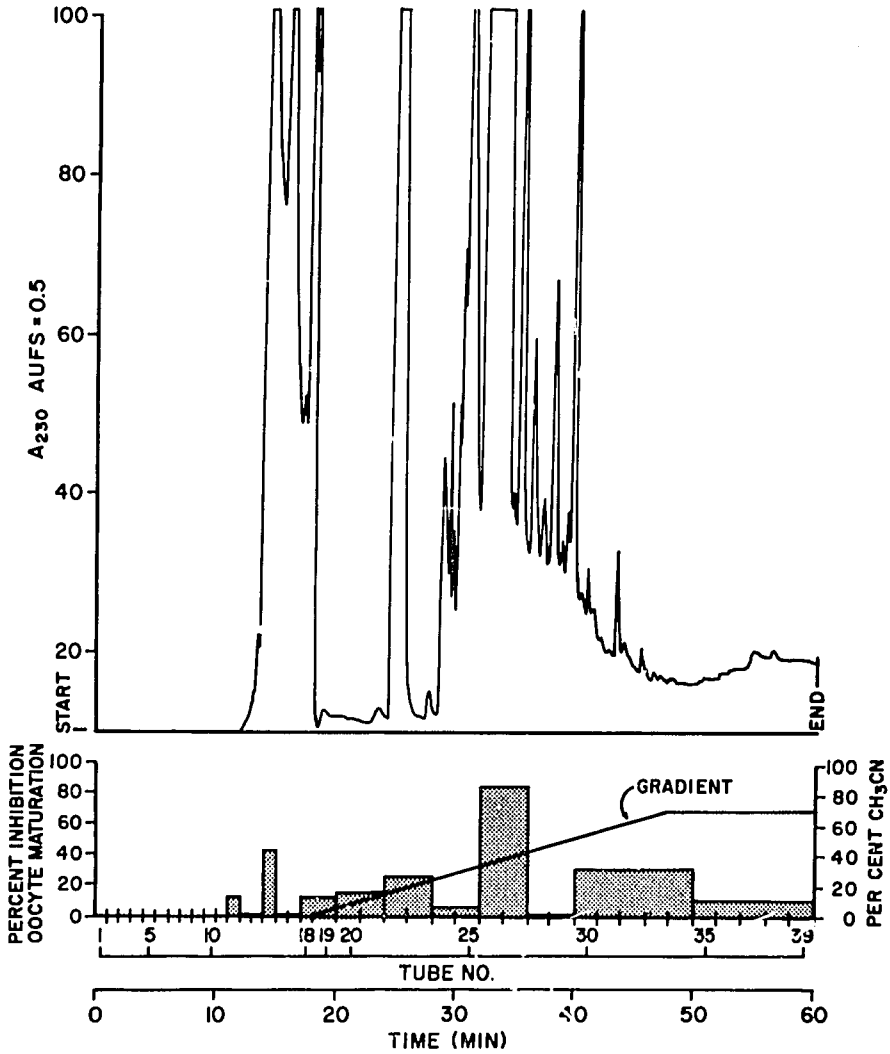


Figure 5. HPLC of fraction B of Figure 2. Fraction B (0.96 mg; 750 units), acidified to 0.1% TFA, was chromatographed in two portions on a Whatman Magnum 9 reverse phase column as described in the legend to Figure 3. Significant inhibition of oocyte maturation was measured in (26 + 27).

mechanism and/or the specific compounds involved. In mice and rats physiological concentrations of a purine may have an inhibitory role in the presence of suboptimal amounts of intracellular cyclic AMP. They appear to have only minimal activity in the absence of agents which increase intracellular cyclic AMP. In the models suggested by Eppig and Downs[21] either the putative inhibitor is activated by cyclic AMP within granulosa and

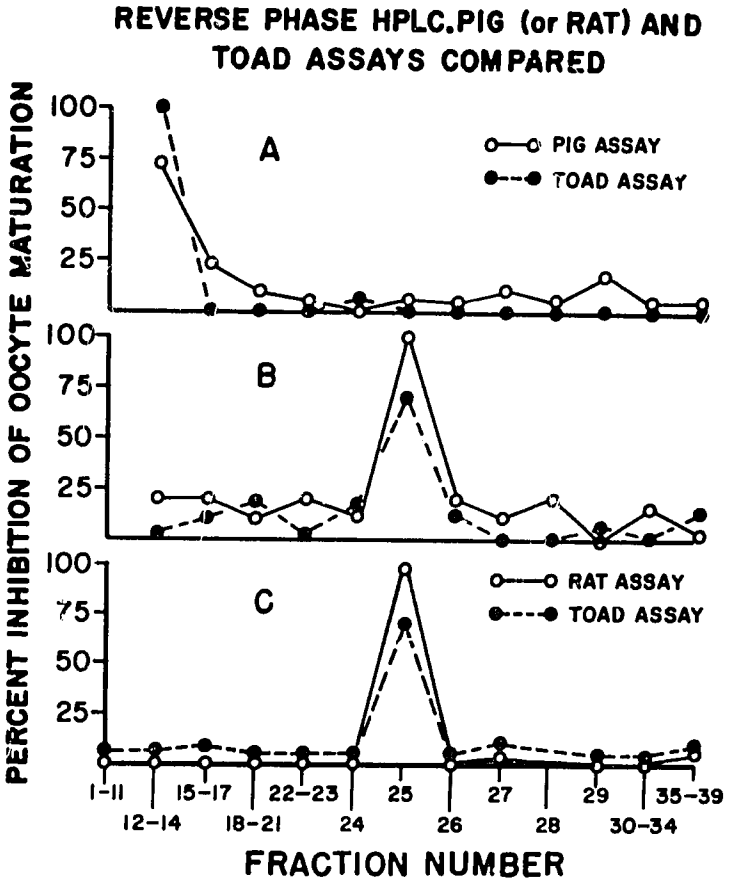


Figure 6. Reverse phase HPLC on fractions from QAE-Sephadex and comparison of mammalian (pig or rat) and Xenopus oocyte assays. In all these diagrams the HPLC run was conducted as described in the legend to Figure 3. The pig oocyte assays in Panels A and B and the rat oocyte assay in Panel C are shown by open circles and the toad assays by filled circles. The samples fractionated in Panels A and B were obtained from QAE-Sephadex chromatography b:: different from the experiments shown in Figure 2.

Panel A: Activity diagram from reverse phase HPLC of a peak corresponding to Fraction A (594 units; 82 units/mg). The pig oocyte assay was conducted at a dilution of (1:25) and the toad assay at (1:50).

Panel B: Activity diagram from reverse phase HPLC of a peak corresponding to Fraction B (706 units; 1474 units/mg). The pig assay was conducted at a dilution of (1:30) and the toad assay at (1:50).

Panel C: Fraction B (different from that of Panel B) was run on reverse phase HPLC and the fraction found to be active in the rat assay (tube 26) was rechromatographed on reverse phase HPLC. The activity diagram shows the results of the rat oocyte assay at a dilution of (1:9) and the toad assay at (1:20).

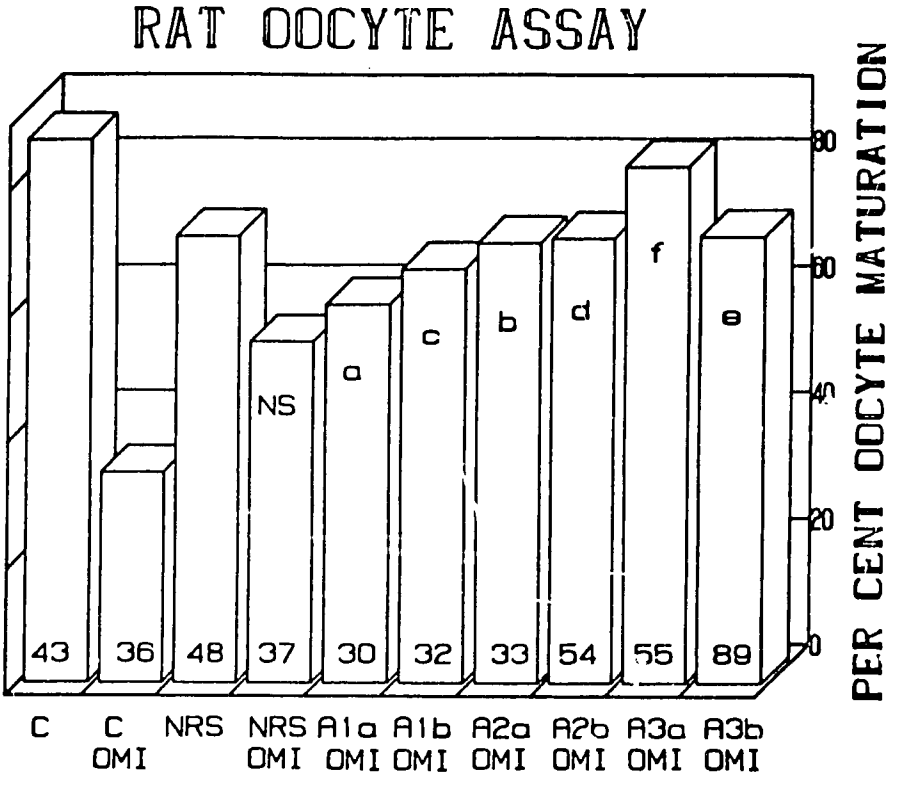


Figure 7. Rat oocyte assay to detect blocking of the action of OMI by rat polyclonal antibodies to crude OMI. Ten groups were included: control medium (C); control medium + OMI; normal rat serum (NRS) (1:25); NRS (1:25) + OMI; and each antibody at (1:50) (a) and (1:200) (b) + OMI. The concentration of NRS was (1:25) rather than (1:50) because its protein concentration was about half that of the antibody preparations. The OMI was at dilution of (1:100) giving a final concentration of peptide from OMI of 10 μg/ml. Antibody preparations and OMI were incubated for 2 hours before addition of oocytes. The number at the bottom of each bar is the total number of oocytes in the group. The percent oocyte maturation in each of the seven groups comprising NRS + OMI or antibody + OMI were compared with control medium + OMI by CHI-squared analysis. The letters, shown near the top of each bar, have the following meanings: NS, not significant; a, $p < 0.05$; b, $p < 0.025$; c, $p < 0.01$; d, $p < 0.005$; e, $p < 0.0005$; f, $p << 0.0005$.

cumulus cells and transferred to the oocyte, or else the inhibitor is transferred to the oocyte and helps maintain elevated levels of cyclic AMP. Either of these mechanisms could explain the inhibitory activities of purines in rodent systems.

Since cyclic AMP itself may also be transferred from granulosa and cumulus cells to the oocyte, the above models should be modified. It is possible that the compound(s) that we call OMI is capable of either acting independently of cyclic AMP or else to enhance

its action within the oocyte. This may be true in rodent as well as in the pig oocyte system. Interactions between OMI, purines, and cyclic AMP might be the underlying theme in the regulation of meiotic arrest.

REFERENCES

1. ABERDAM E, HANSKI E, DEKEL N: Maintenance of meiotic arrest in isolated rat oocytes by the invasive cyclase of Bodetella pertussis, Biol Reprod 36:530, 1987
2. BILLIG H, BRAUNSTROM M, TORNELL J, MAGNUSSON C: Guanosine and hypoxanthine inhibit rat oocyte maturation. Acta Physiol Scand 123:9A, 1985
3. BORNSLAEGER EA, SCHULTZ RM: Regulation of mouse oocyte maturation: Effect of elevating cumulus cell cAMP on oocyte cAMP levels. Biol Reprod 33:698, 1985
4. CAMERON IL, LUM JB, NATIONS C, ASCH RH, SILVERMAN, AY: Assay for characterization of human follicular oocyte maturation inhibitor using Xenopus oocytes. Biol Reprod 28:817, 1983
5. CHANNING CP, POMERANTZ SH, BAE IH, EVANS VW, ATLAS SJ: Actions of hormones and other factors upon oocyte maturation. In CHANNING CP, SEGAL SJ (eds): Plenum Press, Adv Exp Biol Med 147:189, 1982
6. CHARI S, HILLENJSO T, MAGNUSSON C, STURM G, DUAME E: *In vitro* inhibitor of rat oocyte meiosis by human follicular fluid fractions. Arch Gynec. 233:155, 1983
7. CHO WK, STERN S, BIGGERS JD: Inhibitory effect of dibutyryl cAMP on mouse maturation *in vitro*, J Exp Zool 187:383, 1974
8. DANIEL SAJ., KHALIL, MW, ARMSTRONG, DT: 19-Androstenedione (4-estrene-3,17-dione) inhibits porcine oocyte maturation *in vitro*. Gamete Res 13:173, 1986
9. DEKEL N: Hormonal control of ovulation. In LITWACK G (ed): Biochemical actions of hormones, p 50, New York, Academic Press, 1985
10. DEKEL N, BEERS WH: Rat oocyte maturation *in vitro*: Relief of cyclic AMP inhibition with gonadotropins. Proc Natl Acad Sci (US) 75:4369, 1978
11. DEKEL N, BEERS WH: Development of the rat oocyte *in vitro*: Inhibition and induction of maturation in the presence or absence of cumulus oophorus. Devel Biol 75:247, 1980
12. DEKEL N, ABERDAM E, SHERIZLY I: Spontaneous maturation in vitro of cumulus-enclosed rat oocytes is inhibited by forskolin. Biol Reprod 31:244, 1984
13. DEKEL N, LAWRENCE TS, GILULA NB, BEERS, WH. Modulation of cell to cell communication in the cumulus oocyte complex and the regulation of oocyte maturation by LH. Devel Biol 86:356, 1981
14. DOWNS SM, EPPIG JJ: Cyclic adenosine monophosphate and ovarian follicular fluid act synergistically to inhibit mouse oocyte maturation. Endocrinol 114:418, 1984
15. DOWNS SM, EPPIG JJ: Induction of mouse oocyte maturation *in vivo* by perturbarts of purine metabolism. Biol Reprod 36:431, 1987
16. DOWNS SM, COLEMAN DL, EPPIG JJ: Maintenance of murine oocyte meiotic arrest: Uptake and metabolism of hypoxanthine and adenosine by cumulus-cell enclosed and denuded oocytes. Devel Biol 117:174, 1986
17. DOWNS SM, COLEMAN DL, WARD-BAILEY PF, EPPIG JJ: Hypoxanthine is the principal inhibitor of murine oocyte maturation in a low molecular weight fraction of follicular fluid. Proc Natl Acad Sci (US) 82:454, 1985
18. DUMONT JN: Oogenesis in *Xenopus laevis* I. Stages of oocyte development in laboratory maintained animals. J Morph 136:153, 1972. EPPIG J, DOWNS SM. Chemical signals that regulate mammalian oocyte maturation. Biol Reprod 30:1, 1984
19. EKHOLM C, HILLENSJO T, MAGNUSSON C, ROSBERG S: Stimulation and inhibition of rat oocyte meiosis by forskolin. Biol Reprod 30:537, 1984
20. EPPIG JJ: The relationship between cumulus cell-oocyte coupling, oocyte meiotic maturation, and cumulus expansion. Devel Biol 89:268, 1982
21. EPPIG JJ, DOWNS SM: Chemical signals that regulate mammalian oocyte maturation. Biol Reprod 30:1, 1984
22. EPPIG JJ, WARD-BAILEY PF, COLEMAN DL: Hypoxanthine and adenosine in murine ovarian follicular fluid: Concentration and activity in maintaining oocyte meiotic arrest. Biol Reprod 33:1041, 1985

23. EPPIG JJ, FRETER RR, WARD-BAILEY PF, SCHULTZ RM: Inhibition of oocyte maturation in the mouse: Participation of cAMP, steroid hormones, and a putative maturation-inhibitory factor. Devel Biol 100:39, 1983
24. FLEMING C, KHALIL W, ARMSTRONG DT: Porcine follicular fluid does not inhibit maturation of rat oocytes *in vitro*. J Reprod Fertil 69:665, 1983
25. FOOTE WD, THIBAULT C: Recherches experimentales sur la maturation *in vitro* des oocytes de truie et de veau. Annal Biol Anim Biochim Biophys 9:329, 1969
26. GWATKIN RBL, ANDERSEN OF: Hamster oocyte maturation *in vitro*: Inhibition by follicular components. Life Science, 19:527, 1976
27. HILLENSJO T, BATTA SK, SCHWARTZ-KRIPNER A, WENTZ AC, SULEWSKI J, CHANNING CP: Inhibitory effect of human follicular fluid upon maturation of porcine oocytes in culture. J Clin Endo Metab 47:1332, 1978
28. KHALIL MW, WALTON JS: Identification and measurement of 19-norandrostenedione in porcine ovarian follicular fluid using high performance liquid chromatography. J Endocrinol 107:375, 1985
29. LEIBFRIED L, FIRST NL: Effect of bovine and porcine follicular fluid and granulosa cells on maturation oocytes *in vitro*. Biol Reprod 23:699, 1980
30. LIEBFRIED L, FIRST NL: Follicular control of meiosis in the porcine oocyte. Biol Reprod 23:705, 1980
31. MAGNUSSON C, HILLENSJO T: Inhibition of maturation and metabolism of rat oocyte by cyclic AMP. J Exp Zool 201:138, 1977
32. MALLER JL, BUTCHER FR, KREBS EG: Early effect of progesterone on levels of cyclic adenosine 3,'5'-monophosphate in Xenopus oocytes. J Biol Chem 254:579, 1979
33. MILLER JG, BEHRMAN HR: Oocyte maturation is inhibited by adenosine in the presence of follicle-stimulating hormone. Biol Reprod 35:833, 1986
34. MOOR RM, SMITH MW, DAWSON RMC: Measurement of intercellular coupling between oocytes and cumulus cells using intracellular markers. Exp Cell Res 126:15, 1980
35. POMERANTZ SH, BILLELO PA: Inhibition of progesterone-mediated maturation of oocytes of *Xenopus laevis* by oocyte maturation inhibitor from pig follicular fluid. The development of a routine assay for the inhibitor with *Xenopus* oocytes. Gamete Res 17:267, 1987
36. POMERANTZ SH, CHANNING CP, TSAFRIRI A: Studies on the purification and action of an oocyte maturation inhibitor isolated from porcine follicular fluid. In GROSS E, MEIENHOFER J (eds): Peptides: Structure and biological functions. Pierce Chem Co 765, 1979
37. PRESTON SL, PARMER TG, BEHRMAN HR: Adenosine reverses calcium-dependent inhibition of follicle-stimulating hormone action and induction of maturation in cumulus-enclosed rat oocytes. Endocrinol 120:1346, 1987
38. RACOWSKY C: Androgenic modulation of cyclic adenosine monophosphate (cAMP)-dependent meiotic arrest. Biol Reprod 28:774, 1983
39. RACOWSKY C: Effect of forskolin on the spontaneous maturation and cyclic AMP content of rat oocyte-cumulus complexes. J Reprod Fert 72:107, 1984
40. RACOWSKY C: Effect of forskolin on maintenance of meiotic arrest and stimulation of cumulus expansion, progesterone and cyclic AMP production by pig oocyte-cumulus complexes. J Reprod Fert 74:9, 1985
41. RACOWSKY C: Effect of forskolin on the spontaneous maturation and cAMP content of hamster oocyte-cumulus complex. J Exp Zool 234:87, 1985
42. RACOWSKY C, McGAUGHEY RW: Further studies of the effects of follicular fluid and membrane granulosa cells on the spontaneous maturation of pig oocytes. J Reprod Fertil 66:505, 1982
43. RACOWSKY C, SATTERLIE RA: Metabolic, fluorescent dye and electrical coupling between hamster oocytes and cumulus cells during meiotic maturation *in vivo* and *in vitro*. Devel Biol 108:191, 1985
44. RICE C, McGAUGHEY RW: Effect of testosterone and dibutyryl cAMP on the spontaneous maturation of pig oocytes. J Reprod Fertil 62:245, 1981
46. SALUSTRI A, PETRUNGARO S, SIRACUSA G: Effect of adenosine on meiotic resumption *in vitro* is not related to ATP changes in the oocyte. Biol Reprod (Suppl) 36:73, 1987
47. SATO E, ISHIBASHI T: Meiotic arresting action of the substance obtained from cell surface of porcine ovarian granulosa cells. Jap J Zootech Sci 48:22, 1977
48. SCHUETZ AW, ROCK J: Stimulatory and inhibitory effects of human follicular fluid on amphibian oocyte maturation and ovulation *in vitro*. Differentiation 21:41, 1982

49. SCHULTZ RM, MONTGOMERY RR, BELANOFF JR: Regulation of mouse oocyte meiotic maturation: Implication of a decrease in oocyte cAMP and protein dephosphorylation in commitment to resume meiosis. Devel Biol 97:264, 1983
50. SCHULTZ RM, MONTGOMERY RR, WARD-BAILEY P, EPPIG JJ: Regulation of oocyte maturation in the mouse: possible roles of intercellular communication, cAMP and testosterone. Devel Biol 95:294, 1983
51. STONE SL, POMERANTZ SH, SCHWARTZ-KRIPNER A, CHANNING CP: Inhibitor of oocyte maturation from porcine follicular fluid: further purification and evidence for reversible action. Biol Reprod 19:585, 1978
52. TSAFRIRI A, CHANNING CP: An inhibitory influence of granulosa cells and follicular fluid upon oocyte meiosis *in vitro*. Endocrinol 96:922, 1975
53. TSAFRIRI A, POMERANTZ SH: Regulation of the development of meiotic competence and of the resumption of oocyte maturation in the rat. In EVANS CW, DICKINSON HG (eds): Controlling Events in Meiosis, p 25, Cambridge University Press, 1984
54. TSAFRIRI A, POMERANTZ SH: Oocyte maturation inhibitor, Clin Endocrinol Metab 15:157, 1986
55. TSAFRIRI A, POMERANTZ SH, CHANNING CP: Porcine follicular fluid inhibitor of oocyte meiosis: Partial purification and characterization. Biol Reprod 14:511, 1976
56. TSAFRIRI A, CHANNING CP, POMERANTZ SH, LINDNER HR: Inhibition of maturation of isolated rat oocytes by porcine follicular fluid. J Endocrinol 75:285, 1977
57. TSAFRIRI A, WEINSTEIN Y, BARAMI S, CHANNING CP, POMERANTZ SH, LINDNER HR: The control of meiotic maturation of the rat oocyte. In CORTE C (ed): Research on Steroids, Vol. 8, p 193, London, Academic Press, 1979
58. URNER F, HERMANN WL, BAULIEU EE, SCHORDERET-SLATKINE S: Inhibition denuded mouse oocyte meiotic maturation by forskolin, an activator of adenylate cyclase. Endocrinology 113:1170, 1983
59. VIVIRELLI E, CONTI M, DeFELICI M, SIRACUSA G: Meiotic resumption and intracellular cAMP levels in mouse oocytes treated with compounds which act on cAMP metabolism. Cell Differentiation 12:271, 1983
60. WALLACE RA, JARED DM, DUMONT JN, SEGA MW: Protein incorporation by isolated amphibian oocytes III. Optimum incubation conditions. J Exp Zool 184:321, 1973
61. WASSARMAN PM, JOSEFOWICZ WJ, LETOURNEAU GE: Meiotic maturation of mouse oocytes *in vitro*: Inhibition of maturation at specific stages of nuclear progression. J Cell Sci 22:531, 1976

DISCUSSION

CHAIRPERSON — JEFFREY M. SPIELER

DR. ZILBERSTEIN: Were the OMI fractions added to the medium in the *Xenopus* assay?

DR. POMERANTZ: Yes.

DR. ZILBERSTEIN: Did you try injecting the OMI fraction directly into the *Xenopus* oocyte?

DR. POMERANTZ: No.

DR. ZILBERSTEIN: The big advantage of the *Xenopus* oocyte is that you can inject directly into it.

DR. POMERANTZ: Apparently the fractions work when they are added to the medium. I am not so sure if we want to inject into the oocyte. Progesterone, which is the effector required for oocyte maturation in *Xenopus*, has to work on the outside. Unlike all the other steroid receptors, the progesterone receptor in the *Xenopus* is on the surface of the oocyte.

Since I am not sure how OMI works, I am not sure that there would be any advantage to injecting it into the oocyte. I have not tried it, however. It would take a lot of material and much more time. Our assays include 40 to 50 oocytes in each dish for each group.

DR. BUSCAGLIA: We are using a similar *Xenopus* assay for OMI at the Salk Institute. We obtained very good dose responses with pig follicular fluid.

Although I agree with your results, we noticed that this assay must be used only under very, very stringent conditions because the presence of salts in the culture medium may change the ability of progesterone to induce oocyte maturation. For example, low concentrations of sodium have no effect, but even a small amount of potassium may completely inhibit progesterone-induced oocyte maturation in *Xenopus*.

DR. POMERANTZ: I agree with you. The assay appears to be really supersensitive. It is much more sensitive than the pig oocyte assay, especially with pig follicular fluid, the PM-10 filtrate, and the QAE Sephadex fractions. I think that is because there are so many different kinds of compounds in follicular fluid which inhibit oocyte maturation. However, we are not interested in those substances which do not inhibit the maturation of pig oocytes.

Concerning a question about using PMSF in the preparation, we do use it to treat follicular fluid, and it does not have any effect on the pig oocyte assay. We depend on the pig assay for monitoring the early stages of purification. It is only later on that we use the toad assay.

DR. HENZL: I would like to invite Dr. Karten to make a comment on the assay.

DR. KARTEN: I would like to have an open discussion on the kinds of animal models being used. Is there some agreement on a best assay? Is there a system better than the frog assay, and if so, why? I would also like to hear about some of the other problems that the investigators in the audience have had in reproducing the test for the elusive, but real, OMI factor. And finally, I would like to hear something about the variability in the PFF on a batch-to-batch basis in terms of finding the OMI factor.

DR. HENZL: Does anybody care to comment on Dr. Karten's questions?

DR. POMERANTZ: I can say something about the variation in the pig follicular fluid because we certainly found a great deal of variation from one batch to the other. Until most recently, that is, around the first of October 1987, we have been using follicular fluid that we collected ourselves from medium and small-sized pig follicles. And since October, when we were funded by CONRAD, I have been able to get follicular fluid in large quantities through the courtesy of Dr. Bialy at NICHD. I have been able to look at only two batches in more detail and they are quite different in their effect in the pig oocyte assay. With a 50-50 mixture of medium and follicular fluid, the inhibition was about 75 percent with one batch while the other batch only gave 30 percent inhibition. So, I am sure that as we study additional batches we will find a lot of variation.

DR. G. JONES: I would just like to ask if anybody has considered what the change in the granulosa cell from cyclic AMP and protein kinase A to probably pnosphatol and isoltol and protein kinase C may have to do with oocyte maturation? It seems to me that this could have a great deal to do with the cyclic AMP system.

DR. HENZL: Would you care to answer that question, Dr. Pomerantz?

DR. POMERANTZ: I do not have any special observations to make on this point.

21

Anti-Müllerian Hormone in the Ovary

BERNARD VIGIER
JACQUELINE BÉZARD
NATHALIE JOSSO

Anti-Müllerian hormone (AMH), also called Müllerian-inhibiting substance (MIS) or factor (MIF), is a glycoprotein consisting in the assembly, through disulfide bonds, of two identical 72K subunits.[4,5,20] The purified protein contains 13.6% carbohydrate and its aminoacid composition[19] is very close to that specified by its nucleotide sequence.[6] The cDNA[18] and genes[6] coding for bovine[6,18] and human[6] AMH have been cloned, and the gene localized to the tip of the short arm of human chromosome 19.[7]

The most obvious effect of AMH, the one that led Alfred Jost, in 1953[13], to postulate the existence of such a hormone, and which has given it its name, is the inhibition of the growth of Müllerian derivatives in the male fetus. Early in fetal life, Müllerian ducts develop in both sexes, and in females, they differentiate into tubes, uterus, and the upper part of the vagina. In males, AMH-mediated regression of Müllerian ducts is the first sign of masculinization of the internal genital tract. Müllerian ducts are sensitive to AMH only during a short developmental "window," at 14 days in the fetal rat, and before 8 weeks in the human.[12,24] However, since Sertoli cells, which synthesize AMH in the testis[2,10,27] do so far beyond the period of the AMH-sensitive "window" of Müllerian ducts,[31] the possibility that AMH might have extra-Müllerian physiological effects has been entertained by various investigators. The discovery that AMH is also an ovarian hormone in the adult[11,30] gave additional impact to this finalistic view of endocrine physiology. However, proof of extra-Müllerian effects of AMH can be obtained only by directly testing the effect of the hormone, purified to homogeneity, on its supposed target organs. In this chapter, we will review the data relative to the production of AMH in the mammalian ovary, and produce evidence for an effect of AMH on the morphology and steroidogenesis of the fetal ovary.

PRODUCTION OF AMH BY GRANULOSA CELLS

Granulosa cells of the ovarian follicle and Sertoli cells of the seminiferous tubule share many structural and functional characteristics, and Vigier and his colleagues[30] were therefore not surprised to detect AMH, a Sertoli cell protein, in the follicular fluid and

granulosa cell incubation medium of adult cows. The highest concentration (45 ng/ml) was reached in small follicles, large follicles contained approximately four times less. The bioactivity of AMH, purified from bovine follicular fluid, was similar to that of testicular AMH (Fig. 1). Necklaws *et al.*[17] confirmed the higher concentration of AMH in small follicles — 2 μg/ml versus 0.8 — the difference in the absolute values reported by the two groups could be due to contaminants contained in the AMH preparation used as RIA standard by the Boston group.

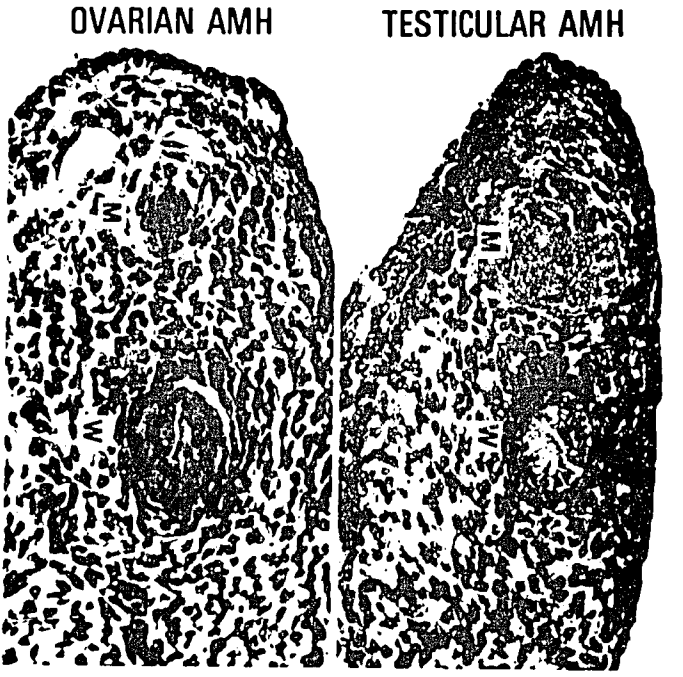


Figure 1. Müllerian duct regression in anterior segment of 14-day-old rat reproductive tract exposed to testicular AMH, purified from incubation medium of bovine fetal testes (0.7 μg/ml) or to ovarian AMH, purified from bovine follicular fluid ((0.9 μg/ml). Periodic acid Schiff, X 350. M : Müllerian duct, W : Wolffian duct.

Messenger ribonucleic acid (mRNA) isolated from bovine ovarian follicles is a 2 kb transcript similar to that prepared from fetal and neonatal testes.[6,18] An additional 1.7 kb species is produced by human granulosa cells in culture.[33] The amount of AMH mRNA produced by the ovary is however extremely low, less than 2% than that isolated from the fetal testis,[18] and its regulation is also different. Voutilainen and Miller[33] have demonstrated that both ovarian and testicular mRNA are insensitive to FSH, and stimulated by cyclic AMP, while hCG increases AMH mRNA fourfold in the ovary, but not in the testis.

DR. WINTERS: I would have expected that the FSH bioassay levels would rise commensurate with the immunoassay levels in men who are older and have damaged testes.

DR. HSUEH: I am not the best person to answer this question, but there are many cases during aging where cells are not functioning as optimally as they are supposed to be. All the earlier studies are based on immunoassay, and this is the first time a bioassay was also done.

DR. WINTERS: For example, in the serum in those specific samples, can the activity be neutralized by FSH antiserum?

DR. HSUEH: I did not have time to present these data. We have characterized the recovery of purified FSH in PEG-treated samples. By adding FSH to the same serum samples before and after PEG treatment we showed that 95 percent of the FSH is recovered.

DR. WINTERS: What is the molecular weight of the bioassayable material in the serum of older men?

DR. HSUEH: There are many different forms with a pH around 5.

DR. CORBIN: Dr. Paulsen, can you help clarify this issue?

DR. PAULSEN: To comment on the clinical material to ease Dr. Winters' concerns, these patients were entirely well, had retention of normal testicular function, and I think they represent a set of men that do not have damaged testes with increased age.

7

Changes in the Serum Concentrations of Inhibin in Mammals

YOSHIHISA HASEGAWA

Differences in the regulatory mechanism of LH and FSH secretion from the pituitary gland has become an attractive field of research in the area of reproductive endocrinology. Dissociated changes in serum LH and FSH levels were often observed in various animals including pigs,[16,27,28,34,35] rats,[3,5,12,14] cows[8] and primates[7] under certain physiological and experimental conditions. It is widely accepted that LHRH from the hypothalamus regulates LH secretion in most cases. On the other hand, FSH secretion seems to be negatively controlled by a gonadal substance, inhibin.[1,10] The physiological importance of inhibin in the regulation of serum FSH levels has been demonstrated *in vivo* using follicular fluid.[13] For an understanding of the feedback system possibly existing between FSH and inhibin *in vivo*, it is extremely important to establish a sensitive and reliable inhibin radioimmunoassay (RIA) system suitable to measure serum inhibin levels. Recent work conducted by several groups,[19,20,29,30] including ourselves,[11,23] on the development of pure inhibin preparations enable one to raise antisera to inhibin and to develop specific RIA's for inhibin.[15,21,36]

As previously reported, we have isolated porcine and bovine follicular fluid 32 kilodalton (KDa) inhibins in fairly good yields.[11,23] They were used as immunogens to raise antisera in rabbits or chickens. Using the antisera and the pure antigens, we have developed homologous RIA systems for pigs and cows. Furthermore, the antiserum against porcine 32 KDa inhibin was found to cross-react with rat inhibin preparations. Similarly, the antisera to bovine 32 KDa inhibin also recognized human and goat inhibin as well. Utilizing such characteristics of the antisera, we next established heterologous RIA systems for rat, human, and goat inhibins. Consequently, as shown in Table 1, a sensitive RIA system that could measure concentrations of inhibin in peripheral blood during normal cycles was established for rat by using an antiserum to porcine inhibin, and for human and goat by using an antiserum to bovine inhibin.

In this chapter, we describe changes in the serum inhibin levels during the estrous or menstrual cycles of pigs, rats, cows, goats, and women. Changes in the serum gonadotropin levels of the respective mammals were also measured to demonstrate a possible feedback system between serum inhibin and FSH levels.

TABLE 1
CHARACTERISTICS OF PORCINE, RAT, BOVINE, HUMAN AND SHIBA-GOAT RIA SYSTEMS.

	Pig	Rat	Cow	Human	Shiba-goat
Antigen for immunization	32K PI	32K PI	BI after immuno-affinity	BI after immuno-affinity	BI after immuno-affinity
Antibody (dilution used)	CA710 ($1x10^4$)	CA710 ($1x10^4$)	RD926 ($2.5x10^4$)	TNDH-1 ($50x10^4$)	TNDH-1 ($50x10^4$)
Tracer	32K PI	32K PI	32K BI	32K BI	32K BI
Castrated serum in standard (μl)	100	100	200	100	100
Binding rate of tracer (%)	26	25	31	29	32
Standard	PI after Sephacryl S 200	PI after Sephacryl S 200	BI after immuno-affinity	hFF	gFF
Slope (Log-Logit)	-1.17	-1.00	-0.88	-0.88	-0.71
Sensitivity (/tube)	eq.32KPI	eq.32KPI	eq.32KBI		
Minimum detectable dose	0.03 ng	0.06 ng	0.01 ng	13 nl	0.8 nl
ED_{50}	0.64 ng	1.36 ng	0.22 ng	230nl	17 nl
Coefficient of variation (%)					
intra-assay	7.7	4.5	6.3	6.1	6.7
Inter-assay	13.2	8.7	5.6	6.7	7.5

PI; purified porcine inhibin, BI;purified bovine inhibin, hFF; human follicular fluid, gFF; goat follicualr fluid.

RADIOIMMUNOASSAY SYSTEMS FOR INHIBIN

PORCINE INHIBIN RIA SYSTEM

High-titer antisera to porcine follicular fluid (pFF) 32 KDa inhibin have been raised in adult male chickens. We have demonstrated that there are several inhibin forms in pFF of different molecular weights.[15,23] One of the antisera (CA710) recognizes the higher molecular weight forms of inhibin as well as the 32 KDa inhibin. A pattern of immunoreactive inhibin was practically identical to that of bioactive inhibin on a SDS-PAGE of pFF, indicating that the present RIA system measures total inhibin molecules including such higher molecular weight forms.

In determining serum inhibin levels, interference of serum with binding of the labeled antigen to the antiserum is the most serious problem. Such serum effects have been reported by McLachlan *et al.*[21] The non-specific effects of serum on the RIA could be minimized by setting the incubation temperature at 30°C during the RIA procedure. However, the serum effects were still observed in the presence of more than 100 μl of serum in the assays even under the incubation conditions at 30°C. Therefore, a constant volume (100 μl) of castrated male pig serum was always included in the standards in order to compensate for such serum effects when measuring serum inhibin levels. Figure 1 shows typical standard curves in the porcine RIA system in the presence or absence of 100 μl of castrated male pig serum, indicating that the serum effects in this RIA system can be satisfactorily canceled when the results were expressed in terms of B/B_0. The mean least detectable amount of inhibin, defined as 2 x SD of maximum binding (B_0) was 30.7 pg/tube. Non-specific binding was always less than 2% of the total readioactivity. Intra- and inter-assay coefficients of variations were 7.7% and 13.2%, respectively.

RAT INHIBIN RIA SYSTEM

Several lines of evidence indicate that the porcine inhibin RIA system described above is also applicable to the measurement of rat serum inhibin levels. First, the antibody used in the porcine RIA system could neutralize rat ovarian inhibin activity which was estimated by an *in vitro* inhibin bioassay using a rat anterior pituitary cell culture system. Second, displacement curves of several rat inhibin preparations, including peripheral serum from PMSG primed immature female rats, were practically parallel to that of the pFF inhibin standard in the porcine inhibin RIA system. Third, it is well documented that serum inhibin levels can be quantified by an *in vitro* bioassay when immature female rats receive PMSG.[18] As can be seen in Figure 2, serum inhibin levels in PMSG-primed rats determined by the *in vitro* bioassay were parallel with those by the porcine RIA. These data indicate that the porcine RIA system is applicable to the measurement of rat inhibin levels in serum. Since we do not have a purified rat inhibin preparation, a purified porcine inhibin was used as a standard and concentrations of inhibin in rat serum were expressed as ng/ml equivalent of porcine 32 KDa inhibin.

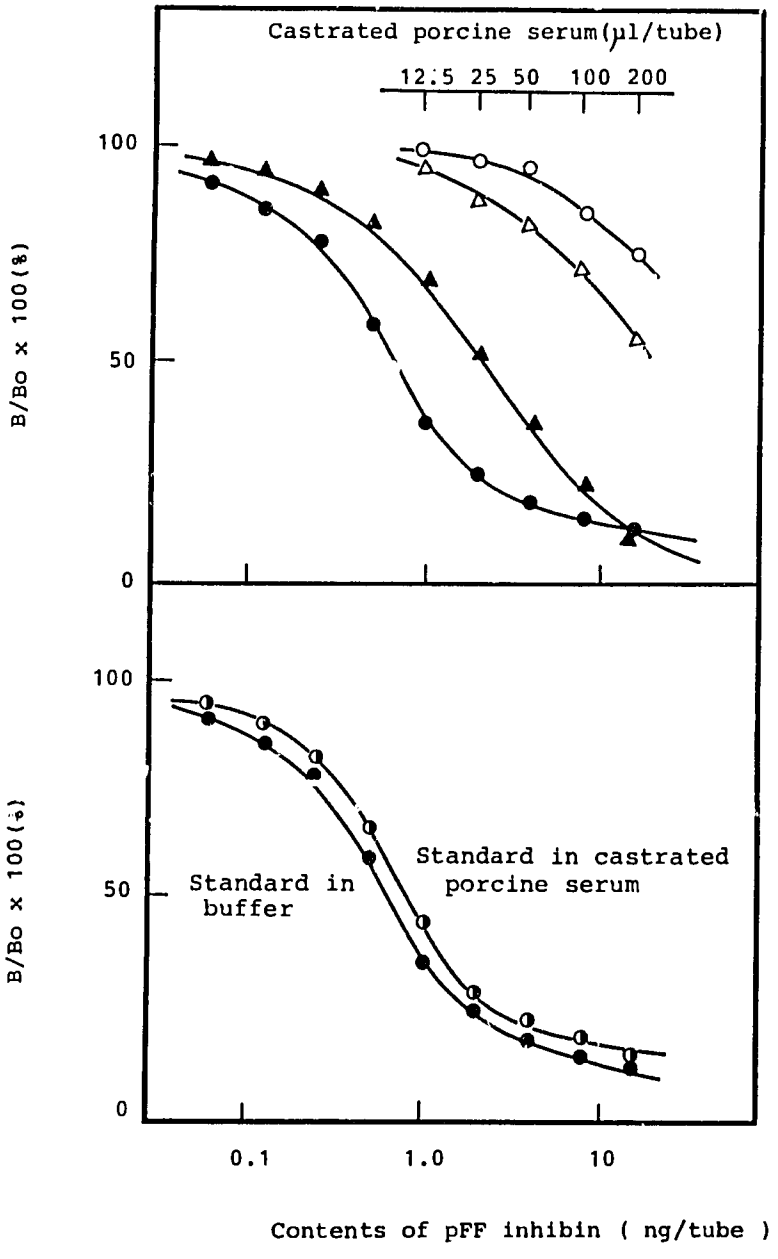


Figure 1(A). The effect of temperature on the RIA standard curves (●:at 30°C and ▲: at 4°C) and on the non-specific displacement of various doses of castrated male serum (○:at 30°C and △: at 4°C).

(B) Comparison of the standards curves obtained with pFF 32K inhibin in the presence (◑) or absence (●) of 100 μλ of serum from castrated pigs (Hasegawa *et al.* J Endocrinol 118 (in press) 1988).

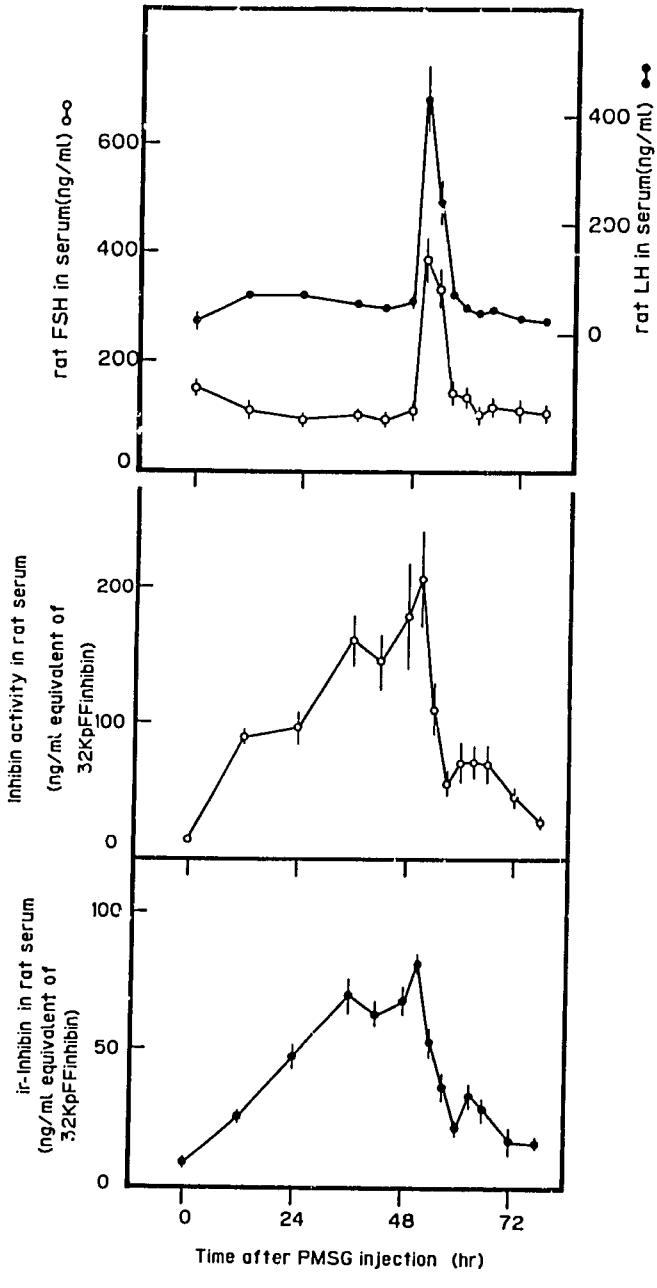


Figure 2. Mean serum concentrations (± SEM) of LH, FSH, and inhibin from immature female rat (N=5) after injection of 30 IU PMSG. Bioassay of inhibin in serum was carried out by using *in vitro* pituitary cell culture system (Hasegawa *et al.* unpublished).

BOVINE INHIBIN RIA SYSTEM

A homologous RIA system for bovine inhibin has also been established. In this case, castrated male rabbits were immunized with bFF inhibin preparations purified by immunoaffinity chromatography[24] to obtain antisera of high-titers. A purified bFF 32 KDa inhibin was labeled by the method of Hasegawa *et al.*[15] and the labeled antigen was then purified on an immunoaffinity column. When measuring serum inhibin levels, 200 μl of castrated male bovine serum were also included in the standards to compensate for the serum effects. Incubation was also carried out at 30°C. A sensitivity of the RIA was 10 pg/tube, average ED_{50} value was 220 $\pm$ 70 pg/tube (n$\pm$11). Intra- and inter-assay coefficients of variation were 6.3% and 5.6% (n=11), respectively.

HUMAN INHIBIN RIA SYSTEM

Fortunately, the bovine inhibin RIA system described above is also applicable to the measurement of human and goat serum inhibin levels. Criteria mentioned in the rat RIA system were also fulfilled in this case. One of the antisera to bovine inhibin (TNDH-1) could neutralize human inhibin activity in follicular fluid. Displacement curves for several lots of human follicular fluid were parallel with that of the bFF inhibin standard. Since no one has purified human inhibin from natural sources as yet, we used a pooled human follicular fluid as a standard. Biological potency of the standard was determined by the *in vitro* bioassay. The ED_{50} value of the standard was 230 nl/tube. Concentrations of inhibin in human serum were expressed nl/ml of the standard human follicular fluid. Statistical data are also shown in Table 1.

GOAT INHIBIN RIA SYSTEM

Serum concentrations of inhibin in goats could also be measured by using the bovine inhibin RIA system. In this case the criteria described in the rat and human inhibin RIA's were also fulfilled. Concentrations of inhibin in goat serum were expressed as nl/ml of a standard goat follicular fluid. Statistics are in Table 1.

MEASURING INHIBIN BY RIA

SERUM INHIBIN LEVELS DURING THE ESTROUS CYCLES OF PIGS

In cycling female pigs, an LH surge, which directly induces ovulation, occurs without a concomitant rise of FSH, while serum FSH levels begin to rise after the LH surge.[27,34,35] It seems essential to determine serum inhibin levels during the estrous cycle in order to understand the regulation of such changes in serum FSH levels.

We collected daily blood samples at 10:00 a.m. from two cycling sows during the ovulatory period of three to five consecutive cycles.[17] The day of ovulation was taken as day 0 of the cycle. Serum inhibin levels, as well as serum LH, FSH, estradiol and progesterone levels, were determined. As shown in Figure 3, serum LH levels showed a sharp peak at day -2, the so-called preovulatory LH surge, while FSH levels showed two gently-

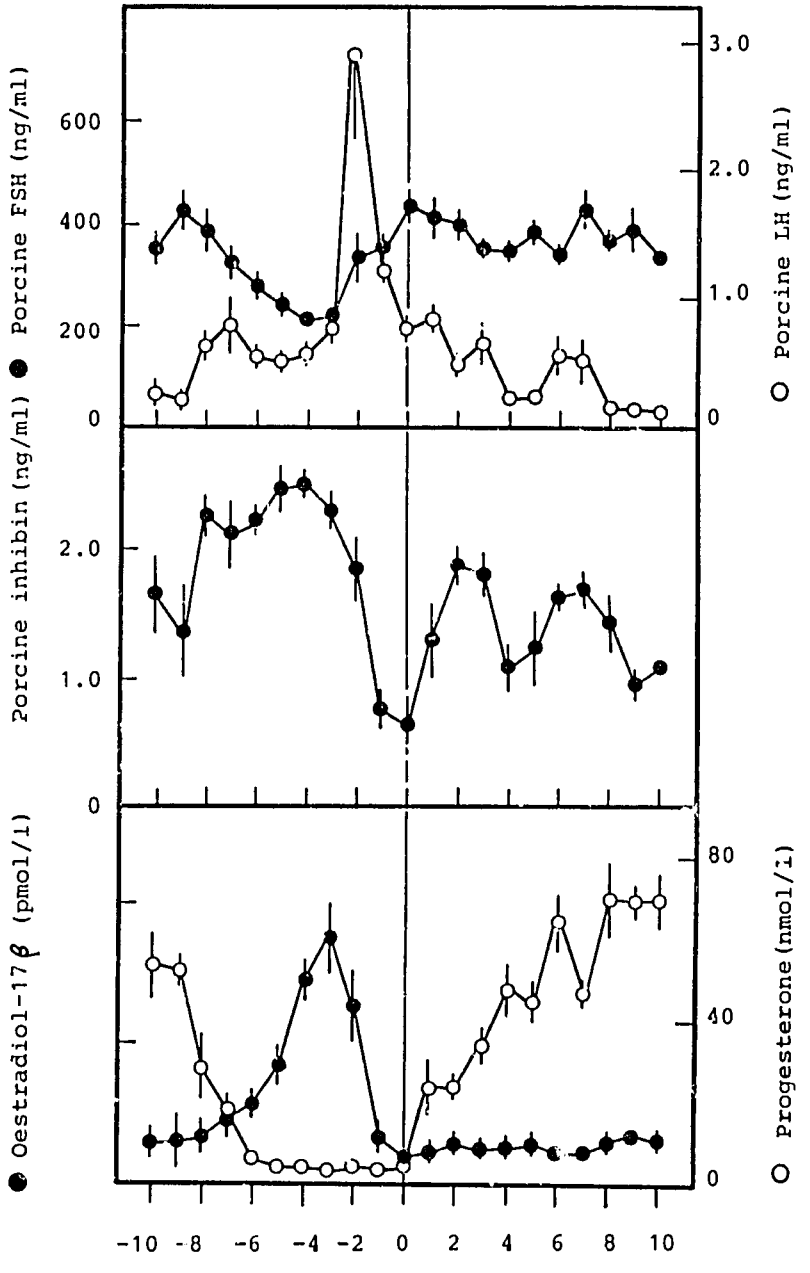


Figure 3. Mean plasma concentrations (± SEM) of porcine LH, FSH, estradiol-17β and progesterone during the estrous cycle of pigs. Day of ovulation designated as Day 0 (Hasegawa *et al.* J Endocrinol 118 (in press) 1988).

sloping peaks around day -9 and day 0. FSH levels reached maximum levels late in the luteal phase (420 ± 44 ng/ml) and then began to decline, reaching minimum at day -4. Although serum FSH levels did not increase together with the LH surge at day -2, a significant rise in FSH levels was seen two days after the LH surge. On the other hand, serum inhibin levels fluctuated between 0.5 ng/ml and 2.5 ng/ml, showing two small peaks during the luteal phase and a long plateau-like burst during the follicular phase. The high inhibin levels during the follicular phase continued for more than 6 days, the duration of which was much longer than that of the estradiol surge. Serum FSH levels during the follicular phase seem to be inversely related to the serum inhibin levels. Inhibin secreted into the peripheral blood may directly regulate FSH secretion from the pituitary gland.

It is also notable that serum inhibin levels fluctuated showing small peaks during the luteal phase, while serum estradiol levels remained low. Parlow *et al.*[26] reported that during the luteal phase all follicles present in the ovary are less than 4 mm in diameter on day 4, but this is followed on day 10 with an average of about 15 follicles per ovary between 4 mm and 8 mm in diameter. Our present results may reflect the development and degradation of such small follicles present in the ovary during the luteal phase which may already be producing inhibin but not yet secreting estradiol.[9] Such dissociated secretion of inhibin and estradiol from rat granulosa cells was also observed under *in vitro* culture conditions.[2,31] Alternatively, the corpus luteum may be producing inhibin during this period, as suggested by Cuevas *et al.* following their immunohistochemical study.[4] High inhibin levels were found during the luteal phase of women, suggesting that inhibin plays an important role during this phase.

SERUM INHIBIN LEVELS DURING THE ESTROUS CYCLES OF RATS

Changes in the serum concentrations of LH and FSH during the estrous cycles of rats have been well characterized. The most striking feature of the serum gonadotropin changes is that following the preovulatory LH/FSH surge which takes place during the afternoon of proestrous, a second discharge of FSH without a rise in LH, the so-called second FSH surge, occurs during the early morning of estrus. We have previously demonstrated that the second FSH surge is not dependent on hypothalamic LHRH.[14] Therefore, changes in serum inhibin levels during the periovulatory period of cyclic rats may hold the key to the solution of what causes the second FSH surge. Our experimental protocol was as follows: female Wistar rats exhibiting more than three consistent 4-day cycles were used in this study. Groups of five rats each were bled and killed at 3-hour intervals throughout the estrous cycle. Anterior pituitaries and ovaries were removed immediately after decapitation. The pituitaries and ovaries were then homogenized and centrifuged to obtain extracts. Sera or supernatants of tissue extracts were stored frozen at -30°C until hormone assays. Figure 4 shows changes in serum and ovarian inhibin levels as well as serum and pituitary FSH, serum progesterone, and serum estradiol levels.

Inhibin levels in the ovarian extracts and in peripheral sera correlated well as shown in Figure 4. Changes in serum inhibin levels followed the changes in the ovarian inhibin levels with a latency of about 6 hours.

Characteristics of the changes in serum inhibin levels are as follows: 1) Levels were high during the morning of proestrous; 2) Levels dropped sharply in the evening of the pro-

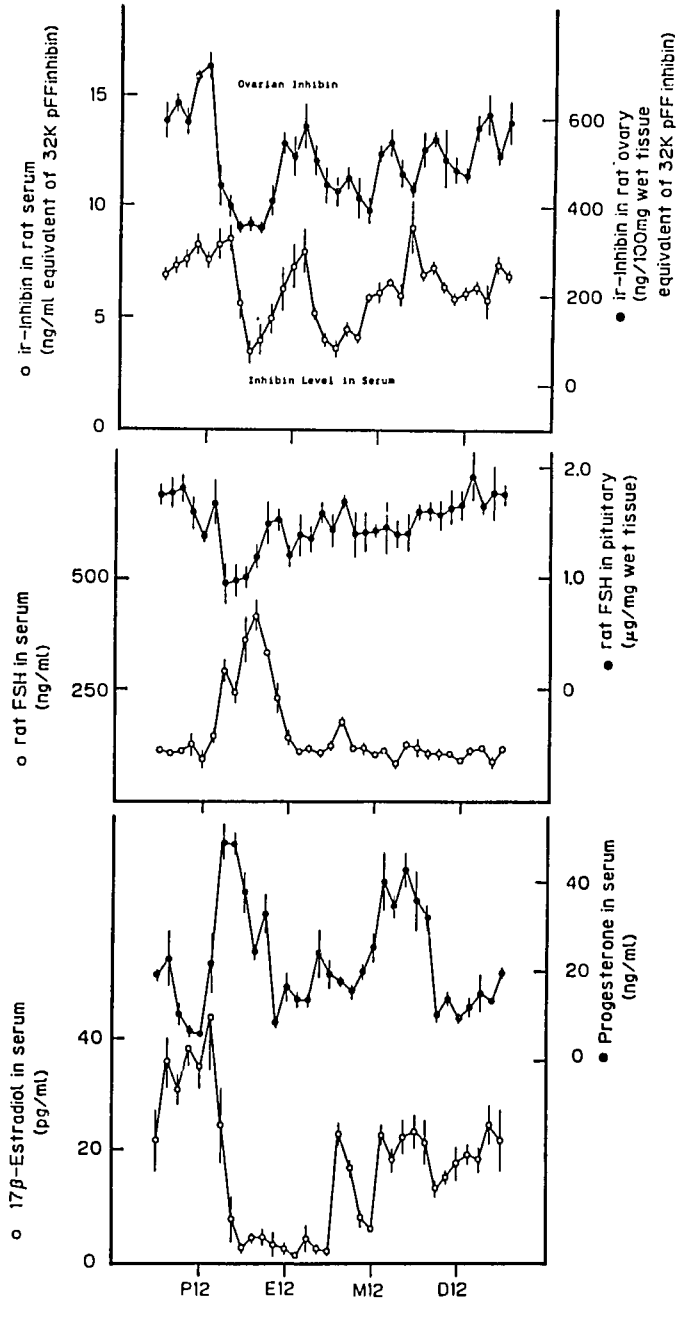


Figure 4. Mean serum concentrations (± SEM) of inhibin, FSH, estradiol-17β and progesterone during 4-day estrous cycle of the rat (N=5). Pituitary FSH and ovarian inhibin contents during cycle were also shown (Hasegawa *et al.* unpublished).

estrous day after the first LH/FSH surge; 3) Levels then recovered rapidly, reaching a peak at 1500 on estrus; 4) Levels then dropped again to record the second lowest levels at 2400 on estrus; and 5) Thereafter, inhibin levels increased gradually with small peaks throughout the metestrous and diestrous day.

The present results clearly show that an inverse relationship exists between the so-called second FSH surge and serum inhibin levels from the proestrous evening to the estrous morning. De Paolo *et al.*,[6] using a qualitative *in vitro* bioassay, reported that inhibin levels in ovarian venous plasma are low from the proestrous evening to the estrous morning. Our present results together with the De Paolo findings suggest that the second FSH surge is caused by the sharp drop in inhibin levels during the proestrous evening. It is surprising that pituitary FSH content showed a gradual increase during the period of the second FSH surge, in spite of the large discharge of FSH into the blood.

It is noteworthy that a small but distinct FSH rise was observed at 0300 on metestrous in association with the second drop in inhibin levels at midnight of estrus. In addition, it is very interesting that serum estradiol levels were not always parallel with serum inhibin levels. For example, a sharp inhibin peak at 1500 during estrus was not associated with a rise in the serum estradiol levels. This suggests that regulation of inhibin production is somewhat different from that of estradiol production. This speculation was also supported by an *in vitro* study using a rat granulosa cell culture system.[31] It can also be argued that concentrations of aromatase substrates, for example testosterone, are not sufficient to produce estradiol on the day of estrus. Indeed, serum testosterone levels during estrus were relatively low, about one third of the maximum levels (Hasegawa *et al.* unpublished). It seems important to identify the mechanism causing the dissociation of inhibin and estradiol production to understand the development and degeneration of follicles in the ovary.

SERUM INHIBIN LEVELS DURING THE ESTROUS CYCLES OF COWS

There have been few reports describing changes in the serum concentrations of LH and FSH during the estrous cycles of cows, probably because of the relatively low concentrations of serum FSH levels during the cycles. Dobson and Kamonpatana reported recently in their review[8] that there is no consistent feature in the changes in serum concentrations of FSH during the estrous cycles of female cows. In order to examine the relationship between serum FSH and inhibin levels during the cycles of cows, it is necessary to show a detailed feature of cycling changes in FSH levels. Figure 5 shows hormonal changes during the estrous cycles of cows. Blood samples were collected from three cycling cows during every two cycles. Cows were bled at 4-hour intervals during the periovulatory period and at 12-hour intervals during the rest of the cycle. Serum concentrations of LH, FSH, and inhibin were determined by respective RIA's.

As far as we know, Figure 5 is the first detailed profile of serum levels of FSH during the estrous cycles of cows. The FSH profile was quite different from those of pigs and rats. In cycling cows, a preovulatory FSH surge, which was associated with an LH surge, was striking, while a second FSH surge 24 hours after the first LH/FSH surge was not as evident as corresponding surges in pigs and rats, though the second FSH surge does indeed take place in cycling cows. Serum FSH levels were relatively high during the luteal phase and then dropped to a low level during the follicular phase.

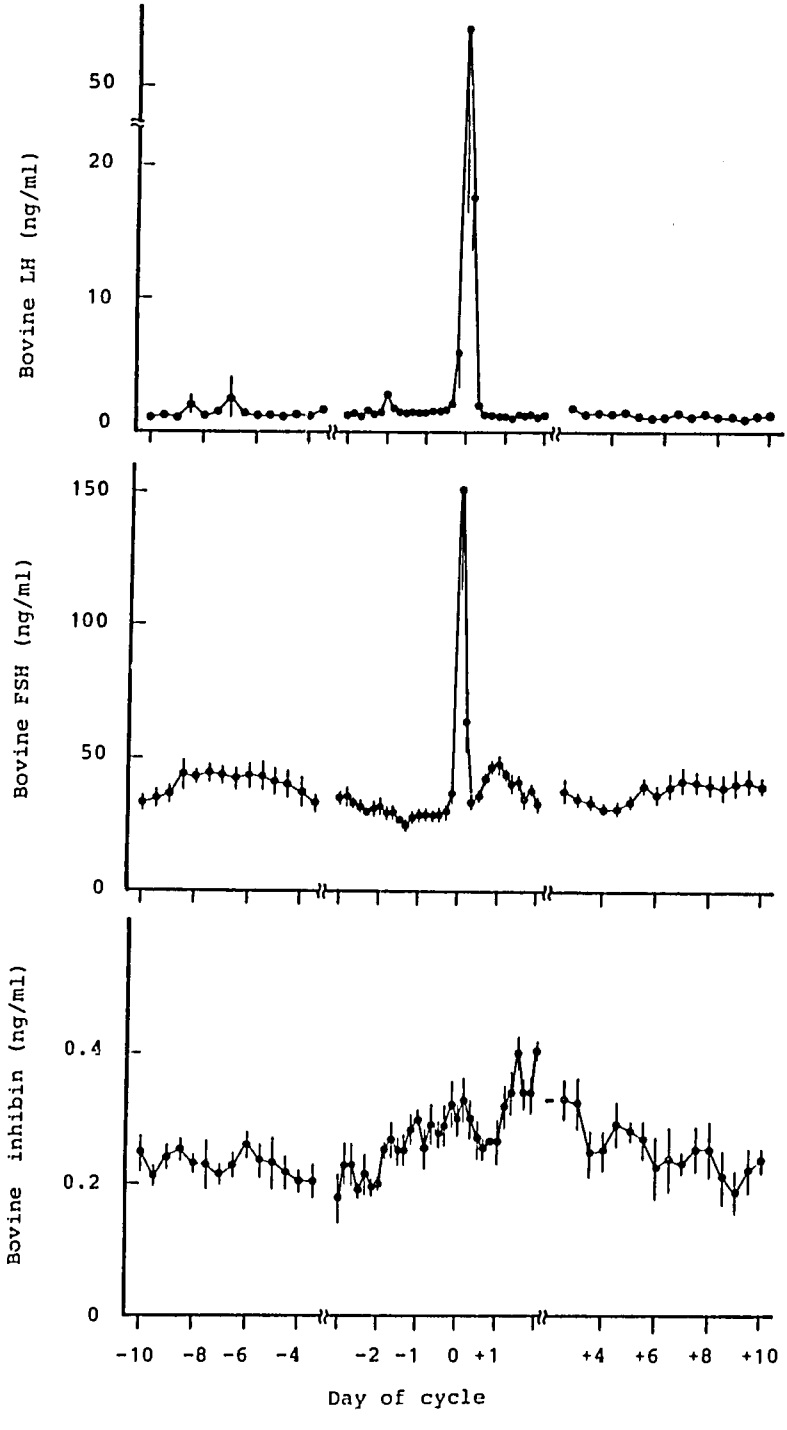


Figure 5. Mean plasma concentrations (± SEM) of LH, FSH, and inhibin during estrous cycle of the cows (N=6). Time of LH surge designated as day 0 (Hasegawa et al. unpublished).

Serum inhibin levels, on the other hand, showed characteristic changes during the cycle as shown in Figure 5. Serum inhibin levels increased gradually during the follicular phase, peaked moderately on the day of the preovulatory LH/FSH surge, then dropped sharply after the preovulatory LH/FSH surge. The small second FSH surge correlated well with the sharp drop in inhibin levels. Surprisingly, inhibin levels then recovered rapidly reaching a maximum 36 to 48 hours after the preovulatory LH/FSH surge. It is well known that ovulation occurs 24 to 36 hours after the gonadotropin surge in the cow, therefore, inhibin levels reached maximum just after ovulation. Inhibin levels then dropped gradually, creating small peaks during the luteal phase; the inhibin levels were relatively low during this period.

In general, serum FSH and inhibin levels did not correlate as well in cows as in rats or pigs. However the second FSH surge seems to be inversely related to serum inhibin levels.

It is quite interesting that the maximum secretion of inhibin occurs just after ovulation when serum progesterone levels still remain at basal levels. This suggests that the highest inhibin levels 36 to 48 hours after the gonadotropin surge are not due to possible inhibin production from differentiated luteal cells or the corpus luteum. Such a rapid recovery of inhibin levels was also observed in cyclic rats just after the second FSH surge. The secretion mechanism and the source of inhibin during this period is entirely obscure, but one can speculate that the second FSH surge is a trigger for such an inhibin burst.

SERUM INHIBIN LEVELS DURING THE MENSTRUAL CYCLES OF WOMEN

Changes in serum gonadotropin levels during the menstrual cycles of women have been well characterized. In this study, we examined changes in serum inhibin levels in relation to gonadotropin changes. Blood samples were collected once daily from six healthy women volunteers. Serum inhibin levels, as well as gonadotropin levels, were determined by RIA's. The day of the preovulatory LH/FSH surge was taken as day 0.

As shown in Figure 6, normal patterns for serum gonadotropin changes were obtained. On the other hand, serum inhibin levels show a characteristic profile that is quite different from those of the other mammals. First of all, inhibin levels were quite high during the luteal phase. The very high inhibin levels during the luteal phase overlapped with the progesterone burst. Such a profile is characteristic of women, since high inhibin levels were not observed during the luteal phase of the other mammals examined thus far. McLachlan *et al.*[22] and Tsonis *et al.*[33] also reported high inhibin levels during the luteal phase in women.

It is well known that the human corpus luteum is able to produce estradiol, while many other mammals cannot secrete this steroid from their corpus luteum. This suggests that the human corpus luteum is somewhat functionally different from that of other mammals. Therefore, by analogy with estradiol, it is conceivable that the human corpus luteum produces inhibin, accounting for the high inhibin levels present during the luteal phase in women. Such an inhibin burst during the luteal phase, when FSH levels remain quite low, may arrest the development of antral follicles until the regression of the corpus luteum takes place.

Inhibin levels dropped sharply in association with the regression of the corpus luteum, and remained low during the early follicular phase. Serum inhibin levels then increased gradually during the late follicular phase reaching a moderate peak on the day of the preovulatory LH/FSH surge.

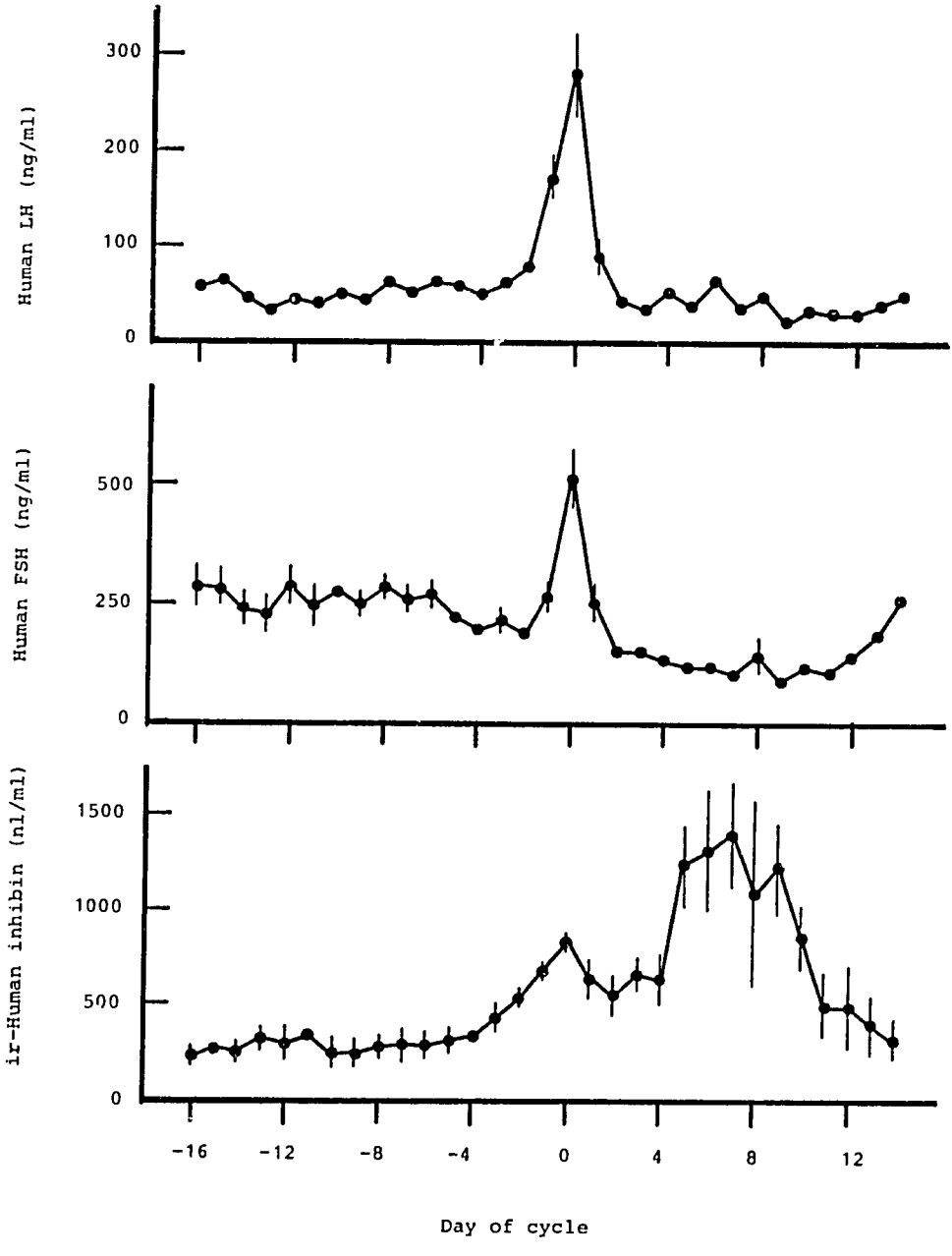


Figure 6. Mean plasma concentrations (± SEM) of LH, FSH, and inhibin during menstrual cycle of six women. Day of LH peak designated as day 0 (Hasegawa *et al.* unpublished).

Serum inhibin and FSH levels correlate quite well in an inverse fashion, except for a few days around the preovulatory gonadotropin surge. During the late follicular phase, antral follicles begin to respond not only to FSH but also to LH to produce estradiol and inhibin.[32,33] Therefore, a strict feedback system between FSH and inhibin may not be feasible during this period. Similarly, since the corpus luteum is responsive only to LH and not FSH, a lack of stimulation by FSH, which might cause a drop in inhibin secretion from the follicles, seems to have no effect on inhibin secretion from the corpus luteum during the luteal phase.

SERUM INHIBIN LEVELS DURING THE ESTROUS CYCLES OF GOATS

As described previously, serum inhibin levels in goats can be measured using a bovine inhibin RIA system. Small Japanese Shiba goats were used in the present experiment.[25] Blood samples were collected from six cycling goats at 2-hour intervals during the periovulatory period and at 24-hour intervals from day 2 to day 16. The day of the preovulatory LH/FSH surge was taken as day 0.

As shown in Figure 7, serum FSH levels presented a fluctuating pattern during these cycles. A small but distinctive second FSH surge was observed about 24 hours after the first LH/FSH surge. During the luteal phase FSH levels were relatively low, but fluctuated with several small peaks.

Serum inhibin levels, as shown in Figure 7, fluctuated along with changes in serum FSH levels in a reverse fashion. The inverse relationship between FSH and inhibin was quite evident during the luteal phase. A drop in FSH levels during the late follicular phase was also associated with an increase in serum inhibin levels. The second FSH surge, which occurs 24 hours after the first LH/FSH surge, seems to be a consequence of a sharp drop in inhibin levels following the first surge.

Unlike human cycles where inhibin levels during the luteal phase are significantly higher than the follicular phase, suggesting the major source of inhibin is the corpus luteum, inhibin levels in the goat were higher during the follicular phase. They then declined significantly during the luteal phase with fluctuations creating small peaks, suggesting that the major source of inhibin in the luteal phase goat is the newly developing ovarian follicle during that period. It would appear that a strict negative feedback mechanism between FSH and inhibin exists during the luteal phase. In other words, during the luteal phase of goats, inhibin is secreted from FSH-responsive components in the ovary, namely follicles, rather than from the corpus luteum, which is not responsive to the FSH stimulation.

CONCLUSIONS

Antisera have been raised against purified porcine and bovine ovarian inhibins. We have now established RIA systems that can be used for the measurement of inhibin concentrations in peripheral sera of cyclic pigs, rats, cows, goats, and women. In cycling pigs, rats, and goats, serum inhibin levels are high during the follicular phase and then drop sharply after the preovulatory LH/FSH surge. In these animals inhibin levels are relatively low during the luteal phase. Several small inhibin peaks are also found during the luteal phase in these animals. In cycling pigs, rats, and goats, fluctuations in inhibin levels that per-

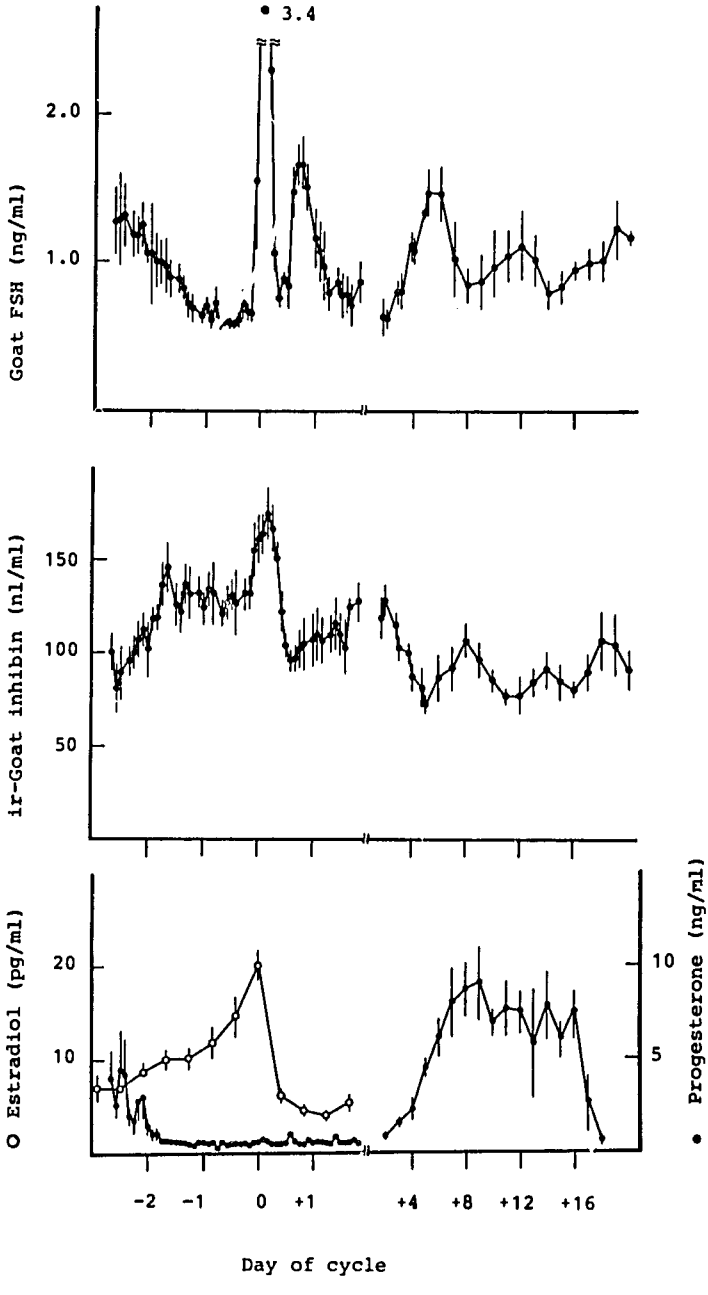
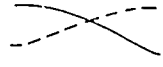
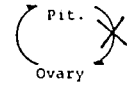
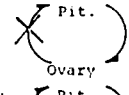
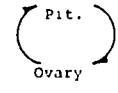
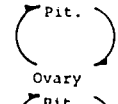
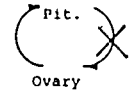


Figure 7. Mean concentrations (± SEM) of LH, FSH, inhibin, estradiol-17β and progesterone during estrous cycle of six Shiba-goats. Time of gonadotropin surge designated as day 0 (Hasegawa *et al.* unpublished).

sisted for a fairly long time after ovulation may reflect the development and degeneration of follicles present in the ovary during the period. In cycling cows, serum inhibin levels reached a maximum 36 to 48 hours after the preovulatory LH/FSH surge, when serum progesterone levels remained low. Inhibin levels then dropped and remained relatively low during the luteal phase. A clear relationship between FSH and inhibin could not be established in cycling cows, except during the period of the second FSH surge. In the menstrual cycles of women, serum inhibin levels were quite high during the luteal phase, in contrast to the other mammals examined thus far. This suggests that a major source of inhibin during the luteal phase is the corpus luteum, at least in women. Therefore, the corpus luteum of women seems to be functionally different from that of the other mammals as it relates to inhibin production.

Table 2 summarizes relationships between FSH and inhibin during the cycles of the mammals examined so far.

TABLE 2
RELATIONSHIPS BETWEEN SERUM CONCENTRATIONS OF INHIBIN AND FSH

Period	Patterns of FSH (——) and inhibin (----)	Animals	Feedback	Souce of inhibin
Follicular phase		Pig, Cow Human, Goat		Growing Follicles
LH/FSH surge		Rat, Pig, Cow Human, Goat		Preovulatory Follicles
Second FSH surge		Rat, Pig, Cow Goat		Non- ovulated Follicles
Luteal phase (A)		Pig, Goat Cow		Small Follicles
Luteal phase (B)		Human		Corpus luteum

ACKNOWLEDGMENTS

The collaboration of Dr. S. Iwamura, the National Institute of Animal Health; Dr. F. Gotoh, Kagoshima University; and Dr. Y. Mori, Tokyo University of Agriculture and Technology is gratefully appreciated. We wish to thank Dr. A.F. Parlow and the National Pituitary and Hormone Distribution Program, NIDDK, NIH for rat and human gonadotropin RIA kits; Dr. D.J. Bolt, Animal Science Institute for the bovine FSH RIA kit; Dr. S. Kanematsu,

Iwate University, for anti-bovine LH and antiporcine LH; and Mr. T. Hanafa, Dr. K. Taya and S. Sasamoto, Tokyo University of Agriculture and Technology for antibovine inhibin.

REFERENCES

1. BAKER HWG, BREMNER WJ, BURGER HG, DE KRETSER DM, DULMANIS A, EDDIE LW, HUDSON B, KEOGH EJ, LEE VWK, RENIE GC: Testicular control of follicle-stimulating hormone secretion. Recent Prog Horm Res 32:429, 1976
2. BICSAK TA, TUCKER EM, CAPPEL S, VAUGHAN J, RIVIER J, VALE W, HSUEH AJW: Hormonal regulation of granulosa cell inhibin biosynthesis. Endocrinology 119:2711, 1986
3. BUTCHER RL, COLLINS WE, FUGO NW: Plasma concentration of LH, FSH, prolactin, progesterone and estradiol-17β throughout the 4-day estrous cycle of the rat. Endocrinology 94:104, 1974
4. CUEVAS P, YING S-Y, LING N, UENO N, ESCH F, GUILLEMIN R: Immunohistochemical detection of inhibin in the gonad. Biochem Biophys Res Commun 142:23, 1987
5. DAANE TA, PARLOW AF: Periovulatory patterns of rat serum follicle stimulating hormone and luteinizing hormone during the normal estrous cycle: Effect of pentobarbital. Endocrinology 88:653, 1971
6. DE PAOLO LV, SHANDER D, WISE PM, BARRACLOUGH CA, CHANNING CP: Identification of inhibin-like activity in ovarian venous plasma of rats during the estrous cycle. Endocrinology 105:645, 1979
7. DI ZEREGA GS, HODGEN GD: Folliculogenesis in the primate ovarian cycle. Endocrine Reviews 2:27, 1981
8. DOBSON H, KAMONPATANA M: A review of female cattle reproduction with special reference to a comparison between buffaloes, cows and zebu. J Reprod Fertil 77:1, 1986
9. FOXCROFT GR, HUNTER MG: Basic physiology of follicular maturation in the pig. J Reprod Fertil [Suppl] 33:1, 1985
10. FRANCHIMONT P, VERSTRAELEN-PROYARD J, HAZEE-HAGELSTEIN MT, RENARD CH, DEMOULIN A, BOURGUIGNON JP, HUSTIN J: Inhibin: From concept to reality. Vitam Horm 37:243, 1979
11. FUKUDA M, MIYAMOTO K, HASEGAWA Y, NOMURA M, IGARASHI M, KANGAWA K, MATSUO H: Isolation of bovine follicular fluid inhibin of about 32K Da. Mol Cell Endocrinol 44:55, 1986
12. GAY VL, MIDGLEY JR AR, NISWENDER GD: Patterns of gonadotrophin secretion associated with ovulation. Fed Proc 29:1880, 1970
13. GRADY RR, CHALESWORTH MC, SCHWARZ NB: Characterization of the FSH-suppressing activity in follicular fluid. Recent Prog Horm Res 38:490, 1982
14. HASEGAWA Y, MIYAMOTO K, YAZAKI C, IGARASHI M: Regulation of the second surge of follicle-stimulating hormone: Effects of antiluteinizing hormone-releasing hormone serum and pentobarbital. Endocrinology 109:130, 1981
15. HASEGAWA Y, MIYAMOTO K, FUKUDA M, TAKAHASHI Y, IGARASHI M. Immunological study of ovarian inhibin. Endocrinol Japan 33:645, 1986
16. HENDRICKS DM, GUTHRIE HD, HANDLIN DL: Plasma estrogen, progesterone and luteinizing hormone levels during the estrous cycle in pigs. Biol Reprod 6:210, 1972
17. IWAMURA S, YOKOKI Y, KAMOMAE H, HARITANI M, OGASA A: A technique for blood collection by chronic catheterization from the auricular vein in the sow. Japan J Anim Reprod 31:165, 1985
18. LEE VWK, MCMASTER J, QUIGG H, FINDLEY J, LEVERSHA L: Ovarian and peripheral blood inhibin concentrations increase with gonadotropin treatment in immature rats. Endocrinology 108:2404, 1981
19. LEVERSHA LJ, ROBERTSON DM, DE VOS FL, MORGAN FJ, HEARN MTW, WETTENHALL REH, FINDLEY JK, BURGER HG, DE KRETSER DM: Isolation of inhibin from ovine follicular fluid. J Endocrinol 113:213, 1987
20. LING N, YING S-Y, UENO N, ESCH F, DONOROY L, GUILLEMIN R: Isolation and partial characterization of a Mr 32,000 protein with inhibin activity from porcine follicular fluid. Pro Natl Acad Sci USA 82:7217, 1985
21. MCLACHLAN RI, ROBERTSON DM, BURGER HG, DE KRETSER DM: The radioimmunoassay of bovine and human follicular fluid and serum inhibin. Mol Cell Endocrinol 46:175, 1986

22. MCLACHLAN RI, ROBERTSON DM, HEALY DL, BURGER HG, DE KRETSER DM: Circulating immunoreactive inhibin levels during the normal human menstrual cycle. J Clin Endocrinol Metab 65:954, 1987
23. MIYAMOTO K, HASEGAWA Y, FUKUDA M, NOMURA M, IGARASHI M, KANGAWA K, MATSUO H: Isolation of porcine follicular fluid inhibin of 32 K Daltons. Biochem Biophys Res Commun 129:369, 1985
24. MIYAMOTO K, HASEGAWA Y, FUKUDA M, IGARASHI M: Demonstration of high molecular weight forms of inhibin in bovine follicular fluid (bFF) by using monoclonal antibodies to bFF 32K inhibin. Biochem Biophys Res Commun 136:1103, 1986
25. MORI Y, KANO Y: Changes in plasma concentrations of LH, progesterone and oestradiol in relation to the occurrence of luteolysis, oestrus and time of ovulation in the Shiba goat. J Reprod Fertil 72:223, 1984
26. PARLOW AF, ANDERSON LL, MELAMPHY RM: Pituitary follicle-stimulating hormone and luteinizing hormone concentrations in relation to reproductive stages of the pigs. Endocrinology 75:365, 1964
27. RAYFORD PL, BRINKLEY HJ, YOUNG EP, REICHERT LE JR: Radioimmunoassay of porcine FSH. J Anim Sci 39:348, 1974
28. REDMER DA, CHRISTENSON RK, FORD JJ, DAY BN: Effects of unilateral ovariectomy on compensatory ovarian hypertrophy, peripheral concentrations of follicle-stimulating hormone, and ovarian venous concentrations of estradiol-17β in prepuberal gilts. Biol Reprod 31:59, 1984
29. RIVIER J, SPIESS J, MCCLIMTOCK R, VAUGHAN J, VALE W: Purification and partial characterization of inhibin from porcine follicular fluid. Biochem Biophys Res Commun 133:120, 1985
30. ROBERTSON DM, FOULDS LM, LEVERSHA L, MORGAN FJ, HEARN MTW, BURGER HG, WETTENHALL REH, DE KRETSER DM: Isolation of inhibin from bovine follicular fluid. Biochem Biophys Res Commun 126:220, 1985
31. SUZUKI T, MIYAMOTO K, HASEGAWA Y, ABE Y, UI M, IBUKI Y, IGARASHI M: Regulation of inhibin production by rat granulosa cells. Mol Cell Endocrinol 54:185, 1987
32. TSONIS CG, HILLIER SG, BAIRD DT: Production of inhibin bioactivity by human-lutein cells: Stimulation by LH and testosterone *in vitro*. J Endocrinol 112:R11, 1987
33. TSONIS CG, MCNEILLY AS, BAIRD DT: Production and secretion of ovarian inhibin. In BURGER HG, DEKRETSER DM, FINDLAY JK, IGARASHI M (eds): Inhibin-non-steroidal regulation of follicle stimulating hormone secretion, p 33, New York, USA, Raven Press, 1987
34. VAN DE WIEL DFM, ERKENS J, KOOPS W, VOS E, VAN LANDEGHEM AAJ: Periestrous and midluteal time courses of circulating LH, FSH, prolactin, estradiol 17-β and progesterone in the domestic pig. Biol Reprod 24:223, 1981
35. VANDALEM JL, BODART CH, PIRENS G, CLOSSET V, HENNEN G: Development and application of homologous radioimmunoassays for porcine gonadotropins. J Endocrinol 81:1, 1979
36. YING SY, CZVIK J, BECKER A, LING N, UENO N, GUILLEMIN R: Secretion of follicle-stimulating hormone and production of inhibin are reciprocally related. Proc Nad Acad Sci USA 84:4631, 1987

DISCUSSION

CHAIRPERSON: ALAN CORBIN, Ph.D.

DR. HSUEH: Regarding the role of the corpus luteum in the production of inhibin, would you comment on the rat versus the human study? I think the human is pretty clear in terms of producing inhibin.

DR. HASEGAWA: I think you are asking about the production of inhibin from a corpus luteum in the pseudopregnant rat. After ovulation, the ovary has a lot of corpora lutea in PMSG-primed immature rats. But inhibin levels do not increase.

DR. DANFORTH: Does your antiserum cross-react with activin or FRP?

DR. HASEGAWA: I tested TGF β and activin. They did not cross-react in our inhibin RIA systems.

DR. DUNWAY: Do you think that progesterone plays a role in the very high levels of inhibin during the luteal phase in the woman?

DR. HASEGAWA: I did not test the effect of progesterone on inhibin production.

DR. SCHENKEN: Have you had the opportunity to determine whether both ovaries secrete inhibin during the luteal phase or is it just secreted from the ovary that contains the corpus luteum?

DR. HASEGAWA: I did not investigate that.

DR. RAJ: You mentioned that you characterized your antiserum by using *in vitro* immunoneutralization. Did you investigate the effect on FSH levels by neutralizing inhibin with antiserum *in vivo*?

DR. HASEGAWA: No, I did not test that because I did not have enough antibody.

8

Regulation of the Subunit mRNAs of Follicle-Stimulating Hormone by Inhibin, Estradiol, and Progesterone

WILLIAM L. MILLER
LEE-WEN LIN
CHARLOTTE L. PHILLIPS
JOYCE C. WU
SUSAN C. LAWS
MICHAEL J. BEGGS
KAREN GUZMAN
AMY MILSTED

Ovine pituitary cells in culture, prepared from the pituitaries of mature ewes, are useful for studying the synthesis and secretion of follicle-stimulating hormone (FSH). They produce and secrete FSH at a rate equal to or slightly in excess of that observed in normal cycling sheep (7 to 43 ng/million pituitary cells/h).[2] The FSH they secrete is as active as the ovine FSH prepared by Sherwood *et al.* (133 x NIH-FSH-S1).[18] Finally, ovine pituitary cultures do not secrete free FSHβ subunit but only FSHβ subunit in combination with α-subunit protein.[18]

Regulation of FSH can be studied in culture. Inhibin, 17β-estradiol (E_2), and progesterone (P_4) negatively regulate FSH production/secretion in ovine pituitary cultures.[3,11,18] Our laboratory has used these cultures to study the effects of inhibin, E_2, and P_4 on A) FSH secretion, B) messenger RNA levels for the α and β subunits of FSH, and C) rates of transcription for the α- and β- subunit genes of FSH. In brief, we have found that inhibin, E_2, and P_4 all share the abilities to decrease both FSH secretion and FSHβ mRNA levels; however, each hormone exhibits distinctive actions that are unique only to itself. We have recently found that inhibin can also increase GnRH binding to ovine pituitary cells in culture. These data are reported here in preliminary form, since they add a new dimension to inhibin action. This chapter reviews our current understanding of inhibin, E_2, and P_4 actions on ovine gonadotropes in mixed cell pituitary culture.

INHIBIN, E_2, AND P_4 DECREASE FSH SECRETION IN OVINE PITUITARY CULTURE

Porcine ovarian inhibin (abbreviated I_p in this text; ED50 = 0.4 ± 0.1 γ/ml), E_2, and P_4 can routinely decrease FSH secretion by 60% to 70% in ovine pituitary cultures within

24 hours (Fig. 1). The initial effects of these hormones (0-24 hours) are difficult to differentiate from each other, but each response is distinctive after 24 hours. This subsection examines each kinetic response and focuses on differences that occur.

Porcine inhibin (I_p) does not significantly change the rate of FSH secretion in culture during the first 4 hours of treatment (Fig. 1; see also Fig. 4A), whereas E_2 and P_4 usually

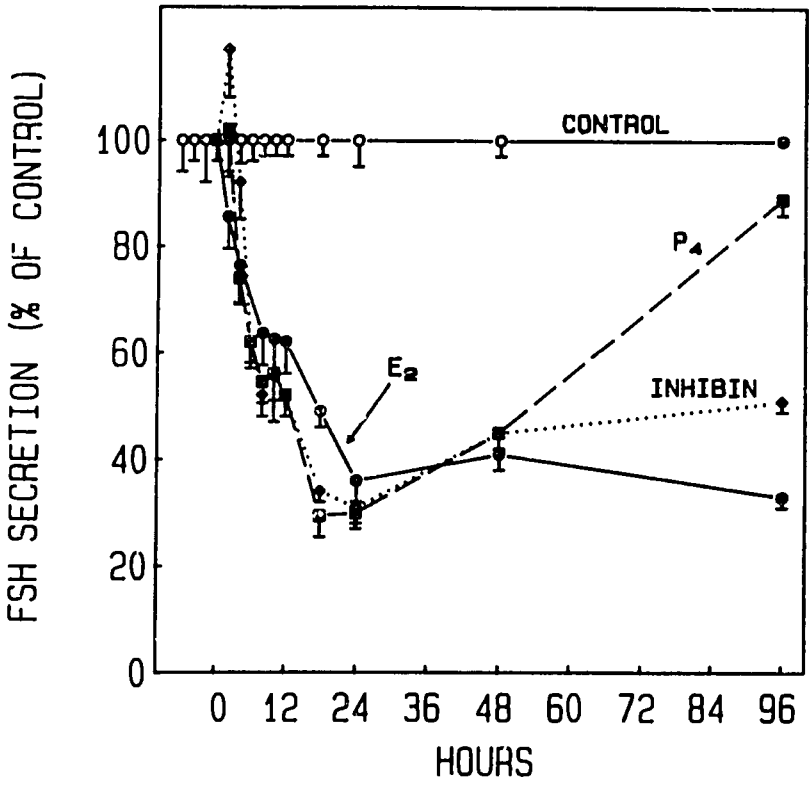


Figure 1. FSH secretion is inhibited by I_p, E_2, or P_4 in ovine pituitary cultures. Ovine pituitary cultures were prepared as described[9] and incubated in 60 mm tissue culture dishes at a concentration of 10 million cells/5 ml. Cultures that were two days old were treated with either normal media (control), 5 μg/ml of I_p, 10 nM E_2, or 100 nM P_4. Culture medium was first changed at 40 hours after cell plating (-8 hours in terms of this experiment). Cultures were rinsed twice with medium 199 and new control medium was added. Culture media were collected from all cultures and stored at -20 °C for radioimmunoassay of FSH at -6 hour, -4, -2, 0, 2, 4, 6, 8, 10, 12, 18, 24, 48, and 96 hours. Treatments began at time = 0 and continued with each media change. Radioimmunoassay (RIA) was performed as described[10] using a highly purified FSH standard with bioactivity of 125 x NIH-FSH-S1. All treatments were done in triplicate and individual samples were assayed in duplicate in one RIA. Results are presented as the mean ± SEM of the Treatment Value divided by the mean ± SEM of the Control Value at each time point x 100.

decrease FSH secretion 10% by 2-3 hours, and up to 20-30% by 4-6 hours (Fig. 1). Because of the time scale and multiple data points clustered around time 0-6 hours in Figure 1, it is easy to overlook this distinction between the inhibin and steroid actions, but it is reproducible and, presumably, reflects a difference in their reaction mechanisms. It should also be noted that it only takes 8-12 hours for I_p to reach its maximal effect once the initial 4-hours delay period passes ($T_{1/2}$ for decrease of FSH secretion [5] 4-6 h). Thus, after a total of 12-16 hours of I_p treatment, a new steady-state level of FSH secretion is usually reached; the new level typically has a tendency to rebound slowly toward control values (Fig. 1; see also Fig. 4B).

Seven separate experiments provided results similar to those reported in Figure 1. Inhibition of FSH secretion by I_p always followed the same kinetics, but the magnitudes of inhibition (at 24 hours) ranged from 32% to 70% with an average of 60% ± 9% (see Figs. 3 and 4 below). One culture in the past 6 years was inhibited 98% by I_p with respect to FSH secretion. The reason(s) for variation is (are) not clear. Animal-to-animal variation may account for the differences, since identical I_p preparations elicited the different responses; alternatively, small changes in culture preparation may cause cellular changes, such as differential degradation of inhibin receptors, that might cause this variation.

The effect of inhibin on ovine gonadotropes can be totally reversed within 24 hours (unpublished results of S. K. Batra, our laboratory). Reversal has already been characterized for a second activity of I_p in ovine pituitary cultures, mainly its ability to increase gonadotrope responsiveness to gonadotropin-releasing hormone (GnRH)[11] (see Fig. 7). The $T_{1/2}$ of reversal is 7 hours once I_p is removed from culture media. These data are consistent with results from O. J. Ginther's laboratory, which showed that decreased levels of FSH rebounded within 24 to 36 hours after treatment with bovine inhibin ceased.[17]

Treatment of cultures with E_2 causes FSH secretion to decrease with a $T_{1/2}$ of approximately 12-15 hours. This is 2-3 times that for I_p (Figs. 1 and 4). The effect of E_2 on FSH secretion is extremely consistent in terms of both kinetics and magnitude of response. E_2 generally causes 75% to 85% inhibition of FSH secretion, and maximal inhibition is always maintained as long as E_2 is present. Removal of E_2 from culture relieves inhibition, but very slowly; FSH secretion does not increase before 48 hours after removal of E_2, and cultures take 6 to 8 days to recover fully.[18]

Treatment with P_4, in contrast to treatments with either I_p or E_2, causes a transient inhibition that is spontaneously reversed, often within 48 to 96 hours. The transient nature of the P_4 response *in vitro* is probably accounted for by turnover and depletion of P_4 receptor molecules in the absence of E_2. It is well established in several cell types that P_4 receptors are dependent on E_2 for their continued synthesis;[2,4] although our complete culture medium contains 10% sheep serum, the serum is charcoal-treated to remove steroids, and the complete culture medium is essentially devoid of E_2.[18]

Treatment of cultures with P_4 consistently causes a more rapid decrease in FSH secretion than treatment with E_2. Inhibition is usually first observed after 2 hours, as with E_2 treatment, but maximum inhibition occurs at approximately 12 hours, giving P_4 action a $T_{1/2}$ of about 5 hours. This is equivalent to that of inhibin. Reversal of P_4 inhibition occurs 3 to 4 days after removal of P_4.[3]

SECRETION OF FSH IS HIGHLY DEPENDENT UPON FSH SYNTHESIS IN OVINE PITUITARY CULTURE

Intracellular FSH is not increased in cultures treated with inhibin (unpublished data, K. Guzman, our laboratory), E_2, or P_4.[3,18] If secretion itself were inhibited, internal storage of FSH should increase unless a compensatory mechanism exists for rapidly degrading intracellular FSH. While there is some evidence for a mechanism that degrades intracellular granules storing gonadotropins,[6] there is also considerable evidence that E_2, P_4, and possibly I_p decrease FSH secretion by blocking FSH synthesis.

FSH secretion is highly dependent upon FSH synthesis. In several typical cultures, gonadotropes secreted the equivalent of the entire internal contents of FSH every 5-8 hours.[2,3] In addition, our laboratory has reported that FSHβ- subunit protein is synthesized, combined with the α subunit, processed, and secreted within 1 h in ovine pituitary cultures.[25] Thus, if synthesis were inhibited, it would be reflected in decreased FSH secretion in about 1 hour.

INHIBIN, E_2, AND P_4 DECREASE LEVELS OF FSHβ mRNA; E_2 AND P_4, BUT NOT INHIBIN, DECREASE LEVELS OF αmRNA; P_4 ALSO DECREASES THE SIZE OF αmRNA

Northern blot analysis of α and FSHβ mRNAs from ovine pituity tissue is shown in Figure 2. Note that each lane contains total RNA isolated from 2.5 million pituitary cells. Figure 2 reveals that ovine pituitary cells in culture contain as much FSHβ mRNA as whole pituitary tissue. Alpha-mRNA levels are lower in culture than *in vivo* by 2.7-fold, but they still exceed FSHβ mRNA levels by approximately 8-fold. Figure 2 also shows that maximally effective treatments with E_2, P_4, and two different preparations of inhibin decreased FSHβ mRNA levels. Finally, the data show that αmRNA is decreased by E_2 and P_4, but not by inhibin. Mercer *et al.*[16] have recently analyzed the effects of inhibin on FSHβ and αmRNAs in ovariectomized, hypothalamic-disconnected, GnRH-pulsed ewes, and have reported results similar to ours.

It should also be noted in Figure 2 that the sizes of the α and FSHβ mRNAs, which are consistent with those reported for bovine and rat α and FSHβ subunits,[14,15] are not changed by any of the treatments except for P_4 treatment. Preliminary studies involving complete digestion of the 3' poly(A) tail using poly-dT hybridization followed by RNase H digestion[23] indicate that P_4 causes shortening of the poly(A) tail.

Finally, two inhibin preparations were used to obtain the data in Figures 2 and 7 (see below). The first preparation of inhibin (I_p) as noted above was of porcine origin and had an ED_{50} of 0.4 μg/ml. Based on its ED_{50}, it appeared to be about 0.1% pure when compared to the most potent preparations currently available.[12,20] The second preparation of inhibin was of bovine origin (I_B) and was generously given to us by Dr. K. Miyamoto;[8] it had an ED_{50} of 0.6 $\pm$ 0.1 ng/ml in ovine pituitary culture. Both inhibin preparations gave essentially the same results, except that I_p seemed to cause a greater decrease in FSHα mRNA than the highly purified I_B (Fig. 2).

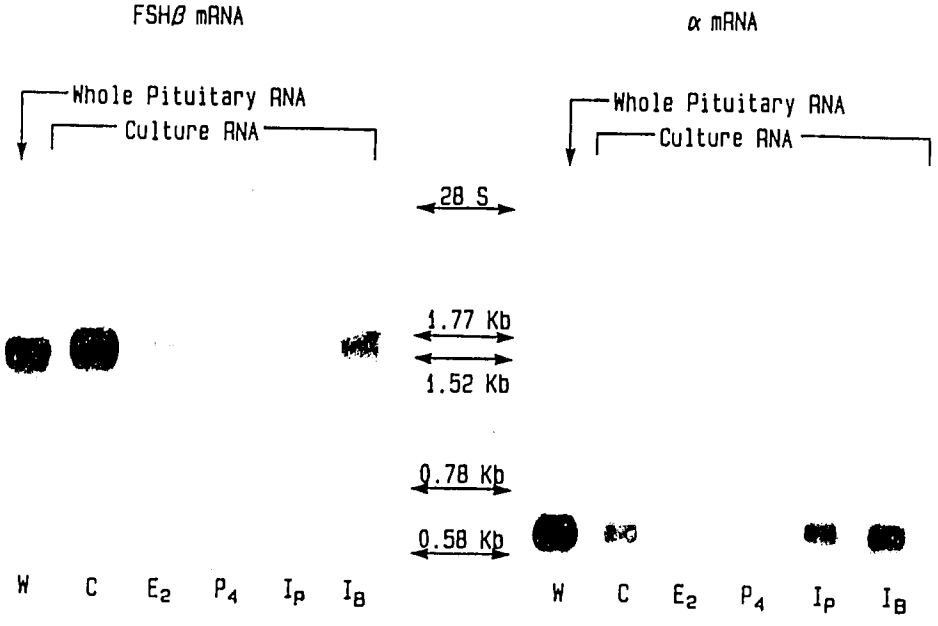


Figure 2. Northern blot analysis of α and FSHβ mRNAs from whole pituitary tissue or pituitary cultures treated with and without I_P, I_B, E_2, or P_4. Total RNA from 2.5 million cells of either whole pituitary or ovine pituitary cultures was isolated, electrophoretically fractionated, and transferred to hybridization membranes. Cultured cells were pretreated for 24 hours with or without 5 μg/ml of I_P, 10 ng/ml of I_B, 10 nM E_2, or 100 nM P_4 (see lanes above), before RNA was isolated. Northern blots were first probed with [^{32}P]FSHβ cDNA which included nucleotides 349-519 of the bovine FSHβ cDNA[15] (autoradiographic exposure =72 hours) and then with [^{32}P]αcDNA from a bovine α clone (palpha-1) (autographic exposure = 1 hour). E_2, P_4, I_P, and I_B decreased FSHβ mRNA by 70%, 71%, 69%, and 64%, respectively. Decreases (increases) for αmRNA in the same respective order were 47%, 44%, (5%), and (23%). (See reference 22 for specific details.)

TREATMENT WITH E_2 OR P_4 CAUSES PARALLEL DECREASES IN FSH SECRETION AND FSHβ mRNA LEVELS; αmRNA IS DECREASED, BUT NOT AS RAPIDLY

While data in Figure 2 are from one time point (24 hours), the kinetics of decrease in FSH secretion and FSHβ mRNA levels after E_2 treatment have been compared at 3, 6, 9, 12, 18, 24, 36, and 48 hours (these data will be published elsewhere).[22] The kinetics of FSH secretion and FSHβ mRNA decreases are essentially identical after the first time point at 3 hours. At 3 hours the decrease in FSHβ mRNA is always greater than the decrease in FSH secretion, which suggests a cause-and-effect relationship. This type of kinetic analysis has been repeated three times with similar results; the results are also consistent with data from cell-free translation studies.[1]

It has also been determined that E_2 decreases αmRNA, but the kinetics of the decrease in αmRNA lag behind the decrease in FSH secretion. For example, as reported in ref. 22, at 24 hours, when FSH secretion decreased by 62% ± 5%, αmRNA decreased only 28% ± 5%. As shown in Figure 2, E_2 decreased FSHβ mRNA and FSH secretion (not shown) by 70% but αmRNA by only 47%. Measurement at 3 days, however, showed that the percent decrease in αmRNA level matched that observed for FSH secretion (70% ± 10%).

Progesterone also causes a rapid drop in FSHβ mRNA followed closely by a similar decrease in FSH secretion during the first 6 hours of treatment. However, FSHβ mRNA levels stop declining after 6 hours while FSH secretion declines even further by 12 hours. Furthermore, the rebounding increase in FSH secretion from its nadir at 12-24 hours (Fig. 1) is not always accompanied by an equally rapid increase in FSHβ mRNA. These data suggest that FSHα levels probably play a role in decreasing FSH secretion, but that P_4 may affect other aspects of gonadotropin function as well, such as secretion or translation.

Progesterone decreases αmRNA levels and FSH secretion in a similar manner. In most cases P_4 does not decrease αmRNA as rapidly or to the same extent as FSHβ mRNA or FSH secretion. This is the case in Figure 2 where FSHβ mRNA and secretion (not shown) decreased by 71% while αmRNA decreased by only 44%. However, after 24 hours there is a general tendency for P_4 to alter αmRNA in a pattern that parallels FSH secretion.[22]

INHIBIN DECREASES LEVELS OF FSHβ mRNA AND FSH SECRETION, BUT NOT ALWAYS WITH THE SAME KINETICS; βmRNA IS NOT AFFECTED BY INHIBIN

Our data indicate that inhibin does not alter αmRNA at any time (Figs. 2 and 4B). These results have been observed 6 or 7 times in culture and have also been observed *in vivo* during recent studies in the laboratory of I. J. Clark.[16] We have also collected much data to indicate that inhibin decreases FSH secretion and FSHβ mRNA to the same extents (Fig. 3). I. J. Clark's laboratory has also observed these results in sheep *in vivo*. Nevertheless, all the data in Figure 3 come from studies at a single time point (24 hour) and, even at this time point, the correspondence of FSHβ mRNA and FSH secretion is not close in several cultures (4 and 5). Thus, more complete time-course studies, like those discussed above for E_2 and P_4, were performed. Their unexpected results are shown in Figure 4A; the results are in contrast to those observed after treatment with either E_2 or P_4. While FSHβ mRNA did decrease, the decrease neither preceded that of FSH secretion (the data points for FSHβ mRNA at 3 and 6 h are not significantly different from control values) nor did it follow the general pattern or slope of FSH secretion. These data have been repeated several times with similar results. The data indicate that decreases in FSHβ mRNA may not be the primary or only cause for the decrease in FSH secretion.

E_2 AND P_4 BLOCK TRANSCRIPTION OF FSH-SUBUNIT GENES; PRELIMINARY EVIDENCE INDICATES THAT INHIBIN DOES NOT ACT AT TRANSCRIPTION

Transcriptional run-off analyses indicate that both E_2 and P_4 decrease transcription of the FSHβ-subunit gene by 85% within 2 hours and 90% thereafter (Fig. 5). Both steroids also

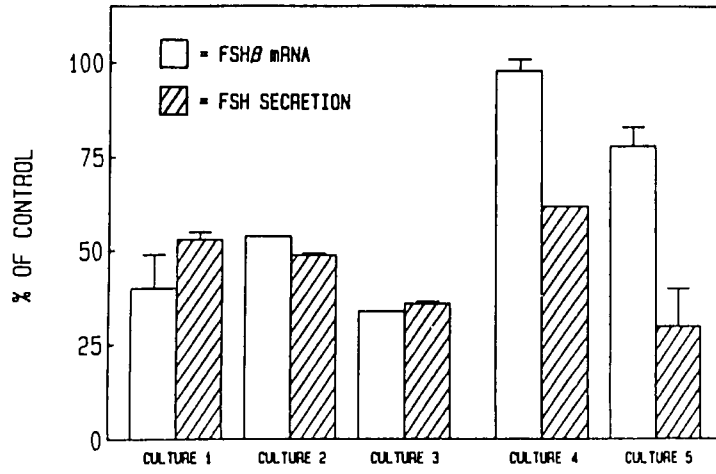


Figure 3. Inhibin can decrease FSH secretion and FSHβ mRNA levels differentially or equally. Ovine pituitary cultures were prepared and treated with I_p for 24 hours as in Figure 2. Media were collected for RIA of secreted FSH and RNA samples were analyzed either by dot-blot (cultures 1, 4, and 5) or Northern blot analysis (cultures 2 and 3).[22]

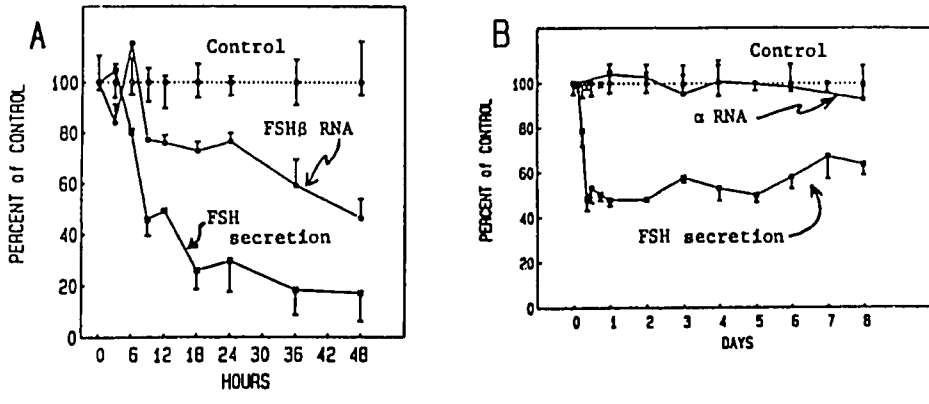


Figure 4. Estradiol decreases FSH secretion and FSHβ mRNA (A), but not αRNA (B). Ovine pituitary cells were cultured with control media or media containing 5 μg/ml of I_p. Media were replaced daily and 3 hours prior to collections for RIA samples. At the indicated times, media were collected and RNAs were isolated. The amount of FSH secreted and the cellular RNA levels of FSHβ (A) and the α subunit (B) were determined by RIA and dot blot analyses; results are expressed as percentages of control values. (See reference 22 for specific details.)

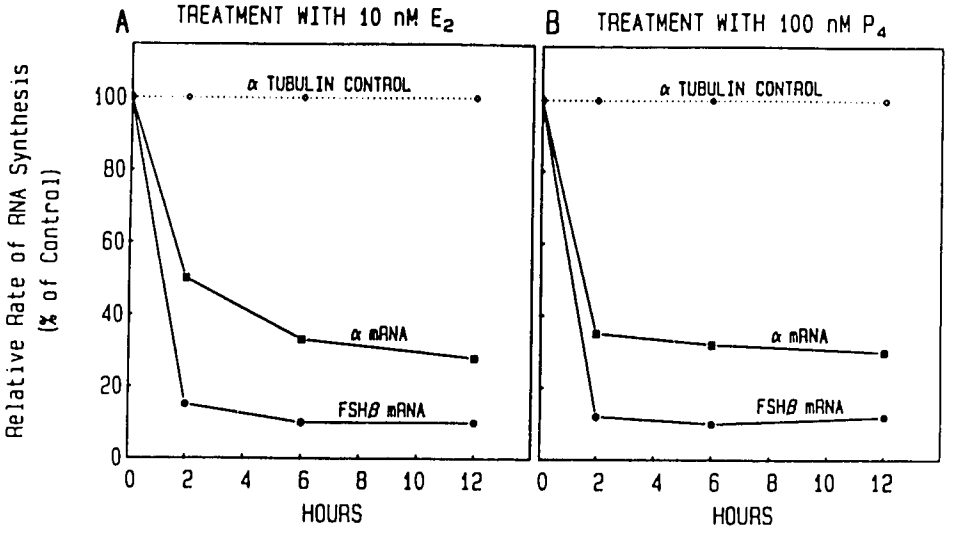


Figure 5. Estradiol (A) and progesterone (B) decrease transcription of FSHβ and α genes in ovine pituitary cultures. Ovine pituitary cell cultures were treated with 10 nM E_2, P_4, or control medium for 0, 2, 6, or 12 hours. At the indicated times, cells were collected, nuclei were isolated, and transcriptional assays were performed as described.[19,22] Each hybridization filter contained 2 μg of pBR322 (control plasmid), and 2 μg each of pBR322 plasmid containing cDNA for human α-tubulin, bovine α-subunit, and bovine FSHβ. RNase- resistant counts bound to filters were detected and quantitated by autoradiography and scanning densitometry.[22]

block transcription of the α gene by 70%, but this level of inhibition takes 12 hours. In all cases the effects of E_2 and P_4 on α- and FSHβ-gene transcription were compared to the rates of transcription of α-tubulin, which is not regulated by E_2 or P_4, and did not change significantly during the treatments.

The effects of inhibin on transcription rates were also studied. Addition of inhibin to ovine pituitary cultures in a manner which normally caused major decreases in FSH secretion by 12 to 18 hours (same conditions used in Figs. 1, 4A, and 4B) did not cause a decrease in the transcription rate of the FSHβ gene. While these studies have been repeated three times at various time intervals (2 to 12 hours), the data show no alteration of FSHβ gene transcription. More studies are planned, however, with longer time intervals.

The lack of evidence for transcriptional regulation by inhibin, plus the fact that inhibin can decrease FSH secretion without first causing a major decrease in FSHβ mRNA, indicates that inhibin acts differently from E_2 and P_4 to decrease FSH secretion. Studies are currently in progress to confirm our preliminary results with inhibin and to initiate studies designed to determine if inhibin acts at translation as suggested by Fukuda *et al.*,[7] subunit processing, or secretion.

INHIBIN INCREASES GnRH RESPONSIVENESS IN OVINE PITUITARY CULTURES: GNRH BINDING IS INCREASED BY INHIBIN

Inhibin is typically associated with its ability to decrease FSH secretion (noted above) and, more recently, with its ability to decrease GnRH-stimulated LH secretion in rat pituitary cell culture.[7] It was reported in 1984, however, that porcine inhibin (I_P) increased the responsiveness of ovine pituitary cultures to GnRH-stimulated LH secretion.[11] This positive activity is not to be confused with FSH-releasing activity found in follicular fluid[13,24] since it does not release LH in the absence of GnRH. In an attempt to understand more about the actions of inhibin and GnRH, our laboratory has recently measured the binding of a GnRH analog (GNRH-A; des-Gly^{10}-[D-Ala^{6}]-LHRH-ethylamide) to ovine pituitary cultures which had been treated with or without I_P or I_B.

Figure 6 shows that I_P increases GnRH responsiveness and GnRH-A binding to ovine pituitary cells to similar extents. Figure 7 shows that the highly purified bovine inhibin I_B is equally effective in increasing GnRH binding.

This section on inhibin action has been included in this chapter to emphasize the fact that inhibin has at least two, and perhaps more, specific effects on pituitary gonadotropes. It will require study at multiple levels of gonadotrope function to determine the mechanism(s) of action of inhibin and the multiple ways inhibin affects gonadotropin synthesis and/or secretion.

CONCLUSION

Studies with ovine pituitary culture have shown that estradiol and progesterone block transcription of both genes encoding the subunits of FSH. The resulting decreases in α and FSHβ mRNAs appear to account entirely for the decrease in FSH secretion that follows treatment with either steroid. FSHβ mRNA is less abundant than αmRNA *in vitro* as well as *in vivo*. Because steroid-induced decreases in FSHβ mRNA precede decreases in FSH secretion which, in turn, precede decreases in αmRNA, it appears that regulation of the FSHβ gene is paramount in regulating overall FSH secretion during abrupt decreases in FSH synthesis. The major decrease in αmRNA may influence FSH synthesis long-term.

Inhibin decreases FSH secretion and FSHβ mRNA levels, but does not alter αmRNA levels. The decrease in FSHβ mRNA does not appear to account for the decrease in FSH secretion, however, and may actually be secondary to that decrease. Inhibin does not significantly alter transcription of the gene encoding the FSHβ subunit.

Finally, since 1984 it has been known that partially enriched preparations of porcine inhibin can increase the responsiveness of ovine pituitary cultures to GnRH.[11] The preliminary studies reported here are the first, however, to show that this activity is also associated with highly purified inhibin (ED_{50} = 0.6 ng/ml) and that inhibin increases GnRH binding in ovine pituitary cultures.

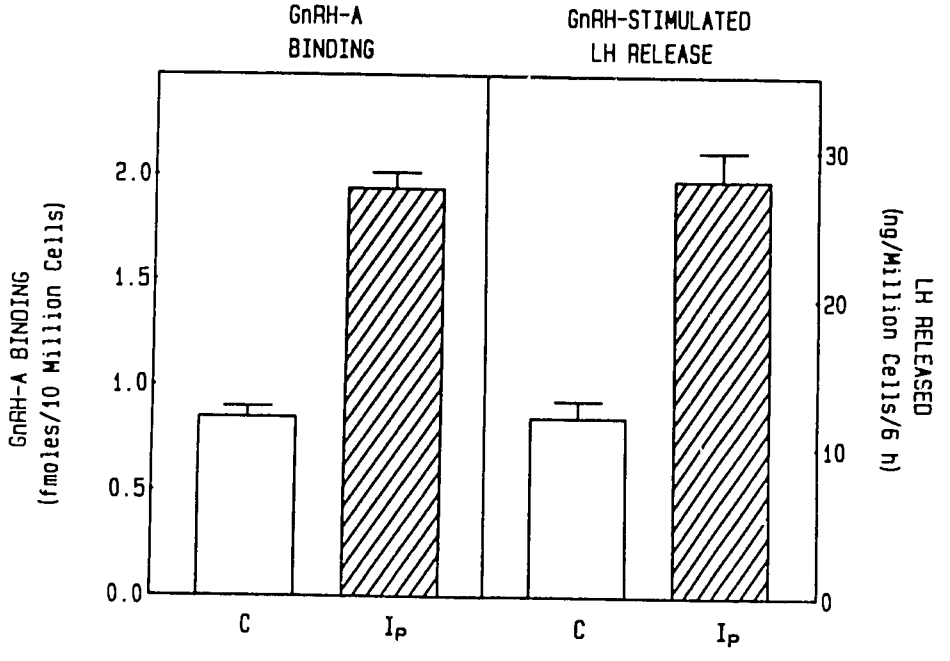


Figure 6. Porcine inhibin (I_p) increases ovine pituitary responsiveness to GnRH and GnRH binding. The data above were obtained by treating 2-day-old cultures (10 million cells/ 5 ml/60 mm culture plate) with or without 5 μg/ml of I_p for 24 hours. GnRH binding was measured by a modification of the procedure by Moss *et al.*[21] Briefly, cells were gently scraped from culture plates and incubated for 5 hours with either 200,000 DPM of monoiodinated des-Gly^{10}-[D-Ala^{6}]-LHRH-ethylamide (designated GnRH-A) or 200,000 DPM of monoiodinated GnRH-A plus 10^{-7} M GnRH-A (non-specific binding). The specific activity of monoiodinated GnRH-A was approximately 1.5 mCi/μg. Each treatment was done in quadruplicate; individual assay tubes contained 7-8 million cells for GnRH binding studies. Responsiveness of ovine pituitary cultures to GnRH was tested using cultures that were identical to those used for binding studies. These cultures were washed twice with medium 199 and treated with 10 nM GnRH-A for 6 hours. Culture media were assayed for LH by RIA.[9] Basal secretion for "C" (7.0 $\pm$ 0.1 ng/million cells/6 h) and for I_p (12.8 $\pm$ 0.75 ng/million cells/6 hour) was subtracted from the LH values prior to plotting (above). Thus, the value plotted above is due to the effect of GnRH-A only. Results are the mean $\pm$ SEM of quadruplicate values.

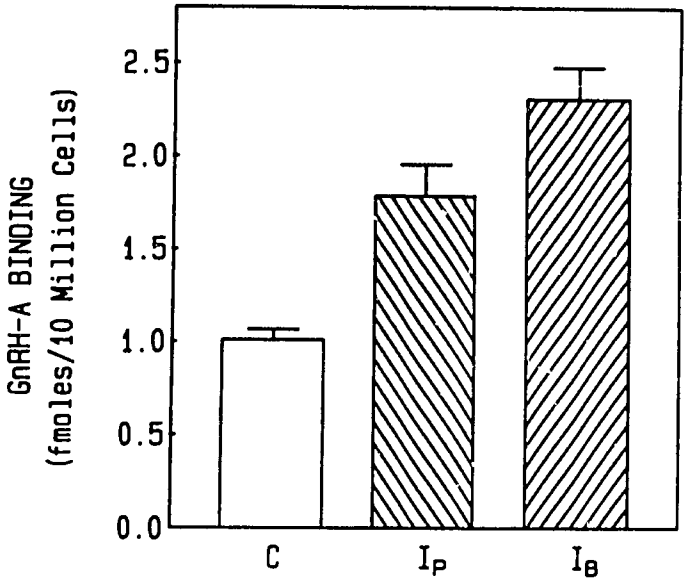


Figure 7. Both I_p and highly purified I_B increased GnRH binding in ovine pituitary cultures. Cultures were treated with I_p (5 μg/ml) or I_B (10 ng/ml) for 24 hours prior to assaying for GnRH-A binding. Analyses were done as described in Figure 6. Results are reported as the mean $\pm$ SEM of quadruplicate value. "C" is significantly different from both I_p and I_B ($p = 0.01$), but I_p is not different from I_B. GnRH binding was determined as described in Figure 6.

ACKNOWLEDGMENTS

The authors wish to thank the following investigators for use of their cDNA plasmids: Dr. R. Maurer, University of Iowa, Iowa City, IA, for bovine αcDNA; Dr. S. Chappel, Integrated Genetics, Framingham, MA, for a fragment of bovine FSHβ cDNA; and Dr. D. Cleveland, Johns Hopkins University, Baltimore, MD, for human α-tubulin cDNA. The authors are also grateful for the generous gift of highly purified 32 Kd bovine inhibin from Dr. K. Miyamoto, Gunma University School of Medicine, Maebashi, Gunma 371, Japan. We thank Dr. John Nilson, Case Western Reserve, Cleveland, OH, for his advice during our analysis of transcription rates, and Mr. Isaac Cotton for his technical assistance with sheep. We also thank Joann Fish for preparation of this manuscript. This work was supported in part by NIH grant HD10773; USDA grant 86-CRCR-1-2181; a grant from the Veterans Administration (A.M.); the U.S. Environmental Protection Agency, Research Triangle Park, NC (S.L.); and grant 85-G-00708 from the N.C. Biotechnology Center.

REFERENCES

1. ALEXANDER DC, MILLER WL: Regulation of ovine follicle-stimulating hormone β-chain mRNA by 17β-estradiol *in vivo* and *in vitro*. J Biol Chem 257:2282, 1982
2. BATRA SK, BRITT JH, MILLER WL: A direct pituitary action of progesterone on basal secretion of follicle-stimulating hormone in ovine cell culture: Dependence on ovaries *in vivo*. Endocrinology 119:1929, 1986
3. BATRA SK, MILLER WL: Progesterone inhibits basal production of follicle- stimulating hormone in ovine pituitary cell culture. Endocrinology 117:2443, 1985
4. CORBANI M, WOLINSKA-WITORT E, COUNIS R, JUTISZ M: Progesterone together with estradiol negatively regulates in the rat the mRNA encoding the pituitary gonadotropins. 69th Annual Meeting of The Endocrine Society, Indianapolis, Abstract No. 475, 1987
5. ERWIN CR, CROYLE ML, DONELSON JE, MAURER RA: Nucleotide sequence of cloned complementary deoxyribonucleic acid for the α subunit of bovine pituitary glycoprotein hormones. Biochemistry 22:4856, 1983
6. FARQUHAR MG, SKUTELSKY EH, HOPKINS CR: Structure and function of the anterior pituitary and dispersed pituitary cells. *In vitro* studies. In TIXIER-VIDAL A, FARQUHAR MG (eds): The Anterior Pituitary Gland, p 83. New York, Academic Press, 1975
7. FUKUDA M, MIYAMOTO K, HASEGAWA Y, IBUKI Y, IGARASHI M: Action mechanism of inhibin *in vitro*-cycloheximide mimics inhibin actions on pituitary cells. Mol Cell Endocrinol 51:41, 1987
8. FUKUDA M, MIYAMOTO K, HASEGAWA Y, NOMURA M, IGARASHI M, KANGAWA K, MATSUO H: Isolation of bovine follicular fluid inhibin of about 32 kDa. Mol Cell Endocrinol 44:55, 1986
9. HUANG ES-R, MILLER WL: Effects of estradiol-17β on basal and luteinizing hormone releasing hormone-induced secretion of luteinizing hormone and follicle stimulating hormone by ovine pituitary cell culture. Biol Reprod 23:124, 1980
10. HUANG ES-R, MILLER WL: Estrogenic and antiestrogenic effects of enclomiphene and zuclomiphene on gonadotropin secretion by ovine pituitary cells in culture. Endocrinology 112:442, 1983
11. HUANG, ES-R, MILLER WL: Porcine ovarian inhibin preparations sensitize cultured ovine gonadotropes to luteinizing hormone-releasing hormone. Endocrinology 115:513, 1984
12. LING N, YING S-Y, UENO N, ESCH F, DENOROY L, GUILLEMIN R: Isolation and partial characterization of a M_r 32,000 protein with inhibin activity from porcine follicular fluid. Proc Natl Acad Sci USA 82:7217, 1985
13. LING N, YING S-Y, UENO N, SHIMASAKI S, ESCH F, HOTTA M, GUILLEMIN R: Pituitary FSH is released by a heterodimer of the β-subunits from the two forms of inhibin. Nature 321:779, 1986
14. MAURER RA: Molecular cloning and nucleotide sequence analysis of complementary deoxyribonucleic acid for the β-subunit of rat follicle stimulating hormone. Mol Endocrinol 1:717, 1987
15. MAURER RA, BECK A: Isolation and nucleotide sequence analysis of a cloned cDNA encoding the β-subunit of bovine follicle-stimulating hormone. DNA 5:363, 1986
16. MERCER JE, CLEMENTS JA, FUNDER JW, CLARK IJ: Rapid and specific lowering of pituitary FSHβ mRNA levels by inhibin. Mol Cell Endocrinol 53:251, 1987
17. MILLER KF, CRITSER JK, GINTHER OJ: Inhibition and subsequent rebound of FSH secretion following treatment with bovine follicular fluid in the ewe. Theriogenology 18:45, 1982
18. MILLER WL, KNIGHT MM, GRIMEK HJ, GORSKI J: Estrogen regulation of follicle-stimulating hormone in cell cultures of sheep pituitaries. Endocrinology 100:1306, 1977
19. MILSTED A, COX RP, NILSON JH: Cyclic AMP regulates transcription of the genes encoding human chorionic gonadotrophin with different kinetics. DNA 6:213, 1987
20. MIYAMOTO K, HASEGAWA Y, FUKUDA M, NOMURA M, IGARASHI M, KANGAWA K, MATSUO H: Isolation of porcine follicular fluid inhibin of 32K daltons. Biochem Biophys Res Commun 129:396, 1985
21. MOSS GE, CROWDER ME, NETT TM: GnRH-Receptor interaction. VI. Effect of progesterone and estradiol on hypophyseal receptors for GnRH, and serum and hypophyseal concentrations of gonadotropins in ovariectomized ewes. Biol Reprod 25:938, 1981
22. PHILLIPS CL, LIN L-W, WU JC, GUZMAN KG, MILSTED A, MILLER WL: 17β-Estradiol and progesterone inhibit transcription of the genes encoding the subunits of ovine follicle-stimulating hormone. Mol Endocrinol (in press)
23. SHIELS BR, NORTHEMANN W, GEHRING MR, FEY GH: Modified nuclear processing of α_1-acid glycoprotein RNA during inflammation. J Biol Chem 262:12826, 1987

24. VALE W, RIVIER J, VAUGHAN J, MC CLINTOCK R, CORRIGAN A, WOO W, KARR D, SPIESS J: Purification and characterization of an FSH-releasing protein from porcine ovarian follicular fluid. Nature 321:776, 1986
25. WHITFIELD GK, MILLER WL: Biosynthesis and secretion of follicle- stimulating hormone β-subunit from ovine pituitary cultures: Effect of 17β-estradiol treatment. Endocrinology 115:154, 1984

DISCUSSION

CHAIRPERSON: KOJI YOSHINAGA, PH.D.

DR. ATTARDI: Can you detect any messenger RNA for LH-β in your cultures?

DR. MILLER: I would have said "no" two years ago. But we recently changed collagenase preparations, and we can detect it now. It is much higher than the FSH-β message. However, we still get no net synthesis of LH in our cultures.

DR. ATTARDI: We have conducted similar studies to those you have described with regard to inhibin, and we used a partially purified inhibin preparation from monkey Sertoli cell culture medium. We looked at the effect on dispersed rat pituitary cells in culture. I have not done as detailed a time-course as you, but I found that there is a parallel decrease in FSH-β mRNA and FSH secretion. The mRNA decreased by about 80% after 24 hours and remained depressed after 72 hours. FSH secretion was decreased by about 60% under those conditions.

The α mRNA in rats also seemed to be slightly decreased, but only by 20%, however. In the rat pituitary cells there was no change in basal LH secretion. There was also no change in the LH-β mRNA, or possibly a slight increase, but there was certainly no suppression.

DR. MILLER: Dr. Ian Clarke's laboratory has also shown that in sheep there is a large decrease in FSH-β mRNA and that there is no decrease in the α mRNA, but apparently, α mRNA goes up and down a little bit, as you saw here.

We were ready to publish that the FSH mRNA did follow the FSH secretion and then put on the brakes when we saw some studies where secretion and FSH-β mRNA did not decrease in parallel. We feel we need to do some more studies on this to determine what is real.

In most cases, the FSH message does go down along with secretion. So the slide that I showed you was an exception. But the exceptions worry me.

DR. SHETH: In your studies on mRNAs, have you tried any combinations of various peptides and steroids in your experiments?

DR. MILLER: If you mean with combinations of estradiol, inhibin, and progesterone, then the answer is "Yes, but only at the secretion level." They all decrease secretion to the same maximal level. Estradiol is maximally effective in these cultures. If you add all three hormones together, the decrease looks like the estradiol decrease.

DR. MASON: Why do you insist that the decrease in mRNA has to be parallel with the decrease in FSH secretion?

DR. MILLER: I guess we thought that if the mRNA decrease accounted for the decrease in secretion it would have to parallel it. It certainly does seem to parallel it in the case of estradiol.

DR. MASON: That does not mean it has to work with the inhibin, does it?

DR. MILLER: The slopes are different. I am just not ready to say yet that message levels account for the decrease in synthesis where inhibin is involved.

DR. MASON: I mean the message does go down, correct?

DR. MILLER: It does.

DR. MASON: Well, therefore, there is going to be less synthesis.

DR. MILLER: But, if it is only 50% down and your synthesis is down 70%, then it does not seem to me to correlate.

DR. MASON: Again, it could be acting in another location as well.

DR. MILLER: That is possible.

DR. KERDELHUE: What is your explanation for the increase in GnRH binding sites after treatment with inhibin?

DR. MILLER: I think there is a possibility that, since inhibin is related to growth factors, it just might be a general stimulus in those cells. On the other hand, some people think that it may interact actually at the membrane surface with GnRH receptors.

Since I do not know yet whether it is increased receptor number or increased affinity, I cannot really tell.

DR. KERDELHUE: You can tell an increase in its number without a change in affinity. In general, nobody ever sees any change in affinity.

DR. MILLER: I suspect that is the case. If that is the case, it would mean increased receptor numbers. We have done some studies with cycloheximide and the effect seems to be cycloheximide sensitive. So, we think it involves increased synthesis of the GnRH receptor.

DR. WARD: On your α subunit, you showed some instances where it went down as much as 70% for your messenger production? How does this fit with the hypothesis that the α levels are not really involved in the mass law control of the $\alpha\beta$ subunit combination? And, how would it relate to, say, other things like FSH in the same cultures?

DR. MILLER: I have had many discussions with John Nilson on this matter. He has cell lines that make α and β together and believes that the α is important in overall production of gonadotropin. I think it should be too.

In our case, however, if you do quantitation on Northern blots, we have about a tenfold excess of α over FSH-β to begin with. If we drop the FSH-β first, it drops even lower, and it may be that α is always in excess in these cultures. Even though the α drops, it may still be in enough excess so it does not really affect overall protein synthesis.

DR. RAJ: I was intrigued by your statement that there was no additive or synergistic action when you added estrogen or progesterone and inhibin together. How would you interpret this? Do you mean to say that they are working through a common pathway?

DR. MILLER: I think the work with progesterone and estradiol indicates that the steroids are working in a similar manner. They may even be working at the same locus as a repressor on the α and FSH-β genes. That would be a common pathway. Inhibin seems to work via a different mechanism that does not involve transcription, but it may be that if you lower transcription enough, you just will not see any additional inhibition. It is hard to inhibit something that is almost totally inhibited.

DR. YOSHINAGA: In your incubation medium, I think you put some indicator. Is that phenol red or something like that? Is there any possibility that an indicator can inhibit FSH secretion?

DR. MILLER: Phenol red has some estrogenic action for MCF-7 cells, but it does not seem to have any action on our primary cultured cells. We detect no action at all. By the way, we can get inhibition with physiologic levels of estradiol and progesterone in ovine pituitary cultures. The maximum inhibition that I showed you here was clearly with hyperphysiologic levels of steroids, but half maximal estradiol and progesterone actions are with physiologic levels that you would normally find in a sheep.

9

Gonadal Inhibitors of Steroidogenesis and Gametogenesis

PAUL FRANCHIMONT
ANNE MATHIEU
MARIE-THERESE HAZEE-HAGELSTEIN
HUGO VAN HEUVERSWYN
ANNE MARMENOUT

The testis and ovary are responsible for the production of sex steroids and maturation of gametes necessary for reproduction, two functions which are interrelated and depend upon endocrine pituitary control involving the secretion of follicle-stimulating hormone (FSH), luteinizing hormone (LH), and prolactin. It is also evident that these gonadal functions are modulated by several autocrine and paracrine mechanisms. The latter not only include mediators such as gonadal steroids which play a local regulatory role, but several peptides which have been identified, isolated, and characterized biologically. This chapter will examine several of these sex steroids and gonadal peptides which play an important role in meiosis and aromatase activity.

REGULATION OF MEIOSIS

METHODS FOR INVESTIGATING MEIOSIS

For the exploration of oocyte meiosis, the culture technique of Tsafriri and Channing[46] based on the spontaneous meiotic resumption of preovulatory oocytes with adhering cumulus cell complexes *in vitro* is often used. Studies of meiosis in male rats can be carried out using flow cytometric methods which rapidly and sensitively measure changes in testicular tissue. As different steps in the process of spermatogenesis involve distinct changes in the DNA content of the germ cells, the method offers the possibility of monitoring such changes on the basis of the incorporation of bromodeoxyuridine, a thymidine analog, into DNA during the S phase of the cell cycle, and the calculation of the total DNA with specific dyes. For instance, the use of the strong, simple intercalating dye propidium iodide provides good estimates of cellular DNA content and is therefore used extensively in estimating ploidy.[4] Fluorescence emission of individual cells can then be measured by a fluorescence-activated cell sorter. In our laboratories, right-angle light scattering (RAS) and propidium iodide fluorescence are useful in distinguishing six populations of sper-

The study described in this chapter was supported by Grant No. 3.4527.86 of the Belgian Scientific Foundation for Medical Research (FRSM).

matogenic cells whose S-phase cells (green fluorescence) can also be discriminated. These include round and elongated spermatids, spermatocytes II, and spermatogonia, young spermatocytes I (leptotene and zygotene), and pachytene spermatocytes I.

SUBSTANCES INVOLVED IN MEIOSIS AT PUBERTY

In the course of preliminary experiments in immature 17-day-old rats, the injection of 5 μg (5 U FSH) pure human FSH (Metrodin-Serono, Rome, Italy) for 10 consecutive days leads to the appearance of haploid cells which are not found in control animals (Table 1). By contrast, LH exerts no such effect. Therefore, FSH seems to be involved in the development of spermatogenesis and the process of meiosis. These results are not completely in agreement with classical observations in the literature whereby LH is considered to be the physiological agent inducing maturation of mammalian oocytes, with FSH being equally effective.[34]

TABLE 1. Effect of Pure FSH on the Repartition of Germinal Cells According to their DNA content

	REPARTITION OF GERMINAL CELLS (mean % ± SE)			
10-Day Treatment	1 n	2 n	4 n	S
Control	0	59.3 ± 2.7	26.8 ± 2.4	13.7 ± 1.3
FSH (5 IU/day)	10.7 ± 1.7	49.6 ± 5.9	27.5 ± 2.4	11.8 ± 4.6

SUBSTANCES ARRESTING SPONTANEOUS RESUMPTION OF MEIOSIS IN OOCYTES

Several substances have been proposed as factors responsible for meiosis inhibition: one (or several) polypeptide(s) termed oocyte maturation inhibitor (OMI), adenosine and cyclic nucleotides, ovarian steroids, and somatostatin. In addition, we have isolated a peptide from ovine rete testis fluid (RTF) with biological properties similar to the follicular OMI isolated by Tsafriri *et al.*[47]

OOCYTE MATURATION INHIBITOR

Several polypeptides have been discovered and termed OMI without knowing if and how they are related to one another. One peptide has been identified by Tsafriri *et al.*[47,48] having a molecular weight ranging between 1000 and 2000 daltons, with the purest preparation being 15,000 times more concentrated than the original follicular fluid. For their part, Sato and Koide[41] have isolated a thermoresistant peptide from bovine granulosa cells with a molecular weight of less than 6000 daltons (oocyte maturation preventing factor or

OMPF). In our laboratory, a factor from RTF has been isolated[15] that is capable of inhibiting the spontaneous resumption of oocyte meiosis and progesterone production by cumulus cells (Fig. 1). Both effects disappeared when the RTF fraction was subjected to pepsin or trypsin digestion. Using polyacrylamide gel electrophoresis, three peptides with molecular weights of 4600, 12,800, and 24,000 daltons were identified. Their possible relationship with the meiosis-preventing substance (MPS), identified in the epididymis and testis of the human male fetus, which prevents germ cells enclosed in seminiferous cords during fetal and infantile life from entering meiosis[18] has not yet been investigated.

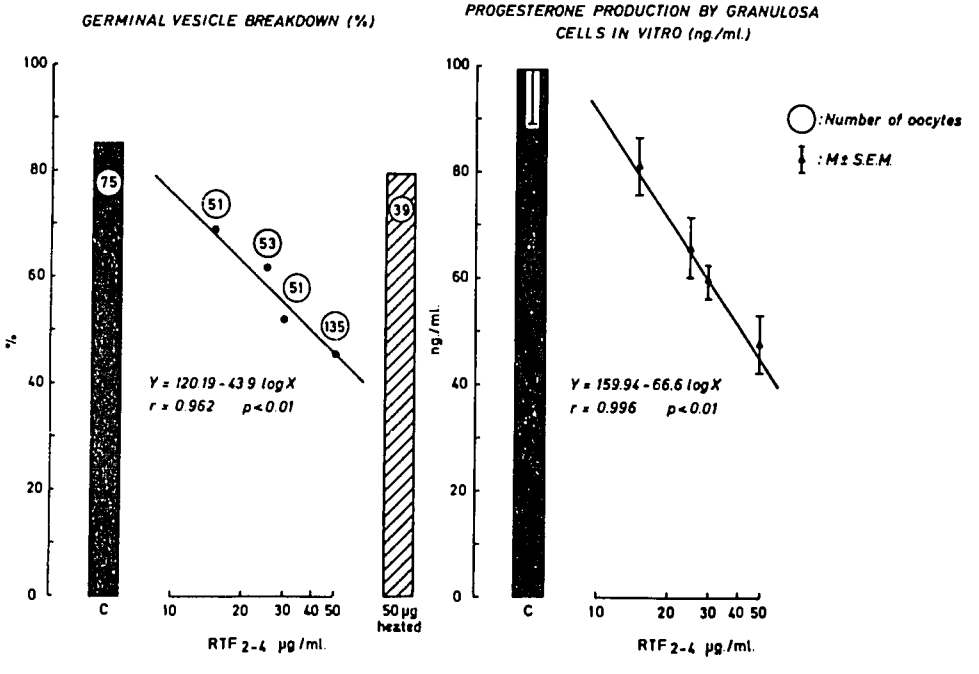


Figure 1. Effect of OMI factor isolated from rete testis fluid on (A) germinal vesicle breakdown and (B) progesterone production by granulosa cells *in vitro*. In both instances, significant inhibition occurs.

ADENOSINE AND CYCLIC NUCLEOTIDES

As described elsewhere, the cyclic AMP analogs, phosphodiesterase inhibitors, adenosine, as well as adenosine monophosphate (AMP), diphosphate (ADP), and triphosphate (ATP) inhibit spontaneous (for cAMP) or FSH-induced oocyte maturation either surrounded by, or denuded from, its cumulus.[13,30,43] Hypoxanthine seems to potentiate the inhibitory activity of cAMP.[43]

STEROIDS

Testosterone, dihydrotestosterone, and progesterone reinforce the inhibitory effect of FSH, phosphodiesterase inhibitors, and cAMP analogs on oocyte maturation.[1,13] However, these steroids are only able to potentiate maturation-arresting activity if sufficient cAMP levels are present inside the oocyte.

SOMATOSTATIN

Recently, Mori *et al.*[32] have reported a pig ovary extract containing a 1-14 and 1-28 somatostatin-like immunoreactivity. These authors[33] have also shown that 1-14 somatostatin was able to inhibit spontaneous meiosis in porcine oocytes under *in vitro* culture. Furthermore, the percentage of oocytes whose germinal vesicles were undergoing breakdown diminished with increasing concentrations of somatostatin. This effect was inhibited by preincubation with anti-somatostatin serum. Thus, this neuropeptide might be secreted by one of the ovarian compartments and play a role in the inhibition of oocyte maturation.

INHIBITION OF ESTROGEN SYNTHESIS

All inhibition of aromatization results in defective folliculogenesis and an evolution to atresia. Several endocrine, paracrine, and autocrine mechanisms reduce the aromatization of androgens into estrogens under the influence of FSH. These involve: 1) the role of hormones such as prolactin, LH, or human chorionic gonadotropin which will not be considered in this chapter; 2) gonadal steroids such as progesterone and reduced non-aromatizable androgens; and 3) polypeptide parahormones including FSH-receptor binding inhibitor (FSH-RBI), follicular regulatory protein(s) also known as aromatase inhibitor (AI), inhibins and related peptides (activins, transforming growth factor β), and epidermal growth factor.

EFFECT OF PROGESTERONE

The granulosa cell aromatizes androgens originating from the stroma and theca interna into estrogens. This process intensifies with the development of the follicle until the preovulatory LH peak; there is a parallel increase of aromatization potential and follicular size.[49] The decrease in aromatization which occurs at the moment of, and following, the preovulatory LH peak appears to be associated with two different phenomena. On the one hand, LH itself inhibits the activity of 17-20 lyase and the 17-α-hydroxylase, reducing the production of androstenedione, an essential aromatization precursor.[45] On the other, progesterone, formed by the granulosa cells in response to the preovulatory LH release, acts as an autocrine and paracrine aromatization inhibitor. Fortune and Vincent[14] have, in fact, shown that progesterone has a specific and irreversible inhibitory effect on 17β-estradiol production by rat granulosa cells. The less differentiated the granulosa cell, the more evident the inhibitory effect of progesterone which could constitute a biochemical vector of follicular dominance. Through progesterone secretion, the dominant follicle maintains its neighboring follicles in an immature state by inhibiting their aromatization potential.

As follicular development evolves, the number of granulosa-cell LH receptors increases; their stimulation by LH induces progesterone production which remains very low during follicular maturation until the preovulatory peak. The autocrine inhibitory action of the estrogens limits progesterone production. Thus, the human granulosa cells of the preovulatory central follicles, although capable of secreting important amounts of progesterone, have their capacity suppressed by physiological concentrations of estradiol found in the follicular fluid (from 0.1 to 3 μg/ml) which inhibit the 3-β-hydroxysteroid dehydrogenase responsible for the transformation of pregnenolone to progesterone.[51]

PARACRINE AND AUTOCRINE INHIBITION OF AROMATIZATION BY OVARIAN ANDROGENS

Non-aromatizable androgens induce atresia.[39] In addition, the follicular fluid of atretic follicles is characterized by high androgen levels and androgen:estrogen ratios above those found in the fluid of normally developing follicles.[28]

Androgen secretion principally takes place in the theca. However, granulosa cells possess 5-α-reductase which can transform testosterone and androstenedione into non-aromatizable androgens: 5-α-dihydrotestosterone and 5-α-androstane-3.17-dione.[2,29] At their physiological concentrations in the follicular fluid (10^{-7} M and 10^{-6} M, respectively) these two non-aromatizable androgens are responsible for an important reduction of aromatization.[19]

THE FSH-RECEPTOR BINDING INHIBITOR

Darga and Reichert[5,6] were instrumental in isolating a peptide from follicular fluid, testicular extracts, and serum which exhibited an inhibitory action on FSH binding to its receptor. In follicular fluid, the concentration of this substance, termed FSH-receptor binding inhibitor (FSH-RBI), diminishes with increasing follicular size: the smaller the follicle, the greater the inhibitory action of the fluid on FSH binding.[44]

In vitro, the inhibition of FSH binding to its receptors is accompanied by a reduced cAMP level and a decrease in the number of LH receptors, phenomena normally induced in the granulosa cell by FSH.[36] FSH-RBI also depresses FSH induced estrogen production by rat granulosa cells.[17] *In vivo*, FSH-RBI prevents the compensatory weight gain of the remaining ovary in the unilaterally ovariectomized mouse.[42]

Using immunofluorescence, FSH-RBI has been identified at the level of granulosa cells present in the antral follicle though not in primordial or preantral follicles.[40] However, such FSH-RBI localization does not indicate whether one is dealing with its site of action, production, or both.

FSH-RBI could be an atretic factor. This hypothesis is supported by the observations of Sluss *et al.*[44] who found a direct and significant relationship between FSH-RBI levels in atretic follicular fluids and the degree of atresia reflected by androgen levels and androgen:estrogen ratios. In another study, ovarian cyst fluid was found to contain much higher FSH-RBI levels than fluid from healthy follicles.[37]

FOLLICULAR REGULATORY PROTEINS (FRP)

While searching for factors involved in follicular dominance, DiZerega *et al.* determined that the Graafian follicle secretes biologically active proteins (FRP) from the fifth day onwards which affect the response of other follicles to gonadotropin stimulation.[12] This regulatory protein was initially found in human[11] and porcine[24] venous blood taken from the ovary containing the dominant follicle as well as in human granulosa cell-culture media.[10] Follicular regulatory proteins suppress the follicular response to gonadotropin stimulation *in vivo*,[9] while inhibiting estrogen and progesterone production of the granulosa cell *in vitro*. In addition, FRP reduces the number of FSH-induced LH/HCG binding sites on the porcine granulosa cell.[31] The protein is destroyed by heat and trypsin treatment, has a molecular weight between 12,500 and 16,000 daltons and an isoelectric point between 6.1 and 4.5. This molecule is devoid of any effect on pituitary cell culture and does not correspond to either inhibins or activins.[17]

The granulosa cell is the source of FRP, whose production diminishes as the cell luteinizes and secretes more progesterone or when it is stimulated by gonadotropin.[10]

A positive correlation has been demonstrated between the follicular fluid concentration of FRP and that of both inhibin and 17β-estradiol.[11] By contrast, an inverse relationship exists between follicular fluid FRP levels and those of progesterone. These data indicate that follicular FRP activity is related to follicular functional differentiation which diminishes as luteinization takes place.

The action of FRP on aromatization differs from that on progesterone production. Follicular regulatory proteins inhibit granulosa cell aromatase at any concentration, an effect which cannot be compensated by addition of excess FSH. Small doses of FRP stimulate progesterone production by activating the 3-β-dehydroxysteroid dehydrogenase, while a large dose provokes an inhibition of progesterone production which can be corrected by adding gonadotropin or eliminating FRP from the culture medium.

The administration of FRP during the first 15 days of the menstrual period in the monkey disturbs the course of follicular maturation as well as that of luteal function: during the abnormally long follicular phase, anovulation and low plasma β estradiol levels are observed, while reduced 17β-estradiol and progesterone levels characterize the luteal phase.[7]

INHIBINS, ACTIVINS, AND THE TRANSFORMING GROWTH FACTOR β (TGF β)

The nature and structure of inhibin has only recently been discovered.[26,38] It consists of a heterodimer composed of an α and β subunit. The former has a molecular weight of 18,000 daltons, while the latter is in fact made up of two 14,000-dalton subunits termed β A and β B. Beta subunits may also be linked by disulfide bridges as a homodimer β A-β A[50] termed FSH-releasing protein (FRP) and also known as activin A, or as the heterodimer β A-β B,[27] termed activin B. Both activins have molecular weights of 28,000 daltons. Finally, TGF-β is a homodimer consisting of two identical subunits of 12,500 daltons. Both inhibin and TGF-β have biochemically related structures — the β-subunits of inhibin and TGF-β subunit possess 9 cysteine and 33 other identical amino acids found in identical positions.[54] These similarities indicate that the two subunits may be derived from a common ancestral gene.

Inhibin not only inhibits FSH secretion but, via an endocrine mechanism, is able to reduce aromatization, thereby favoring follicular involution. However, studies by Ying *et al.*[53] have also shown that in the rat granulosa cell, inhibin exerts a direct inhibitory effect on aromatization.

More recently, Hsueh *et al.*[23] demonstrated that LH-induced production of androgens, the substrates for aromatization, is stimulated by gonadal inhibin at the Leydig and thecal cell level. In contrast, TGF-β stimulates FSH release from pituitary cells and induces an important increase in aromatization of androgens to estrogens in the granulosa cell.[53,54] This transforming growth factor is produced by ovarian thecal cells and inhibits epidermal growth factor- (EGF) stimulated growth-promoting effects and regulates functional differentiation.

It has also been recently shown that activin A, a homodimer of the β-A subunit capable of stimulating FSH secretion, is a powerful inhibitor of aromatization by rat granulosa cells,[17] and of androgen synthesis by Leydig and thecal cells.[23]

The N-terminal sequence of the inhibin α subunit (α 1-26 Gly-27-Tyr 28-OH) has been tested on granulosa cells from immature female rat ovaries cultured with FSH and testosterone.[20] Although an inhibitory effect by this sequence has been described on progesterone, cAMP, and 17β-estradiol production, we have been unable to confirm such results in a similar biological model.[17]

EPIDERMAL GROWTH FACTOR

This factor is known to exert a mitogenic effect on a great variety of epithelial and non-epithelial cells.[3] When added to bovine and rat granulosa cell cultures, EGF produces an increase in DNA synthesis resulting in an increase in mitosis and cellular protein content.[16,22] This mitogenic effect is accompanied by a reduction in progesterone and inhibin synthesis[16] as well as by a direct depression of aromatization.[21] The inhibitory effect of EGF on follicular differentiation is also seen by a reduction of FSH-induced receptor production.[25]

Although EGF could be produced locally, no formal evidence exists at present to support this hypothesis. By contrast EGF receptors have been found on the membranes of bovine granulosa cells.[52] The EGF effect occurs after the step involving cAMP synthesis since it is able to reduce aromatization induced by adenylcylase activation (e.g., by cholera toxin) and by dibutyrl cAMP.[21]

ACKNOWLEDGMENTS

We thank Mr. and Mrs. F. Shapiro (SYNTAXIS-BELGIUM) for their editorial and technical assistance with the manuscript.

REFERENCES

1. ARMSTRONG DT, DANIEL SAJ: Intraovarian paracrine regulation. International Symposium on Cell to Cell Communication in Endocrinology. Florence, Italy, Abstract No. 19, 1987
2. BOGOVICH K, RICHARDS JS: Androgen synthesis during follicular development: Evidence that rat granulosa cell 17 ketosteroid reductase is dependent on hormonal regulation. Biol Reprod 31:122, 1984
3. CARPENTIER G, COHEN S: Epidermal growth factor. Annu Rev Biochem 48:193, 1979
4. CLAUSEN OPF, PURVIS K, HANSSON V: Application of micro-flow fluorometry to studies of meiosis in the male rat. Biol Reprod 17:555, 1977
5. DARGA NC, REICHERT LE Jr: Some properties of the interaction of follicle stimulating hormone with bovine granulosa cells and its inhibition by follicular fluids. Biol Reprod 19:235, 1978
6. DARGA NC, REICHERT LE Jr: Evidence for the presence of a low molecular weight follitropin binding inhibitor in bovine follicular fluid. Adv Exp Med Biol 112:383, 1979
7. DIZEREGA GS, WILKS JW: Inhibition of the primate ovarian cycle by a porcine follicular fluid protein(s). Fertil Steril 41:635, 1984
8. DIZEREGA GS, GOEBELSMANN U, NAKAMURA R: Identification of proteins secreted by the preovulatory ovary which suppresses the follicle response to gonadotropins. J Clin Endocrinol Metab 54:1091, 1982
9. DIZEREGA GS, MARRS RP, CAMPEAU JD, KLING OR: Identification of proteins in pooled human follicular fluid which suppress follicular response to gonadotropins. J Clin Endocrinol Metab 56:35, 1983
10. DIZEREGA GS, MARRS RP, CAMPEAU JD, KLING OR: Human granulosa cell secretion of protein(s) which suppress follicular response to gonadotropins. J Clin Endocrinol Metab 56:147, 1983
11. DIZEREGA GS, CAMPEAU JD, NAKAMURA RN, UJITA EL, LOBO R, MARRS RP: Activity of a human follicular fluid protein(s) in spontaneous and induced ovarian cycle. J Clin Endocrinol Metab 57:838, 1983
12. DIZEREGA GS, CAMPEAU JD, UJITA EL, SHREIBER JR, BATTIN DA, MONTZ FJ, NAKAMURA RN: Follicular regulatory proteins: paracrine regulators of follicular steroidogenesis. Proc Satellite Symposium of Montebello, p 422, 1985
13. EPPIG JJ, DOWNS SM: Chemical signals that regulate mammalian oocyte maturation. Biol Reprod 30:1, 1984
14. FORTUNE JE, VINCENT SE: Progesterone inhibits the induction of aromatase activity in rat granulosa cells *in vitro*. Biol Reprod 28:1078, 1983
15. FRANCHIMONT P: Inhibin and gonadal parahormones: possible contraceptive agents. In ZATUCHNI G, GOLDSMITH A, SPIELER J, SCIARRI J (eds): Male Contraception: Advances and Future Prospects, p 408, Philadelphia, Harper and Row, 1985
16. FRANCHIMONT P, HAZEE-HAGELSTEIN MT, CHARLET-RENARD CH, JASPAR JM: Effect of mouse epidermal growth factor on DNA and protein synthesis, progesterone and inhibin production by bovine granulosa cells in culture. Acta Endocrinologica 111:122, 1986
17. FRANCHIMONT P, JASPAR JM, HAZEE-HAGELSTEIN MT, CHARLET-RENARD CH, VAN HEUVERSWYN H, DE WAELE P, MARMENOUT A, BOSMAN F, VAN DE VOORDE A: Effect of inhibin alpha and beta subunit and their polymers on aromatase activity of rat granulosa cells. (submitted)
18. GRINSTED J, BYSKOV AG: Meiosis inducing and meiosis preventing substances in human male reproductive organs. Fertil Steril 35:199, 1981
19. HILLIER SG, VAN DEN BOOGAERD AMJ, REICHERT L, VAN HALL EV: Intraovarian sex steroid hormone interactions and the regulation of follicular maturation: aromatization of androgens by human granulosa cells *in vitro*. J Clin Endocrinol Metab 50:640, 1980
20. HILLIER SG, TSONIS CG, EIDNE KA: Inhibition of FSH-stimulated granulose cell function by a synthetic fragment of the porcine inhibin α subunit: evidence for involvement of GnRH receptors. J Endocrinol 113:R3-R5, 1987
21. HSUEH AJW, WELSH TH, JONES PBC: Inhibition of ovarian and testicular steroidogenesis by epidermal growth factor. Endocrinology 108:2002, 1981
22. HSUEH AJW, ADASHI EY, JONES PBV, WELSH TH Jr: Hormonal regulation of the differentiation of cultured ovarian granulosa cells. Endocr Rev 1:76, 1984
23. HSUEH AJW, DAHL KD, VAUGHAN J, TUCKER E, RIVIER J, BARDIN CW, VALE W: Heterodimers and homodimers of inhibin subunits have different paracrine action in the modulation of luteinizing hormone-stimulated androgen biosynthesis. Proc Natl Acad Sci USA 84:5082, 1987

24. KLING OR, ROCHE PC, CAMPEAU JD, NISHIMURA K, NAKAMURA RM, DIZEREGA GS: Identification of porcine follicular fluid fraction which suppresses follicular response to gonadotropin. Biol Reprod 30:564, 1984
25. KNECHT M, CATT K: Epidermal growth factor and gonadotropin-releasing hormone inhibit cyclic AMP-dependent luteinizing hormone receptor formation in ovarian granulosa cells. J Cell Biochem 21:201, 1983
26. LING N, YING SY, UENO N, ESCH F, DENOROY L, GUILLEMIN R: Isolation and partial characterization of a MW 32,000 protein with inhibin activity from porcine follicular fluid. Proc Natl Acad Sci USA 82:7217, 1985
27. LING N, YING SY, UENO N, SHIMASAKI S, ESCH F, HOTTA M, GUILEMIN R: Pituitary FSH is released by a heterodimer of the beta subunits from the two forms of inhibin. Nature 321:779, 1986
28. McNATTY KP: Cyclic changes in antral fluid hormone concentrations in the human. Clin Endocrinol Metab 7:577, 1978
29. McNATTY KP, MAKRIS A, DE GRAZZIA O, OSTHANONDH R, RYAN KJ: The production of progesterone, androgen and oestrogen by human granulosa cells *in vitro* and *in vivo*. J Steroid Biochem 11:755, 1979
30. MILLER JGO, BEHRMAN HR: Oocyte maturation is inhibited by adenosine in the presence of follicle stimulating hormone. Biol Reprod 35:833, 1986
31. MONTZ FJ, UJITA EL, CAMPEAU JD, DIZEREGA GS: Inhibition of LH/HCG binding to porcine granulosa cells by a follicular fluid protein(s). Am J Obstet Gynecol 148:436, 1984
32. MORI T, SAITO H, OHNO Y, IRAHARA M, HOSOI E, SAITO S: Evidence for existence of somatostatin-like immunoreactivity with molecular heterogeneity in porcine ovaries. Acta Endocrinol 10:254, 1984
33. MORI T, IRAHARA M, SATO H, OHNO Y, HOSOI E: Inhibitory action of somatostatin on meiotic maturation of cultured porcine oocytes. Acta Endocrinol 110:408, 1985
34. NEAL P, BAKER TG: Response of mouse graafian follicles in organ culture to varying doses of follicle-stimulating hormone and luteinizing hormone. J Endocrinol 65:27, 1975
35. REICHERT LE Jr, ABOU-ISSA H: Studies on a low molecular weight testicular factor which inhibits binding of FSH to receptor. Biol Reprod 17:614, 1977
36. REICHERT LE Jr, SANZO MA, DARGA NS: Studies on a low molecular weight follicle-stimulating hormone binding inhibitor from human sperm. J Clin Endocrinol Metab 49:866, 1979
37. RETTORI V, VAN SICKLE M, STEINBERGER A, ASCH RH: Follicle-stimulating hormone binding inhibiting activity and inhibin properties of human ovarian cystic and porcine follicular fluid. Acta Eur Fertil 15:93, 1984
38. RIVIER J, SPIESS J, McCLINTOCK J, VAUGHAN J, VALE W: Purification and partial characterization of inhibin from porcine follicular fluid. Biochem Biophys Res Common 133:120, 1985
39. ROSS GT, HILLIER SG: Experimental aspects of follicular maturation. Eur J Obstet Gynecol Reprod Biol 9:169, 1979
40. SATO E, ISHIBASHI T: Immunohistochemical localization of a follicle-stimulating hormone (FSH) inhibitor from bovine follicular fluid. Adv Bioscien 34:203, 1982
41. SATO E, KOIDE SS: A factor from bovine granulosa cells preventing oocyte maturation. Differentiation 26:59, 1984
42. SATO E, ISHIBASHI T, IRITANI A: Purification and action sites of follicle-stimulating hormone inhibitor from bovine follicular fluid. J Anim Sci 55:873, 1982
43. SATO E, KOIDE SS: Biochemical transmitters regulating the arrest and resumption of meiosis in oocytes. Int Rev Cytol 106:1, 1987
44. SLUSS PM, REICHERT LE Jr: Porcine follicular fluid contains several low molecular weight inhibitors of follicle-stimulating hormone binding to receptors. Biol Reprod 30:1091, 1984
45. SUZUKI K, TAMAOKI BI: Post-ovulatory decrease in estrogen production is caused by the diminished supply of aromatizable androgen to ovarian aromatase. Endocrinology 107:211, 1980
46. TSAFRIRI A, CHANNING CP: An inhibitory influence of granulosa cells and follicular fluid upon porcine oocyte meiosis "*in vitro*". Endocrinology 96:922, 1975
47. TSAFRIRI A, BAR-AMI S, DEKEL N: Follicular regulation of oocyte maturation. In FUDJU T, CHANNING CP (eds): Non-steroidal regulators. Reproductive Biology and Medicine, p 123 Oxford, Pergamon Press, 1982
48. TSAFRIRI A, DEKEL N, BAR-AMI S: The role of oocyte maturation. J Reprod Fert 64:541, 1982

49. TSONIS CG, CARSON RS, FINDLAY JK: Relationship between aromatase activity follicular fluid, oestradiol, 17-β-oestradiol and testosterone concentrations and diameter and atresia of individual ovine follicles. J Reprod Fertil 72:153, 1984
50. VALE W, RIVIER J, VAUGHAN J, McCLINTOCK R, CORRIGAN A, WOO W, KARR D, SPIESS J: Purification and characterization of an FSH-releasing protein from porcine ovarian follicular fluid. Nature 321:776, 1986
51. VELHUIS JD, KLASE PA, SANDOW A, KOLP LA: Progesterone production by highly differentiated human granulosa cells isolated from preovulatory graafian follicles induced by exogenous gonadotropins and human chorionic gonadotropin. J Clin Endocrinol Metab 57:847, 1983
52. VLODAVSKY I, BROWN KD, GOSPODAROWICZ D: Comparison of the binding of epidermal growth factor to cultured granulosa and luteal cells. J Biol Chem 253:3744, 1978
53. YING SH, BECKER A, BAIRD A, LING N, UENO N, ERSCH F, GUILLEMIN R: Type β transforming growth factor (TGF β) is a potent stimulator of the basal secretion of follicle stimulating hormone (FSH) in a pituitary monolayer system. Biochem Biophys Res Commun 135:950, 1986
54. YING SH, BECKER A, LING N, UENO N, GUILLEMIN R: Inhibin and β type transforming growth factor (TGF β) have opposite modulating effects on the follicle stimulating hormone (FSH) induced aromatase activity of cultured rat granulosa cells. Biochem Biophys Res Commun 136:969, 1986

DISCUSSION

CHAIRPERSON: KOJI YOSHINAGA, Ph.D.

Due to illness, Dr. Franchimont was unable to attend the Workshop. Dr. Marmenout presented on his behalf.

DR. BERGER: I found your results very interesting, but I am puzzled by your results with activin on aromatase activity.

This group may be familiar with work that was published by Laura Hutcherson which suggested that activin actually acts as an anti-luteinization factor for granulosa cells, as it was shown that activin stimulates aromatase and inhibits progesterone production.

I wonder if you have any explanation for what obviously is a contradictory finding?

DR. MARMENOUT: I have no immediate answer, but it is repeatedly found and if we make a comparison with other cell lines which produce no activin, then we do not find this activity. So it is a reproducible feature with activin cDNA harvested COS cells.

DR. NEKOLA: Am I correct that the best oocyte maturation studies showed a 30 to 50 percent inhibition of germinal vesicle breakdown? If so, do you have an explanation of why it is difficult to show better inhibition?

DR. MARMENOUT: You are right, maximum inhibition is around 30 to 40 percent of the initial value in the absence of OMI. The reason is not evident. Perhaps a synergistic effect of several factors is needed to get 100% inhibition.

DR. SCHOMBERG: My question relates to the FSH receptor binding inhibitor. Since FSH receptor binding and FSH stimulated EGF (epidermal growth factor) production do go down as the follicle develops, I would like to know why you associated this activity with follicle atresia.

DR. MARMENOUT: Being a molecular biologist, I am not in the position to give a satisfactory answer to your question. But, since it inhibits aromatase activity, you have depressed the levels of estrogens, and it has been suggested that this phenomenon can lead to atresia.

DR. SCHOMBERG: That is a good comment with respect to aromatase activity. I was just thinking of the FSH receptor population per se, and that population does go down as a function of normal follicle development.

DR. KHAN: With reference to the peptide you found in rete testis fluid which inhibits progesterone production and oocyte maturation, does that peptide act similarly in the testis? Have you done any studies on whether it inhibits androgenesis or related cell aromatase activity of the Sertoli cells in the testis?

DR. MARMENOUT: No, but studies are presently underway on meiosis appearance in pubertal rats, but the results are not yet available.

DR. MANBERG: As a follow-up to Dr. Burger's comment, it seems very contradictory that you have a compound that would increase FSH on the one hand and inhibit its activity on the other. Have you tried doing an experiment where you increase the FSH in the granulosa cell assay to see whether it can overcome the inhibition of activin?

DR. MARMENOUT: No.

DR. MANBERG: Can you speculate on the physiological significance of an activin having two effects that are very contradictory?

DR. MARMENOUT: There may be modulating effects. Activin could be involved in follicle recruitment under the influence of its action on FSH secretion without affecting estrogen production. It could also be involved in the genesis of the mid-cycle FSH peak. However, this is only speculation on my part.

DR. TSAFRIRI: I was very impressed with the studies that you presented on OMI activity. Could you comment on the nature and conditions of the assay?

DR. MARMENOUT: Again, since I am a molecular biologist I am not that familiar with the bioassays. But I presume that the assay can be vouched for.

DR. MASON: In the aromatase assay that you did with the recombinant activin, were you using crude supernatants from tissue culture cells?

DR. MARMENOUT: Yes.

DR. MASON: So, is it possible that the discrepancy in the results may be explained by the presence of another peptide that is encoded in the precursor regions of the activin that may be having that effect on aromatase activity? That peptide, presumably, would be in your supernatant. There may be another biologically-active peptide in the precursor.

DR. MARMENOUT: Yes, coming from the precursor.

DR. MASON: So the activity that you are seeing may not be activin.

DR. MARMENOUT: I cannot exclude that possibility right now.

10

Inhibin — Regulation and Mechanism of Action

HENRY BURGER
ZHANG ZHIWEN
SUSAN DAVIS
VICTOR LEE
RONALD CARSON
ADRIAN HERINGTON
JOCK FINDLAY
PAUL FARNWORTH
QI-FA WANG
SIMON AU
DAVID DE KRETSER
DAVID ROBERTSON

Within the past three years, there has been rapid progress in knowledge of the physiology of inhibin and inhibin-related peptides. Inhibin was first purified to homogeneity from bovine follicular fluid in our laboratories,[23] an achievement based substantially on the development of a sensitive and specific bioassay, utilizing the selective suppression of FSH content in dispersed, primary cultures of rat anterior pituitary cells.[26] Studies of inhibin regulation utilized that assay and a subsequent modification based on measurement of basal FSH release.[16] This modification provided a more sensitive system, applicable to the measurement of inhibin in rat peripheral blood. Following the purification and characterization of inhibin, radioimmunoassays were developed which opened the way for more detailed and extensive studies.[19] The present review deals with two aspects of inhibin-regulation of its secretion in the rat and its mechanism of action on the pituitary.

FACTORS REGULATING INHIBIN SECRETION IN THE RAT

FOLLICLE-STIMULATING HORMONE

The major hypothesis underlying our studies on the regulation of inhibin secretion *in vivo* and *in vitro* was that inhibin is part of a classic endocrine closed-loop feedback system in which gonadal inhibin is an important negative regulator of pituitary FSH secretion and gonadal inhibin secretion is in turn under FSH control. Based on the knowledge that the administration of pregnant mare serum gonadotropin (PMSG) to immature female rodents results in stimulation of multiple follicular growth followed by ovulation and the develop-

ment of corpora lutea, we reasoned that the immature female rat would be a useful model in which to study the regulation of inhibin secretion.

We established dose-response and time course data for both ovarian and peripheral venous concentrations of inhibin in response to gonadotropin stimulation in 21-day old immature female Sprague-Dawley rats. Inhibin bioactivity was measured in extracts from homogenates of the ovaries and extracts made from peripheral venous blood.[17] Peripheral and ovarian inhibin concentrations rose in a dose-related fashion following the administration of 10, 20 and 40 IU PMSG. At the same time, serum FSH levels fell in an inverse relationship with circulating inhibin. The time course of the response was studied in groups of animals given 20 IU PMSG.[18] Inhibin levels rose from undetectable basal levels to a peak of 452 U/ml at 64 hours. Ovarian inhibin rose from basal values of 2.4 U/mg to 21.7 U/mg. Groups of immature female rats were also treated with either highly purified ovine FSH or highly purified LH in doses of 2 μg each. Circulating inhibin levels rose to reach a peak at 6 hours after the purified FSH, whereas there was no detectable inhibin response to the injection of LH. The time course of the response to purified FSH was much shorter than that of the response to PMSG, which we interpreted as being due to the difference in biological half-life of the two gonadotropin preparations. Further evidence that FSH was the specific tropic agent for inhibin secretion was obtained in experiments undertaken in immature female rats which were hypophysectomized and subsequently treated with purified ovine FSH and LH. Animals hypophysectomized on day 25 of age received twice daily subcutaneous injections of ovine gonadotropins on days 28 and 29 prior to removal of the ovaries on day 30. The inhibin content of ovarian supernatants was measured by specific radioimmunoassay. Injection of 0.6 to 5 μg ovine FSH resulted in a dose-dependent increase in ovarian inhibin and estradiol content, while they were not altered significantly by injection of ovine LH, suggesting that inhibin levels were reflecting follicular rather than luteal activity.

The stimulation of inhibin production by PMSG was shown to involve an increase in inhibin gene expression. The amount of messenger RNA coding for the inhibin α subunit was significantly increased following PMSG treatment of immature female rats.[5] Recent experiments in the PMSG-primed rat have indicated parallelism between ovarian inhibin α-subunit messenger RNA levels, serum inhibin and ovarian estradiol concentrations with concomitant suppression of serum FSH levels.

The evidence for FSH regulation of ovarian inhibin production *in vivo* was confirmed by studies *in vitro*. Rat granulosa cells were cultured in the presence or absence of purified gonadotropins and inhibin release was assessed both by bioassay and radioimmunoassay.[29] Granulosa cells were isolated from immature female Sprague-Dawley rats at 25 days of age after treatment for 3 days with diethylstilbestrol, a model in which granulosa cells proliferate but do not differentiate. Cells were washed twice in McCoys' 5a medium supplemented with 2 mML-glutamine, 100 U penicillin per ml, 100 U streptomycin sulphate per ml and seeded into 48-well culture plates at 0.5 x 10^6 cells per well in 1 ml culture medium. The cells were cultured under serum-free conditions for 48 hours in the presence of rat FSH in doses from 3 to 300 ng/ml. A dose-related increase in the inhibin concentration in the media was observed following 48 hours incubation, inhibin levels increasing approximately sixfold at the highest FSH concentration. These results were in agreement with those obtained by other investigators.[3]

We hypothesized that FSH was acting through cyclic AMP as a second messenger. Exposure of granulosa cells to either a non-hydrolysable cAMP analog (8-bromo cyclic AMP) or to prostaglandin E_2, which is known to increase endogenous cellular adenylate cyclase activity, stimulated inhibin (measured by RIA) in a dose-dependent manner. The results are consistent with the hypothesis that FSH-stimulated inhibin production may be mediated through the classic second messenger pathway.[31]

In addition to the evidence concerning granulosa cells, we have obtained similar evidence for FSH regulation of Sertoli cell inhibin secretion. In 90-day old male Sprague-Dawley rats, we examined the effects of hypophysectomy at 16 hours, 3, 7 and 42 days after surgery.[2] Unilateral efferent duct ligation was performed 16 hours before death to measure seminiferous tubule fluid production and the increment in testicular inhibin values (inhibin production). There was a decrease in testicular inhibin content and seminiferous tubule fluid production by 7 days and a decrease in inhibin production by 42 days. Serum FSH and LH were suppressed three days after surgery. In 30-day chronically hypophysectomized adult rats treated for 3 days with twice-daily subcutaneous injections, FSH with or without testosterone stimulated an increase in testicular inhibin content, but not inhibin or tubule fluid production. Neither hCG nor testosterone acting alone had any effect on these parameters. These studies suggested that FSH alone stimulates an increase in testicular inhibin, again consistent with the regulatory role of FSH on inhibin production. In further experiments,[1] rats were given subcutaneous implants of high or low dose testosterone and 10 days later, hypophysectomy or sham operation was performed. The rats were sacrificed 50 days later, 16 hours after unilateral efferent duct ligation. The low dose testosterone implants maintained serum testosterone at control values and decreased testicular weight, whereas the high dose implants raised serum testosterone threefold and maintained testicular weight at 75 to 85% of pretreatment values. In intact rats, the low dose implants caused no change in testicular inhibin content, but decreased production. No significant changes occurred with high dose implants. After hypophysectomy, both values were suppressed significantly and could not be maintained at control levels either by high or low dose implants. However, the high dose implants partly restored inhibin production despite a lack of effect on testicular inhibin content. In contrast, tubule fluid production depended mainly on intratesticular testosterone levels and occurred normally in intact or hypophysectomized rats with high, but not low, dose implants. The results indicated that inhibin and seminiferous tubule fluid production, which are both Sertoli cell functions, are under different hormonal control. The maintenance of inhibin production by the testis required the support of pituitary hormones, presumably FSH, while seminiferous tubule fluid production required testosterone, presumably through LH stimulation of Leydig cells. The findings are again consistent with the hypothesis that inhibin is produced in response to tropic stimulation by FSH. Data obtained with Sertoli cell cultures *in vitro*[13] also indicated that they were able to produce inhibin (measured by bioassay) and could be stimulated to increase their inhibin production in the presence of FSH.

LUTEINIZING HORMONE — HUMAN CHORIONIC GONADOTROPIN

In addition to FSH, LH is also capable of regulating inhibin production in some circumstances. In our initial studies in the immature female rat, highly purified LH in a dose of 2 μg had no effect on circulating or ovarian inhibin concentrations.[18] Similarly, in cultured

rat granulosa cells, under basal conditions, LH and hCG were without significant effect.[30] However, LH/hCG in low doses was capable of increasing inhibin secretion in cultured granulosa cells which had been pretreated with FSH.[3,30] Addition of higher doses of hCG (0.6-3.0 U/ml) or purified rat LH to cultures containing half-maximal or maximal concentrations of PMSG/FSH resulted in a dose-dependent inhibition of PMSG/FSH-induced inhibin production. These data were in agreement with observations in the PMSG-treated immature female rat.[14] Animals given 20 IU of PMSG and 40 hours later given 20 IU hCG showed a rapid decline in circulating inhibin concentrations from 227 down to 66 U/ml at 8 hours post-hCG. A similar inhibition was observed 6 hours following the administration of 40 ng rat LH to PMSG-primed animals.[15] Thus, it appears that LH/hCG is capable of stimulating inhibin secretion at low doses, following FSH priming, but of inhibiting it at high doses. This could explain the fall in circulating inhibin seen following the mid-cycle LH surge in the human menstrual cycle.[20] Data recently reported for human luteal cells in long-term culture indicate that inhibin secretion from this tissue is stimulated by LH (and testosterone), but not by FSH.[27]

From the above data, it can be conluded that in the granulosa cell, FSH is the predominant regulator of inhibin production. LH is able to release inhibin from granulosa cells after their exposure to FSH. Luteal cells, on the other hand, appear to be resistant to FSH, but to respond to LH, at least in the human cycle. Sertoli cell inhibin secretion is predominantly under FSH control.

OTHER FACTORS

A number of other factors have been shown to influence inhibin secretion *in vitro*. We and others[3,29] have shown that somatomedin-C (IGF-I) is capable of stimulating inhibin production by rat granulosa cells *in vitro*. Somatomedin-C alone was effective over the physiological dose range of 3-100 ng/ml.[29] Concomitant addition of FSH (100 ng/ml) and somatomedin-C (3-100 ng/ml) resulted in a significant increase in inhibin production at all doses of somatomedin-C. The dose-dependent effects of FSH and somatomedin-C were also time-dependent, with a synergistic effect apparent after 48 hours of culture. Insulin was also shown to be effective, but at 100-fold higher concentration,[3,4] whereas fibroblast growth factor had no effect. Epidermal growth factor (EGF) has been shown by others to inhibit FSH-stimulated inhibin production.[3] We have confirmed this observation and also demonstrated an inhibitory effect of EGF on basal inhibin secretion after 2 days of culture.[31] This was sustained over the subsequent 2 days. The effect on basal inhibin secretion was dose-dependent and maximal inhibition to 50% of control was observed at a dose of 100 ng EGF/ml at day 4. EGF also inhibited basal progesterone secretion in a similar manner. EGF caused a dose-dependent inhibition of FSH-stimulated inhibin secretion with an ID_{50} (0.5 ng/ml, 0.08 nM), about one-eighth that in the absence of FSH. In addition, EGF inhibited the stimulation of inhibin production by 8-bromo cyclic AMP and prostaglandin E_2. To exclude the possibility that EGF was toxic to the granulosa cells, several biochemical parameters related to cell growth were measured. EGF treatment did not alter cell number, but slightly increased [^{3}H]-thymidine incorporation into cellular DNA. The effect of EGF on ^{35}S-methionine incorporation into cellular protein was biphasic, being stimulatory at doses less than 10 ng/ml, but inhibitory at 100 ng/ml. There is thus a direct inhibitory effect of EGF both on basal and FSH-stimulated inhibin production by granulosa

cells, suggesting an important regulatory role of this growth factor on ovarian function; the demonstration of EGF in follicular fluid by radioreceptor assay is consistent with this concept.[12]

The responses of cultured cells to other anterior pituitary hormones were also studied. Treatment with prolactin and TSH caused no change in basal inhibin production. Limited studies on the direct effects of steroids have been undertaken. Androstenedione and estradiol at 10^{-7}M enhanced inhibin secretion stimulated by 0.1 U/ml PMSG, but not by higher PMSG concentrations. Progesterone (10^{-7}M) inhibited inhibin production at 10 mU/ml PMSG.[15]

The above data are consistent with the inhibin hypothesis, namely that FSH is the major regulator of inhibin secretion by the gonad. LH may be particularly important in the regulation of secretion by luteal cells, at least in man, and its role in the male requires further study. Other growth factors are clearly involved in the regulation of inhibin secretion, with somatomedin-C/IGF-I being stimulatory and EGF being inhibitory.

MECHANISM OF ACTION OF INHIBIN

Initial studies on the mechanism of action of inhibin require cautious interpretation because of the impure nature of the preparations used. Our early studies on the mechanism of action of ovine testicular inhibin proteins in cultured anterior pituitary cells[25] led us to suggest that the action of inhibin is specifically on FSH synthesis and does not influence LH production. We also found that crude inhibin-containing proteins of testicular origin may cause inhibition of FSH release, both in the presence and absence of LHRH, an effect independent of the primary action of inhibin on FSH production. The recent availability of highly purified inhibin has allowed a re-examination of the mechanism of action of inhibin and has considerably extended our original studies.[7]

Pituitary cell cultures were prepared from anterior pituitary glands obtained from 4-month old male Sprague-Dawley rats. The pituitaries were diced, washed and enzymatically digested with 0.15% trypsin containing 0.01% DNase, followed by disaggregation of the tissue to produce a single cell suspension. The cells were dispersed at 250,000 viable cells per ml in the presence of 10% fetal bovine serum. Highly purified bovine follicular fluid inhibin, in the 31kDa form, with an approximate specific activity of 750,000 U/mg was added at final concentrations from zero to 5.6 U/ml on day 2 of culture. Cultures were terminated on days 0, 1, 2, 3, 4 and 5 and then at increasing intervals up to day 28. For cultures which continued beyond 5 days, the medium was removed at regular intervals (4 or 5 days) and saved to be replaced immediately with fresh medium with or without inhibin. At termination, the medium from each culture was saved, cells were washed with warm DMEM and lysed with 0.5 ml 0.1% Triton-X100. The levels of FSH and LH in samples of culture media and cell lysates were determined by radioimmunoassay.

Basal release of FSH from untreated rat pituitary cells occurred at a constant rate of 1.90 $\pm$ 0.24 ng/10^6 cells per hour (mean $\pm$ SE, n= 6 different cell dispersions) during the first 10 days of culture and thereafter continued at a decreasing rate for up to 28 days (Fig. 1).

Addition of inhibin on day 2 reduced the rate of basal FSH release over the subsequent 8 days in a concentration-dependent manner to reach complete inhibition at inhibin levels > 2.5 U/ml, with an IC_{50} of 0.16 U/ml. Basal release of LH in control cultures was initially 1.5 ng/10^6 cells per hour, decreasing during the first 2 days to become constant at

0.15 ± 0.02 ng/10^6 cells/hour between days 2 and 12 of culture (Fig. 1D). In the presence of inhibin, a significant concentration-dependent reduction of basal LH release was observed with an inhibin IC_{50} of 0.66 U/ml. Basal LH release was suppressed to 25% of control by the highest concentrations of inhibin under the conditions used. Pituitary cell content of FSH and LH decreased in culture to approach undetectable levels by day 28. Inhibin

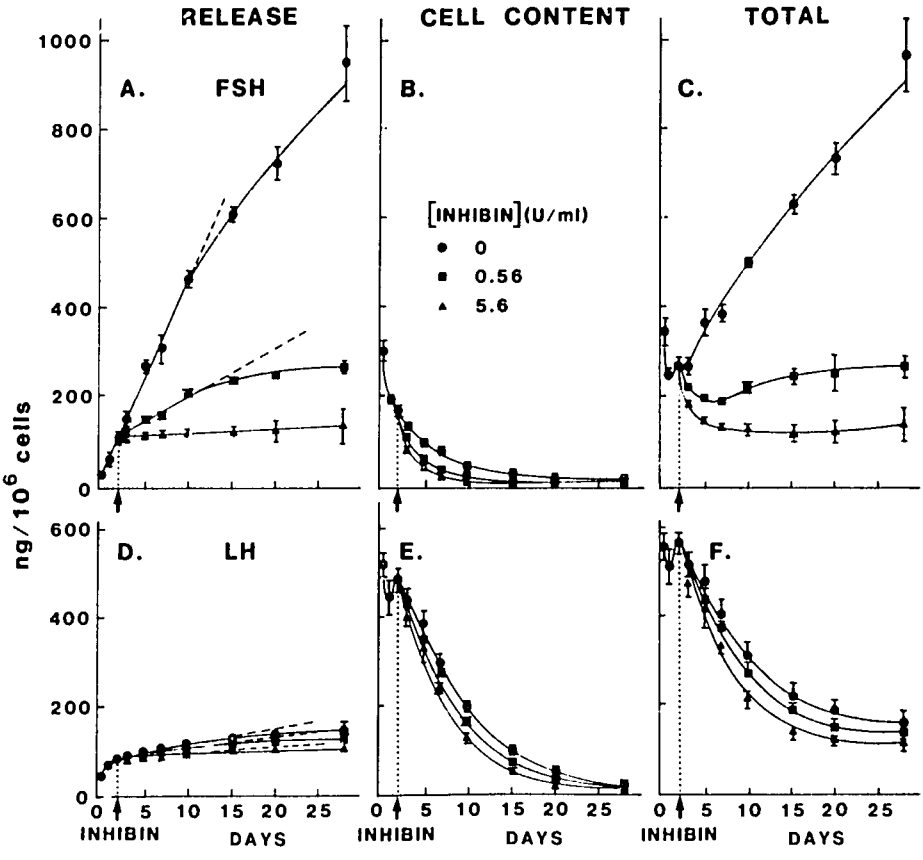


FIGURE 1. Temporal changes in the levels of FSH (A-C) and LH (D-F) in rat pituitary cell cultures before and after the addition on day 2 (indicated by arrows and dotted lines) of medium containing 0, 0.56, or 5.6 U/ml of 31 kDa bovine inhibin. Hormone release (A and D), cell content (B and E), and total (C and F) (all in nanograms per 10^6 cells) are plotted on a common scale to facilitate comparison. Each point represents the mean ± SD of single measurements for quadruplicate wells from one representative experiment and error bars are given except where they are smaller than the symbols. The medium ± inhibin was replaced on days 5, 10, 15, 20 and 25 so that the released and total hormone data after day 5 include contributions from medium-change samples. In cases where mean results were combined, the SD given is the sum of estimates for the individual contributing means. (Reproduced with permission from Farnworth PG et al.)

hastened the decrease of intracellular FSH and LH in a concentration-dependent manner (Fig. 1B and 1E). The declines in cell content were first-order processes. The $t_{1/2}$ for FSH disappearance from control cultures was 5 days and fell progressively with increasing inhibin concentrations to a minimum of 1.8 days. The inhibin IC_{50} was 0.81 U/ml. The $t_{1/2}$ for the reduction in LH cell content decreased in a similar manner, but less dramatically, from 5.8 to 3.6 days with an inhibin IC_{50} of 0.83 U/ml.

In the absence of inhibin, the total measured FSH rose steadily above initial levels after day 4 of culture (Fig. 1C). After 10 days, this increase largely reflected released FSH as the cell content became negligible. After the addition of maximally suppressing concentrations of inhibin (5.6 U/ml), total FSH fell in parallel with FSH cell content, until, after day 10, it reflected the amount of FSH released into the medium before the addition of inhibin on day 2. Total LH fell progressively after day 2 in control and inhibin-treated cultures and primarily reflected basal LH release by day 28 in culture as stored LH became negligible.

The results of this study showed that purified 31 kDa bovine inhibin potently suppressed basal FSH synthesis and release by rat anterior pituitary cells in culture, in accordance with the previous findings for impure preparations. The investigation revealed less potent actions of inhibin on suppression of basal LH release and acceleration of the normal exponential decline in intracellular stores of both gonadotropins.

An important aspect of the study was the demonstration that FSH synthesis by rat pituitary cells continued for many days *in vitro* in the absence of GnRH. In contrast, total LH in untreated pituitary cell cultures did not increase, consistent with the findings in other studies[11] that synthesis of LH is dramatically reduced in the absence of GnRH. The tonic synthesis of FSH in these circumstances contributed little to its storage and was mainly coupled to release of the hormone, as previously concluded from rat pituitary cell cultures.[22] The basal rate of LH release declined tenfold during the first 2 days of culture, analogous to the rapid fall in circulating LH levels to the barely measurable range which follows termination of hypothalamic input to the pituitary in gonadectomized animals.[11]

Against the background of changing levels of FSH and LH in untreated pituitary cell cultures, the predominant effect of purified 31kDa bovine inhibin *in vitro* was the potent inhibition of basal FSH release with an IC_{50} of 7 pM. We concluded that this blockade in turn reflected suppression of tonic FSH synthesis by inhibin. Had FSH release been blocked without a parallel inhibition of FSH synthesis, higher cell contents of FSH in treated than in control cells would have been expected. This was not observed. This primary effect of inhibin to suppress both FSH tonic synthesis and basal release accords with previous findings for impure preparations and correlates with the well established preferential suppression of plasma FSH by inhibin *in vivo*.[9]

Inhibin accelerated the normal exponential decline of FSH and LH cell contents during the prolonged culture of pituitary cells, but at a higher (though still physiological) concentration than its effect on FSH synthesis and release. This action may contribute to the reduction by inhibin of gonadotropin stores available for release by GnRH.

Most of the LH release during the first 5 days of culture occurs before inhibin is added on day 2. Hence, demonstration of an inhibin effect on LH release is difficult, but an effect was demonstrable in long-term cultures with an IC_{50} of 28 pM, significantly higher than for the effect on FSH. The similarity of the inhibin dose-response relationships for

suppression of FSH/LH cell content and basal LH release suggests that there may be a common action of inhibin underlying all three effects. Its site of action appears to involve the mobilization and/or degradation of both gonadotropins. It is possible that basal FSH release is also suppressed by inhibin in the manner of basal LH release, but that this action of inhibin is masked by its more potent inhibition of basal FSH synthesis.

The conditions under which *in vitro* bioassays for inhibin have been conducted do not result in demonstrable suppression of LH release and this criterion still remains useful under those circumstances for the assessment of assay specificity.

The results of this study showed that purified inhibin inhibits FSH synthesis and the release and degradation of both gonadotropins in rat pituitary cell cultures under basal conditions. The predominant mechanism involves suppression of tonic FSH synthesis which is coupled to FSH release and the secondary mechanisms involve FSH/LH degradation, mobilization, or both.

Further experiments have evaluated the effects of purified 31kDa bovine inhibin on the release of FSH and LH stimulated by GnRH or its agonist analog, Buserelin, in 5-day cultures of pituitary cells from adult male Sprague-Dawley rats.[6] Exposure of the cultures to a range of inhibin concentrations for 3 days before and during a 4 hour stimulation with GnRH resulted in total suppression of basal FSH release at the highest concentrations used and suppression of stimulated FSH release by 80-98% (IC_{50} = 0.16 to 0.25 U/ml, depending on GnRH concentration). Basal LH release was inhibited by >75% and stimulated LH release was maximally inhibited by 50-80% (IC_{50} = 0.51 to 1.1 U/ml). Comparable data were obtained for Buserelin-stimulated release. The results showed that purified bovine inhibin antagonized the release of both FSH and LH stimulated either by GnRH or its potent agonist analog *in vitro*. Inhibin reduced the apparent potency of GnRH agonists and decreased the proportion of total available gonadotropin that could be released by excess GnRH agonist. Higher concentrations of inhibin were required for these common actions against GnRH-stimulated gonadotropin release than for the inhibition of FSH tonic synthesis/basal release indicating one or more secondary sites of inhibin action in addition to its primary selective action to suppress tonic FSH synthesis. Additional possible mechanisms for inhibin action include an alteration in GnRH receptor number or affinity; we have recently obtained data suggestive of the former possibility.[28]

Consistent with the effect of inhibin on FSH synthesis is the demonstration that cycloheximide, an inhibitor of protein synthesis, completely mimicked the action of purified porcine inhibin on gonadotropin secretion by cultured rat pituitary cells.[10] Inhibin may affect FSH gene expression directly; it was recently shown that bovine follicular fluid has a rapid and specific effect on FSH-β mRNA levels in the pituitary glands of ovariectomized, hypothalamo-pituitary disconnected sheep given 2-hourly pulses of GnRH.[21]

Other gonadal peptides may also inhibit FSH production from the pituitary; we have recently isolated and characterized a protein from bovine follicular fluid, different from inhibin, which has such properties.[24]

CONCLUSIONS

The data presented are consistent with the hypothesis that FSH and inhibin form a classic closed-loop endocrine feedback system. FSH is a major stimulator of the gonadal secretion of inhibin, and inhibin in turn inhibits FSH basal release by a mechanism predominantly

affecting FSH synthesis. FSH synthesis and release are closely coupled and can proceed in the absence of GnRH stimulation. It can be hypothesized that inhibin is the principal and inhibitory regulator of FSH secretion, and therefore has potential as a contraceptive.[8] However, under certain circumstances, LH is able to stimulate inhibin production, particularly from luteal cells. Inhibin in turn can be shown to have a small effect on LH basal release, but more clearly inhibits GnRH-stimulated LH secretion.

The data on interactions between LH and inhibin presented in this review provide a new dimension to our knowledge of the physiology of inhibin which was not previously appreciated when considering inhibin as a potential contraceptive.[8]

REFERENCES

1. AU CL, IRBY DC, ROBERTSON DM, de KRETSER DM: Effects of testosterone on testicular inhibin and fluid production in intact and hypophysectomized adult rats. J Reprod Fertil 76:257, 1986
2. AU CL, ROBERTSON DM, de KRETSER DM: Effects of hypophysectomy and subsequent FSH and testosterone treatment on inhibin production by adult rat testes. J Endocrinol 105:1, 1985
3. BICSAK TA, TUCKER EM, CAPPEL S, VAUGHAN J, RIVIER J, VALE W, HSEUH AJW: Hormonal regulation of granulosa cell inhibin biosynthesis. Endocrinology 119:2711, 1986
4. BURGER HG, CARSON RS, DAVIS S, ZHIWEN Z: Regulation of inhibin secretion from the rat ovary. In BURGER HG, de KRETSER DM, FINDLAY JK, IGARASHI M (eds): Inhibin - Non-Steroidal Regulation of FSH, p 191. New York, Raven Press, 1987
5. DAVIS SR, DENCH F, NIKOLAIDIS I, CLEMENTS JA, FORAGE RG, BURGER HG, KROZOWSKI Z: Inhibin A subunit gene expression in the ovaries of immature female rats is stimulated by pregnant mare serum gonadotropin. Biochem Biophys Res Commun 138:1196, 1986
6. FARNWORTH PG, ROBERTSON DM, DEKRETSER DM, BURGER HG: The effects of 31 kDa bovine inhibin on FSH and LH in rat pituitary cells *in vitro:* Antagonism of GnRH and buserelin. J Endocrinol (in press) 1988
7. FARNWORTH PG, ROBERTSON DM, DEKRETSER DM, BURGER HG: Effects of 31 kilodalton bovine inhibin on follicle-stimulating hormone and luteinizing-hormone in rat pituitary cells *in vitro:* actions under basal condition. Endocrinology 122:207, 1988
8. FINDLAY JK: The nature of inhibin and its use in the regulation of fertility and diagnosis of infertility. Fertil Steril 46:770, 1986
9. FINDLAY JK, ROBERTSON DM, CLARKE IJ: Influence of dose and route of administration of bovine follicular fluid and the suppressive effect of purified 31 kilodalton bovine inhibin on plasma FSH in ovariectomized ewe. J Reprod Fertil 80:455, 1987
10. FUKUDA M, MIYAMOTO K, HASEGAWA Y, IBUKI Y, IGARASHI M: Action mechanism of inhibin *in vitro:* Cycloheximide mimics inhibin actions on pituitary cells. Mol Cell Endocrinol 51:41, 1987
11. HAMERNIK DL, CROWDER ME, NILSON JH, NETT TM: Measurement of messenger ribonucleic acid for luteinizing-hormone β-subunit, α-subunit, growth hormone and prolactin after hypothalamic pituitary disconnection in ovariectomized ewes. Endocrinology 119:2704, 1986
12. HSU C-J, HOLMES SD, HAMMOND JM: Ovarian epidermal growth factor-like activity. Concentrations in porcine follicular fluid during follicular enlargement. Biochem Biophys Res Commun 147: 242, 1987
13. LE GAC F, de KRETSER DM: Inhibin production by Sertoli cell cultures. Mol Cell Endocrinol 28:487, 1982
14. LEE VWK: PMSG-treated immature female rat - a model system for studying control of inhibin secretion. In GREENWALD GS, TERRANOVA PF (eds): Factors Regulating Ovarian Function, p 157. New York, Raven Press, 1983
15. LEE VWK: Control of inhibin secretion in polytocous animal species. In SAIRAM SR, ATKINSON LE (eds): Gonadal Proteins and Peptides and Their Biological Significance, p 141. Singapore, World Scientific Publishing Co., 1984
16. LEE VWK, COLVIN N, QUIGG H, ATLEY L, McMASTER J, LEVERSHA L, BURGER HG: A rapid, sensitive and reliable assay for inhibin bioactivity. Aust J Biol Sci 40:105, 1987

17. LEE VWK, McMASTER J, QUIGG H, FINDLAY J, LEVERSHA L: Ovarian and peripheral blood inhibin concentrations increase with gonadotropin treatment in immature rats. Endocrinology 108:2403, 1981
18. LEE VWK, McMASTER J, QUIGG H, LEVERSHA L: Ovarian and circulating inhibin levels in immature female rats treated with gonadotropin and after castration. Endocrinology 111:1849, 1982
19. McLACHLAN RI, ROBERTSON DM, BURGER HG, de KRETSER DM: The radioimmunoassay of bovine and human follicular fluid and serum inhibin. Mol Cell Endocrinol 46:175, 1986
20. McLACHLAN RI, ROBERTSON DM, HEALY DL, BURGER HG, DEKRETSER DM: Circulating immunoreactive inhibin levels during the normal human menstrual cycle. J Clin Endocrinol Metab 65:954, 1987
21. MERCER JE, CLEMENTS JA, FUNDER JW, CLARKE IJ: Rapid and specific lowering of pituitary FSHβ mRNA levels by inhibin. Mol Cell Endocrinol 53:251, 1987
22. MILLER WL, WU J: Estrogen regulation of follicle-stimulating hormone production *in vitro:* Species variation. Endocrinology 108:673, 1981
23. ROBERTSON DM, FOULDS LM, LEVERSHA L, MORGAN FJ, HEARN MTW, BURGER HG, WETTENHALL REH, de KRETSER DM: Isolation of inhibin from bovine follicular fluid. Biochem Biophys Res Commun 126:220, 1985
24. ROBERTSON DM, KLEIN R., de VOS FL, McLACHLAN RI, WETTENHALL REH, HEARN MTW, BURGER HG, de KRETSER DM: The isolation of polypeptides with FSH suppressing activity from bovine follicular fluid which are structurally different to inhibin. Biochem Biophys Res Commun 149:744, 1987
25. SCOTT RS, BURGER HG: Mechanism of action of inhibin. Biol Reprod 24:541, 1981
26. SCOTT RS, BURGER HG, QUIGG H: A simple and rapid *in vitro* assay for inhibin. Endocrinology 107:1636, 1980
27. TSONIS CG, HILLIER SG, BAIRD DT: Production of inhibin bioactivity by human granulosa-lutein cells: stimulation by LH and testosterone *in vitro.* J Endocrinol 112:R11, 1987
28. WANG Q-F, FARNWORTH PG, FINDLAY JK, BURGER HG: Effect of purified 31 kDa bovine inhibin on specific binding of GnRH to rat anterior pituitary cells in culture. Endocrinology (in press) 1988.
29. ZHIWEN Z, CARSON RS, HERINGTON AC, LEE VWK, BURGER HG: Follicle stimulating hormone and somatomedin C stimulate inhibin production by rat granulosa cells *in vitro.* Endocrinology 120:1633, 1987
30. ZHIWEN Z, LEE VWK, CARSON RS, BURGER HG: Selective control of rat granulosa cell inhibin production by FSH and LH *in vitro.* Mol Cell Endocrinol (in press)
31. ZHIWEN Z, HERINGTON AC, CARSON RS, FINDLAY JK, BURGER HG: Direct inhibition of rat granulosa cell inhibin production by epidermal growth factor *in vitro.* Mol Cell Endocrinol 54:213, 1987

DISCUSSION

CHAIRPERSON: KOJI YOSHINAGA, Ph.D.

DR. KERDELHUE: Do you think that normal concentrations of FSH and LH can account for the production of inhibin?

DR. BURGER: I am sorry if I did not make myself clear. In the vast majority of circumstances in which we have looked at the effect of FSH, it is only stimulatory. It is only when you go to very high doses of FSH in some circumstances that the dose response curve turns over in a manner which is similar to that in many other biological systems.

LH is biphasic in the presence of pretreatment with FSH. At low doses it stimulates; at higher doses it inhibits. My interpretation of the *in vivo* data is that the preovulatory LH surge presumably exposes the granulosa cells to LH concentrations which are in that high range and which are inhibitory. Incidentally, we have some preliminary data showing switch-off of the α subunit message in that circumstance as well. At low doses, one does not know whether during the follicular phase, for example, there might be some

stimulation by LH. The situation, as you will see in the next paper in the male, is somewhat different and suggestive of a dual role for the gonadotropins in control.

DR. KERDELHUE: Even when using very high doses of inhibins, you never reach zero in terms of FSH production. Do you think that there is some autonomous FSH secretion which is not inhibin sensitive or is there another mechanism?

DR. BURGER: I think that is a phenomenon that is very variable and that perhaps reflects the duration of exposure of the cell, as well as the dose of inhibin used. There are circumstances in some of the cell culture experiments where you occasionally do really get down to say 20% of the basal level, but you are quite correct that it is very unusual to see complete suppression. I can only speculate, as you did, that there may be a level of autonomous production. And, as I indicated before, certainly the concept which we are finding useful is that FSH production appears to be constitutive property of at least some gonadotropes.

DR. DORFLINGER: Have you been able to identify whether anything can change the ED_{50} for inhibin-induced inhibition of FSH at the pituitary level? For instance, would estrogen or other steroids alter the ED_{50}?

DR. BURGER: We have not really done those experiments. The ED_{50} for some of the phenomena, of course, is shifted to the right by increasing amounts of GnRH or buserelin. But whether you can change it under the sorts of circumstance you have mentioned is something which has not yet been studied.

DR. MOUDGAL: In the 1987 Serono Symposium on Inhibin in Tokyo, there was a clear indication that the corpus luteum secretes inhibin. How would you implicate LH? Would it be the primary stimulus?

DR. BURGER: I do not really want to pre-empt Robert McLachlan's paper because he is going to discuss the corpus luteum secretory phenomenon. But you may have seen a short paper recently from David Baird's group in Edinburgh which suggested that if you took granulosa cells that were harvested from the follicles of women in an *in vitro* fertilization program and cultured them for ten to twelve days *in vitro*, that under those circumstances where they are presumably thoroughly luteinized, it was only LH and testosterone which were stimulatory of inhibin production and FSH was without effect. The concept we are developing is that the granulosa cell is primarily an FSH-sensitive cell, and that the luteal cell may well be an LH-sensitive cell, rather than FSH-sensitive.

DR. BARDIN: I just wanted to point out that it is interesting that in the testis, some of the regulators are different and even opposite. FSH, of course, is still stimulatory, but EGF is also stimulatory. Testosterone has no effect. Estradiol and androstenedione inhibit, and LH does not have any effect at all that we can tell from our studies so far. I guess this really should not surprise us. Since this is obviously a ubiquitous hormone, it is going to have a different regulatory mechanism in almost every tissue. However, we thought that the testis and ovary might be more similar than they actually are.

DR. BURGER: I think that is absolutely so. I guess we are plagued by the fact that we have used cultured granulosa cells, and a lot of the data that you mentioned, and data that we also obtained — we being David deKretser and people in his laboratory — have used cultured Sertoli cells. I suspect, as with so many other things in the testis, the interaction between Leydig cells, or whatever other cells you might like to nominate in the interstitial space, and the Sertoli cells, and perhaps the germ cells, may play a very important role in the final expression of inhibin, or any other product of Sertoli cell synthesis. As you will see from the data in the next paper, there is certainly some fairly persuasive evidence that LH is a regulator of testicular inhibin production, even though it has no effect on cultured Sertoli cells on their own.

DR. PETRAGLIA: Can you summarize the data on the effect of steroids on granulosa cell production of inhibin? Also, do you have *in vivo* data on the possible effect of steroids on inhibin plasma levels?

DR. BURGER: As far as the latter question is concerned, we really have very little in the way of data on administration of steroids on *in vivo* control mechanisms. These are experiments that we are currently considering. We do have some suggestive data, but it is very preliminary, that testosterone may be able to turn inhibin off in the male. But this is yet to be confirmed and expanded.

As far as the *in vitro* studies about which I alluded, these were studies in which we used inhibitors of steroid biosynthesis, such as cyano-ketone, to inhibit various steps in the steroidogenic pathway. The basic gist of the data was that where we effectively inhibited, for example, progesterone secretion, we also inhibited inhibin secretion in parallel.

Victor Lee did some studies, which he published several years ago, looking at the effects of androstenedione, estradiol and progesterone, and from my memory he was only able to show effects in the presence of low doses of PMSG in his granulosa cell cultures. If I recall correctly, I think that androstenedione showed a degree of enhancement of the PMSG effect. I also think that progesterone was mildly inhibitory. The effects of steroids in that system were not very striking; they were very small effects.

11

The Physiology of Inhibin in Women

ROBERT I MCLACHLAN
DAVID M. ROBERTSON
HELEN M. BUCKLER
SUSAN R. DAVIS
GARY L. WARNE
GEORGE A. WERTHER
DAVID L. HEALY
DAVID M. deKRETSER
HENRY G. BURGER

The physiology of inhibin in humans has previously been studied to a limited extent due primarily to the inability to reliably measure circulating inhibin levels. Information has therefore been obtained in humans and nonhuman primates by the measurement of inhibin *in vitro* bioactivity in tissue fluids (especially follicular fluid, FF), in cytosolic extracts of gonadal tissue and in the spent media of cultured gonadal cells. In agreement with studies performed in subprimate species regarding the cellular origin of inhibin, cultured human granulosa cells secreted inhibin bioactivity and this secretion was increased by FSH.[8] FSH administration also increased the levels of inhibin in FF in both women[9,24] and primates.[42] The proposed inhibitory action of inhibin on FSH secretion *in vivo* has been examined in primates. The administration of steroid-free porcine FF to female rhesus monkeys inhibited the post-castration rise of FSH and, to a lesser extent, LH.[43] Porcine FF treatment during the follicular phase of the primate menstrual cycle has been shown to decrease FSH levels and to result in impaired folliculogenesis and subsequent luteal function.[13,45] These observations await confirmation using purified inhibin preparations.

Significant limitations of these previous studies have included: 1) their reliance on rat pituitary cell culture inhibin bioassay systems with their associated problems of insensitivity and limited specificity; 2) the indirect nature of studies examining FF inhibin levels in relation to ovarian inhibin secretion into serum and, therefore, of the proposed feedback effect of inhibin upon FSH; 3) the doubtful specificity of experiments using crude FF preparations to examine the effect of inhibin *in vivo*; and 4) the limited number of experimental paradigms that can be explored in humans and monkeys using *in vitro* bioassay of FF and tissue extracts. The measurement of circulating inhibin levels was clearly essential to allow further understanding of its role in reproductive physiology in man.

We have described sensitive and specific heterologous radioimmunoassay (RIA) systems for inhibin applicable to human serum based on purified bovine FF inhibin.[30,31] This paper will briefly outline the development of these RIA systems and then discuss their applica-

tion to the study of inhibin physiology in women under a range of physiological and pathological conditions. Much of the data is consistent with previous animal and primate studies which have used *in vitro* bioassay. Nonetheless, several novel concepts emerged relating to inhibin as a product of the human corpus luteum and the feto-placental unit.

RADIOIMMUNOASSAY OF SERUM INHIBIN

We have developed three heterologous RIA systems for human serum inhibin based on purified bovine FF inhibin isolated by the procedures of Robertson *et al.*[40,41] All were specific to intact inhibin while each was more sensitive than its predecessor allowing the study of a wider range of physiological conditions.

INHIBIN ANTISERA

Antisera have been raised in rabbits to purified 58 kDa (As 474, Ref 31) and subsequently to 31 kDa (As 749, Ref 30; As 1989, unpublished) bovine FF inhibin. All antisera have shown the ability to neutralize the *in vitro* inhibin bioactivity of bovine and human FF.

IODINATION OF INHIBIN

Purified 31 kDa bovine FF inhibin was iodinated by the chloramine T procedure and purified on a column of the dye ligand Matrex Red A to a specific activity of 40 μCi/ug.[31] This material showed the physiochemical characteristics of the non-iodinated hormone and was stable during incubation with serum under RIA conditions.

RIA PROCEDURES

Double antibody RIA systems were developed for the measurement of human serum inhibin. Relative to 31 kDa bovine FF inhibin, a wide range of glycoproteins, growth factors and proteins structurally related to inhibin (transforming growth factor β, Mullerian inhibiting substance, bovine activin A and free inhibin subunits) all showed $<1\%$ cross-reactivity. Human postmenopausal serum (PMS), serum from women with gonadal failure (Turner's syndrome, oophorectomy, premature ovarian failure) and serum from castrate men showed either no (As 474, As 1989) or minimal (As 749) immunoreactivity. In order to compensate for any nonspecific effects of serum in the RIA systems, PMS was used as diluent for all samples and standards. Extended incubation times (2 to 7 days) and delayed tracer addition were performed to maximize assay sensitivities.

RIA STANDARD

In the absence of purified human inhibin, we have examined various preparations as potential RIA standards with the aim of achieving parallelism between the dose-response lines of the standard and the serum samples. A partial purification of human FF was performed by gel filtration under neutral and acidic conditions followed by reversed-phase HPLC in an analogous way to that described for 31 kDa bovine FF inhibin.[30] This material produced parallel dose-response lines to human female and pregnant sera and was calibrated

in an *in vitro* bioassay itself ultimately calibrated in terms of an ovine testicular lymph preparation of defined unitage Iu/mg.[44] In view of the usage of different antisera and standard preparations, some differences in inhibin values under the same physiological settings make it difficult to compare inhibin levels between the separate studies described below.

STUDIES OF INHIBIN PHYSIOLOGY IN WOMEN

THE EFFECTS OF MANIPULATION OF GONADOTROPIN LEVELS ON SERUM INHIBIN

The effect of gonadotropin administration and withdrawal on human ovarian inhibin secretion have been examined.

Exogenous Gonadotropin Administration

The administration of FSH activity (as human menopausal gonadotropin) to women during ovulation induction for *in vitro* fertilization (IVF) resulted in marked increases in both serum inhibin and estradiol (E_2) levels.[32] That both inhibin and E_2 represented markers of follicular development was supported by their correlation with both the number of follicles detected on ultrasound and the number of oocytes collected at subsequent laparoscopy. We concluded that inhibin represented a circulating protein marker of granulosa cell function under the tropic influence of FSH. Similarly, studies in rats have reported that serum inhibin bioactivity[22] and immunoactivity[38] rose sharply following pregnant mare serum gonadotropin administration.

LHRH Agonist Administration: Stimulation and Withdrawal of Endogenous Gonadotropins

The repetitive administration of an LHRH agonist provides a clinical model for gonadotropin stimulation and withdrawal and therefore allows an evaluation of their effects on ovarian inhibin secretion.[3] Initially, LHRH agonist treatment results in a transient hypersecretion of gonadotropins accompanied by increased E_2 secretion.[26] With continued administration, functional gonadotropin withdrawal occurs as the result of diminished circulating levels of bioactive gonadotropins and from the loss of pituitary responsiveness to LHRH.[26] Based on the reports of Fleming *et al.*[14] we have employed a protocol of ovulation induction using the LHRH agonist buserelin (D-SER (TBu)$_6$-EA-LRH, Suprefact^R, Hoechst, Frankfurt, FRG) to suppress endogenous gonadotropin secretion and enable simplified control of subsequent follicular development using exogenous gonadotropin therapy. Buserelin was commenced in the late luteal phase of menstruating subjects following reports in humans and primates that this procedure more rapidly induced hypogonadism, avoided unopposed estradiol action on the endometrium and lessened the incidence of breakthrough bleeding in the following cycle.[16,17] Buserelin (100 μg 6 times daily by nasal insufflation) was administered until patients were hypogonadal as assessed by serum E_2 levels ($<$ 150pmol/L) and by the absence of follicular development on ovarian

ultrasound. Human menopausal gonadotropin (hMG, 225 U im daily; Pergonal, Serono, Rome) was co-administered with buserelin and hMG doses increased in order to produce multiple folliculogenesis.[18]

This treatment protocol was administered in 14 subjects without polycystic ovary syndrome (PCOS) who had been resistant to conventional ovulation induction treatments.[47] Transient increases in serum gonadotropin levels were observed during the first two days of buserelin treatment and were accompanied by rises in serum E_2 and inhibin (Fig. 1). In 12 cycles, serum E_2 then fell into the postmenopausal range by day 12 of buserelin treatment and inhibin levels fell in parallel into, or below, the early follicular phase range of normal cycles. In 2 cycles, E_2 remained above the postmenopausal range and similarly inhibin was not suppressed. A significant ($p < 0.05$) correlation was found between serum inhibin and E_2 levels in these 14 subjects. Serum progesterone (P) also rose transiently following buserelin treatment and showed a significant ($p < 0.01$) correlation within inhibin (Fig. 1).

This study supported the tropic role of endogenous gonadotropins in ovarian inhibin secretion in women. As buserelin was commenced in the late luteal phase, this study also suggested that the human corpus luteum secreted inhibin in addition to E_2 and P and that all were to some degree under gonadotropic control. This concept is discussed later.

Another model of gonadotropic suppression in women is combined oral contraceptive (OC) pill usage. We have examined daily blood samples in several women over one cycle of OC treatment and found inhibin levels to be in the early follicular phase range or suppressed below the assay limit of detection.

NORMAL MENSTRUAL CYCLE

Improvement in RIA sensitivity was obtained using antisera raised to 31 kDa bovine FF inhibin (As 749, As 1989). This has allowed examination of serum inhibin levels during the normal menstrual cycle. We have recently described inhibin levels during the menstrual cycle in 6 normal women who were ovulating regularly (mean cycle 28.0 days; range 25 to 35 days) and had luteal phase serum progesterone levels > 25 nM/L.[30] The profiles of inhibin, LH, FSH, E_2 and P during the menstrual cycle are shown in Figure 2.

Follicular Phase

Serum inhibin levels were low during the early follicular phase consistent with the limited inhibin biosynthetic capacity of the small follicles present at this time. The well described perimenstrual FSH rise was observed during this period and is thought to lead to recruitment of a cohort of follicles from which the dominant follicle will subsequently arise.[2] During the mid-follicular phase, inhibin levels remained low. This was an unanticipated finding as the number of granulosa cells and their capacity to secrete E_2 and respond to FSH increase during follicular development.[33] Inhibin, as a protein marker of follicular development, may similarly have been expected to increase during this period. In addition, a rise in serum inhibin levels would have been expected along with the significant fall in serum FSH that was observed during this time. The failure to observe a rise in inhibin levels could be attributed to 1) the wide subject variation and small sample number

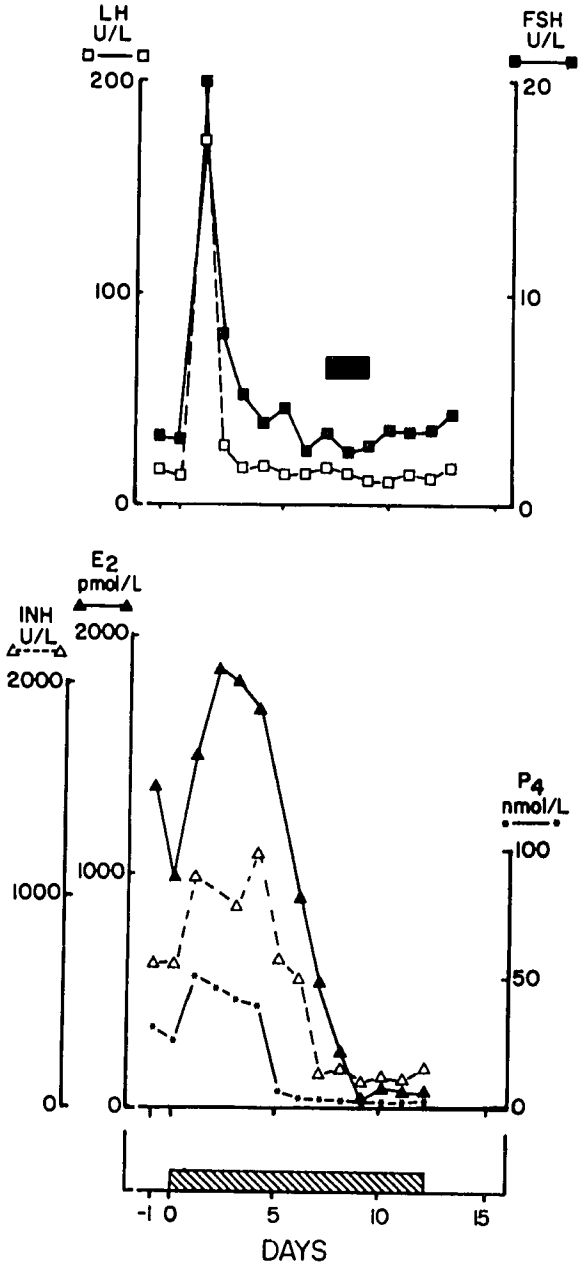


Figure 1. Serum LH, FSH, E_2, P and inhibin in a non-PCOS subject during buserelin administration (100 μg/6 times daily, intranasally, hatched bar) commenced on day 0. Menses is indicated by the solid bar. Note the transient rise of E_2, P and inhibin following the initial gonadotropin rise induced by buserelin. During continued treatment, functional gonadotropin withdrawal is followed by the decline of E, P, and inhibin to low levels. Reproduced from Buckler *et al.*, J. Clin Endocrinol Metab 66:798, 1988 with permission.

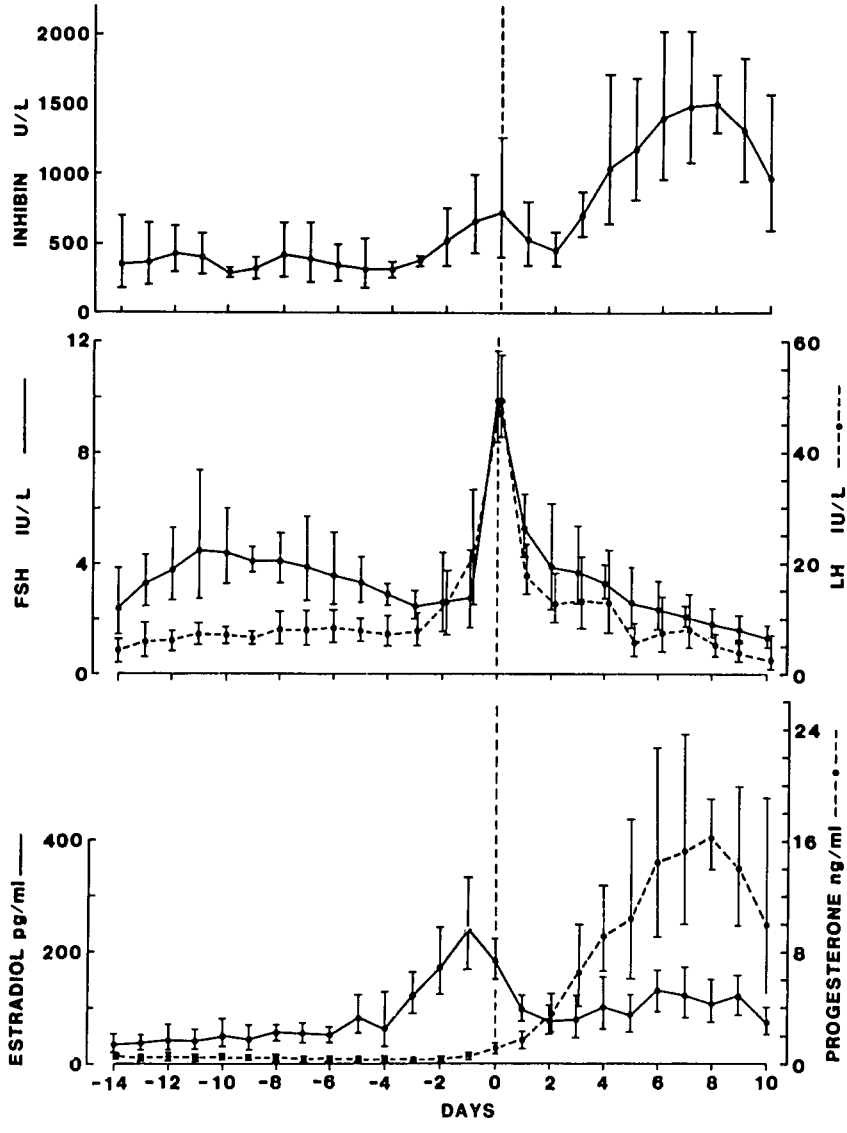


Figure 2. Serum inhibin, FSH, LH, E_2 and P concentrations during the normal menstrual cycle. Data from 6 subjects is normalized around the end of the day of LH surge (broken line). The data are expressed as a geometric mean =67% confidence intervals between 4 and 6 observations per point. Reproduced from McLachlan *et al.*, J Clin Endocrinol Metab 68:954, 1987 with permission.

in this study or 2) other factors operating during the mid-follicular phase that operated to progressively reduce serum FSH levels despite stable inhibin concentrations (for example increased pituitary sensitivity to inhibin feedback, the combined effect of a small rise in serum E_2).

During the late follicular phase serum inhibin and E_2 levels increased in parallel. This observation was consistent with studies showing granulosa cells from large follicles produced more inhibin[6] and the finding of inhibin bioactivity in human ovarian venous blood during this phase of the cycle.[7] The finding was also similar to that during the follicular phase of ovulation-induced cycles mentioned previously.[32] It was interesting that E_2 reached a peak on the day prior to the LH surge while inhibin continued to increase to a midcycle peak coincident with the LH surge suggesting different regulation of secretion of these two parameters of follicular growth.

Periovulatory Phase

Despite the mid-cycle peak of inhibin, FSH also rose at this time, indicating disruption of the proposed reciprocal relationship between these two hormones. We speculated that the FSH rise occurred despite increasing inhibin levels as a result of changes in LHRH pulse frequency and amplitude observed at midcycle.[36,50] In addition, the rising E_2 and P levels, which are also important in the induction of the midcycle gonadotropin surge [23] may be involved in the loss of inhibin feedback on FSH.

Luteal Phase

immediately following the midcycle gonadotropin surge, inhibin levels fell slightly perhaps as a result of disruption of the follicular architecture at ovulation and the formation of the corpus hemorrhagicum.

A major finding of the study was the rise of inhibin levels during the luteal phase to a peak two-fold higher than that at mid-cycle. Other significant correlations were observed between inhibin and both P and E_2 suggesting that inhibin, in addition to the sex steroids, was being produced by the corpus luteum. We reported similar findings during the luteal phase of ovulation-induced cycles in which inhibin levels rose to a mid-luteal peak of similar magnitude to the high levels achieved in the late follicular phase.[29] Inhibin levels then fell sharply at the time of menses and showed significant correlations with P throughout the luteal phase (Fig. 3a). These findings were unanticipated in view of previous reports that bovine[19] and rat[5] luteal cells *in vitro* do not secrete inhibin.

There is now other persuasive evidence to support the corpus luteal production of inhibin. In a report by Tsonis *et al.*,[48] human granulosa cells were collected during oocyte retrieval for IVF and cultured for up to 12 days. These cells luteinized *in vitro* and were found to secrete inhibin bioactivity, although at low levels. Of interest was the observation that both LH and testosterone, but not FSH, increased inhibin secretion. The possibility exists that inhibin secretion by the corpus luteum, in a way similar to P secretion, may be at least partially under LH control. In both the rat[11] and the human female,[12] mRNA for the α subunit of inhibin has been identified in corpus luteal tissue. Both inhibin immuno- and bioactivity was also found in cytosolic extracts of human corpora lutea.[12] Im-

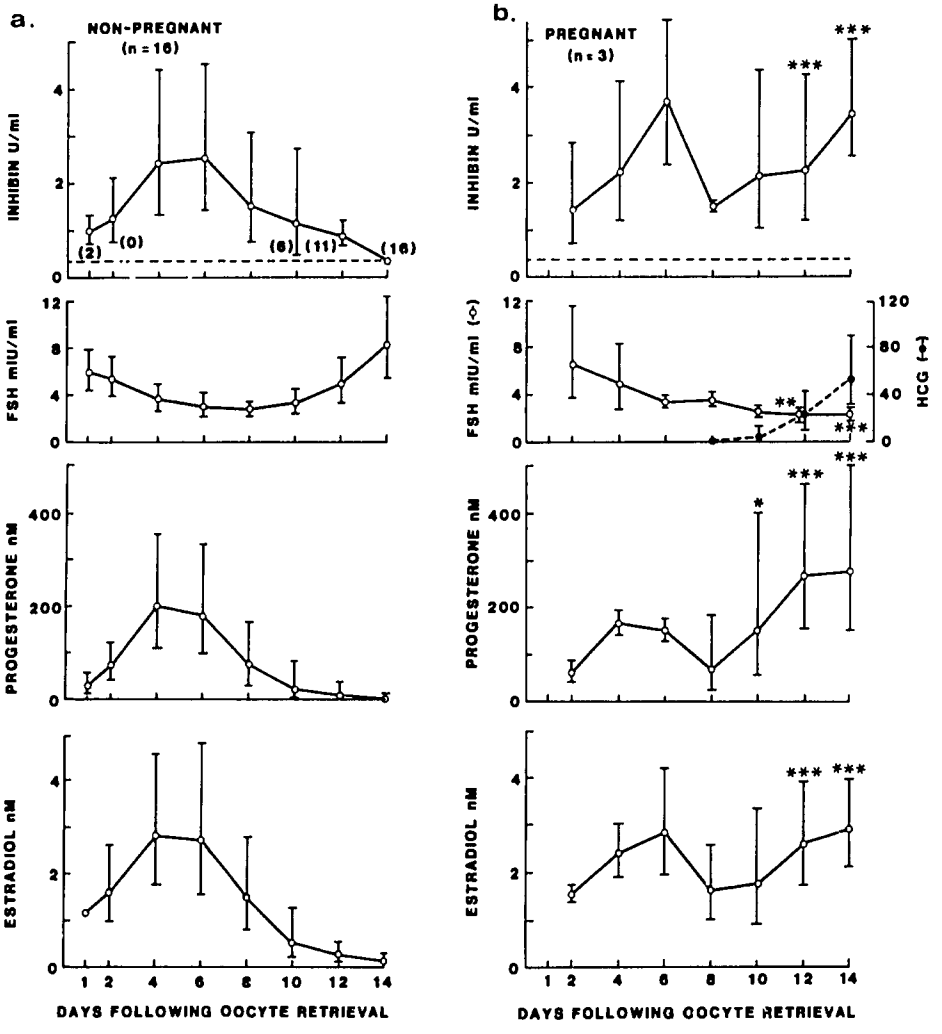


Figure 3. Serum inhibin, FSH, P and E_2 levels in the sera of (a) nonpregnant patients (n=16) and (b) pregnant patients (n=3) on the days following oocyte retrieval for IVF. Results are expressed as the geometric mean =67% confidence intervals. The broken line indicates the limit of sensitivity on inhibin RIA (0.37 U/ml). The number of subjects showing non-detectable inhibin values is shown in parenthesis. Non-detectable values were given the limit of assay detection in the calculation of the mean % confidence intervals. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ comparing hormone values from the pregnant and non-pregnant groups on the same day. Significance values in the second panel of (b) refer to serum FSH. Reproduced from McLachlan *et al.* Fertil and Steril 48:1101, 1987 with permission.

munohistochemical staining in the rat corpus luteum has been reported using an antiserum raised to a synthetic fragment of the NH_2-terminal region of the porcine α inhibin subunit.[10] Finally, inhibin *in vitro* bioactivity has been detected in human serum during the follicular and early luteal phases of ovulation-induced cycles and during the luteal phases of both ovulation-induced and normal cycles.[49]

FUNCTIONS OF INHIBIN DURING THE MENSTRUAL CYCLE

Potentially inhibin may have an endocrine role in the control of FSH secretion and/or intragonadal regulatory actions.

FSH Regulation

An inverse relationship was observed between inhibin and FSH during the mid to late follicular phase of normal cycles consistent with the proposed feedback effects of inhibin on FSH secretion. It could be proposed that the dominant follicle secretes increasing amounts of inhibin, and possibly E_2, in order to limit further FSH secretion thereby controlling its own rate of development and preventing the development of new asynchronous follicles.

A significant inverse relationship was also observed during the luteal phase of both normal[30] and ovulation-induced[29] cycles, suggesting a hitherto unsuspected role for inhibin in the regulation of FSH during the luteal phase. The suppression of gonadotropins during the luteal phase has been attributed to the sex steroid products of the corpus luteum while the perimenstrual rise of FSH has been attributed to their withdrawal. This concept is in conflict with data in monkeys showing that P, when administered in a manner designed to maintain P levels in the peak luteal range, is not sufficient to prevent the perimenstrual rise of FSH.[37] Inhibin must now be considered as a further candidate for the regulation of luteal phase FSH secretion. It could be postulated that inhibin secretion by the corpus luteum is important in suppressing FSH levels and therefore in preventing the development of new follicles prior to regression of the corpus luteum.

As yet no direct evidence for these postulates exists in man or primates.

Paracine Regulation

Evidence has accumulated in lower animals, primarily using rat granulosa cells in culture, in support of a paracrine role for inhibin and the structurally related protein transforming growth factor β(TGF-β). For example, TGF-β has been shown to modulate the expression of LH receptors in response to FSH exposure[21] and to increase FSH-stimulated aromatase activity and P secretion.[20,51] Inhibin has been reported to decrease aromatase activity[51] but not consistently.[20] Activin A enhanced aromatase activity but decreased P systhesis in an "anti-luteinization" action.[20] Clearly, during the follicular phase, inhibin and/or activin and/or TGF-β may have important roles in the regulation of follicular growth, function and maturation. As yet such studies have not been extended to corpus luteal physiology nor to man or primates.

PUBERTY

During normal puberty, the levels of gonadotropins and sex steroids increase and a closed-loop feedback system is established between the hormones of the hypothalamo-pituitary unit and the gonad.[15] Based on animal studies, a similar relationship has been postulated for inhibin and FSH. In female rats, inhibin levels rose in ovarian homogenates[1] and in serum[39] during puberty while a sharp fall in serum FSH was observed.

We have recently described inhibin levels in a cross-sectional study of 102 normal school girls and examined the relationship of inhibin to the gonadotropins, E_2 and pubertal stage[4] (Fig. 4). Inhibin levels were low during Tanner stage I-II then rose 2.3 fold between stages II-IV into the normal early follicular phase range of adult menstrual cycles. Inhibin levels

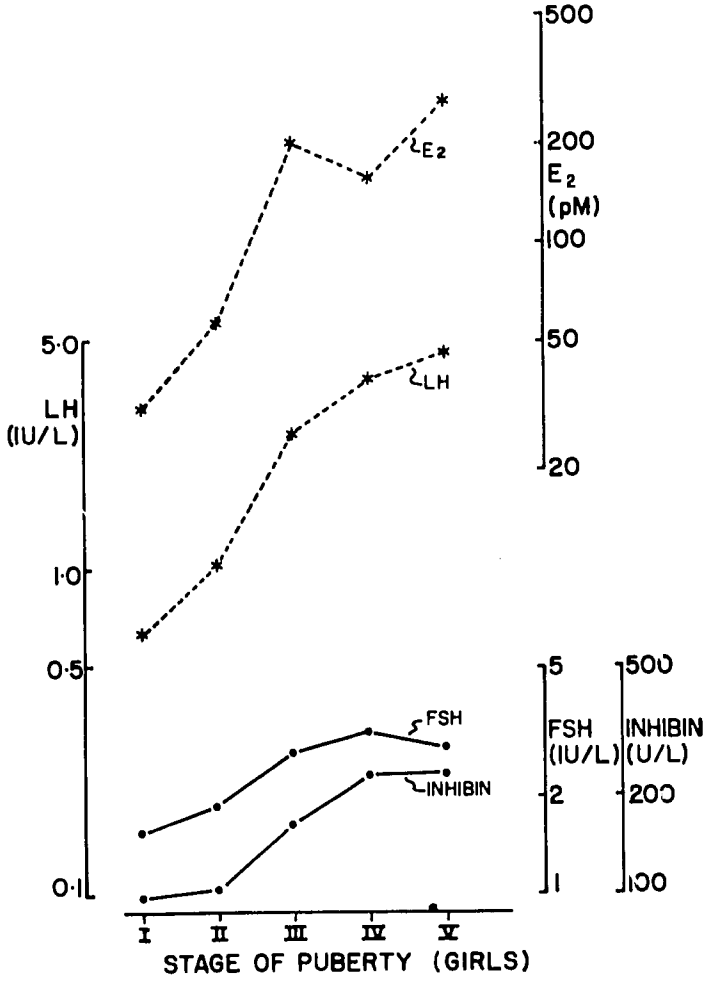


Figure 4. Serum LH, FSH, E_2 and inhibin as a function of the stage of puberty in 102 girls. Reproduced from Burger *et al.*, J. Clin Endocrinol Metab (in press) with permission.

did not increase further between stages IV-V. Significant correlations were found between inhibin and each of LH, FSH and E_2. Similar data was also obtained in 99 normal boys during puberty in that a 3.1 fold increase in inhibin was observed and significant correlations were detected between inhibin and each of LH, FSH and testosterone. Of interest was the fact that absolute levels of inhibin were approximately twofold higher in males as compared to females.

It is postulated that the rise in FSH during puberty stimulates ovarian folliculogenesis and, as a consequence, inhibin secretion. With the establishment of normal menstrual cycles, inhibin levels rise and the negative feedback relationship between FSH and inhibin is established. Nonetheless, it should be noted that there is no direct evidence in support of this postulate in humans.

POLYCYSTIC OVARY SYNDROME

The polycystic ovarian syndrome (PCOS) represents a heterogenous group of clinical disorders featuring oligo- or amenorrhea, infertility, hirsutism, obesity and bilateral polycystic ovaries. Serum LH levels are often inappropriately elevated in relation to FSH levels. It has not been clear whether this abnormal gonadotropin profile resulted from a primary defect in the hypothalamic-pituitary axis and/or resulted from inappropriate ovarian steroid or inhibin feedback. It has been speculated that in PCOS, elevated inhibin levels may be involved in producing the discordant gonadotropin levels by selectively inhibiting FSH release.[46]

We have recently examined 5 PCOS subjects (age 28 to 36 years) diagnosed on the basis of 1) oligo- or amenorrhea and infertility; 2) an inappropriately elevated LH level in the presence of normal or low FSH level in the presence of follicular phase levels of serum P; 3) normal serum prolactin and; 4) the presence of a number of other recognized PCOS features including an elevated body mass index, hirsutism and the presence of polycystic ovaries on ultrasound examination.[3] Subjects were bled twice weekly for 4 weeks. Serum inhibin levels in these anovulatory women with PCOS were found to be similar to those in the early or mid-follicular phase of normal menstrual cycles. Therefore the abnormality of gonadotropin secretion in PCOS cannot be explained simply by the selective suppression of FSH secretion by high inhibin levels. Supportive data comes from morphological examination of polycystic ovaries which have shown numerous small follicles with hyperplasia of the theca interna but very few granulosa cells.

We have also examined inhibin levels during ovulation-induction with the LHRH agonist buserelin and hMG as described earlier in this paper.[16] PCOS subjects responded similarly to their non-PCOS counterparts. Inhibin and E_2 levels rose during initial gonadotropin stimulation while P levels remained low consistent with the absence of corpus luteal tissue in these anovulatory subjects. During continued buserelin administration, inhibin levels fell but rose during follicular development induced with hMG. In conclusion, no evidence has been found to support a primary defect in PCOS inhibin physiology either in terms of basal- or gonadotropin-stimulated (exogenous or endogenous) secretion.

INHIBIN DURING HUMAN GESTATION

A previously unanticipated area of inhibin research has been the physiology of inhibin during gestation. With the knowledge that the placenta secretes a wide range of steroid and protein hormones, we examined charcoal-treated cytosolic extracts from normal full-term human placenta for inhibin immuno- and bioactivity. Both activities were detected and gave parallel dose response lines to the human FF standard in both assay systems.[28] Inhibin immunoactivity was also detected in umbilical cord serum. In addition, Mayo *et al.*[25] identified the mRNA for the human inhibin α subunit in a full-term placental cDNA library and we have demonstrated α and β subunit mRNA in placental extracts.[12] Clearly then the full-term placenta is a source of inhibin.

We have recently reported inhibin immunoreactivity in the serum of women during conception cycles in an IVF program. [29] Rather than falling in the late luteal phase, inhibin levels rose to become significantly higher than non-pregnant subjects by day 12 following oocyte retrieval (Fig. 3b). Thereafter, inhibin levels increased gradually during gestation and showed a twofold increase between the first and second halves of gestation. A similar early rise, and comparable absolute levels, of inhibin have also been found in 3 subjects with premature ovarian failure who conceived as part of a donor oocyte IVF program[27] (Fig. 5). In these subjects the absence of ovarian tissue was confirmed at subsequent cesarian

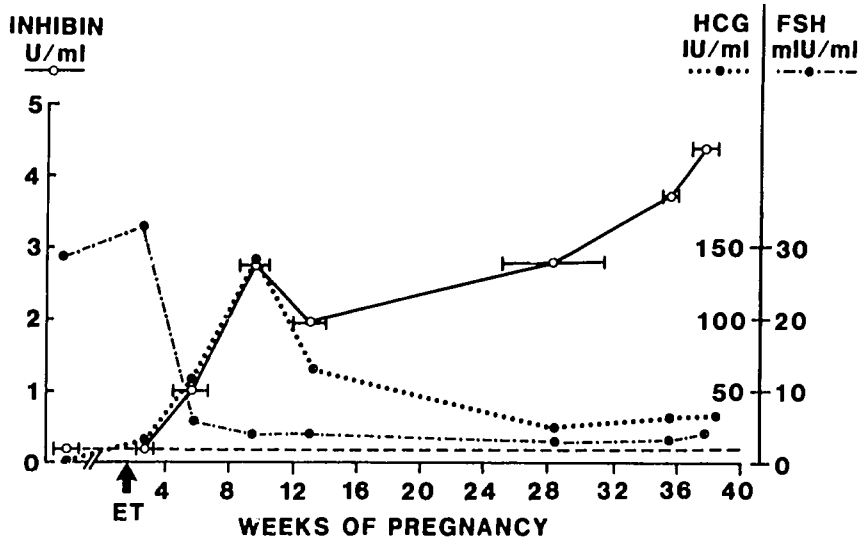


Figure 5. Serum inhibin, FSH and hCG concentration at various times during pregnancy in a patient with premature ovarian failure who conceived following donor oocyte *in vitro* fertilization. ET indicates the time of embryo transfer. Repeated serum samples were drawn throughout pregnancy, however, due to scarcity of samples, individual aliquots were pooled to give 8 pools throughout pregnancy. The horizontal bar at each time point indicates the duration over which the serum samples were pooled for the individual result. The horizontal broken line indicates the limit of detection of inhibin RIA (0.37 U/ml). Reproduced from McLahlan *et al.*, Clin Endocrinol 27:663, 1987 with permission.

section. These studies have shown that inhibin appears in the maternal circulation in early gestation at approximately the same time as hCG. Inhibin levels continue to rise during gestation while hCG levels fall from mid-gestation onward, suggesting differing regulation of these two placental proteins. The maternal ovary is unlikely to be a major source of inhibin during pregnancy in view of the study of donor oocyte pregnancies.

Potential roles for inhibin during pregnancy could include the suppression of maternal FSH which has generally been attributed to the high circulating levels of sex steroids. A second, and probably more likely, role would involve a local function for inhibin within the fetal-placental unit. In a recent study Petraglia *et al.*[35] have presented immunohistochemical evidence for the localization of inhibin in the cytotrophoblast layer of the human placenta at term and in primary cultures of human trophoblasts using an antiserum raised to a fragment of the NH_2-terminal fragment of the porcine α subunit. High concentrations of hCG stimulated inhibin secretion by cultured placental cells, probably by a cAMP-dependent mechanism. Interestingly, incubation of placental cells with inhibin antiserum increased the secretion of hCG and LHRH-like immunoreactivity from trophoblastic cells, suggesting that endogenous inhibin may have a local tonic inhibitory action upon the secretion of these two proteins. Finally, partial primary structural homology has been reported between inhibin (especially the β subunit) and the DPP-C gene product in *Drosophila*.[34] The latter has a key role in embryogenesis in that organism. In view of all of the above studies it is tempting to speculate an important growth regulatory role for inhibin during human gestation.

CONCLUSIONS

The study of human inhibin physiology has advanced rapidly in the past few years following the purification of ovarian inhibin. With the advent of specific radioimmunoassays, mRNA probes and sensitive *in vitro* bioassays, it is now possible to examine both circulating inhibin levels and inhibin gene expression in various tissues. In this paper, data have been presented from studies in adult women relating to the effects of gonadotropin administration and withdrawal on ovarian inhibin secretion, the profiles of serum inhibin during normal and ovulation-induced cycles and the corpus luteal production of inhibin. During human puberty, inhibin levels have been shown to rise progressively as ovarian function is established. During pregnancy serum inhibin levels are elevated in maternal serum and inhibin has been localized to the trophoblast/placenta. As yet, the physiological role of inhibin under these various settings is not clear, although indirect evidence suggests it may have both endocrine and paracrine functions.

Such data obtained under normal physiological conditions provide the essential background for the future study of inhibin in disorders of reproduction. For example, in the polycystic ovary syndrome serum inhibin levels are not elevated as previously suspected and cannot therefore be directly responsible for the abnormal gonadotropin profile observed in that condition. Inhibin, as a protein marker of follicular development, may represent a valuable measurement in the study of human infertility and a clinically useful parameter in the monitoring of ovulation induction.

REFERENCES

1. AU CL, ROBERTSON DM, DE KRETSER DM: Measurement of inhibin and an index of inhibin production by rat testes during post-natal development. Biol Reprod 35:37, 1986
2. BAIRD DT: Factors regulating the growth of the preovulatory follicle in the sheep and human. J Reprod Fertil 69:343, 1983
3. BUCKLER HM, MCLACHLAN RI, MACLACHLAN VB, HEALY DL, BURGER HG: Serum inhibin levels in polycystic ovary syndrome: Basal levels and response to LHRH antagonist and exogenous gonadotropin administration. J Clin Endocrinol Metab 66:798, 1988
4. BURGER HG, MCLACHLAN RI, BANGAH M, QUIGG H, FINDLAY JK, ROBERTSON DM, deKRETSER DM, WARNE G, WERTHER G, HUDSON IL, COOK J, FIEDLER R, GRECO S, YONG A, SMITH P: Serum inhibin concentrations rise throughout normal male and female puberty. J Clin Endocrinol Metab 1988 (in press)
5. CARSON RS, LEE VWK: Inhibin content and production by antral, preovulatory and luteinized rat ovarian follicles. 26th Annual Meeting of the Endocrine Society of Australia, Canberra, Australia 1983 (Abstract 33)
6. CHANNING CP, ANDERSON LD, HOOVER DG, KOLENA J, OSTEEN KG, POMERANTZ SH, TANABE K: The role of nonsteroidal regulators in control of oocyte and follicular maturation. Recent Prog Horm Res 38:331, 1982
7. CHANNING CP, GAGLIANO P, TANABE K, FORTUNY A, CORTES-PRIETO J: Demonstration of a gradient in inhibin activity, estrogen, progesterone and Δ4-androstenedione in follicular fluid, ovarian vein blood, and peripheral blood of normal women. Fertil Steril 43:142, 1985
8. CHANNING CP, TANABE K, CHACON M, TILDON JT: Stimulatory effects of follicle-stimulating hormone and luteinizing hormone upon secretion of progesterone and inhibin activity by cultured infant human ovarian granulosa cells. Fertil Steril 42:598, 1984
9. CHANNING CP, TANABE K, JONES SG, LEBECH P: Inhibin activity of preovulatory follicles of gonadotropin-treated and untreated women. Fertil Steril 42:243, 1984
10. CUEVAS P, YING S-Y, LING N, UENO N, ESCH F, GUILLEMIN R: Immunohistochemical detection of inhibin in the gonad. Biochem Biophys Res Commun 142:53, 1987
11. DAVIS SR, DENCH F, NIKOLAIDIS I, CLEMENTS JA, FORAGE RG, BURGER HG: Inhibin α-subunit gene expression in the ovaries of immature rats is stimulated by pregnant mare serum gonadotropin. Biochem Biophys Res Commun 138:1196, 1986
12. DAVIS SR, KROZOWSKI Z, MCLACHLAN RI, BURGER HG: Inhibin gene expression in the human corpus luteum. J Endocrinol 115:R21, 1987
13. DIZEREGA GS, TURNER CK, STOUFFER RL, ANDERSON LD, CHANNING CP, HODGFN GD: Suppression of follicle-stimulating hormone-dependent folliculogenesis during the primate ovarian cycle. J Clin Endocrinol Metab 52:451, 1981
14. FLEMING R, HAXTON MJ, HAMILTON MPR, MCCURNE GS, BLACK WP, MACNAUGHTON MC, COUTTS JRT: Successful treatment of infertile women with oligomenorrhea using a combination of an LHRH agonist and exogenous gonadotropins. Brit J Obstet Gynaecol 92:369, 1985
15. FRANCHIMONT P, BURGER HG: Human Growth Hormone and Gonadotropins in Health and Disease, Amsterdam-Oxford: North Holland Publishing Co., 1985
16. FRASER HM, SANDOW J: Suppression of follicular maturation by infusion of a luteinizing hormone releasing hormone agonist started during the late luteal phase in the stumptailed macaque monkey. J Clin Endocrinol Metab 60:579, 1985
17. HEALY DL, LAWSON SR, ABBOTT M, BAIRD DT, FRASER HM: Towards relieving uterine fibroids without surgery: Subcutaneous infusion of a luteinizing hormone agonist commencing in the luteal phase. J Clin Endocrinol Metab 63:619, 1986
18. HEALY DL, MORROW L, JONES M, BESANKO M, ROGERS DAW, OKAMATO S, THOMAS A, MACLACHLAN B, MARTINEZ F: Contributions of *in vitro* fertilization to knowledge of the reproductive endocrinology of the menstrual cycle. Baillieres Clin Endocrinol Metab 1(1):133, 1987
19. HENDERSON KM, FRANCHIMONT P: Regulation of inhibin production by bovine ovarian cells *in vitro*. J Reprod Fertil 63:431, 1981

20. HUTCHINSON LA, FINDLAY JK, DE VOS FL, ROBERTSON DM: Effects of bovine inhibin, transforming growth factor-β and bovine activin-A on granulosa cell differentiation. Biochem Biophy Res Commun 146:1405, 1987
21. KNECHT M, FENG P, CATT KJ: Transforming growth factor-beta regulates the expression of luteinizing hormone receptors in ovarian granulosa cells. Biochem Biophys Res Commun 139:800, 1986
22. LEE VKW, MCMASTER J, QUIGG H, FINDLAY JK, LEVERSHA L: Ovarian and peripheral blood inhibin concentrations increase with gonadotropin treatment in immature rats. Endocrinology 108:2403, 1981
23. LIU JH, YEN SSC: Induction of midcycle gonadotropin surge by ovarian steroids in women: A critical evaluation. J Clin Endocrinol Metab 57:797, 1983
24. MARRS RP, LOBO R, CAMPEAU JD, NAKAMURA RM, BROWN J, UJITA EL, DIZEREGA GS: Correlation of human follicular fluid inhibin activity with spontaneous and induced follicle maturation. J Clin Endocrinol Metab 58:704, 1984
25. MAYO KE, CERELLI GM, SPIESS J, RIVIER J, ROSENFELD MG, EVANS RM, VALE W: Inhibin A-subunit cDNAs from porcine ovary and human placenta. Proc Nat Acad of Science USA 83:5849, 1986
26. MCLACHLAN RI, HEALY DL, BURGER HG: Clinical aspects of LHRH analogues in Gynecology: A Review. Brit J Obstet Gynaecol 93:431, 1986
27. MCLACHLAN RI, HEALY DL, LUTJEN PJ, FINDLAY JK, DE KRETSER DM, BURGER HG: The maternal ovary is not the source of circulating inhibin levels during human pregnancy. Clin Endocrinol 27:663, 1987
28. MCLACHLAN RI, HEALY DL, ROBERTSON DM, BURGER HG, DE KRETSER DM: The human placenta: a novel source of inhibin. Biochem Biophys Res Commun 140:485, 1986
29. MCLACHLAN RI, HEALY DL, ROBERTSON DM, BURGER HG, DE KRETSER DM: Circulating immunoactive inhibin in the luteal phase and early gestation on women undergoing ovulation induction. Fertil Steril 48:1101, 1987
30. MCLACHLAN RI, ROBERTSON DM, HEALY DL, BURGER HG, DE KRETSER DM: Circulating immunoreactive inhibin levels during the normal human menstrual cycle. J Clin Endocrinol Metab 65:954, 1987
31. MCLACHLAN RI, ROBERTSON DM, BURGER HG, DE KRETSER DM: The radioimmunoassay of bovine and human follicular fluid and serum inhibin. Mol Cell Endocrinol 46:175, 1986
32. MCLACHLAN RI, ROBERTSON DM, HEALY DL, DE KRETSER DM, BURGER HG: Plasma inhibin levels during gonadotropin-induced ovarian hyperstimulation for IVF: A new index of follicular function? Lancet i:1233, 1986
33. MCNATTY KP, SMITH DM, MAKRIS A, OSTHANODH R, RYAN KJ: The microenvironment of the human antral follicle: interrelationships among the steroid levels in antral fluid, the populaton of granulosa cells, and the status of the oocyte *in vivo* and *in vitro*. J Clin Endocrinol Metab 49:851, 1979
34. PADGETT RW, ST JOHNSTON RD, GELBART WM: A transcript from a Drosophila pattern gene predicts a protein homologous to the transforming growth factor-β family. Nature 325:81, 1987
35. PETRAGLIA F, SAWCHENKO P, LIM ATW, RIVIER J, VALE W: Localization, secretion, and action of inhibin in human placenta. Science 237:187, 1987
36. REAME N, SAUDER SE, KELCH RP, MARSHALL JC: Pulsatile gonadotropin secretion during the human menstrual cycle: evidence for altered frequency of gonadotropin-releasing hormone secretion. J Clin Endocrinol Metab 59:328, 1984
37. RESKO JA, NORMAL RL, NISWENDER GD, SPIES HG: The relationship between progestins and gonadotropins during the late luteal phase of the menstrual cycle in rhesus monkeys. Endocrinology 94:128, 1974
38. RIVIER C, RIVIER J, VALE W: Inhibin-mediated feedback control of follicle-stimulating hormone secretion in the female rat. Science 234:205, 1986
39. RIVIER C, VALE W: Inhibin: Measurement and role in the immature female rat. Endocrinology 120:1688, 1987
40. ROBERTSON DM, de VOS FL, FOULDS LM, MCLACHLAN RI, BURGER HG, MORGAN FJ, HEARN MTW, DE KRETSER DM: Isolation of 31 kDa form of inhibin from bovine follicular fluid. Mol Cell Endocrinol 44:271, 1986
41. ROBERTSON DM, FOULDS LM, LEVERSHA L, MORGAN FJ, HEARN MTW, BURGER HG, WETTENHALL REH, DE KRETSER DM: Isolation of inhibin from bovine follicular fouid. Biochem Biophys Res Commun 126:220, 1985
42. SCHENKEN RS, ANDERSON WH, HODGEN GD: Follicle-stimulating hormone increases ovarian vein nonsteroidal factors with gonadotropin-inhibiting activity. Fertil Steril 42:785, 1984

43. SCHENKEN RS, ASCH RH, ANDERSON WH: Effects of porcine follicular fluid on the post-castration secretion of gonadotropins in Rhesus monkeys. J Clin Endocrinol Metab 59:436, 1984
44. SCOTT RS, BURGER HG, QUIGG H: A simple and rapid *in vitro* bioassay for inhibin. Endocrinology 107:1536, 1980
45. STOUFFER RL, HODGEN GD: Induction of luteal phase defects in Rhesus monkeys by follicular fluid administration at the onset of the menstrual cycle. J Clin Endocrinol Metab 51:669, 1980
46. TANABE K, GAGLIANO P, CHANNING CP, NAKAMURA Y, YOSHIMURA Y, IIZUKA R, FORTURY A, SULEWSKI J, REZAO N: Levels of inhibin-F activity and steroids in human follicular fluid from normal women and women with polycystic ovarian disease. J Clin Endocrinol Metab 57:24, 1983
47. TROUNSON AO: *In vitro* fertilization at Monash University Melbourne, Australia. In CROSIGNANI PY, RUBIN BL (eds): *In Vitro* Fertilization in Embryo Transfer. p 315, New York, Academic Press, 1983
48. TSONIS CG, HILLIER SG, BAIRD DT: Production of inhibin bioactivity by human granulosa-lutein cells: stimulation by LH and testosterone *in vitro*. J Endocrinol 112:R11, 1987
49. TSONIS CG, MCNEILLY AS, BAIRD DT: Production and secretion of ovarian inhibin. In BURGER HG, de KRETSER DM, FINDLAY JK, IGARASHI M (eds): Inhibin-Non-Steroidal Regulation of Follicle Stimulating Hormone Secretion. Vol 42, p 203. New York, Serono Symposia from Raven Press, 1987
50. VELDHUIS JD, BEITINS IZ, JOHNSON ML, SERABIAN MA, DUFAU ML: Biologically active luteinizing hormone is secreted in episodic pulsations that vary in relation to stage of the menstrual cycle. J Clin Endocrinol Metab 58:1050, 1984
51. YING S-Y, BECKER A, LING N, UENO N, GUILLEMIN R: Inhibin and beta type transforming growth factor (TGF-β) have opposite modulating effects on the follicle stimulating hormone (FSH)-induced aromatase activity of cultured rat granulosa cells. Biochem Biophys Res Commun 136:969, 1986

DISCUSSION

CHAIRPERSON: KOJI YOSHINAGA, Ph.D.

DR. CROWLEY: Are there any particular tricks to facilitate iodinating inhibin? I heard it is very difficult to do. Do you know what the half-life of inhibin is in the circulation? Finally, can you convert your RIA units into picomoles of standard for comparative purposes?

DR. McLACHLAN: To answer the last question first, since we do not have purified human inhibin we have used a partially purified preparation calibrated according to its *in vitro* bioactivity. However, we can made a quess. If we assume that the specific activity of human inhibin is similar to bovine inhibin, then we can calculate that the sensitivity of the assays are between 0.2-1.0 ng/ml.

Concerning the iodination of inhibin, it did, in fact, take us a long time to develop the methodology. The iodination is fairly routine using a chloramine procedure, but the purification of the iodinated material was difficult. We found the dye ligand Matrix Red A to be highly effective, as described in our first RIA paper. That procedure yields a very pure material with identical physicochemical characteristics to the non-iodinated form.

Finally, we have examined the half life of inhibin in circulation in rats and have found a biphasic disappearance curve. The first $t_{1/2}$ is about 15 minutes, and that is in support of Dr. Victor Lee's early studies using the bioassay of serum following castration of PMSG-stimulated female rats. There is a second decay component with a much lower $t_{1/2}$ of about 20 hours. The proportion of these two $t_{1/2}$ values varies between males and females.

DR. CROWLEY: Have you looked for evidence of pulsatile secretion of inhibin by the corpus luteum, in a similar way to that shown for progesterone?

DR. McLACHLAN: No, we have not as yet. That is clearly a very interesting point, and I suspect that we may demonstrate pulsatility of inhibin as with progesterone.

MR. SPIELER: I would like a little more discussion on the temporal relationship between inhibin and FSH secretion. You demonstrated that there was an increase in inhibin as the estrogen level increased which was followed by simultaneous surges of inhibin and FSH. Is this a result of the feedback mechanism whereby FSH induces inhibin release which, in turn decreases FSH, or do the two peaks coincide as a result of infrequent sampling?

In other words, do you think that the FSH peak actually occurs at the same time as the inhibin peak in the normal menstrual cycle?

DR. McLACHLAN: In all the normal menstrual cycles that we have studied, we see a midcycle surge of inhibin and, obviously, of FSH. As Dr. Hasegawa mentioned earlier, they also have recently observed the same phenomenon using a different assay system. I cannot explain why FSH and inhibin peak simultaneously, whereas during the preceding two to four days they had varied inversely. I suspect that at midcycle it related to interactions between LHRH and inhibin at the pituitary level, as previously mentioned by Dr. Burger.

I think that the time frame from minus 48 to plus 24 hours after the LH surge is very interesting and requires careful study of inhibin and the other hormonal dynamics. I think that daily sampling, as performed in our study, is not frequent enough.

DR. HUTCHISON: Is it your impression that inhibin is always paralleling progesterone secretion? And, if you give hCG during the luteal phase for corpus luteum support, do you see an inhibin response?

DR. McLACHLAN: Inhibin and progesterone change in parallel in all subjects examined. We have not found a discordant profile yet. With regard to the response of inhibin to hCG during the luteal phase, we are currently examining this point.

DR. GOODMAN: About ten years ago Gary Hodgen and I were looking at the role of the corpus luteum in regulating follicle growth during the ovarian cycle. We removed the monkey corpus luteum and then replaced it with a silastic capsule releasing luteal phase levels of progesterone. We found in that model that we could completely reverse the effects of luteectomy with progesterone alone. The inference we drew was that progesterone was the principal luteal hormone regulating follicle growth, and that luteal phase estradiol was not very important, which was an issue that Dr. Barrett had been raising at the time.

Besides the evidence you have that there is mRNA being expressed in luteal tissue, do you have any evidence that inhibin is coming from an extra-luteal component of the ovary? If it is coming from the corpus luteum, then what is the compelling reason to believe that luteal inhibin is important in the regulation of the menstrual cycle?

DR. McLACHLAN: I am aware of your previous publication. There is evidence, on the other hand, from Dr. Resko suggesting that the replacement of progesterone alone, in the monkey, was not capable of preventing the inter-cycle FSH rise. So, there is some controversy on this issue.

We do not have any evidence for an extra-corpus luteum site of inhibin secretion. We believe, at this stage of the cycle, that all of the inhibin is coming from the corpus luteum. I cannot explain the discrepancy that you are mentioning from the previous study using the silastic implants after luteectomy. Nonetheless, apart from the high levels of inhibin and its inverse correlation with FSH during the luteal phase, there is currently no other compelling evidence for a role for inhibin in controlling luteal phase gonadotropin secretion.

DR. ZILBERSTEIN: Did you have the opportunity to look at what happened to inhibin levels after pregnancy or in cases where there was termination of pregnancy?

DR. McLACHLAN: No.

DR. MILLER: Concerning the questions about inhibin and FSH both increasing at mid-cycle, there has been some reported evidence from Tom Landerfelt's laboratory that the FSH beta message decreases at that time. So, I would suspect that inhibin probably is causing the reduction in FSH beta and the increase in FSH observed is due to GnRH release.

DR. McLACHLAN: That is an interesting point.

DR. SHETH: I would like to point out that both Professor Li and our group reported localization of inhibin in the Leydig cells of the human, monkey, and dog testis. The stimulation of inhibin in men by FSH and LH that you observed may be due to this.

DR. McLACHLAN: What sort of inhibin did you examine for?

DR. SETH: Human testicular inhibin and antibodies to this peptide. The above data are shortly to be published in the Anatomical Record.

DR. MANBERG: You mentioned that inhibin levels during follicular stimulation might be a good marker of oocyte development. Are there any situations where measuring inhibin might be advantageous to measuring estradiol or give you additional information?

DR. McLACHLAN: There was a minority of subjects in our initial study where there was discordance between inhibin and estradiol levels in the late follicular phase. In about 90 percent of ovulation-induced cycles there is concordance throughout the follicular phase of ovulation-induced cycles. In a minority, we have found that inhibin began to fall while estradiol was still increasing. This might suggest that the two can be discordant under certain circumstances. We do not know the significance of that observation. Perhaps inhibin is a better marker of oocyte maturation, and even the occurrence of pregnancy. This is something that needs to be looked into further.

DR. LAMBERT: But have you found, for example, any cases where the inhibin levels correlate better to oocyte number than estrogen?

DR. McLACHLAN: No, they all correlate about the same, but the numbers are small. Concerning differences between inhibin levels in conception and non-conception cycles, I think we will have to look at a large number of IVF cycles since the conception rate is about 15 percent per cycle.

DR. PETRAGLIA: Have you measured plasma inhibin levels in postmenopausal women? And, how do you explain the low inhibin levels found at the begining of pubertal maturation?

DR. McLACHLAN: Postmenopausal serum does not contain any inhibin immunoactivity. In fact, we include postmenopausal serum as diluent for all standards and samples.

Inhibin levels in early puberty, although low, are still measurable being just above the limit of assay detection. In addition, serum from castrate and premature ovarian failure patients also contain no inhibin immunoactivity.

12

How Does Inhibin Regulate LH and FSH Secretion? Studies in Perifused Rat Pituitary Cells

FUMIKAZAU KOTSUJI
STEPHEN J. WINTERS
HUGH S. KEEPING
BARBARA ATTARDI
HIROYUKI OSHIMA
PHILIP TROEN

Inhibin has been defined as a glycoprotein hormone of gonadal origin which selectively suppresses FSH synthesis and secretion.[7] In contrast to this view, a few studies in experimental animals have found that circulating LH levels also fall after inhibin administration.[2,11] However, these findings were generally attributed to contaminants in the impure preparations of inhibin used. Nevertheless, the actions of inhibin are only partly understood.

Although the physiological importance of inhibin in the regulation of gonadotropin secretion in the male is unknown, a deficiency of inhibin has been proposed to explain the increased FSH secretion in men with severe oligospermia and azoospermia.[9,13,15,16,18,19,23] Interestingly, LH secretion is also increased in these men.[9,13,15,16] While there is evidence that a reduction in testosterone production could explain this increase in LH levels,[3,15,16] these data could also reflect an effect of inhibin on LH secretion.

The use of dispersed pituitary cells for the bioassay of inhibin has produced important advances in our understanding of inhibin's actions. Most studies of inhibin effects *in vitro* in the absence of GnRH have demonstrated a pronounced inhibition of FSH secretion and little or no change in LH secretion.[8,10,12,20] However, under normal conditions the pituitary is stimulated intermittently by GnRH. Thus the physiological significance of changes observed in the absence of GnRH can be questioned. In the presence of GnRH, both LH and FSH secretion are usually decreased by inhibin,[8,10,12,21] although higher concentrations of inhibin are needed to suppress LH than FSH (Fig. 1). Moreover, the ED_{50}'s for the effects on GnRH-induced secretion are greater than those for basal secretion.

Previous studies of the effects of inhibin on GnRH-stimulated gonadotropin secretion *in vitro* have used cells continuously exposed to GnRH. These static systems ignore the fundamental role of the intermittent GnRH signal in normal pituitary function. We have therefore used dispersed rat pituitary cells perifused with pulses of GnRH, a model which more closely simulates physiological conditions,[1] to study the time course and selectivity of the effect of inhibin on gonadotropin secretion.

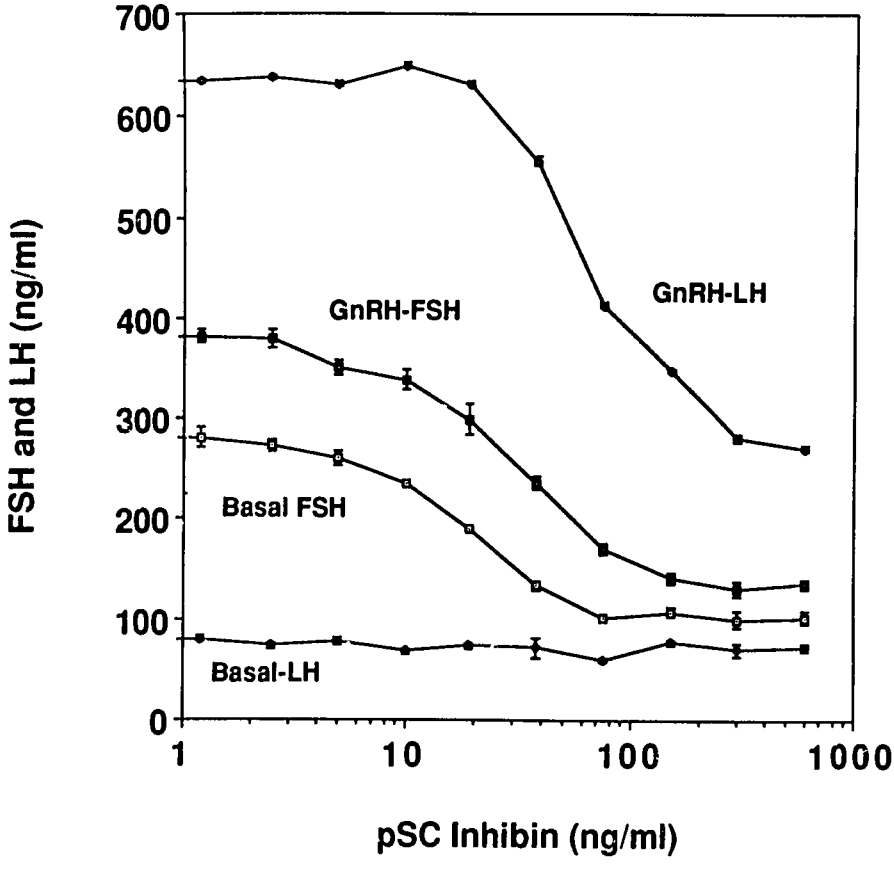


Figure 1. Influence of inhibin from primate Sertoli cells on FSH and LH secretion by rat anterior pituitary cells in monolayer culture. Cells were treated with inhibin at the indicated concentrations for 24 hours (basal secretion), followed by the addition of GnRH (10nM) for 6 hours. (From Kotsuji F, Winters SJ, *et al.*: Effects of Inhibin from Primate Sertoli Cells on Follicle-Stimulating Hormone and Luteinizing Hormone Release by Perifused Rat Pituitary Cells, Endocrinology, 122:2796 1988).

MATERIALS AND METHODS

Inhibin was partially purified from cynomolgus monkey Sertoli cell cultures, as described previously.[17] Sertoli cell culture medium was concentrated with an Amicon apparatus and desalted on a Bio-Gel P-6DG column. The eluted protein was then chromatographed on a Concanavalin A (Con A)-Sepharose affinity column, since we have previously shown[17] that primate Sertoli cell inhibin is completely retained by Con-A and can be eluted with 0.2M methyl-D-glucoside. Inhibin-containing fractions were purified further on a Synchropak RP-4 silica reverse phase HPLC column; activity eluted at approximately 50% acetonitrile. This preparation had an effective concentration (EC_{50}) of approximately 10 ng/ml based upon its ability to suppress basal FSH release from rat pituitary cells cultured for 72 hours.

Pituitary cell cultures were established using anterior pituitaries from 7-8 week old male rats. Pituitaries were rinsed with Hanks-HEPES and divided into 8-10 pieces. Cells were dispersed by treatment with 0.4% collagenase (Boehringer Mannheim), 0.03% DNase (Sigma) in Hanks-HEPES buffer (pH 7.3) containing 0.4% BSA and 0.2% glucose. The minced tissue was mixed for 90 minutes at 80 cycles/minute in a shaking water bath at 37°C. Then cells were digested with 0.25% pancreatin (Sigma) for 8 minutes, washed 3 times with Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal calf serum, and filtered through 100 μm stainless steel mesh. The filtrate was centrifuged at 300 x g and the cells were resuspended in DMEM containing 10% fetal calf serum. The cell yield was 5-9 x 10^5 per pituitary, and viability was 94-97% based upon trypan blue exclusion.

Cells were precultured with Cytodex microcarrier beads type 3 (Pharmacia) to increase the culture surface area for perifusion experiments. Cells were mixed with beads in a ratio of 3.0 x 10^7 cells: 320 mg beads in 100 ml DMEM and cultured in 10 cm siliconized glass Petri dishes for 48 hours at 37°C in 10% CO2-90% air. Adhesion of the cells to the microcarriers was facilitated by transferring the mixture in fresh medium to a culture vessel containing a bulb-shaped rod and stirring at 50 rpm for 24 hours. The cells-beads mixtures containing approximately 10 million cells were packed into Lucite columns (LDC/Milton Roy) of 6.3 mm internal diameter. The columns were placed in a 37°C incubator and perifused at a flow rate of 0.3 ml/minute with DMEM-F12 containing 14.8 mM NaHCO3, 0.2% glucose (pH 7.3) and gassed with 5%CO_2-95%O_2 at 37°C.

RESULTS AND DISCUSSION

The data from a representative experiment are shown in Figure 2. Cells were stimulated with 10nM GnRH for 2 minutes every 60 minutes. Fractions were collected at 10 minute intervals. GnRH produced a rapid and concurrent increase in FSH and LH release and a return to basal levels within 30 minutes, as is characteristic of pituitary cells from male

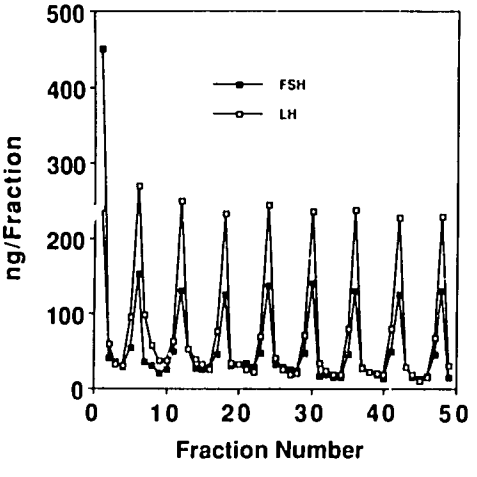


Figure 2. FSH and LH secretion by male rat anterior pituitary cells stimulated with 10 nM GnRH every 1 hour cultured with Cytodex beads for 72 hours before beginning the perifusion. Fractions were collected every 10 minutes.

rats.[22] However, the proportional response of LH was consistently greater than the proportional response of FSH. In some experiments, both basal and GnRH-stimualted secretion declined over the course of the perfusion.

To examine the selectivity and time course of the inhibin effect on LH and FSH secretion by pituitary cells, an infusion of Sertoli cell inhibin was superimposed upon the intermittent stimulation of cells by GnRH. A second column prepared under identical conditions from the same cell suspension was perifused simultaneously with GnRH, but was not treated with inhibin. Cells were stimulated for 8-12 hours with hourly pulses of 10 nM GnRH delivered over 2 minutes. A dose-response effect of inhibin was observed. Higher doses of inhibin led to a significant reduction in both FSH and LH release (Fig. 3A), whereas lower doses of inhibin suppressed GnRH-stimulated FSH secretion but did not affect LH (Fig. 3B).

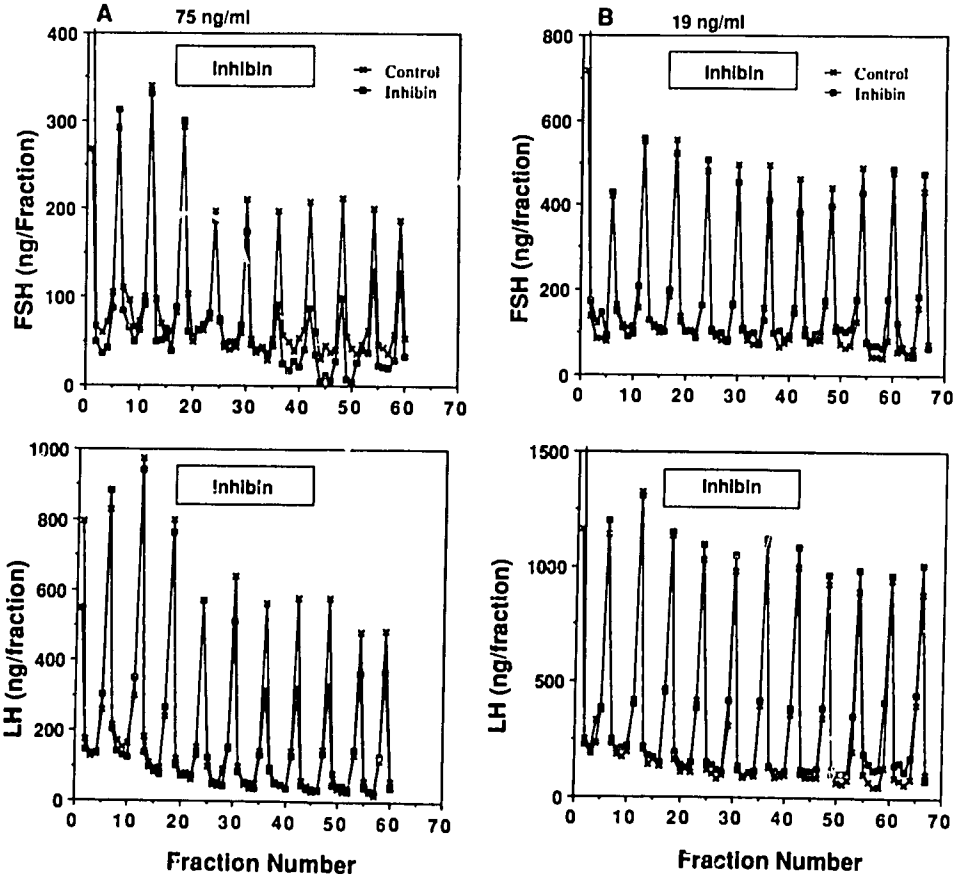


Figure 3. Effect of inhibin on GnRH-induced FSH and LH secretion by perifused anterior pituitary cells. Two columns containing cells from the same dispersion were perifused simultaneously. One column contained cells treated with inhibin for 5 hours, and the second column was an untreated control.

In 3A, the dose of inhibin was 75 ng/ml; Figure 3B, 19 ng/ml.

Figure 4 is a summary of the results of three experiments at the higher dosage of inhibin (75 ng/ml). The effect on gonadotropin pulse amplitude was evident within 2 hours (pulse 5) after adding inhibin to the perifusion medium. At 3 hours (pulse 6) the amplitude of FSH pulses was maximally reduced to 46 ± 9% (M ± SE) of control ($p < 0.01$). The temporal pattern of the effect on LH was similar, but the maximal reduction was to 71 ± 8% of control. The effect on LH was less ($p < 0.025$) than on FSH. Following removal of inhibin from the perifusion medium, gonadotropin secretion began to increase toward control levels after a lag of approximately 1 hour (pulses 10 and 11). The relative amplitude of FSH pulses reached 82 ± 8% of control and LH rose to 88 ± 8% of control at the completion of the experiment 3 hours after removing inhibin from the perifusion medium.

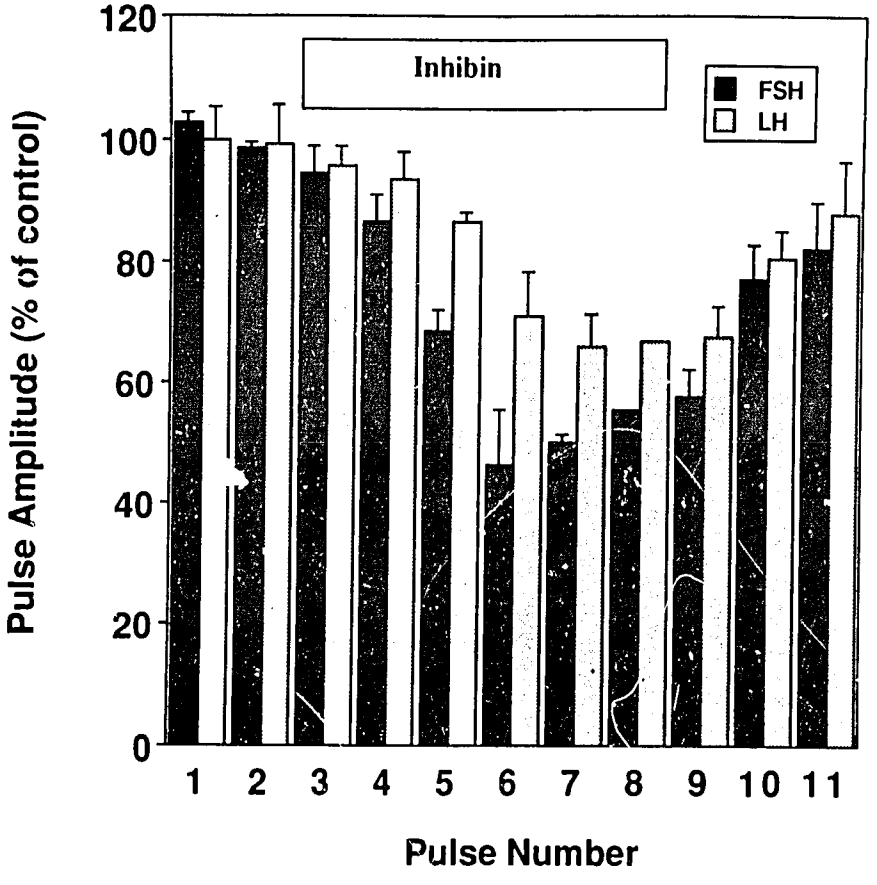


Figure 4. Effect of inhibin on FSH and LH pulse amplitude. Each pulse from the pituitary cell column exposed to inhibin was compared to the simultaneous pulse from the control column, which was designated 100%. The amplitude of a hormone pulse was calculated by subtracting the basal concentration before adding GnRH from the hormone concentrations in each of the three ten-minute fractions after addition of GnRH, which were then summed. The data are the mean ± SEM of three experiments. (from Kotsuji F. *et al.*: *ibid*).

We found that longer exposure to inhibin decreased its suppressive effect on GnRH-induced gonadotropin release. Figure 5 illustrates the results of a representative experiment in which pituitary cells were pretreated with Sertoli cell inhibin (at a dose of 75 ng/ml

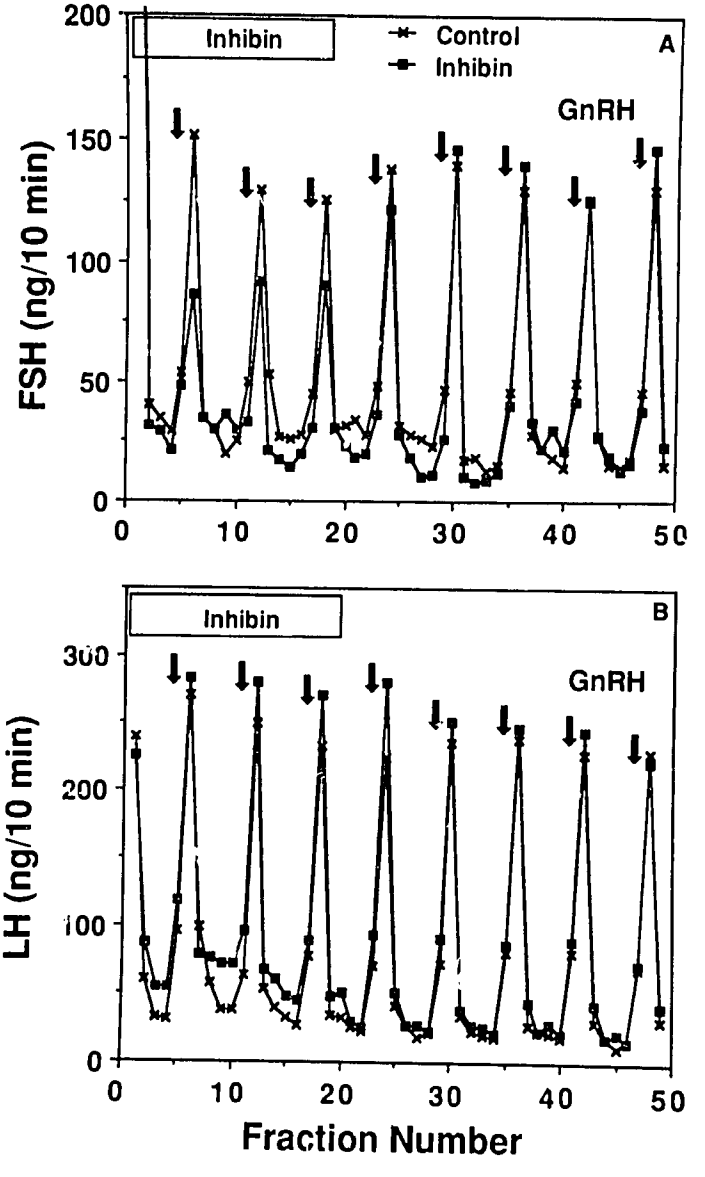


Figure 5. FSH and LH secretion following pulsatile GnRH stimulation of rat pituitary cells exposed to inhibin (75ng/ml) for 24 hours before beginning the perifusion. Fresh inhibin was added to the perifusion medium for 3 hours, and then the cells were treated with medium alone. The control column was not treated with inhibin. (from Kotsuji *et al.: ibid)*

as in Figure 3A), for 24 hours before beginning the perifusion and then perifused with fresh inhibin for 3 hours. Figure 6 is a summary of the results of three such experiments. Maximum suppression of FSH was to 68 ± 1% of control; this suppression was less ($p < 0.01$) than that found during the short-term (5 hours) treatment (Fig 3A). Further, this dose of inhibin suppressed LH secretion in the short-term experiment but had no effect on GnRH-induced LH secretion by cells pretreated with inhibin for 24 hours. After removal of inhibin from the perifusate, FSH secretion returned to control levels within 2 hours. The decreased effectiveness of inhibin when present for 24 hours prior to perifusion may represent a refractoriness of the pituitary cells due to prolonged continuous receptor occupancy like that which occurs with other peptide hormones.[4] Another possibility is that inhibin was metabolized under the conditions of this study. However, fresh inhibin was added during the perifusion.

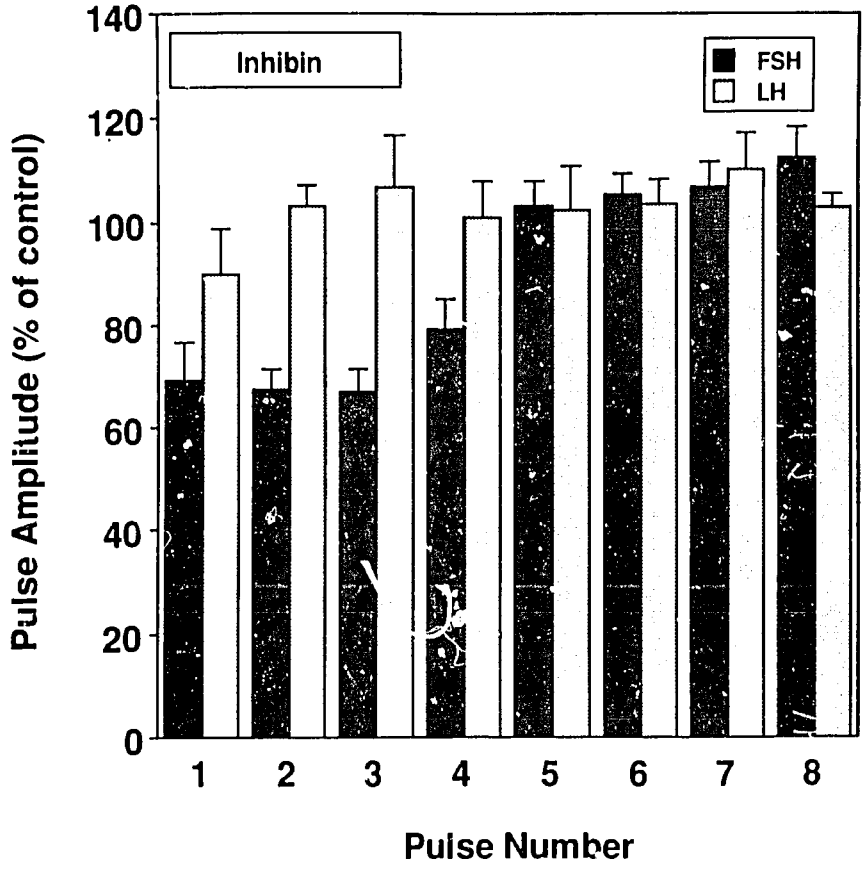


Figure 6. Effect of inhibin on FSH and LH pulse amplitude in cells exposed to inhibin for 24 hours before beginning the perifusion. The results are mean ± SEM of three experiments (from Kotsuji *et al.: ibid).*

We also examined the effect of inhibin on interpulse gonadotropin secretion, defined as the hormone measurable during the 30 minute intervals between GnRH-induced pulses. It is presently unclear whether LH and FSH secretion during these intervals is related to the GnRH stimulus and how these findings relate to gonadotropin secretion *in vivo*. We found a selective effect of inhibin on interpulse FSH secretion in both the short-term studies and in the experiments in which pituitary cells were pretreated for 24 hours with inhibin prior to beginning the perifusion (Fig. 7). This effect on FSH but not LH is similar to the effect of inhibin we observed on basal secretion from monolayer cultures for 24 hours (Fig. 1), as well as to the results of many previous studies with various preparations of inhibin.[8,10,12,20,21]

Based upon our results, we constructed a schematic diagram illustrating the effects of inhibin on interpulse and GnRH-stimulated FSH and LH secretion (Fig. 8). Clearly, *in vitro* factors such as duration of culture and relative purity of the RIA standards used influence this comparison, whereas *in vivo* the intensity and frequency of the GnRH stimulus as well as other central and negative feedback factors such as steroids will modify this relationship. We depict total FSH and LH secretion as equivalent in the absence of inhibin. In agreement with the results of studies *in vivo*,[5] we found that GnRH-independent (interpulse) FSH secretion comprised a greater proportion of total FSH secretion than did interpulse LH secretion. According to this model, the negative feedback actions of inhibin limit interpulse FSH but not interpulse LH secretion. Further, both GnRH-stimulated FSH and LH release are reduced by inhibin; however, the effect on FSH is greater than on LH. Together, these effects produce a pronounced suppression of FSH secretion and a smaller decline in LH secretion. Thus, we believe that two separate mechanisms mediate inhibin action, one relates to a decline in FSH synthesis,[5] and the second is coupled to the action of GnRH.

From these studies, we conclude that inhibin is a gonadal glycoprotein hormone which preferentially, but not selectively, suppresses GnRH-stimulated FSH secretion by perifused rat pituitary cells. Our experiments show that the magnitude and specificity of the inhibin effect on FSH and LH are dependent upon both the dose and duration of inhibin treatment. However, the applicability of these findings to our understanding of the physiological function of inhibin in males must await a comparison of the dosages used *in vitro* with circulating inhibin levels *in vivo*.

The rapid onset of the inhibin effect and the escape from inhibin suppression suggest that the effect of inhibin on FSH release from the pituitary can not be completely explained by changes in pituitary FSH content resulting from FSH synthesis. Taken together with the observed suppression of GnRH-induced LH release, we believe that the action of inhibin on the pituitary appears in part to be related to that of GnRH. Further, the rapidity of the inhibin suppression suggests that inhibin could influence ultradian gonadotropin secretion *in vivo*.

Finally, the observation that inhibin selectively suppressed interpulse FSH secretion was possible only through the use of a cell perifusion model. This model is applicable to the study of the various factors thought to influence the ability of the pituitary to respond to GnRH, and its further use should broaden our understanding of the mechanisms underlying gonadotropin synthesis and secretion. The effect we observed on interpulse FSH release may be due to a reduction in FSH synthesis[5] and a decrease in FSHβ gene expression.[14] Whether interpulse gonadotropin release *in vitro* is a valid model representing constitutive

secretion *in vivo*, or whether all *in vivo* gonadotropin secretion is coupled to GnRH stimulation remains to be clarified.

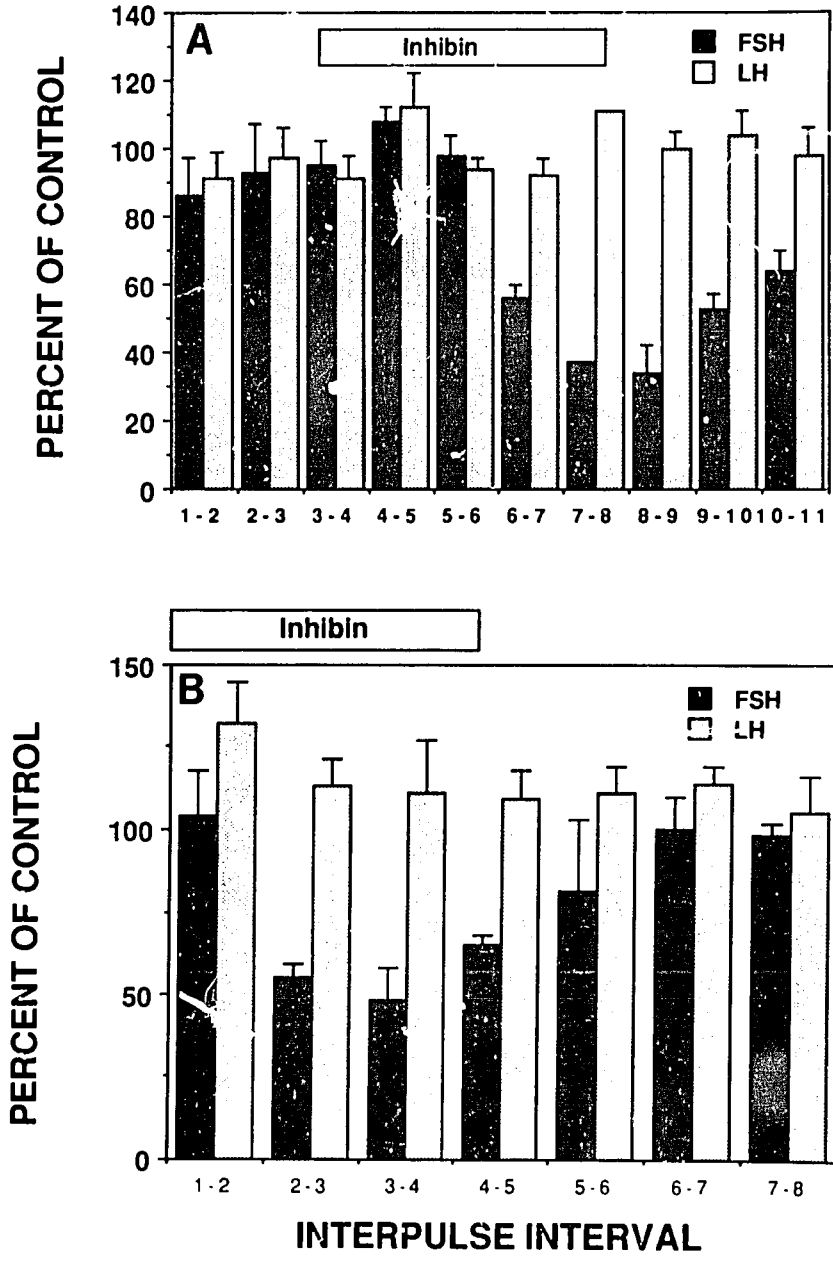


Figure 7. Effects of inhibin on interpulse secretion of FSH and LH. The top panel represents results during the short-term addition of inhibin; the lower panel depicts the results when inhibin was added to the cells for 24 hours before beginning the perifusion.

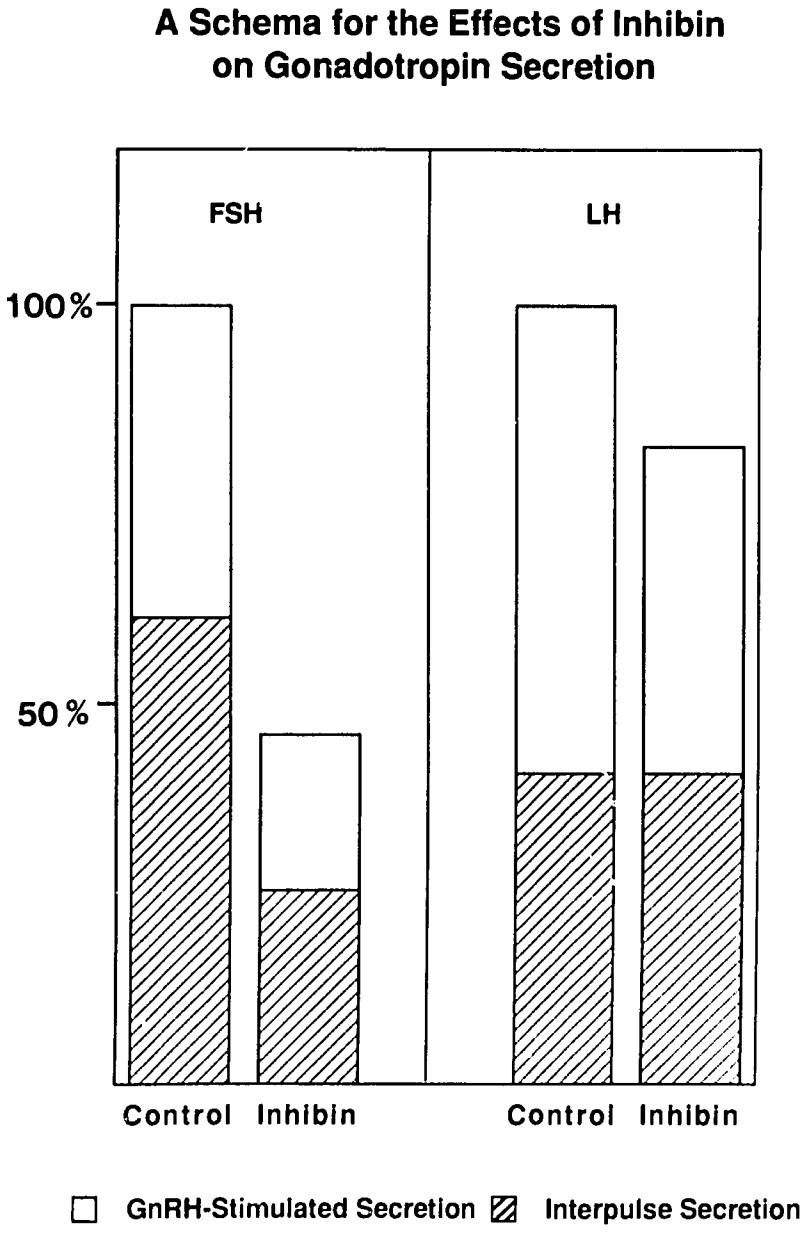


Figure 8. Diagramatic representation of the effects of inhibin on gonadotropin secretion. Total LH and FSH secretion in the absence of inhibin was arbitrarily drawn to be equivalent (100%). Total LH and FSH secretion during perifusion was calculated as the area under the pulse profiles. That portion of each pulse due to the interpulse level was subtracted from the GnRH-induced pulse amplitude; GnRH-independent release was summed and subtracted from the total hormone release during the study.

ACKNOWLEDGMENTS

The authors wish to acknowledge the able technical assistance of Ms. Joyce Szczepanski, Carol Weis and Dana Cline. We also thank Dr. Douglas Metzler, School of Library and Information Science, University of Pittsburgh, for assistance in the statistical analysis of these data. RIA Reagents were provided by Dr. A.F. Parlow through the National Hormone and Pituitary Program. This work was supported in part by NIH grants ROI-19546 (to S.J.W.) and ROI-12099 (to B.A.).

REFERENCES

1. BADGER TM: Perifusion of anterior pituitary cells: Release of gonadotropins and somatotropins. Methods Enzym 124:79, 1986
2. BLANC MR, HOCHEREAU-DE REVIERS MTH, CAHOREAU C, COUROT M, DACHEUX JL: Inhibin: effects on gonadotropin secretion and testis function in the ram and rat. In Intragonadal Regulation of Reproduction FRANCHIMONT P, CHANNING CP, (eds) p 299, London Academic Press, 1981
3. BOOTH JD, MERRIAM GR, CLARK RV, LORIAUX DL, SHERINS RJ: Evidence for Leydig cell dysfunction in infertile men with a selective increase in plasma follicle-stimulating hormone. J Clin Endocrinol Metab 64:1194, 1987
4. CATT KJ, HARWOOD JP, AGUILERA G, DUFAU ML: Hormonal regulation of peptide receptors and target cell responses. Nature 280:109, 1979
5. CHOWDHURY M, STEINBERGER A, STEINBERGER E: Inhibition of *de novo* synthesis of FSH by the Sertoli cell factor (SCF). Endocrinology 103:644, 1978
6. CULLER MD, NEGRO-VILAR A: Evidence that pulsatile follicle-stimulating hormone secreton is independent of endogenous luteinizing hormone-releasing hormone. Endocrinology 118:609, 1986
7. DEJONG FH, ROBERTSON DM: Inhibin: 1985 update on action and purification. Mol Cell Endocrinol 42:95, 1985
8. DEJONG FH, SMITH SD, VANDERMOLEN HJ: Bioassay of inhibin-like activity using pituitary cells *in vitro*. J Endocrinol 80:91, 1979
9. HUNTER WM, EDMONDS D, WATSON GS, MCLEAN N: Plasma LH and FSH levels in subfertile men. J Clin Endocrinol Metab 39:740, 1974
10. LEGAC F, DEKRETSER DM: Inhibin production by Sertoli cell cultures. Mol Cell Endocrinol 28:487, 1982
11. MARTIN GB, WALLACE JM, TAYLOR PL, FRASER HM, TSONIS CG, MCNEILLY AS: The roles of inhibin and gonadotrophin-releasing hormone in the control of gonadotrophin secretion in the ewe. J Endocrinol 111:287, 1986
12. MASSICOTTE J, LAGACE L, LABRIE F, DORRINGTON JH: Modulation of gonadotropin secretion by Sertoli cell inhibin, LHRH and sex steroids. Am J Physiol 247:E495, 1984
13. MECKLENBURG RS, SHERINS RJ: Gonadotropin response to luteinizing-hormone releasing hormone in men with germinal aplasia. J Clin Endocrinol Metab 38:1005, 1974
14. MERCER JE, CLEMENTS JA, FUNDER JW, CLARKE IJ: Rapid and specific lowering of pituitary FSHβ mRNA levels by inhibin. Mol Cell Endocrinol 53:251, 1987
15. NANKIN HR, CASTANEDA E, TROEN P: Endocrine profiles in oligospermic men. In TROEN P, NANKIN HR (eds.): The Testis in Normal and Infertile Men, p 529. New York, Raven Press, 1977,
16. NANKIN HR, TROEN P: Endocrine profiles in idiopathic oligozoospermic men. In HAFEZ (ed): Human Semen and Fertility Regulation in men, p 370 St Louis, Mosby, 1976
17. NOGUCHI K, KEEPING HS WINTERS SJ, SAITO H, OSHIMA H, TROEN P: Identification of inhibin secreted by cynomolgus monkey Sertoli cell cultures. J Clin Endocrinol Metab 64:783, 1987
18. ROSEN SW, WEINTRAUB BD: Monotropic increase of serum FSH correlated with low sperm count in young men with idiopathic oligospermia and aspermia. J Clin Endocrinol Metab 32:410, 1971
19. SCOTT RS, BURGER HG: An inverse relationship exists between seminal plasma inhibin and serum follicle-stimulating hormone in man. J Clin Endocrinol Metab 52:796, 1981

20. SCOTT RS, BURGER HG, QUIGG H: A simple and rapid *in vitro* bioassay for inhibin. Endocrinology 107:1536, 1980
21. SHANDER D, ANDERSON LD, BARRACLOUGH CA, CHANNING CP: Interactions of porcine follicular fluid with ovarian steroids and luteinizing hormone-releasing hormone on the secretion of luteinizing hormone and follicle-stimulating hormone by cultured pituitary cells. Endocrinology 106:237, 1980
22. SMITH MA, VALE WW: Desensitization to gonadotropin-releasing hormone observed in superfused pituitary cells on cytodex beads. Endocrinology 108:752, 1981
23. VAN THIEL DH, SHERINS RJ, MYERS GH JR, DE VITA VT JR: Evidence for a specific seminiferous tubular factor affecting follicle-stimulating hormone secretion in man. J Clin Invest 51:1009, 1987

DISCUSSION

CHAIRPERSON: DO WON HAHN, Ph.D.

DR. HOFFMAN: In your profiles FSH pulse amplitude was decreased when inhibin was added. However, FSH pulse amplitude was decreased in control studies as well. Could you explain?

DR. WINTERS: That is true. In certain of our experiments, the pituitary cells became less responsive to GnRH over the course of the study. This effect has been observed by others who have done cell perifusion experiments. For this reason, we perifused two columns simultaneously in each experiment. One column was treated with inhibin and the second was not. Both columns contained cells from the same dispersion, and both columns were pulsed with GnRH. All comparisons were drawn between the inhibin-treated and its paired untreated column.

DR. GOODMAN: When you culture cells on Cytodex 3, which is Sephadex in a macrospherical form, is it possible that inhibin is trapped within the Sephadex explaining the decreased effect with longer incubation times?

DR. WINTERS: We have not yet measured immunoreactive inhibin levels in our incubation media to determine whether inhibin was metabolized by the cells, or perhaps as you suggest, that inhibin was adsorbed on to the beads. However, we doubt that adsorption occurred since the anterior pituitary cells were allowed to attach to the beads for 48 hours in culture medium containing 10% fetal calf serum prior to adding inhibin. Serum glycoproteins would attach to the beads and should block the subsequent adsorption of inhibin. Cytodex 3 beads also have a layer of denatured collagen on their surface. Finally, fresh inhibin was added during the perifusion.

DR. MANBERG: Have you looked at the kinetics of the tolerance you observed? Do you think this phenomenon will limit any therapeutic applications of inhibin?

DR. WINTERS: We have only performed the 24 hour and short-term experiments I presented today. Therefore, we do not know what duration of exposure is needed for the inhibin effect to decline. I believe that the possibility of desensitization to the effect of inhibin is one with potential clinical importance. Both the dose and method of delivery of inhibin may be of significance.

DR. MANBERG: Both the data from Dr. Burger's group and your data indicate that inhibin blocks GnRH-induced FSH and LH secretion. Dr. Burger also showed that inhibin reduced GnRH receptor binding by the pituitary cells. Yet this morning, Dr. Miller indicated that inhibin increased GnRH-induced FSH secretion. How are these data reconciled?

DR. WINTERS: I believe that this dichotomy results from species differences in gonadotropin secretion *in vitro*. Dr. Miller's convincing data were obtained from cultured ovine pituitary cells whereas our studies used pituitary cells from male rats. Dr. Miller has previously shown that the control of FSH secretion *in vitro* is very different in rat compared to ovine cells. We have not yet measured GnRH receptor binding. It will be of considerable interest to investigate both receptor and post-receptor events in inhibin-treated anterior pituitary cells.

QUESTION: Dr. Burger, was the inhibin effect on GnRH receptor number rather than a change in affinity?

DR. BURGER: Yes, that is correct.

13

Role of Inhibin-Related Peptides in Human Placenta

FELICE PETRAGLIA
WYLIE VALE

The human placenta plays an important role in the initiation and maintenance of pregnancy. In particular, the placenta produces a large variety and quantity of peptide and steroid hormones which influence several functions of the maternal-fetal unit. The trophoblast cells have endocrine competence and form the placental villi. Each villus consists of an outermost layer of syncytiotrophoblast cells and an inner layer of cytotrophoblast cells. Syncytiotrophoblast cells are multinucleated and are rich in the structure required for protein synthesis and secretion (rough endoplasmic reticulum, Golgi complex and mitochondria). Cytotrophoblast cells are mononucleated and seem to represent the stem cells from which the syncytiotrophoblast cells derive. Hormones produced by the trophoblast cells are measurable in the maternal blood, but lower concentrations of placental hormones are also present in the fetal circulation. The regulatory mechanisms for the production of placental protein and steroid hormones are well defined.[17,33,50] In the present study we investigated the presence of inhibin-related peptides in human placenta and their possible role in regulating the secretion of placental gonadotropin.

HUMAN CHORIONIC GONADOTROPIN

From the first days of pregnancy the cells for the trophoblast synthesize and secrete hormones. The most specific placental hormone detectable in maternal circulation immediately after implantation is the human chorionic gonadotropin (hCG). This glycoprotein hormone is composed of two subunits (α=97 amino acids, identical to that of pituitary LH, FSH, and TSH; β=145 amino acids) and is secreted by the syncytiotrophoblast cells. At the time of implantation, the blastocyst covered by the trophoblast penetrates into the endometrial stroma, projecting the microvilli. The intimate contact between trophoblast cells and uterine spiral arterioles explains the presence of hCG in maternal circulation. Between 9 and 12 weeks of gestation, plasma hCG levels reach their peak values. The exponential rise of hCG values is typical during the first trimester of pregnancy, with a doubling time of 1.7 to 2.0 days during the first 4 week.[5,24] Subsequently, the production rate of hCG by placental cells decreases, with a concomitant fall in maternal plasma levels. The synthesis and secretion of hCG is fundamental to maintain the function of the corpus luteum during the first few weeks of pregnancy.[52,55] In fact, placental hCG stimulates progesterone release

by the corpus luteum activating a cAMP-dependent protein kinase.[45] Moreover, a local immunosuppressive action of hCG has been suggested, either modulating the activity of maternal lymphocytes or representing an immunologically protective layer on the trophoblast's cell surface,[8,38] or stimulating the secretion of progesterone, which is known to facilitate the implantation also via an immunosuppressive effect to prevent the rejection of the fetal allograft by the maternal host.[4,11]

The importance of hCG in pregnancy is supported by evidence that the measurement of plasma hCG correlates with the outcome of pregnancy in patients with threatened abortion or in cases of *in vitro* fertilization embryo transfer.[7,9]

The mechanisms that control hCG secretion are very complex. Following the isolation of an immunoreactive gonadotropin-releasing hormone (irGnRH) in human placenta, the presence and action of a local axis similar to the hypothalamus-pituitary-gonadal axis has been suggested.

PLACENTAL GnRH

Placental irGnRH has a stimulatory role in hCG secretion. The addition of synthetic GnRH increases hCG release from human placental preparation.[2,13,19,34,47] The evidence that a GnRH antagonist is able to decrease hCG secretion *in vitro* suggests that local GnRH has a constant stimulatory role in hCG secretion from human placenta.[49] Placental GnRH has the same chemical, immunological and biological activities as the hypothalamic decapeptide[20,46,51] and binding sites for GnRH have been found on placental cell membranes.[2,10] Cytotrophoblast cells contain irGnRH and the intensity of the immunostaining decreases during pregnancy.[31] An irGnRH has been found also in rat placenta, with concentrations decreasing progressively through gestation.[42] The release of GnRH from culture trophoblast cells seems to be mediated by an adenylate cyclase/cyclic AMp pathway.[6] The addition of forskolin, an adenylate cyclase activator, or of dybutyrill cAMP, a synthetic analog of cAMP, increases the release of irGnRH from placental cultures. Beta adrenergic receptor agonists, epinephrine and isoproterenol, stimulate the secretion of irGnRH from placental cells activating the formation of cAMP (Fig. 1). Because β adrenergic acting substances also increase progesterone release from placental tissue,[6] a role for the cathecolamines in the control of the placental hormone secretions may be hypothesized.

The possible existence of a functional axis among GnRH, hCG and progesterone is supported by several lines of evidence. However, after the 12-14th week of pregnancy, plasma GnRH and hCG dissociate from plasma progesterone levels in natural circulation. While peptide hormones reach a steady state concentration, progesterone levels continue to increase until the end of the gestation. This pattern suggests a different mechanism of control regulating the secretion of hCG and progesterone. The second messenger involved in the release of hCG and progesterone is the cAMP[15] and the activity of placental adenylate cyclase is known to be modulated by several factors.[29,43,44] Endocrine and paracrine interactions may locally regulate placental hormonogenesis, but maternal and fetal products may also influence the activity of the trophoblast cells. This particular system of control is unique in nature. The metabolic, endocrine, immune and behavioral changes described in the maternal compartment during gestation may be due to the secretory activity of

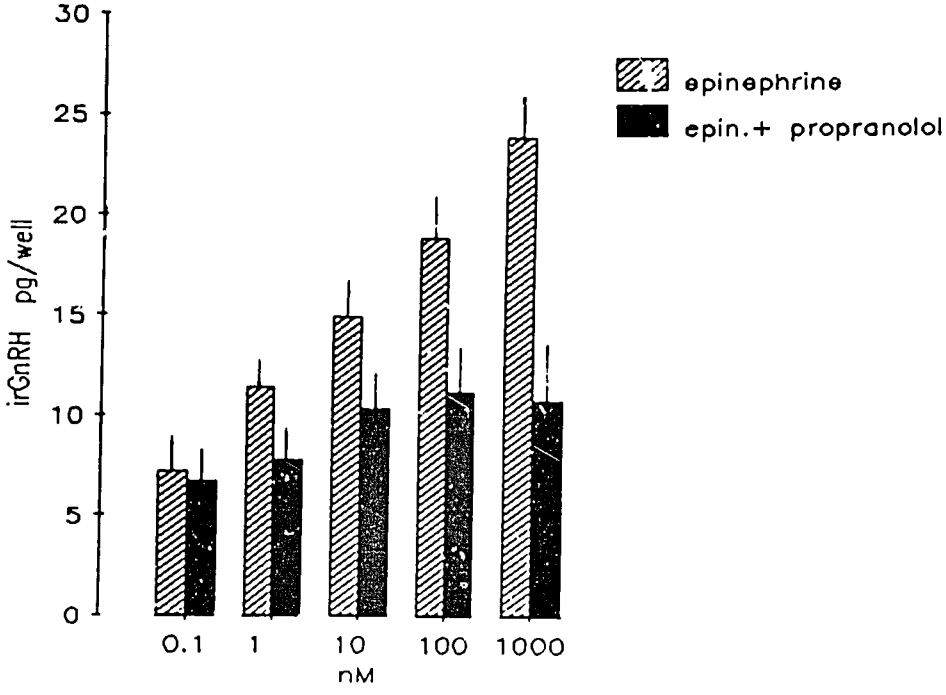


Figure 1. Dose-related effect of epinephrine on gonadotropin-releasing hormone (irGnRH) release from human cultured cells. Propranolol (1 um), a β adrenergic receptor antagonist, reverses this effect.

placental cells and, vice versa, the mother may communicate the feedback signals to the placenta. However, intraplacental regulation seems to play major role in the control of placental hormone secretions. The administration of a GnRH antagonist for 7 days in 3 pregnant baboons at the first month of pregnancy resulted in 2 stillbirths and 1 small liveborn at delivery.[55] This result suggests a possible important role of a GnRH receptors in the maintenance of pregnancy in primates. These *in vivo* data agree with the evidence that the addition of a GnRH antagonist in human placental cultures inhibits the hCG and progesterone release, suggesting a tonic stimulatory action of GnRH on these hormonal placental secretions.[49]

PLACENTAL INHIBIN-RELATED PEPTIDES

The recent purification of inhibin from the gonads of various species[12,21,25,32,40,41] allowed study of the possible presence of a placental inhibin. Two forms of inhibin are recognized, inhibin A and B, both of which are heterodimeric glycoproteins with a molecular weight of about 32,000 daltons consisting of an α and β subunit. The α subunit is common in both forms and there are many homologies among porcine, bovine and human forms. The β subunit is variable and the two forms (A and B) are characterized as inhibin A and B, respec-

tively. There is a very high degree of homology between the βA and the βB chains of inhibin and for each chain between species. Disulfide bridges link the α and β subunits and they are encoded by different messenger RNAs. No difference has been described in the biological properties of inhibins A and B: they both significantly decrease FSH secretion from cultured pituitary cells.[12,21,25,40,41]

Moreover, recent studies revealed that the homodimers composed by two βA chains or by a βA and βB chain have a complete opposite action, stimulating the pituitary FSH release. These polypeptides have a molecular weight of about 28,000 daltons and have been called FSH—releasing peptide (FRP) or activins.[22,23,53] The homology between the β subunits of inhibin and the transforming growth factor-B (TGF-B), the Mullerian duct inhibiting factor (MIF) or the erythroid differentiation factor (EDF) suggests an interesting correlation in the evolution of these proteins. The sources of inhibins in all previously mentioned studies were the seminal and follicular fluids suggesting the gonads as major sources for these peptides.

McLachlan *et al.* and our group have recently shown the presence of bioactive and immunoreactive inhibin in human placenta.[27,35] The existence of a placental inhibin was suggested by the presence of FSH—inhibiting activity in rabbit and human placentas.[1,46] In their study, McLachlan *et al.*[27] have shown that extracted placenta inhibin decreases pituitary FSH content in a dose-dependent manner, and this effect was parallel to that of human follicular fluid inhibin. The same parallelism between placental and follicular fluid inhibin was found in the radioimmunoassay, suggesting an analogy between placental and gonadal inhibins. This similarity was also supported by the evidence that the preincubation of placental extracts with inhibin antiserum completely blocked the inhibin bioactivity of the extracts. Mayo *et al.* reported the presence of inhibin a subunit mRNA in the cDNA library of placenta at term.[26]

LOCALIZATION OF PLACENTAL INHIBIN

Using an antiserum directed against the first 27 amino acids (N-terminal portion) of porcine inhibin, we have found many immunoreactive cells in the cytotrophoblast layer of the human placental villi. The fluorescent staining was localized in the inner cell and not on the periphery of the villi. The cytotrophoblast cells are those positive also for GnRH, somatostatin and CRF immunostaining.[26,54] The observation that these cytotrophoblast-produced peptides have the common characteristic to regulate the secretion of other placental hormones produced by syncytiotrophoblast cells supports the classification of trophoblast cells in two distinct functional populations, with a different hormonal competence. The regulatory peptides secreted by cytotrophoblast cells may play an endocrine and/or a paracrine role, modulating both maternal and placental hormone secretions.

SECRETION OF PLACENTAL INHIBIN

Using a system of cultured human placental cells, we have studied the possible mechanisms of secretion of placental inhibin and the possible action on the secretion of irGnRH and hCG. Placentas collected from pregnant women at term were used to prepare the trophoblast cultures. The cells were dispersed enzymatically and mechanically and suspended in culture

medium.[34] The experiments were done one week after plating and inhibin-LI concentrations were measurable in the culture medium following 48 hours incubation. The addition of a cAMP analog, 8 bromo cAMP, or cholera toxin, increased the release of inhibin-LI from cultured placental cells, suggesting that cAMP may be the second messenger also in the release of placental inhibin. A significant release of inhibin-LI was observed when hCG was added to placental cell cultures.[35] An increase of plasma inhibin-LI following hCG injection has been also shown in immature female rats,[39] and this effect seems to reflect the action of hCG on cultured rat granulosa cells.[3] Therefore, the regulation of inhibin secretion in the placenta is identical to that recently shown in the gonads, stimulated by gonadotropins via the cAMP pathway.

We further evaluated the effect of various neuropeptides on the inhibin release from placental cultures. Vasoactive intestinal peptide (VIP) and neuropeptide Y (NPY) increased the inhibin release in a dose-related manner (Fig. 2). No effects have been observed following the addition of somatostatin, oxytocin or substance P (SP). These results show that the activity of placental inhibin may also be influenced by various circulating or placental substances.

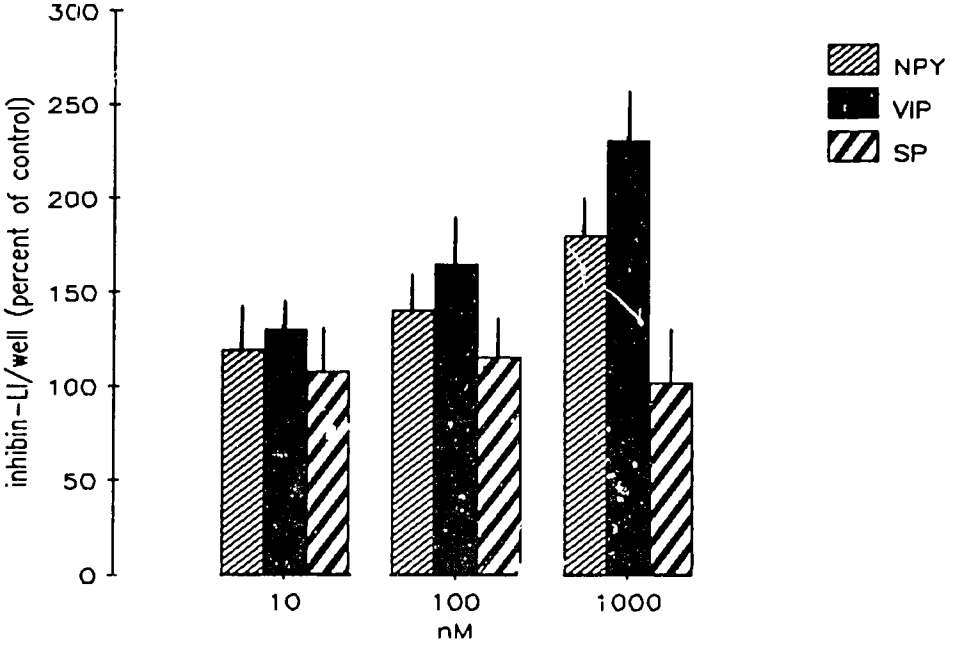


Figure 2. Dose-related effect of chorion neuropeptides (neuropeptide Y (< NPY >; vasoactive intestinal peptide <VIP>; substance P < SP >) on inhibin-like immunoreactivity (II) release from human placental cell cultures.

ACTION OF INHIBIN ON PLACENTAL HORMONOGENESIS

Because the gonadal inhibin acts to inhibit FSH release from the pituitary gland, we hypothesized a possible action of placental inhibin on hCG release. The addition of an antiserum to inhibin in the placental cell cultures significantly increased hCG release, a dose-related and time-dependent effect.[14] This evidence suggests that placental inhibin tonically inhibits the release of hCG in human placenta at term. Therefore cytotrophoblast inhibin may have a local paracrine action regulating the syncytiotrophoblast release of hCG. However, high plasma inhibin levels are present in pregnant women. Also in cord blood inhibin-LI is two/threefold higher than in the peripheral circulating blood of non-pregnant subjects, suggesting also an endocrine role of the placental inhibin.[27,35] Indeed, plasma FSH levels in pregnant women are very low from the first 10 weeks of gestation and the FSH response to GnRH administration is almost absent in pregnant women.[16,17,30] The increase in circulating inhibin, also released by the placenta, may explain this endocrine change in the maternal physiology. As shown before, the control of hCG release is also under the stimulatory action of placental GnRH and the inhibitory action of inhibin is also mediated by a decrease in GnRH release. Indeed, the addition of an antiserum to inhibin in the placental cell cultures increases GnRH release, thus suggesting an inhibitory action of inhibin on GnRH. The evidence that the antiserum-induced release of hCG from placental cultures is blunted by a GnRH antagonist indicates that inhibin acts both on hCG and GnRH secretion from the placenta. However, a purified inhibin preparation did not significantly decrease hCG or GnRH release from placental cell cultures.[55] This result may seem contradictory with the hypothesis that inhibin plays a role in the inhibitory control of hCG secretion, but in our culture system it is difficult to detect the decrease of hCG or GnRH concentrations. The GnRH-induced increase in hCG release from placental cells was reversed by inhibin (Fig. 3), supporting the view that placental inhibin may act on the local GnRH-hCG axis.[28]

PLACENTAL FRP

The presence of a placental FRP has been suggested following the identification of the human β subunit homodimer mRNA in a term placenta.[28] We have investigated the possible effects of purified FRP on irGnRH, hCG, and progesterone release from cultured human placental cells. The addition of FRP for 48 hours to the placental cultures significantly increased the concentrations of irGnRH and progesterone in the culture medium.[37] Moreover, FRP augmented the GnRH-induced release of hCG (Fig. 3), suggesting that this inhibin-related peptide may also play a role in the mechanism regulating the hCG secretion.

CONCLUSIONS

The present findings show that inhibin and FRP play an important role in the regulation of placental hormonogenesis *in vitro*. It is difficult to understand the role of these heterodimeric and homodimeric proteins in the maternal-fetal physiology. However, because of the effect of hCG and progesterone in the initiation and maintenance of human

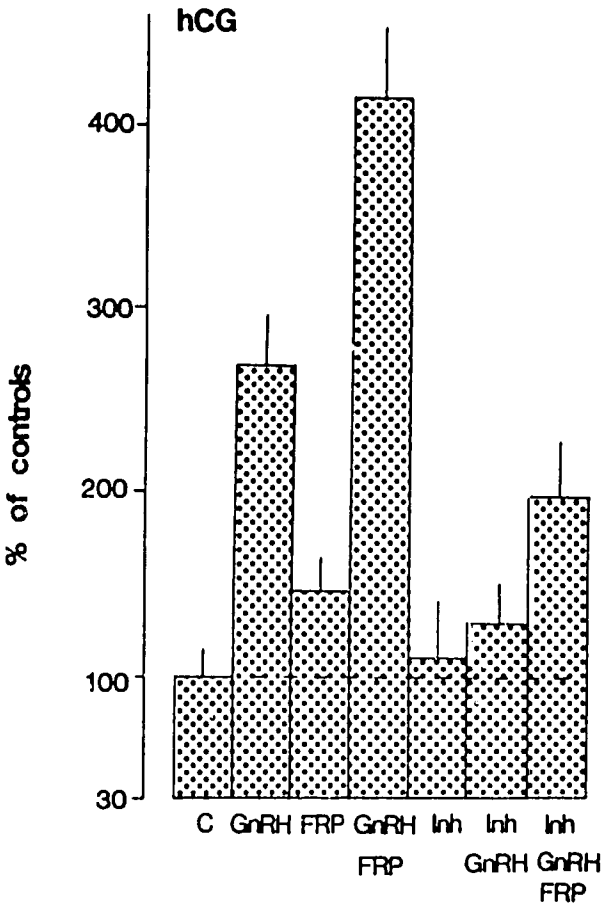


Figure 3. Inhibin (3 ng/ml) decreases and FRP (FSH releasing peptide) (5 ng/ml) increases the GnRH-induced (100nM) release of hCG from human cultured placental cell. Inhibin also antagonizes the FRP-GnRH effect on hCG. Inhibin and FRP do not change significantly basal hCG release.

pregnancy, the importance of inhibin and FRP derives from their role of regulatory peptides. The actual knowledge suggests a complex model of interaction among GnRH, inhibin and FRP in influencing the release of hCG and progesterone (Fig. 4). It can be proposed that during gestation the locally produced releasing factors (GnRH and FRP) may have a positive influence on hCG secretion, and inhibin counteracts this effect. From the balance among these three factors may depend the typical pattern of maternal plasma hCG level changes, with the peak values during the first trimester and stable levels in the remaining part of gestation. Moreover, inhibin-related peptides regulating hCG secretion may also affect the biological functions related to hCG, possibly contributing to the pathogenesis of women's infertility states. Therefore, a clinical use for inhibin to antagonize

ONTOGENY OF OVARIAN AMH PRODUCTION

The ontogeny of testicular and ovarian AMH is also quite different. Whereas AMH production begins in Sertoli cells immediately after they differentiate in the fetal testis,[28] AMH is not detectable in ovine fetal granulosa cells, until the day before birth.[3] The ontogeny of AMH production by granulosa cells has been studied by immunocytochemical methods in the ovary of the developing cow[25] and ewe.[3] AMH production by granulosa cells is influenced both by the age of the animal — AMH synthesis begins at birth, but is maximal in the adult ovary — and by the degree of follicular development — only those granulosa cells populating growing and antral follicles are stained by the immunocytochemical reaction. As the follicle grows, peripheral granulosa cells progressively lose their capacity for AMH synthesis, in contrast to those lining the antral cavity and the oocyte, which contain a high amount of immunoreactivity[3] (Fig. 2). Staining of primordial follicles was never observed, whatever the age of the animal.[3,25]

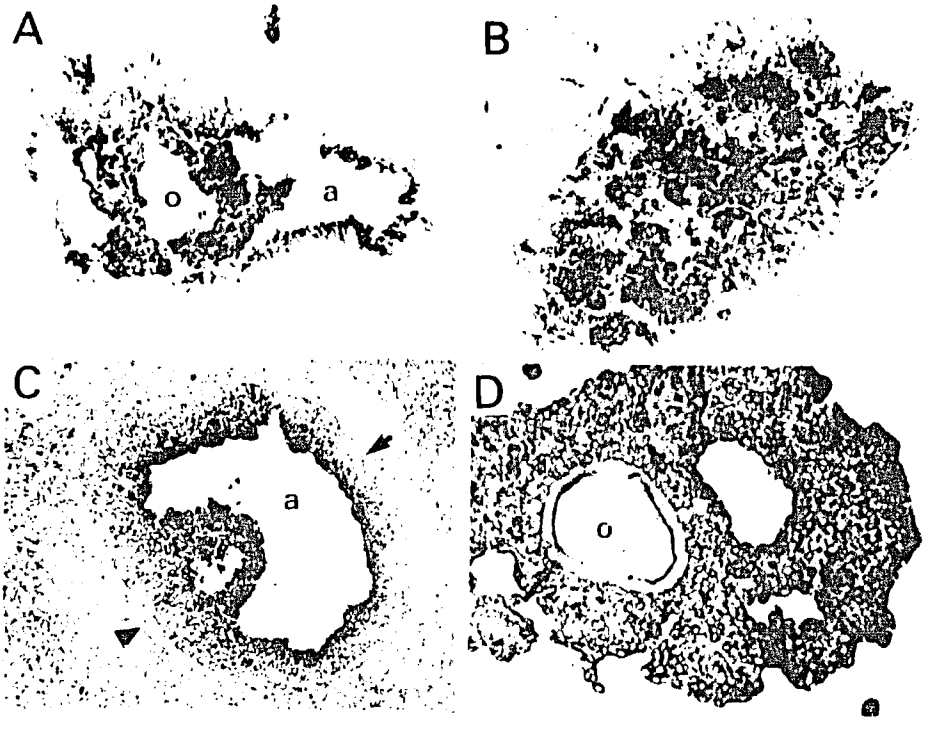


Figure 2. Immunocytochemical reaction for AMH in ovaries of developing ewes, using a monoclonal antibody and an avidin-biotin technique. (A) Small antral follicle, the day preceding birth : O: oocyte, A: antrum. X 200. (B) Growing follicle in a 97 day-old, immature animal : strong immunoreactivity is observed. x 200. (C) Antral follicle in a 97-day-old animal : note the AMH gradient from the basal membrane to the antrum and the oocyte. No immunoreactivity is observed in thecal cells (X 125). (D) Antral follicle in an adult ovary : note AMH immunoreactivity in cumulus cell protrusions across the zona pellucida (arrow) O: oocyte X 200.

EFFECT OF AMH ON OOCYTE MATURATION

Since AMH is produced by granulosa cells of the antral follicle of the adult[3,25] and particularly by those in contact with the oocyte,[3] and since hCG increases this production,[33] efforts have been made to find a physiological effect for ovarian AMH. Takahashi *et al.*[26] have reported that "fraction Green-3," a partially purified preparation of bovine AMH[4,5] at concentrations of 15 fg to 150 pg/ml, is capable of inhibiting the resumption of meiosis in denuded and cumulus-cell enclosed rat oocytes. We have seen no such effect using up to 9 ug/ml of bioactive bovine AMH purified to homogeneity by immunoaffinity chromatography on a monoclonal antibody as described by Picard and Josso.[20] Under the same conditions, a partially purified preparation of oocyte meiosis inhibitor from porcine follicuar fluid exerted an approximately 50% inhibition of meiotic resumption of rat oocytes. Obviously, fraction Green-3, which causes partial regression of the anterior tip of the rat fetal Müllerian duct when added to organ culture medium at a concentration of 6 μg/ml, contains other proteins in addition to pure AMH, which, at a concentration of 1 to 1.5 μg/ml, wipes out the Müllerian epithelium on its entire length (Fig. 1). The discrepancy between the effects of fraction Green-3 and pure AMH could conceivably be due to these contaminants, or to differences in assay conditions.

EFFECT OF AMH ON OVARIAN DIFFERENTIATION

Since AMH is not produced by prenatal granulosa cells, the developing ovary is not exposed to it under normal physiological conditions. Freemartinism[15] is an exception to this rule. When heifers are united to a male twin by chorionic vascular anastomoses, their ovaries cease to grow, become depleted of germ cells, and eventually, in approximately half the cases, develop seminiferous tubules.[14] Vigier *et al.*[32] have recently shown that purified bovine AMH produces the same effects on developing fetal rat ovaries, strongly suggesting that AMH is the testicular factor responsible for triggering the morphological abnormalities of freemartin gonads. Fourteen-day-old fetal rat ovaries, exposed 5 days to AMH in organ culture, lose 80% of their germ cell population, compared to ovaries cultured in control medium (Fig. 3). AMH acts essentially by blocking oogonial replication between days 14 and 17, meiotic maturation is not affected, except in extreme conditions, after 10 days in culture.[32] AMH also mimics the freemartin effect by inducing formation of seminiferous cord-like structures, with polarized epithelioid cells aligned along a basement membrane (Figs. 4 and 5). After 20 days post-coitum, fetal ovaries are no longer sensitive to AMH, therefore endogenous hormone, produced by postnatal granulosa cells, cannot affect ovarian maturation, just as it is unable to inhibit the development of Müllerian derivatives.[30]

EFFECT OF AMH ON OVARIAN AROMATASE ACTIVITY

Although estrogen is not essential to female sex differentiation, endocrine differentiation of the fetal ovary leading to estrogen formation from appropriate precursors has been described in various species, including man,[8] rabbit,[9,16] and cow.[21,22] Estrogen production occurs very early, at the time testosterone synthesis begins in the fetal testis, granulosa

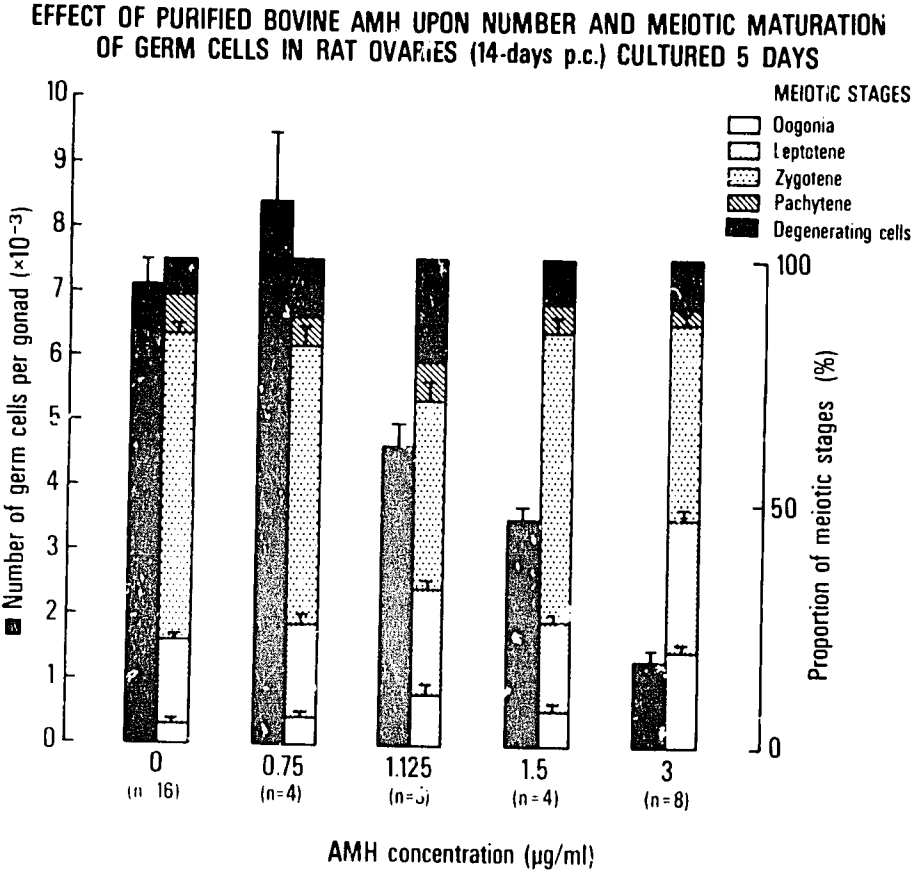


Figure 3. Effect of increasing concentrations of purified bovine AMH upon number and meiotic maturation of germ cells in 14-day-old fetal rat ovaries cultured for 5 days. AMH reduces the total number of germ cells in a dose-dependent manner. In contrast, the progression of meiotic prophase is affected only at the highest dose used. From Vigier *et al.*, 1987, with permission.

cells are not yet differentiated. The capacity for estrogen synthesis can be jeopardized by exposure to fetal testicular secretion: the gonads of freemartin heifers produce testosterone instead of estradiol.[23] To determine whether disruption of steroidogenic pathways in fetal ovaries could be mediated by AMH, we have measured testosterone and estradiol production in ovine fetal ovaries exposed to AMH in organ culture. As shown in Figure 6, 29-day-old fetal ovine ovaries exposed to 2.25 µg/ml AMH underwent endocrine sex reversion: the amount of immunoreactive testosterone released was comparable to that produced by fetal testes under similar conditions, and estradiol secretion was abolished (Fig. 6).

Since the main enzymatic difference in the steroidogenic pathway required for testosterone and estradiol synthesis is the presence of aromatase P450 in the ovary, allow-

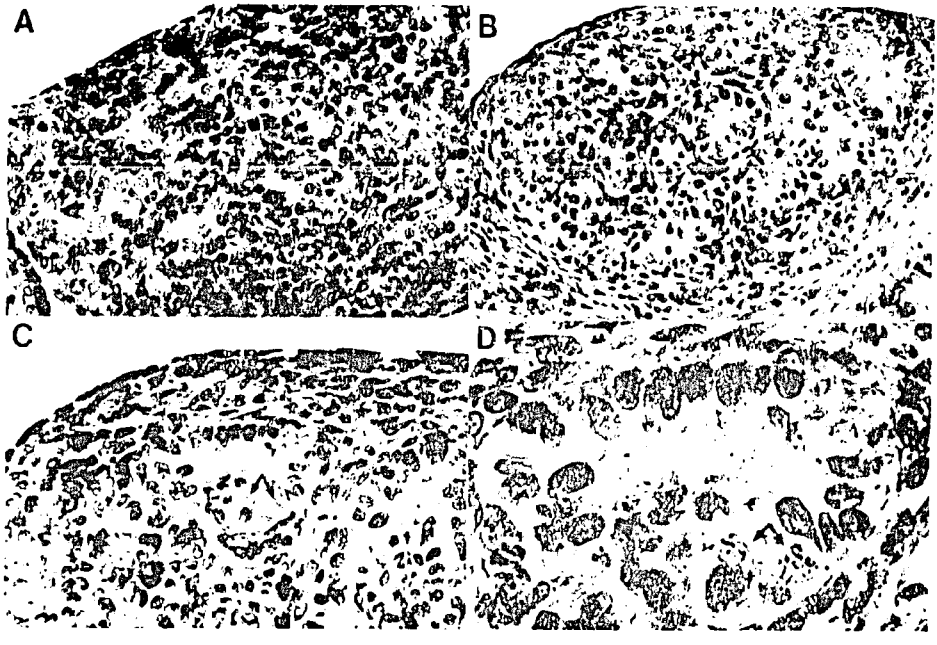


Figure 4. A X 290, Ovary cultured in control medium. Germ cells, in leptotene and zygotene stages of meiotic prophase, are scattered in an undifferentiated blastema. B X 290,C X 450, D X 1125 Effect of AMH (1.5 μg/ml) on the structure of 14.5-day-old rat fetal ovaries explanted 10 days in organ culture. Note conspicuous cord-like structures, delineated by a basement membrane, lined by fusiform mesenchymal cells. Epithelioid somatic cells are polarized along the basement membrane. Note the presence of two germ cells in meiotic prophase (arrows). Hematoxylin-eosin. From Vigier *et al.*, 1987, with permission.

ing estradiol to be formed from either testosterone or androstenedione, it appeared logical to investigate the effect of AMH on aromatase activity. To this effect, undifferentiated fetal ovine and rabbit ovaries were cultured in the presence or absence of AMH, and development of aromatase activity was estimated by conversion of tritiated testosterone or androstenedione to either estradiol (Fig 7) or tritiated water (Fig. 8), as described respectively by Milewich *et al.*,[16] and by Ackerman *et al.*[1] Both techniques revealed a dramatic inhibition of aromatase activity by AMH, 2.25 μg/ml. Experiments are underway to determine whether AMH can also inhibit aromatase activity in adult granulosa cells.

CONCLUSIONS

In conclusion, AMH, produced by postnatal granulosa cells, has been shown to exert various deleterious effects upon fetal ovarian development. Fetal ovaries exposed to AMH lose their germ cell population, and become masculinized both from a morphological and

Figure 5. Seminiferous cord-like structures in the anterior pole of a 14-day-old fetal rat ovary, cultured 5 days in the presence of AMH (3 μg/ml). A Semithin section (X 285), showing seminiferous cord-like structures, separated by dense fusiformo mesenchymal cells. B Electron micrograph showing somatic epithelial-like cells, with abundant, clear cytoplasm, joined by interdigitations (arrows) and grouped in cord-like structures. These cells resemble developing Sertoli cells, except for the lack of rough endoplasmic reticulum. Connective tissue is differentiating outside the cords. (X 3115). g : germ cell. From Vigier *et al.*, 1987, with permission.

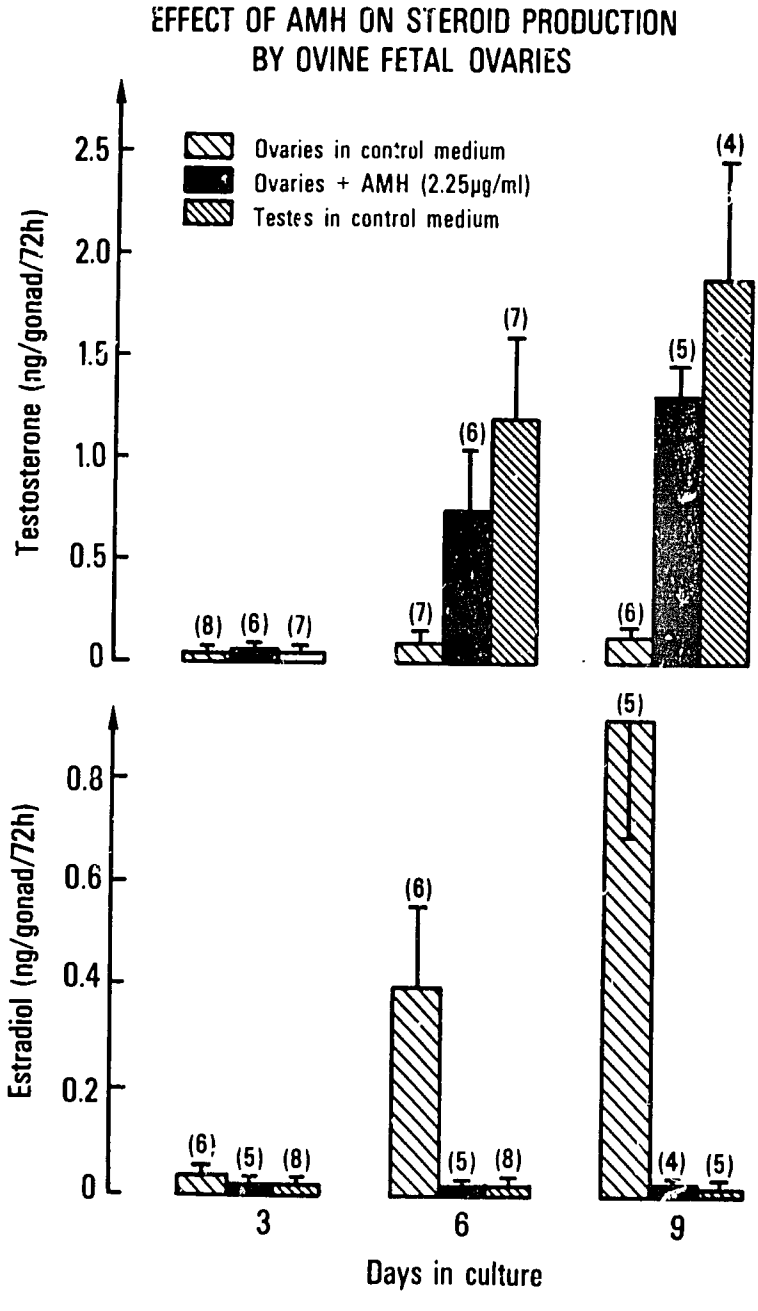


Figure 6. Steroid production, over a 3 day period, by 29-30-day-old ovine fetal gonads after 3, 6, or 9 days in organ culture, in the presence or absence of AMH. A) Testosterone production by fetal ovaries is significantly increased by AMH treatment, and nearly reaches male levels B) Estradiol production is annihilated by AMH treatment, down to male levels.

endocrine standpoint. Fortunately, except in bovine freemartin fetuses, developing ovaries are seldom exposed to AMH, and their sex-reversion can hardly be taken as a physiological achievement. The effect of AMH on the adult ovary is a controversial issue, since, in contrast to others, we ourselves have been unable to influence oocyte maturation using purified bioactive AMH. Teleological minds insist that AMH must fulfill a physiological function in the adult ovary, since it is produced by adult granulosa cells. Another teleological view can however be suggested. Since male and female gonadal somatic cells produce the same proteins, the ovary should produce AMH only after the female reproductive tract has become impervious to its sexist assault. Adult Sertoli and granulosa cells produce a minimal amount of AMH, but at that point, AMH can no longer harm either ovaries or Müllerian duct derivatives. By postponing initiation of AMH secretion by the ovary, Mother Nature protects females from turning into freemartins.

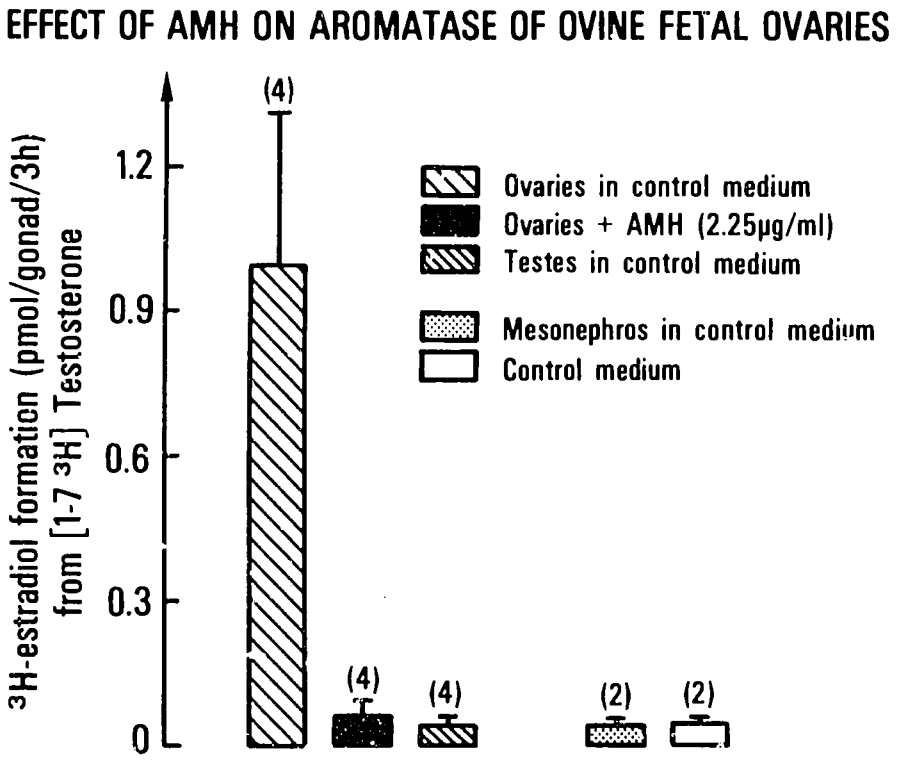


Figure 7. Effect of AMH on ovarian aromatase activity in 29-day-old sheep fetuses, after 9 days in organ culture in the presence or absence of AMH. Aromatase activity is estimated through tritiated estradiol formation from (7-^{3}H) testosterone precursor (0.5 μM), in the course of a 3 hour incubation. Aromatase activity is clearly decreased by AMH treatment

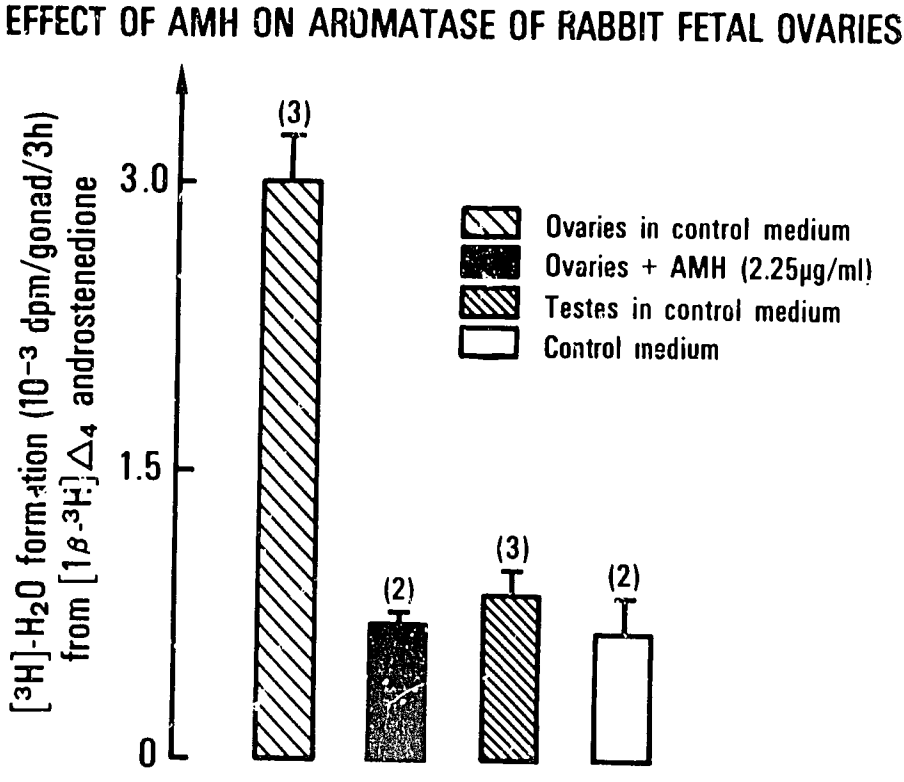


Figure 8. Effect of AMH on ovarian aromatase activity in 16-17-day-old rabbit fetuses, after 3 days in organ culture, in the presence or absence of AMH. Aromatase activity is estimated from tritiated water formation from (1β-^{3}H) Δ-4-androstenedione (0.5 μM) as a precursor. AMH dramatically decreases aromatase activity also in this species.

REFERENCES

1. ACKERMAN GE, SMITH ME, MENDELSON CR, MACDONALD PC, SIMPSON ER: Aromatization of androstenedione by human adipose tissue stroma cells in monolayer culture. J Clin Endocrinol Metab 53:412,1981
2. BLANCHARD MG, JOSSO N: Source of the anti-Müllerian hormone synthesized by the fetal testis : Müllerian-inhibiting activity of fetal bovine Sertoli cells in tissue culture. Pediatr Res 8:968,1974
3. BÉZARD J, VIGIER B, TRAN D, MAULÉON P, JOSSO N: Immunocytochemical study of anti-Müllerian hormone in sheep ovarian follicles during fetal and postnatal development. J Reprod Fertil 80:509, 1987
4. BUDZIK GP, DONAHOE PK, HUTSON JM: A possible purification of Müllerian inhibiting substance and a model for its mechanism of action. Developmental mechanisms: Normal and abnormal, Prog Clin Biol Res, JW LASH, L SAXEN, ALAN R LISS, New York 171:207,1985
5. BUDZIK GP, POWELL SM, KAMAGATA S, DONAHOE PK: Müllerian inhibiting substance fractionation by dye affinity chromatography. Cell 34:307,1983
6. CATE RL, MATTALIANO RJ, HESSION C, TIZARD R, FARBER NM, CHEUNG A, NINFA E, G, FREY AZ, GASH DJ, CHOW EP, FISHER RA, BERTONIS JM, TORRES G, WALLNER BP, RAMACHANDRAN KL, RAGIN RC, MANGANARO TF, MACLAUGHLIN DT, DONAHOE PK: Isolation of the bovine

and human genes for Müllerian inhibiting substance and expression of the human gene in animal cells. Cell 45:685,1986

7. COHEN-HAGUENAUER O, PICARD JY, MATTEI MG, SERERO S, NGUYEN VC, DE TAND MF, GUERRIER D, HORS-CAYLA MC, JOSSO N, FRÉZAL J: Mapping of the gene for anti-Müllerian hormone to the short arm of human chromosome-19. Cytogenet Cell Genet 44:2,1987
8. GEORGE FW, WILSON JD, : Conversion of androgen to estrogen by the human fetal ovary. J Clin Endocrinol Metab 47:550,1978
9. GEORGE FW, WILSON JD: Endocrine differentiation of the fetal rabbit ovary in culture. Nature 283:861,1980
10. HAYASHI H, SHIMA H, HAYASHI K, TRELSTAD RL, DONAHOE PK: Immunocytochemical localization of Müllerian inhibiting substance in the rough endoplasmic reticulum and Golgi apparatus in Sertoli cells of the neonatal calf testis using a monoclonal antibody. J Histochem Cytochem 32:649,1984
11. HUTSON JM, IKAWA H, DONAHOE PK: The ontogeny of Müllerian inhibiting substance in the gonads of the chicken. J Pediatr Surg 16:822,1981
12. JOSSO N, PICARD JY, TRAN D: The anti-Müllerian hormone. Rec Prog Horm Res 33:117,1977
13. JOST A: Problems of fetal endocrinology: The gonadal and hypophyseal hormones. Recent Prog Horm Res 8:379,1953
14. JOST A, VIGIER B, PREPIN J, PERCHELLET JP: Studies on sex differentiation in mammals. Recent Prog Horm Res 29:1,1973
15. LILLIE FR: The freemartin, a study of the action of sex hormones in foetal life of cattle. J Exp Zool 23:371,1917
16. MILEWICH L, GEORGE FW, WILSON JD, : Estrogen formation by the ovary of the rabbit embryo. Endocrinology 100:187,1977
17. NECKLAWS EC, LAQUAGLIA MP, MACLAUGHLIN D, HUDSON P, MUDGETT-HUNTER M, DONAHOE PK: Detection of Müllerian inhibiting substance in biological samples by a solid phase sandwich radioimmunoassay. Endocrinology 118:791,1986
18. PICARD JY, BENAROUS R, GUERRIER D, JOSSO N, KAHN A: Cloning and expression of cDNA for anti-Müllerian hormone. Proc Natl Acad Sci USA 83:5464,1986
19. PICARD JY, GOULUT C, BOURRILLON R, JOSSO N: Biochemical analysis of bovine testicular anti-Müllerian hormone . FEBS Lett 195:73,1986
20. PICARD JY, JOSSO N: Purification of testicular anti-Müllerian hormone allowing direct visualization of the pure glycoprotein and determination of yield and purification factor. Mol Cell Endocrinol 34:23,1984
21. ROBERTS JD, WARREN JC: Steroid biosynthesis in the fetal ovary. Endocrinology 74:846,1964
22. SHEMESH M, HANSEL W: Hormone production by the early bovine embryo. J Steroid Biochem 19:979,1983
23. SHORE L, SHEMESH M, : Altered steroidogenesis by the fetal bovine freemartin ovary J Reprod Fertil 63:309,1981
24. TAGUCHI O, CUNHA GR, LAWRENCE WD, ROBBOY SJ: Timing and irreversibility of Müllerian duct inhibition in the embryonic reproductive tract of the human male. Dev Biol 106:394,1984
25. TAKAHASHI M, HAYASHI M, MANGANARO TF, DONAHOE PK: The ontogeny of Müllerian inhibiting substance in granulosa cells of the bovine ovarian follicle. Biol Reprod 35:447,1986
26. TAKAHASHI M, KOIDE SS, DONAHOE PK:Müllerian inhibiting substance as oocyte meiosis inhibitor. Mol Cell Endocrinol 47:225,1986
27. TRAN D, JOSSO N: Localization of anti-Müllerian hormone in the rough endoplasmic reticulum of the developing bovine Sertoli cell using immunocytochemistry with a monoclonal antibody. Endocrinology 111:1562,1982
28. TRAN D, MEUSY-DESSOLE N, JOSSO N: Anti-Müllerian hormone is a functional marker of foetal Sertoli cells. Nature 269:411,1977
29. TSAFRIRI A, PICARD JY, JOSSO N: Immunopurified anti-Müllerian hormone does not inhibit spontaneous resumption of meiosis *in vitro* of rat oocytes. Biol Reprod, 38:681, 1988
30. VIGIER B, PICARD JY, TRAN D, LEGEAI L, JOSSO N: Production of anti-Müllerian hormone : Another homology between Sertoli and granulosa cells. Endocrinology 114:1315,1984
31. VIGIER B, TRAN D, DU MESNIL DU BUISSON F, HEYMAN Y, JOSSO N: Use of monoclonal antibody techniques to study the ontogeny of bovine anti-Müllerian hormone. J Reprod Fertil 69:207,1983
32. VIGIER B, WATRIN F, MAGRE, TRAN D, JOSSO N: Purified bovine AMH induces a characteristic freemartin effect in fetal rat prospective ovaries exposed to it *in vitro*. Development 100:43,1987
33. VOUTILAINEN R, MILLER WL: Human Müllerian inhibitory factor messenger ribonucleic acid is hormonally regulated in the fetal testis and in adult granulosa cells. Mol Endocrinol 1:604,1987

DISCUSSION

CHAIRPERSON: MILAN R. HENZL, M.D., Ph.D.

DR. SEGAL: You showed that the AMH appears to affect germ cell multiplication during the fetal ovary. Does it also affect the rate of atresia in the fetal ovary?

DR. JOSSO: Not significantly so. You could see that the slope of decrease in the number of germ cells was slightly steeper in the treated ovaries than in the controls. But the difference was not significant.

DR. SEGAL: What do you believe is the role that AMH plays in the testis after sex differentiation?

DR. JOSSO: I have no idea, but this is a question that everybody asks. They say that since AMH is produced by Sertoli cells after Mullerian ducts are regressed, and since it is also made in the ovary, it must have a function. Well, I do not know what it is. We have tried to answer the question using passive immunization. We have immunized a female rabbit against AMH and caused her to reproduce; most of her male fetuses were born with retained Mullerian ducts. We had reasoned that if AMH in the male was responsible for other functions, then we should be able to find them. However, other than retained Mullerian ducts, these male fetuses were completely normal. So at this point we do not know whether AMH has an extra-Mullerian effect in the male fetus and what that effect is.

DR. UENO: We are working with Dr. Donahoe using the rat. I would like to know more about your rat oocyte method.

DR. JOSSO: Could I pass on the technical question to Alex Tsafriri, who is in the audience?

DR. TSAFRIRI: The oocytes were collected from PMSG treated immature rats, namely superstimulated to have multiple follicles, and then isolated within 15 minutes. Only oocytes that had intact cumuli were used. Under the same circumstances we could use the porcine preparations of OMI and show the standard inhibition.

DR. UENO: Dr. Josso, you used the bovine AMH probe in the rat testis. As far as I remember from your slide, the rat testis did not show any cross-reactivity within the bovine AMH DNA; is that right?

DR. JOSSO: I agree that on the slide it did not, but on the film it did. Anyway, our bovine AMH probe does cross-react with rodent AMH since we were able, using this bovine probe, to localize the gene to the human chromosome 19 using a somatic cell hybridization technique. With this probe we were able to see the AMH DNA restriction fragment on the mouse and the hamster parent of our cell. (Cohen-Hagvenauer *et al.*, Cytogenetics 66:2, 1987.)

I agree with you that probably a longer exposure in this particular case would have been

necessary to see the hybridization. But as I did not show the slide for any species comparison, I did not think it was very important.

DR. HASELTINE: I have a question in two parts. The first thing is that it is very interesting to note that you are able to get some seminiferous tubular-like structures with the factor.

Now, what I wanted to ask you is have you looked at these new cultures, these ovarian transformed cultures, to see if they produce estrogen and testosterone?

DR. JOSSO: The histological slides I showed were from rats, and we do not find endocrine changes in the rat. As you know, not all species make estrogen very early in embryonic development.

DR. HASELTINE: No, that is not my question. In the bovine ovaries where you are now measuring the different endocrine changes, are they now making the Mullerian inhibiting substance or factor? Have they obtained that ability, or are they just responding to external stimuli?

DR. JOSSO: We do have very new results in collaboration with G. Charpentior showing that the ovaries transformed by AMH are themselves able to induce regression of Mullerian ducts. It is a sort of autocrine thing. You put in AMH, and it induces the ovary to make AMH in turn. As you know, we have shown this already with the freemartin gonad. (Vigier *et al.*, J Reprod Fert, 70:673, 1984.) The freemartin ovary does have anti-Mullerian activity, and its seminiferous tubules are immunoreactive.

DR. HASELTINE: The second question gets back to what it is doing to the germ cell. You are able to show that there are fewer meiotic divisions and, therefore, the new gonad is depleted of oocytes. The oocytes that are present appear to be under normal meiotic arrest and surrounded by granulosa cells, or are they just not even present?

DR. JOSSO: No, those that survive are more or less normal. Those that survive undergo normal maturation except at the highest doses. But at the highest dose, 3 μg/ml, very, very few cells survive, and the surviving cells do not look very healthy. At 1.5 μg/ml, the cells that survive are maturing normally.

DR. FAIMAN: Could you comment on the autogenesis of AMH presence in the ovary? AMH does have an effect on the fetal ovary as you have shown, but when does it normally appear?

DR. JOSSO: In normal circumstances, a fetal ovary does not come in contact with AMH, since AMH begins to be made the week before birth in ovine fetuses (Bézard *et al.*, J Reprod. Fert. 80:509, 1987.) At that time, Mullerian ducts no longer respond to the Mullerian inhibiting effect. If you take a fetal ovary that is slightly older, it will not respond either. So it all fits in very nicely. At the time when AMH would be dangerous in the fetus, AMH is not there, and AMH appears just when it will not harm the ovary.

22

Mullerian Inhibiting Substance in the Ovary: Function and Mechanism of Action

SHIGERU UENO
MASAHIKO TAKAHASHI
THOMAS F. MANGANARO
PATRICIA K. DONAHOE

Mullerian Inhibiting Substance in the embryo testis was originally described by Jost,[33,34] who ingeniously deduced that it caused regression of the Mullerian duct, the anlagen of the uterus, fallopian tube and upper vagina. Although originally thought to be present only in the Sertoli cells of the fetal and early postnatal testicular tissue,[3,13,14,15,16,33,35] subsequent observations indicated that MIS was also present in the ovary. We first detected MIS in avian ovary throughout fetal and adult life;[31] subsequently it was detected in follicular fluid of the bovine ovary[39,52] where immunohistochemical techniques localized MIS throughout reproductive life to the granulosa cells of the follicles.[46] These findings led us to further study the function of MIS in the ovary.

MIS activity in the rat ovary was tested throughout fetal, post-natal, and adult life,[19] using a standard organ culture assay. High MIS activity coincided with the period of oocyte meiotic arrest and could be correlated with a high number of Graafian follicles, which led us to the hypothesis that MIS might act as an oocyte meiosis inhibitor in the ovary. Initially, bovine MIS, purified from calf testicular tissue, was tested for its affect on spontaneous rat oocyte meiosis *in vitro*.[47] After we cloned both the bovine and human MIS gene and succeeded in producing human recombinant MIS using CHO cells transfected with the human MIS gene,[8] we have tested human MIS for its effect on rat oocyte meiosis. Since the mechanism of action of MIS appears to be related to EGF receptor tyrosine kinase activity,[10] we studied the interaction of EGF and on MIS in the oocyte.[50] These recent observations will be discussed in the context of known physiologic modulators of oocyte meiosis, and the physiological role of MIS and its mechanism of action in oocytes will be considered.

MIS IN THE AVIAN OVARY

During an ontogeny study of MIS production in the chicken testis, before and after birth, we also assayed ovary of similar ages for regression of the rat embryonic Mullerian duct.[14,31] The chick ovary to be assayed was placed adjacent to the urogenital ridge of a 14-1/2 day female rat fetus on an agar-coated stainless steel grid of an organ culture dish (Falcon 3037, Becton Dickinson, Cockeysville, Md.) over a well containing 1 ml of medium [CMRL (Gibco, Grant Island, N.Y.), 10% fetal calf serum 200 U penicillin and 200 mcg streptomycin] at 37°C for 72 hour in a humidified atmosphere of 95% air and 5% CO_2.

Specimens were then fixed, dehydrated, cleared and embedded, and the cranial end of the urogenital ridge was cut in serial cross-section, and stained with hematoxylin and eosin, after which multiple sections were studied by light microscopy and regression of the rat Mullerian duct graded on a scale of 0 to 5 (0—no regression; 5—complete regression). Embryonic, postnatal, and adult ovary incubated with the urogenital ridge all caused considerable Mullerian duct regression,[31] (Fig. 1) and raised the question of why Mullerian ducts in the female are retained in spite of the presence of MIS.

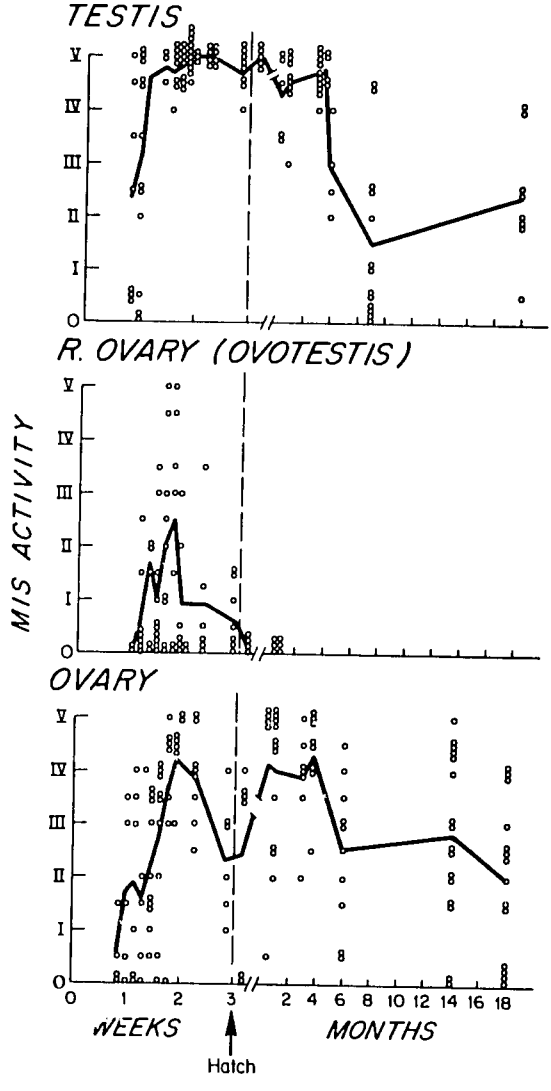


Figure 1. MIS activity of chick testis, ovotestis and ovary from 6 days of embryonic life until 18 months after hatching was graded on a semiquantitative scale from 0 to 5. The solid line represents the mean of observations at each age. The open circles represent the results of each observation (Permission from Journal of Pediatric Surgery).

Retention of the Mullerian duct can be produced by estrogens,[53] suggesting that estrogen might inhibit the action of MIS. Consequently, we assayed the testes for MIS activity after diethylstilbestrol (DES) was injected either into the yolk sac or air sac of the chick embryo. Since the Mullerian ducts did not regress, and MIS bioactivity in the estrogen treated testes was comparable to that of nontreated or normal testes (Fig. 2), we assumed that estrogen inhibited MIS at the receptor level, rather than by modulating MIS synthesis.[32] Subsequently, Newbold *et al.*[40] inhibited Mullerian duct regression in the mouse by pretreatment with DES. Testes in these pretreated animals similarly continued to cause Mullerian duct regression, indicating that the estrogen exerted its effect at the receptor level.

Using 3H estradiol binding, we then measured estrogen receptors (cytosolic, and nuclear type I and II) in the Mullerian ducts of the embryonic chick just before regression occurs.[36]

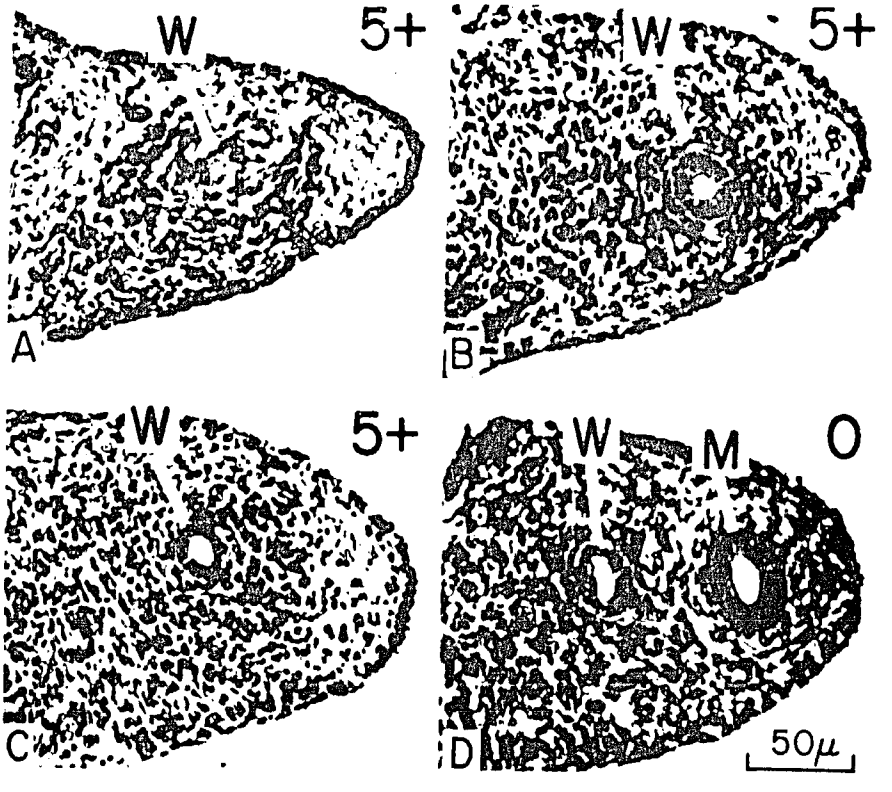


Figure 2. Regression of the rat Mullerian duct (M) *in vitro* by 16-day chick testes treated with DES at 5 days of incubation. (A) Normal testis (5+ regression), (B) DES treated left testis (histologic appearance of an ovary) (5+ regression). (C) DES treated left ovary (5+ regression). (D) DES-treated mesonephros (no regression-Mullerian duct is normal). (Hematoxylin and eosin, X230, W:Wollfian duct). (Permission from Journal of Pediatric Surgery).

Large numbers of right and left Mullerian ducts were collected from 8- and 9-day-old chick embyros. Type II nuclear estrogen receptor was significantly elevated in the retained or left Mullerian duct in the female, while cytosolic and type I nuclear receptors were equal in both regressed (right) and retained (left) ducts (Fig. 3). When sex linked, feather colored chick embryos were injected at 5 days of gestation with steroid synthesis inhibitors and antiestrogens, endogenous MIS in the ovary resulted in regression of the left Mullerian duct[29] when the embryos were examined at 14 days of gestation. In these same studies, Tamoxifen and the LillyR antiestrogens, known to inhibit Type I estrogen nuclear receptors, did not allow MIS to be expressed, whereas norethindrone did. We therefore proposed that norethindrone might be a nuclear Type II inhibitor. Thus MIS is indeed produced in the chick ovary, but regression of the left duct is prevented by estrogen, due to interaction between MIS and estrogen at the receptor level.

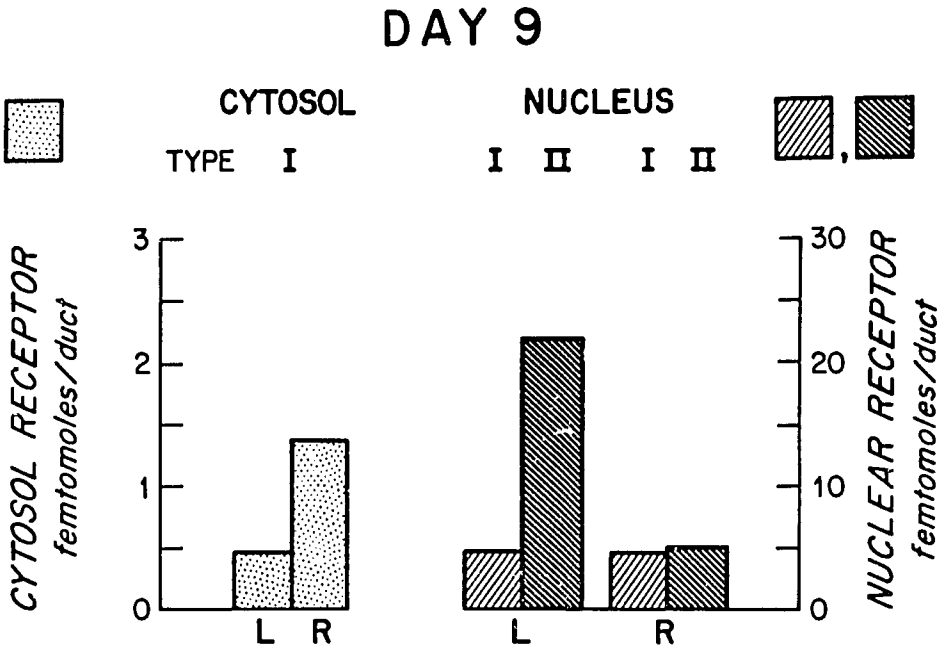


Figure 3. Total specific estradiol binding in cytosol and nuclear preparations of male and female, right (R) and left (L) Mullerian ducts in a pool of 9-day-old chick embryos in 3-hour 37 °C incubations (bars). Data are expressed as fentomoles per duct for cytosolic type I and nuclear type I and II binding sites. In this study, 146 female and 92 male embryos were used. The symbols represent the mean and ranges of this and two similar binding experiments using 3 separate pools of 9-day-old Mullerian ducts. The 2 additional pools consisted of 146 right or left and 240 right or left ducts, respectively (Permission from Endocrinology).

MIS LOCALIZATION IN THE BOVINE OVARY

Bovine MIS was purified from newborn calf testes by sequential column chromatography,[5,6,7] and used to develop monoclonal antibodies.[38,44] These were then used to develop a radioimmunoassay with which MIS was detected in the follicular fluid of mature bovine ovary.[39,52] One of these monoclonal antibodies localized MIS in ovarian follicles by immunocytochemical techniques using avidin-biotin[28] enhancement of the peroxidase-antiperoxidase techniques that had been so successful in localizing MIS in the Sertoli cells of the newborn calf testis.[25]

Fresh bovine ovarian tissues obtained from the newborn period throughout reproductive life,[46] were fixed immediately in Zamboni's solution[54] for 3 hours at 4 °C. After washing in 0.15 M phosphate buffer containing 7% sucrose, they were embedded in cryomolds and frozen at -80 °C. Eight micron sections were mounted on gelatin coated slides. Endogenous peroxidase activity was eliminated and sections then covered with diluted normal horse serum and a monoclonal antibody against bovine MIS, followed by incubation for 45 minutes at room temperature. After washing they were covered with diluted biotinylated second antibody (affinity purified horse antimouse IgG) for another 30 minutes. Another washing step was followed by incubation for 30 minutes at room temperature with VECTASTAIN ABC Reagent (avidin DH and biotinylated horseradish peroxidase H). Peroxidase was detected by reacting with a mixture of 0.05% 3,3'-diaminobenzidine tetrahydrocholoride (DAB) in 0.05 M Tris-HCl buffer and 0.01% hydrogen peroxide for 7 minutes. The sections were counterstaining with hematoxylin and mounted in Permount for study.

MIS could be easily detected by this method in the cytoplasm of Sertoli cells of the calf newborn testis which was used as a positive control. Specific localization of MIS was seen in the cytoplasm of ovarian granulosa cells of the Graafian follicles from 1 day to 5 years of age (Fig. 4), while no staining was found in primary follicles, growing follicles, or corpus luteum, or in the theca cells which surrounded the positively stained granulosa cells.

MIS ACTIVITY IN THE RAT OVARY

We next studied the rat, to more precisely outline the ontogeny of MIS production in a species which, with the right probes, can be used to uncover factors which control both MIS production and response. Ovarian fragments of 1-2 mm total size, from midgestation to adulthood, were assayed for MIS bioactivity in the rat urogenital ridge bioassay, and the results compared to that produced by testicular fragments from rats of the same age. The number of primary, secondary, and mature follicles were analyzed. Primary or primordial follicles are ones which the oocyte is surrounded by a single layer of flattened epithelial cells; secondary or growing follicles are composed of an oocyte encompassed by at least two layers of follicular cells; Graafian follicles have multiple layers of granulosa cells, and cumulus cells surrounding an eccentrically located germ cell, with multilaminated theca cell layer surrounding the granulosa cell layer. The number of these three follicles was counted in five sequential 8 micron sections from a pole of each ovary on each day. Only primordial follicles were observed in the ovaries of fetal and postnatal rats until 2 days after birth. Growing follicles begin to appear at 3 days of age, and Graafian follicles

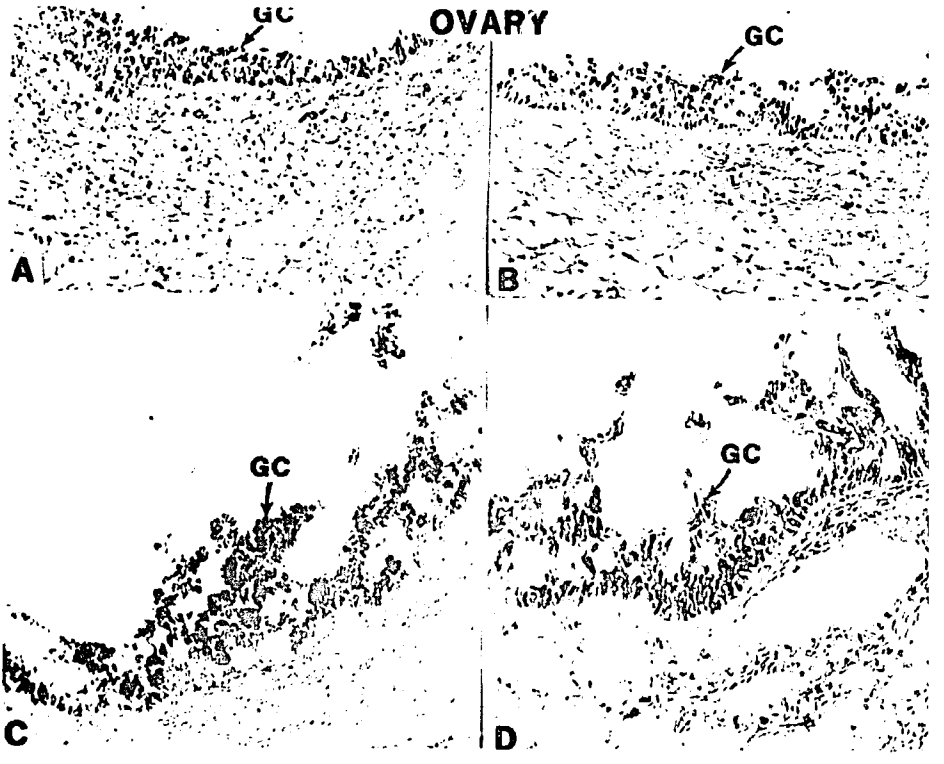


Figure 4. The cytoplasm of the granulosa cells in Graafian follicles of various ages stained specifically using autonomous monoclonal antibody and second antibody conjugated to avidin-biotin horseradish peroxidase for enhancement. The peroxidase is then developed with diaminobenzidine (see text) GC=granulosa cells. A) One-day-old (X190). B) Three-mo-old (X200). C) Two-and-a-half-yr-old (X120). D) Five-yr-old (X200) (Permission from Biology of Reproduction).

at 5 days, the proportional number of each type increasing gradually from 5 until 19 days of age, and thereafter decreasing gradually. After 4 months of age, Graafian follicles were no longer seen (Fig. 5).

MIS activity was expressed in rat testes and ovaries at very different times. MIS activity is high (Grade V) in the testes of fetal and neonatal rat until 4 days of age, after which it decreases dramatically, remaining detectable, but low (Grade I-II) between age 6 and 21 days.[13] After 5 months of age, MIS activity is no longer detected. Conversely, MIS activity was almost never detected in fetal ovaries. After birth, however, MIS activity increases gradually until 20 days of age, and then decreases again. Between age 15 days and 21 days, MIS activity in rat ovaries was high (Grade II-IV) but was characterized by extreme individual variation. After 5 months of age, ovarian MIS activity could no longer be detected in this organ culture system assay (Fig. 6).

Since the 11-day rat ovaries showed the widest range of MIS activity, we used the follicle counts on this day and correlated the grade of MIS activity with the number of mature

Figure 5. The qualitative and quantitative analysis of follicles in rat ovaries from 14 days of embryonic life until 6 months after birth. The numbers of each type of follicle were generated by averaging the number of follicles on each slide (n=5) and multiplying the mean of the slides by 5 for each tissue ovarian sample at each day. The number of ovaries for each day is given in Figure 6. The bar graphs indicate the percentages of primordial, growing and Graafian follicles in the total number of follicles counted on each day (permission from Alan R. Liss).

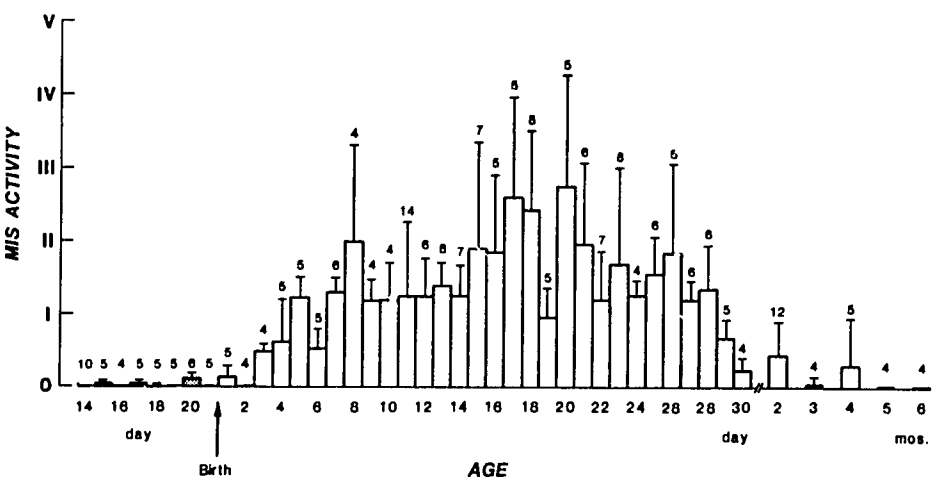


Figure 6. MIS activity (mean +SD) in rat ovaries at various ages as determined by a semi-quantitative organ culture assay which measures Mullerian duct regression on a scale from 0 to 5. Numbers over each bar refer to the number of animals providing ovaries for each day (permission from Alan R. Liss).

Graafian follicles. When the number of follicles per section was high, MIS bioactivity was high; if the number of follicles was meager, then bioactivity was low (Fig. 7). This bioassay observation in the rat corroborates immunohistochemical evidence in the bovine species using monoclonal antibodies that do not cross species, and indicated that MIS in the ovary is predominantly produced in the Graafian follicles.

MIS AS AN OOCYTE MEIOSIS INHIBITOR

The mammalian oocyte enters the first meiotic division during fetal life, becomes arrested right after birth,[2] and does not resume meiosis until the gonadotropin surge shortly prior to ovulation at puberty.[1,9,11,43,48,51] The factors responsible for maintaining the oocyte in the state of meiotic arrest are unknown, but constituents of follicular fluid and granulosa cells[20,42,48] have been causally implicated in oocyte meiotic arrest. Since our ontogeny and morphology studies in the rat showed a correlation between high MIS production and the largest number of Graafian follicles, and since both occurred during the period of meiosis arrest, we hypothesized that a function of MIS in the ovary might be to inhibit the resumption of oocyte meiosis.

BOVINE MIS

It is well known that when oocytes in meiotic arrest are released from the follicles and incubated *in vitro* they resume meiosis spontaneously.[22,24,41] We used rat oocytes to deter-

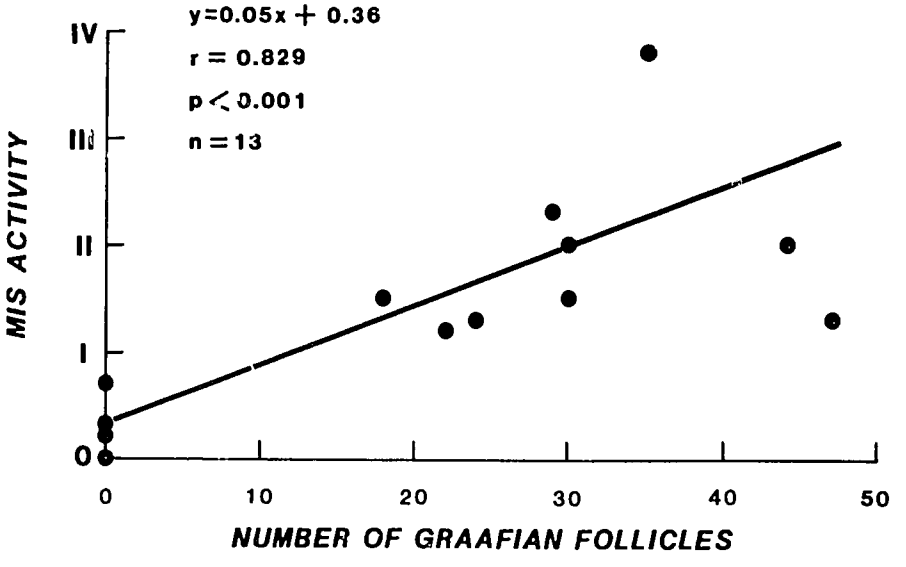


Figure 7. A linear relationship is seen between MIS activity and the number of Graafian follicles in each rat ovary assayed at 11 days after birth with a significant positive slope. Each data point represents the mean of 65 observations. r=0.829 by linear regression analysis with $p<0.001$ (permission from Alan Liss).

mine whether natural bovine MIS could inhibit this spontaneous resumption of oocyte meiosis *in vitro*.[47] Briefly, ovaries were dissected from prepubertal rats and placed in modified Krebs-Ringer's Bicarbonate solution at 35°C and oocytes were released from the follicles by teasing with needles to produce cumulus-enclosed oocytes. To yield denuded oocytes, adhering cumulus cells were stripped from released oocytes by rapidly drawing them in and out of a pipet. Fully grown oocytes containing an intact germinal vesicle either with or without cumulus cells were selected and pipeted into 0.2 ml of media under mineral oil and cultured for 5 hours, after which oocytes were scored immature or mature depending upon whether the germinal vesicle persisted. MIS containing fractions were obtained in our laboratory by sequential ion exchange, carbohydrate affinity and/or dye affinity chromatography as described previously.[5,6,7] After 5 hours of incubation in control media, spontaneous maturation or germinal vesicle breakdown (GVBD) occurred in approximately 80% of either denuded or cumulus-enclosed oocytes. On the other hand, a significant and dose-dependent inhibition of spontaneous GVBD occurred when MIS-containing fractions were added to the media of both denuded and cumulus-enclosed oocytes (Fig. 8).

HUMAN RECOMBINANT MIS

Human and bovine genes for MIS were recently cloned. The human MIS gene was cotransfected with a dehydrofolic reductase (DHFR) gene into DHFR deficient Chinese Hamster Ovary (CHO) cells and selected after methotrexate driven amplification by the ability of the conditioned media to cause regression of the rat embryonic Mullerian duct in organ culture.[8] Conditioned α-MEM media of the transfected, selected, and amplified CHO clones were collected, and MIS was purified, initially from media containing 10% calf serum, and subsequently from media without serum. Recombinant MIS was purified from the conditioned media containing serum by a method similar to bovine MIS that we used to purify from testicular tissue, *i.e.*, by sequential ion exchange, carbohydrate affinity and/or dye affinity chromatography.[5,6] Spontaneous rat oocyte meiosis *in vitro* was then inhibited after incubation in MIS samples purified from this 10% serum containing media. After adapting CHO clones to serum-free conditions and scaling up production, larger volumes of media were collected and loaded onto a Q-Sepharose fast flow (Pharmacia) ion-exchange column, and then loaded on a Lentil Lectin Sepharose 4B (Pharmacia) carbohydrate affinity column.[8] Twenty MIS preparations purified from conditioned medium without serum were added to the oocyte culture medium. The first 10 biologically-active MIS preparations failed to inhibit spontaneous rate oocyte meiosis. However, when a second group of 10 biologically active MIS preparations purified from serum-free conditioned medium was tested for oocyte meiosis inhibitory activity using KRB medium containing NP-40 at 10^{-7} g/ml, spontaneous rat oocyte meiosis *in vitro* was significantly inhibited (Table 1). When the rabbit antiserum raised against human MIS was added to the medium containing MIS, the inhibition of spontaneous rat oocyte meiosis was blocked when compared to the significant inhibition caused by the medium containing MIS, or medium containing MIS with non-immunized rabbit serum. Recently, MIS was purified to near homogeneity (>90%) from conditioned medium by immunoaffinity chromatography using mouse monoclonal antibody raised by Biogen Corporation to the MIS 140 kD dimer eluted from an electrophoresis gel. This homogenous immunopurified MIS had no significant

activity without NP-40. When NP-40 at 10^{-7} g/ml, was added to this highly purified MIS preparation, however, the inhibitory effect on spontaneous rat oocyte meiosis (Table 2) was again observed.

FACTORS WHICH MODULATE MIS ACTIVITY

To determine whether MIS inhibitory action against spontaneous rat oocyte meiosis is synergistic or antagonistic with other substances, we added the following to the media with or without bovine MIS: dibutyryl cyclic AMP, follicular-stimulating hormone (FSH), luteinizing hormone (LH), progesterone, 17β-estradiol, or testosterone. Addition of dbc-

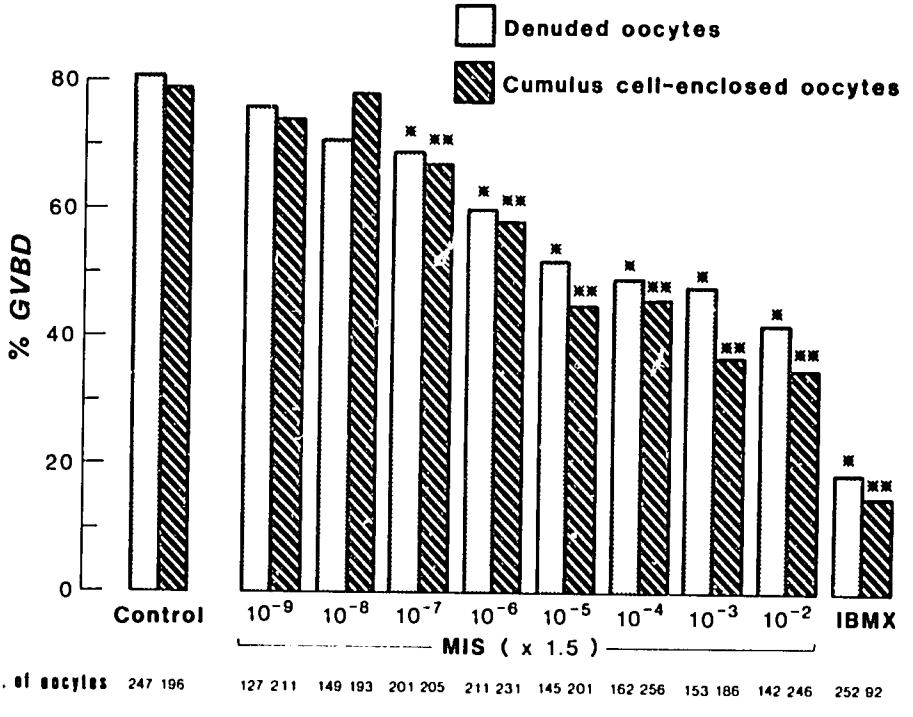


Figure 8. Effect of MIS and isobutylmethylxanthine (IBMX) on the spontaneous germinal vesicle breakdown (GVBD) of denuded and cumulus cell-enclosed rat oocytes. Denuded or cumulus cell-enclosed oocytes of rats were cultured for 5 hours in KRB medium without (control), with 10^{-4}M IBMX alone, or with various concentrations (1.5×10^{-9} to 10^{-2} mg protein/ml) of an MIS preparation. Assuming approximately 80% purity of MIS in the active preparation, the molar concentration required for half-maximal inhibition is 8.5 pM for the MIS preparation. *Indicates a significant ($P < 0.01$ by X2 analysis) difference from the denuded oocyte control; **significant ($P < 0.01$) difference from the cumulus cell-enclosed oocyte control. No. =number of oocytes examined. MIS inhibits both cumulus cell-enclosed and denuded oocytes (permission from Journal of Molecular and Cellular Endocrinology).

TABLE 1
Oocyte Meiosis Inhibition by MIS Samples Purified From Serum Free Conditioned Medium

#Samples	Mullerian Duct Regression*	Mean Protein Concentration (range) mcg/ml	NP-40 g/ml	% Inhibition
10	4.00	78.3 (13-120)	0	4
10	4.35	62.5 (29-160)	10^{-7}	21

*Mean of Mullerian duct regression in organ culture (0-None; 5-Complete)

Two different groups of MIS samples were tested on spontaneous rat oocyte meiosis. MIS samples were purified from serum-free conditioned medium from CHO cells transfected with the human MIS gene. Bioactivity was determined in the standardized organ culture assay at 1:40 dilution. The final protein concentration of the MIS samples used for the oocyte assay was 78.3 mcg/ml (range 13 to 120 mcg/ml) in the first group and 63.5 mcg/ml (range 29 to 160 mcg/ml) in the second group. On reduced gels, the characteristic MIS 70 kD band represented 1-20% of the protein on the g[illegible] samples in group I were tested without adding NP-40 to culture medium for oocytes while those i[illegible] II were tested in the medium containing NP-40 at 10^{-7} g/ml.

AMP had neither synergistic nor antagonistic effects on the MIS inhibition of germinal vesicle breakdown in the oocytes (Fig. 9). FSH, LH, progesterone, estradiol, and testosterone failed to effect the MIS-induced inhibition of rat oocyte meiosis or to independently inhibit or enhance oocyte maturation.

EGF was added at 25 ng/ml a dose which had no effect on oocyte meiosis, to determine if EGF could reverse the inhibitory effect of human recombinant MIS against spontaneous germinal vesicle breakdown in the oocyte. Medium containing MIS samples significantly inhibited spontaneous rat oocyte meiosis *in vitro* (20% inhibition, $p < 0.01$) (Table 3). When EGF was added to the medium before addition of the MIS sample, oocyte meiosis inhibition by MIS was blocked (5% enhancement).

DISCUSSION

Although the function that MIS may subserve in the female still remains a mystery, its presence during the entire reproductive life of the female, but only in the mature Graafian follicle, and its diminished production as both reproductive function and the number of Graafian follicles wane, suggests that MIS may make a significant contribution to the reproductive function of the ovary. The coincidence of high MIS activity in the rat ovary with the period of the oocyte meiotic arrest led us to the hypothesis that MIS might function as an oocyte meiosis inhibitor, which was supported by the observation that a preparation of MIS partially purified from newborn calf testes produced a significant inhibition of rat oocyte meiosis *in vitro* in a dose dependent manner.[48] Human recombinant MIS, initially purified from serum-containing medium by a method similar to that used to purify natural bovine MIS, recapitulated the effect produced by bovine MIS by significantly inhibiting rat oocyte maturation *in vitro*.

When human MIS was later purified from serum-free conditioned medium, although the preparations had high biological activity in the organ culture assay[14] (Table 1), they caused virtually no inhibition of oocyte meiosis. Only when the oocyte incubation media contained MIS plus Nonidet-P 40 (NP-40), a non-ionic detergent[26] used to reduce nonspecific losses during the final chromatography steps in the purification both of natural bovine MIS and human recombinant MIS from conditioned medium containing calf serum, and to stabilize the molecule during long-term storage,[5,6] was spontaneous meiosis inhibited by MIS (Table 1).

The fact that antiserum or antibody raised against human MIS could antagonize the action of the human MIS, however, attests to the specificity of the MIS effect, even in less purified but effective preparations. Most importantly, however, the most highly homogeneous preparation purified by immunoaffinity chromatography, whose MIS bands

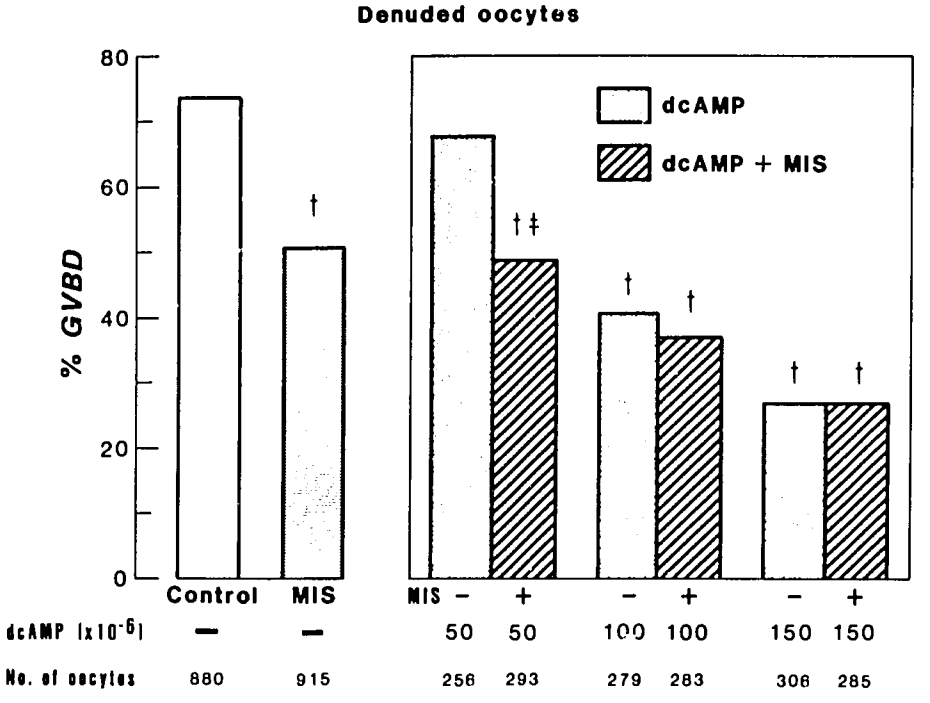


Figure 9. Effect of dbcAMP on MIS inhibition of spontaneous GVBD of denuded and cumulus cell-enclosed rat oocytes. Denuded oocytes (A) and cumulus cell-enclosed oocytes of rats (B) were cultured for 5 hours in culture medium (control), in medium with 1.5 X 10^{-3} mg protein/ml MIS (MIS), in medium with 50, 100 and 150 X 10^{-6} M dbcAMP alone or with 1.5 X 10^{-3} mg protein/ml MIS. † At the top of the bar indicates significant ($P<0.01$) difference from the control group, ‡ indicates significant ($P<0.01$) difference from the group treated with 50 X 10^{-6} below the bars. The MIS-induced and the cAMP-induced inhibition are not additive or antagonistic in either cumulus-enclosed or denuded oocytes (permission from the Journal of Molecular and Cellular Endocrinology).

TABLE 2

Effect of Highly Purified MIS on Rat Oocyte Meiosis

Medium	# of Oocytes examined	NP-40 g/ml	% Inhibition compared to KRB alone
KRB alone	468	0	0
	139	10^{-7}	+4
KRB + MIS	142	0	0
	169	10^{-7}	-21

A preparation purified to near homogeneity by immunoaffinity chromatography using a monoclonal antibody raised to MIS, showed grade IV to V bioactivity in the organ culture assay at 12.5 mcg protein/ml (estimated amount of MIS is 90% of total protein). Spontaneous oocyte meiosis was not inhibited by medium containing the MIS sample alone at 5 mcg protein/ml (0% inhibition compared to KRB solution). Only when MIS was added with NP-40 at 10^{-7} g/ml did the medium containing MIS inhibit oocyte meiosis (21% inhibition, $P < 0.01$). NP-40 itself at 10^{-7} g/ml caused no significant inhibitory activity on oocyte meiosis (4% enhancement).

TABLE 3

Epidermal Growth Factor (EGF) Reversal of MIS Inhibition of Oocyte Meiosis

Medium	# of Oocytes examined	EGF	% GVBD	% Inhibition Compared to control
KRB(with NP40)	530	0	78.3	—
	449	25 mg/ml	75.1	—
KRB(with NP40)	535	0	62.2	-20
+ MIS	457	25 mg/ml	79.4	+ 5

Five MIS samples purified from serum-free conditioned medium were used. Protein concentrations ranged from 23 to 520 mcg/ml and MIS represented 1-20% of the protein on the gel. KRB with NP-40 (at 10^{-7} g/ml) and containing the MIS samples, inhibited oocyte meiosis significantly (20% inhibition compared to KRB medium with NP-40, $p < 0.01$). By adding EGF at 25 ng/ml to the incubation medium containing MIS, the inhibitory effect was antagonized (5% enhancement), while KRB medium containing EGF at 25 ng/ml had no significant effect on rat oocyte meiosis when added alone.

on gel electrophoresis represent 90% of the loaded protein, was also effective in inhibiting spontaneous rat oocyte meiosis, which unequivocally confirms that the oocyte meiosis inhibitory effect observed was due to MIS.

We do not know which population of oocytes, from the heterogeneous array of oocytes present at each stage of the menstrual cycle and at each stage of development in the ovary, is most responsive to MIS. It is conceivable that only oocytes from younger and smaller follicles may react to MIS. This possibility may explain why higher quantities of MIS were detected in smaller rather than larger Graafian follicles in the bovine species,[39,52] and why

inhibition of oocyte meiosis of this heterogeneous population, as measured by germinal vesicle breakdown, rarely exceeds 50%.[47]

Oocyte meiosis inhibitors have been purified in other laboratories. A low molecular weight polypeptide appears to act on cumulus cells and to mediate its effect on the oocyte via a cAMP dependent protein kinase.[4,23,27,49] The mechanism of action by which MIS exerts its effects has been studied extensively in our laboratory,[17,18] where MIS has been shown to antagonize an EGF receptor tyrosine kinase.[9,10,30] Since bovine MIS preparations inhibited spontaneous maturation of both types of oocytes, cumulus enclosed and denuded, and since dibutyryl cAMP did not enhance or antagonize this inhibition,[47] we speculated from these divergent observations that cumulus cells do not mediate the MIS effect, and that MIS also might act as an oocyte meiosis inhibitor by a cAMP-independent mechanism. The present experiments show another antagonism between MIS and EGF, *i.e.*, the reversal of the MIS-induced inhibition of spontaneous rat oocyte germinal vesicle breakdown by EGF, which suggests that an MIS and EGF receptor tyrosine kinase interaction may take place in the oocyte as it does in the embryo during regression of the Mullerian duct[30] and in the MIS sensitive A-431 cell membrane.[9,10] A large body of evidence exists to implicate tyrosine kinase activity as important in oocyte maturation.[23,37,45] For example, EGF itself, in higher concentrations than used in our experiments, can initiate oocyte maturation when added to follicle enclosed oocytes.[12]

We have presented evidence that rat oocyte meiosis is inhibited by both bovine and human recombinant MIS and that this inhibition is blocked by EGF. These findings may elucidate not only the MIS-related events that take place during the process of oocyte meiosis, but may also provide some clues regarding the role that MIS might play in the regulation of the crucial events in cellular proliferation in other embryonic or tumor systems. It is clear that much work needs to be done to access the physiological significance of the MIS-related events and to evaluate its importance in meiotic arrest and maturation.

REFERENCES

1. AYALON D, TSAFRIRI A, LINDNER HR, CORDOVA T, HARELL A: Serum gonadotropin levels in proestrous rats in relation to the resumption of meiosis by the oocytes. J of Reprod Fertil 31:51, 1972
2. BEAUMONT HM, MANDL AM: A quantitative and cytological study of oogonia and oocytes in the fetal and neonatal rat. Proceedings of the Royal Society of London. Series B. Biological Sciences. 155:557, 1962
3. BLANCHARD MG, JOSSO N: Source of the anti-Mullerian hormone synthesized by the fetal testis: Mullerian inhibiting activity of fetal bovine Sertoli cells in tissue culture. Pediatr Res 8:968, 1974
4. BORNSLAEGER EA, MATTEI P, SCHULTZ RM: Involvement of cAMP-dependent protein kinase and protein phosphorylation in regulation of mouse oocyte maturation. Dev Biol 114:453, 1986
5. BUDZIK GP, SWANN DA, HAYASHI A, DONAHOE PK: Enhanced purification of Mullerian inhibiting substance by lectin affinity chromatography. Cell. 21:909, 1980
6. BUDZIK GP, POWELL SM, KAMAGATA S, DONAHOE PK: Mullerian inhibiting substance fractionation by dye affinity chromatography. Cell. 34:307, 1983
7. BUDZIK GP, DONAHOE PK, HUTSON JM: Purification of mullerian inhibiting substance and a model for its mechanism of action. In LASH JW (ed): Developmental Mechanisms: Normal and Abnormal, p 207. New York, Alan R. Liss Inc., 1985
8. CATE RL, MATTALIANO RJ, HESSION C, TIZARD R, FARBER NM, CHEUNG A, NINFA EG, FREY AZ, GASH DJ, CHOW EP, FISHER RA, BERTONIS JM, TORRES G, WALLNER BP, RAMACHANDRAN KL, RAGIN RC, MANGANARO TF, MACLAUGHLIN DT, DONAHOE PK: Isolation of the bovine and human gene for mullerian inhibiting substance and expression of the human gene in animal cells. Cell. 45:685, 1986a

9. CIGARROA F, COUGHLIN JP, DONAHOE PK, CATE RL, MACLAUGHLIN DT: Human recombinant mulierian inhbiting substance inhibits EGF receptor tyrosine kinase. J Cell Biochem 1988 (submitted)
10. COUGHLIN JP, DONAHOE PK, BUDZIK GP, MACLAUGHLIN DT: Mullerian inhibiting substance blocks autophosphorylation of the EGF receptor by inhibiting tyrosine kinase. Mol Cell Endocrinol, 49:75, 1986
11. DEKEL N, HILLENSJO T, KRAICER PF: Maturational effects of gonadotropins on the cumulus-ooycte complex of the rat. Biol Reprod, 20:191, 1979
12. DEKEL N, SHERIZLY I: Epidermal growth factor induces maturation of rat follicle-enclose oocytes. Endocrinology, 116:406, 1985
13. DONAHOE PK, ITO Y, MARTATIA S, HENDREN WH: The production of mullerian inhibiting substance by the fetal, neonatal and adult rat. Biol Reprod, 15:329, 1976
14. DONAHOE PH, ITO Y, HENDREN WI: A graded organ culture assay for the detec › of mullerian inhibiting substance. J Surg Res, 23:141, 1977a
15. DONAHOE PK, ITO Y, MORIKAWA Y, HENDREN WH: Mullerian inhibiting substance in human testes after birth. J Pediatr Surg, 12:323, 1977b
16. DONAHOE PK, ITO Y, PRICE M, HENDREN WH: Mullerian inhibiting substance activity in bovine fetal, newborn and prepubertal testes. Biol Reprod, 16:238, 1977c
17. DONAHOE PK, HUTSON JM, FALLAT ME, KAMAGATA S, BUDZIK GP: Mechanism of action of mullerian inhibiting substance. Annu Rev Physiol 46:53, 1984
18. DONAHOE PK, CATE RL, MACLAUGHLIN DT, EPSTEIN J, FULLER AF, TAKAHASHI M, COUGHLIN JP, NINFA EL, TAYLOR LA: Mullerian inhibiting substance: Gene structure and mechanism of action of a fetal regressor. Recent Prog in Horm Res, 43:430, 1987
19. DONAHOE PK, TAKAHASHI M, UENO S, MANGANAROT: Mullerian inhibiting substance in the ovary. In HAZELTINE F (ed): Meiotic Inhibition: Mol Contr of Meiosis. New York, Alan R. Liss, 1988 (in press)
20. DOWNS SM, COLEMAN DL, WARD-BAILEY PF, EPPIG JJ: Hypoxanthine is the principal inhibitor of murine oocyte maturation in a low molecular weight fraction of porcine follicular fluid. Proc Natl Acad Sci USA 82:454, 1985
21. EPPIG JJ, DOWNS SM: Chemical signals that regulate mammalian oocyte maturation. Biol Reprod 30:1, 1984
22. EDWARDS RG: Meiosis in ovarian oocytes of adult mammals. Nature 196:446, 1962
23. ERIKSON E, MALLER JL: Purification and characterization of a protein kinase from *Xenopus* eggs highly specific for ribosomal protein S6. J Biol Chem 261:350,1986
24. FOOTE WD, THIBAULT C: Recherches experimentales sur la maturation *in vitro* des oocytes de truie et de vear. Annales de Biologie Animale Biochemie Biophysique (Paris), 9:329, 1969
25. HAYASHI M, SHIMA H, HAYASHI K, TRELSTAD RL, DONAHOE PK: Immunocytochemical localization of Mullerian inhibiting substance in the rough endoplasmic reticulum and golgi apparatus in Sertoli cells of the neonatal calf testis using a monoclonal antibody. J Histochem Cytochem 32:649, 1984
26. HELENIUS A, SIMONS K: Solubilization of membranes by detergents. Biochimica et Biophysica Acta 415:29, 1975
27. HILLENSJO T, CHANNING CP, POMERANTZ SH, SCHWARTZ-KRIPNER A: Intrafollicular control of oocyte maturation in the pig. *In Vitro*, 15:32, 1979
28. HSU SM, RAINE L, FANGER H: Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques. J of Histochem Cytochem 29:577, 1981
29. HUTSON JM, DONAHOE PK, MACLAUGHLIN DT: Steroid modulation of Mullerian duct regression in chick embryo. Gen Comp Endocrinol 57:88, 1985
30. HUTSON JM, FALLAT ME, KAMAGATA S, DONAHO PK, BUDZIK GP: Phosphorylation events during Mullerian duct regression. Sci 223:586, 1984
31. HUTSON JM, IKAWA H, DONAHOE PK: The ontogeny of Mullerian inhibiting substance in the gonads of the chicken. J Pediatr Surg 16:822, 1981
32. HUTSON JM, IKAWA H, DONAHOE PK: Estrogen inhibition of Mullerian inhibiting substance in the chick embryo. J Pediatr Surg 17:953, 1982
33. JOST A: Sur la differentiation sexuelle de l'embryon de lapin: Experiances de parabiose. Comptes Rendus Societe de Biologie 140:463, 1946
34. JOST A: Sur les derives Mulleriens d'embryons de lapin des deux sexes castres a 21 jous. Comptes Rendus Societe de Biologie 141:135, 1947
35. JOST A, MAGRE S, CRESCENT M: In MANCINI RE, MARTINI L (eds): Male Fertil Steril, p 1. New York, Academic Press, 1974

36. MACLAUGHLIN DT, HUTSON JM, DONAHOE PK: Specific estradiol binding in embryonic Mullerian ducts: A potential modulator of regression in the male and female chick. Endocrinol 113:141, 1983
37. MALLER JL, FOULKES JG, ERIKSON E, BALTIMORE D: Phosphorylation of ribosomal protein S6 on serine after microinjection of the Abelson murine leukemia virus tyrosine-specific protein kinase into *Xenopus* oocytes. Proc Natl Acad Sci USA 82:272, 1985
38. MUDGETT-HUNTER M, BUDZIK GP, SULLIVAN M, DONAHOE PK: Monoclonal antibody to Mullerian inhibiting substance. J Immunol 128:1327, 1982
39. NECKLAWS E, LAQUAGLIA M, MACLAUGHLIN D, HUDSON P, MUDGETT-HUNTER M, DONAHOE PK: Detection of Mullerian inhibiting substance in biological samples by a solid phase sandwich radioimmunoassay. Endocrinol 2:791, 1986
40. NEWBOLD R, SUZUKI Y, MCLACHLIN JA: Mullerian duct maintenance in heterotypic organ culture *in vivo* exposure to diethylstilbestrol. Endocrinol 115:1863, 1985
41. SATO E, ISHIBASHI T, IRITANI A: Meiotic arresting substance separated from porcine ovarian granulosa cells and hypothetical arresting mechanism of meiosis. In CHANNING CP, SEGAL SJ (eds): Intraovar Contr Mech, p 161. New York, 1982
42. SATO E, KOIDE SS: A factor from bovine granulosa cells preventing oocyte maturation. Differ 26:59, 1984
43. SCHULTZ AW: Oogenesis: Processes and their regulation. Adv Reprod Physiol 4:99, 1969
44. SHIMA H, DONAHOE PK, MUDZIK GP, KAMAGATA S, HUDSON P, MUDGETT-HUNTER M: Production of monoclonal antibodies for affinity purification of bovine Mullerian inhibiting substance activity. Hybridoma 3:201, 1984
45. SPIVACK JG, ERIKSON RL, MALLER JL: Microinjection of pp60v-src omtp *Xenopus* oocytes increases phosphorylation of ribosomal protein S6 and accelerates the rate of progesterone-induced meiotic maturation. Mol Cell Biochem 4:1631, 1984
46. TAKAHASHI M, HAYASHI M, MANGANARO TF, DONAHOE PK: The ontogeny of Mullerian inhibiting substance in granulosa cells of the bovine ovarian follicle. Biol Reprod 35:447, 1986a
47. TAKAHASHI M, KOIDE SS, DONAHOE PK: Mullerian inhibiting substance as oocyte meiosis inhibitor. Mol Cell Endocrinol 47:225, 1986b
48. TSAFRIRI A: Oocyte maturation in mammals. In JONES RE (ed): The Vertebrate Ovary, p 409. New York, Plenum Press 1975
49. TSAFRIRI A, CHANNING CP: An inhibiting influence of granulosa cells and follicular fluid upon porcine oocyte meiosis *in vitro*. Endocrinol 96:922, 1975
50. UENO S, MANGANARO TF, DONAHOE PK: Human recombinant Mullerian inhibiting substance inhibition of rat oocyte meiosis is reversed by epidermal growth factor *in vitro*. Endocrinol 1988 (submitted)
51. VERMEIDEN JPW, ZEILMAKER GH: Relationship between maturation, division, ovulation and luteinization in the female rat. Endocrinol, 95:341, 1974
52. VIGIER B, PICARD JY, TRAN D, LIEGEAL L, JOSSO N: Production of anti-Mullerian hormone: Another homology between Sertoli and granulosa cells. Endocrinol, 114:1315, 1984
53. WOLFF E: L'action du diethylstilboestrol sur les organes genitauz de l'embryon de poulet. C.R. Acad Sci (Paris), 208:1532, 1939
54. ZAMBONI L, DEMARTIN C: Buffered picric acid-formaldehyde: A new, rapid fixative for electron microscopy. J Cell Biol 35:148A, 1967

DISCUSSION

CHAIRPERSON: MILAN R. HENZL, M.D., Ph.D.

DR. MASON: You mentioned that EGF reversed the effects of MIS in the OMI assay. In the concentration range used in that situation, does MIS block EGF receptor phosphorylation?

DR. DONAHOE: The concentrations that we used in the membrane preparations are in the same order of magnitude as in the OMI assay.

DR. MASON: But it seems a little confusing, because then you could assume that EGF would not have any effect, because it could not stimulate the EGF receptor to autophosphyorylate.

DR. DONAHOE: I have to think about that, but it is probably a timing phenomenon related to the relative ontogeny of both ligand and receptor.

DR. SCHOMBERG: I have a follow-up to that question. If EGF is in the granulosa cell, and granulosa cells are also producing MIS, and MIS is inhibiting EGF tyrosine kinase activity, it is somewhat difficult to see how the granulosa cells grow.

DR. DONAHOE: At this time, we do not have enough information to answer your questions, nor do we have sufficient quantities of MIS to completely address the question. It is necessary to determine MIS concentrations in small, medium, and large follicles.

I think it is clear that the concentration of the EGF receptor varies throughout the cell cycle. How MIS and EGF interact *in vivo* remains to be seen. We do have good evidence, however, that MIS may be a natural substance that inhibits EGF-stimulated tyrosine kinase activity.

It is of interest that there is one other tyrosine kinase inhibitor, a yeast protein, PKP, which also requires a protein co-factor.

23

Insulin-Like Growth Factor I: A Paradigm For Putative Intraovarian Regulators

ELI Y. ADASHI
CAROL E. RESNICK
ELEUTERIO R. HERNANDEZ
MARJORIE E. SVOBODA
E. HOYT
DAVID R. CLEMMONS
PAULINE K. LUND
JUDSON J. VAN WYK

The cyclic process of ovarian folliculogenesis is an exponential rather than a linear process marked by dramatic proliferation and differentiation of the developing granulosa cell. Although the central role(s) of gonadotropins[49] and of gonadal steroids[39] in this explosive agenda is well accepted, the variable fate of follicles afforded comparable gonadotropic stimulation suggests the existence of additional intraovarian modulatory systems.[37] Among potential novel intraovarian regulators, Somatomedin-C/Insulin-like growth factor I (Sm-C/IGF-I) has been the subject of increasingly intense investigation.[2,32] Considered in aggregate, these studies strongly suggest the existence of an intraovarian autocrine control mechanism, wherein (Sm-C/IGF-I) may serve as the central signal, and the granulosa cell its site of production, reception, and action. In this capacity, Sm-C/IGF-I may promote the replication and/or cytodifferentiation of the developing granulosa cell, acting largely (but not exclusively) as an amplifier of gonadotropin action. In addition, Sm-C/IGF-I (presumptively of granulosa cell origin) may also provide paracrine input to the adjacent theca-interstitial cell compartment in the interest of coordinated follicular development.

THE GRANULOSA CELL AS A SITE OF Sm-C/IGF-I PRODUCTION

Ovarian production of Sm-C/IGF-I was initially suggested by studies revealing that the immunoreactive (i) Sm-C/IGF-I content of *porcine* follicular fluid substantially exceeded that encountered in serum.[31] Further evidence consisted of the demonstration of cycloheximide-inhibitable, gonadotropin (and estradiol)-dependent[36] iSm-C/IGF-I in serum-free media conditioned by cultured *porcine* granulosa cells.[31] Although the *rat*

Supported in part by NIH Research Grant HD-19998 (EYA) and USPHS Research Career Development Award 1-K04-HD-00697 from the NICHHD, NIH.

ovarian content of iSm-C/IGF-I appears to be growth hormone-dependent,[23] a direct effect of growth hormone at the level of the rat granulosa cell remains to be demonstrated. Extending the investigation to the transcriptional level, we have recently shown that the adult and immature rat ovary (as well as the isolated, immature granulosa cell) is a site of Sm-C/IGF-I gene expression[34] and that it may be subject to gonadotropic regulation. Significantly, of all adult rat organs tested,[43] the ovary (O) displays the third highest level of Sm-C/IGF-I gene expression, the uterus (U) and liver (Li) being the most active in this regard (Fig. 1). In contrast, *human* granulosa cells may be a site of IGF-II rather than Sm-C/IGF-I gene expression.[48,59] These observations and the lack of IGF-II gene expression in the adult *rat* ovary[43] suggest possible species specificity. However, profound differences in the experimental conditions may favor IGF-II gene expression as a dedifferentiation (fetal) marker.

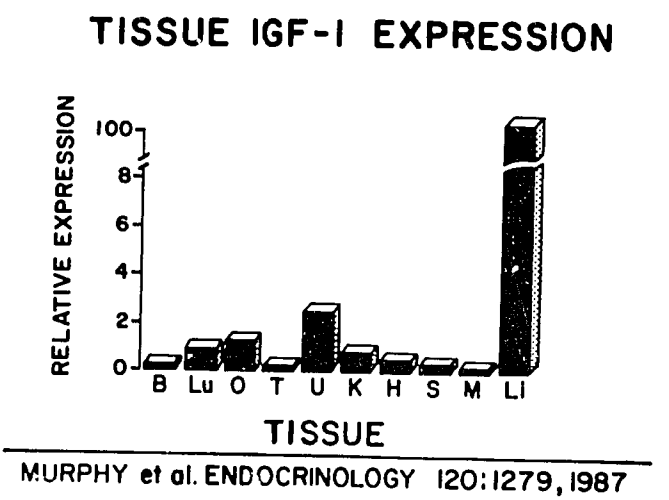


Figure 1: Relative expression of Sm-C/IGF-I gene in various tissues. L = liver; U = uterus; O = ovary.

OVARIAN Sm-C/IFG-I GENE EXPRESSION

To evaluate the possibility of ovarian Sm-C/IGF-I gene expression, polyadenylated RNA from the ovaries of immature (21-23 days old; DES-treated) or randomly cycling adult (70-80 days old; non-DES-treated) rats was hybridized with a ^{32}P-labelled rat Sm-C/IGF-I genomic DNA probe[38] (Fig. 2). As in liver,[38] northern blot analysis revealed three major RNA species sized at 1.7, 4.7, and 7.5 kb. Significantly, however, comparison of hybridization signals indicates a substantially greater abundance of specific Pre Pro Sm-C/IGF-I RNAs (mostly 7.5 and 1.7kb) in ovaries of immature DES-treated (Lane 2) as compared with adult (Lane 1) rats. Given that the ovaries of immature DES-treated rats are comprised largely (>90%) of granulosa cells, these findings suggest measurable Sm-C/IGF-I gene

expression at the level of the ovarian granulosa cell. Reprobing of the blots with a ubiquitin cDNA probe[40] revealed a similar abundance of ubiquitin mRNA in all samples (not shown) indicating that the observed differences are not due to differences in the polyadenylated message load. The significance and the translational competence of the multiple relatively high molecular weight RNA transcripts (the smallest of which is of sufficient size to encode Pre Pro Sm-C/IGF-I) remains uncertain. However, it is likely that these RNA transcripts represent processing variants since Pre Pro Sm-C/IGF-I is encoded by a single gene.[50,51,52] Regardless, these observations are in keeping with the notion that the ovary is a site of Sm-C/IGF-I gene expression and that locally rather than circulatory-derived Sm-C/IGF-I may play a role in ovarian physiology. The above notwithstanding, additional studies will be required to determine whether the differences observed between immature and adult rats are in effect age-dependent or whether DES, applied to immature but not adult rats, may play a role in this regard.

Ovarian Sm-C/IGF-I Gene Expression: Effect of FSH

Given the pivotal role of FSH in the promotion of granulosa cell differentiation,[37] FSH-regulated Sm-C/IGF-I expression is a distinct possibility. Indeed, FSH did prove capable of stimulating porcine granulosa cell iSm-C/IGF-I release.[36] To explore the role of FSH,

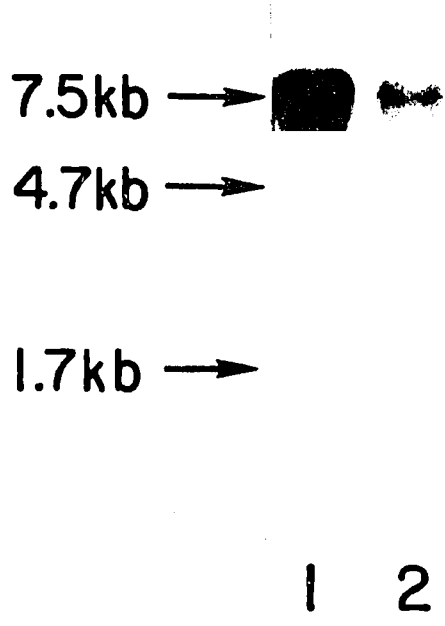


Figure 2: Polyadenylated RNA (20mg/lane) samples are from (1) immature (21-23 days old), DES-treated, or (2) randomly cycling adult (70-80 days old) rats.

increasing amounts of total RNA from the ovaries of FSH (0, 5, or 10mg/rat/day; bid)-treated, immature hypophysectomized rats were dotted onto Genescreen and hybridized with a ^{32}P-labelled rat Pre Pro Sm-C/IGF-I_A cDNA probe.[50] As shown in the dot blot analysis (Fig. 3), treatment with increasing concentrations of FSH produced dose-dependent increments in the intensity of the Pre Pro Sm-C/IGF-I (but not ubiquitin; not shown) hybridization signal. Although these findings do not conclusively implicate the granulosa cell as a site of Sm-C/IGF-I gene expression, the very fact that the granulosa cell (rather than the theca-interstitial cell) is a site of FSH reception and action supports such a possibility.

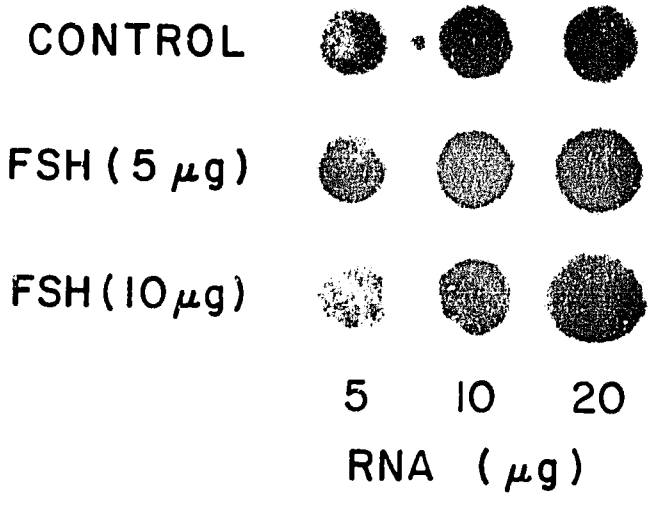


Figure 3: Ovarian Sm-C/IGF-I gene expression: Effect of FSH

Pre Pro Sm-C/IGF-I mRNA Variants: Implications For Regulation and Action

Recent studies on Sm-C/IGF-I gene expression suggest the existence of a complex array of variably sized messenger RNA species.[19,21,50,51,52] In the human, only two types of Sm-C/IGF-I mRNA have thus far been defined by molecular cloning studies and shown to be the consequence of alternative splicing of the primary (single copy) gene transcript.[51,52] More recently, a similar phenomenon has been uncovered in the rat[50] wherein differential splicing produces mRNAs coding for precursor molecules homologous with respect to the mature Sm-C/IGF-I protein but different in the length and sequence of the E domain (trailer) peptide (Fig. 4). Both encode distinct Sm-C/IGF-I precursors of 127 and 133 amino acids containing the same initial 108 residues. The two corresponding cDNAs differ by the presence or absence of a 52-base pair insert (probably coded for by its own exon) as well as in the 5'UT region. As a whole, these studies suggest that Sm-C/IGF-I biosynthesis may not only be subject to transcriptional control but that as in

the case of other genes (e.g. calcitonin/calcitonin gene-related peptide), tissue-specific regulation of RNA and/or protein processing may also be at play. In addition, these observations raise the possibility that Pre Pro Sm-C/IGF-I may be a polyprotein and that its E peptide(s) may subserve discrete tissue-limited biological functions. At this time, the full significance of these developments to Sm-C/IGF-I physiology remains unknown.

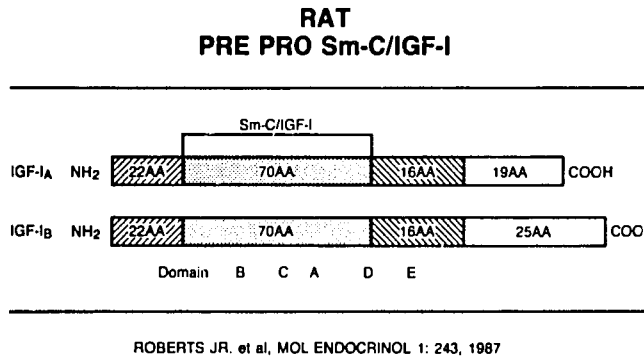


Figure 4: Rat Pre Pro Sm-C/IGF-I variants

THE GRANULOSA CELL AS A SITE OF Sm-C/IGF-I RECEPTION

Studies carried out in the last several years have clearly established the granulosa cell as a site of Sm-C/IGF-I reception.[19,51,52]

Receptor Characterization

Freshly obtained porcine granulosa cells possess high affinity (Kd = 0.69-2.1x10^{-9}M), low capacity binding sites.[37] Binding to FSH-primed rat granulosa cells proved time-, temperature-, and pH-dependent, optimal steady state conditions being achieved following an 8 hour incubation at 15 °C and a pH of 8.0.[52] Although subject to regulation by the cellular density of plating, the binding of [^{125}I]Sm-C/IGF-I to its receptor proved saturable (apparent Kd = 3.3x10^{-9}M) as well as reversible, complete or partial tracer displacement being effected by competitive inhibition and dilution, respectively.[3] Scatchard and Hill analyses yielded linear plots consistent with a single class of non-interacting binding sites. Specificity studies revealed the competition for [^{125}I]Sm-C/IGF-I binding to follow a rank order of potency of Sm-C/IGF-I > MSA > insulin, a pattern compatible with a Type I IGF receptor. Limited or no displacement was observed for a series of chemically-related and unrelated polypeptides as well as by a human insulin receptor antiserum.[3] Using affinity cross-linking, we have also been able to observe that whole ovarian membranes of untreated (or FSH-treated) immature hypophysectomized, DES-treated rats are endowed with specific Type-I IGF receptors.[2] Similar results have later been obtained with isolated granulosa cells from the same experimental model (unpublished).

Receptor Heteroregulation

We have recently reported[3,7,9] FSH and LH to be capable of upregulating granulosa cell Sm-C/IGF-I binding, an effect further augmented by growth hormone but not prolactin. Specifically, we were able to show that FSH is capable of upregulating Sm-C/IGF-I binding in a time- and dose-dependent fashion and that cAMP, its purported intracellular second messenger, may play an intermediary role in this regard.[7] Indeed, granulosa cell Sm-C/IGF-I binding was enhanced following elevation of the intracellular cAMP content by a series of cAMP-generating agonists, inhibition of cAMP-phosphodiesterase activity, or the provision of non-degradable cAMP analogs. High dose forskolin (10^{-5}M), like FSH, proved capable of augmenting Sm-C/IGF-I binding by itself, while an essentially inert dose (10^{-7}M) synergized with FSH in this regard. Significantly, heterologous receptor upregulation was not limited to FSH, similar increments being observed for luteotropic, b_2-adrenergic, but not lactogenic granulosa cell agonists.[3] Related *in vivo* studies using immature, hypophysectomized, DES-treated rats revealed that the ability of FSH to upregulate granulosa cell Sm-C/IGF-I binding: a) is not strictly an *in vitro* phenomenon in that it can be fully reproduced *in vivo;* b) is due to enhancement of Sm-C/IGF-I binding capacity rather than affinity; c) may be subject to diametrically opposed modulation by somatogenic and GnRH-like granulosa cell agonists (up- and down-regulation, respectively); and d) is best maintained by gonadotropins but no prolactin.[9] Inasmuch as gonadotropin-dependence constitutes a unique attribute of the ovarian granulosa cell, our findings further suggest that the granulosa cell Sm-C/IGF-I receptor may have thoroughly adapted to its unique environment, providing the first example of a cell type for which the complement of Sm-C/IGF-I receptors may be cAMP-dependent.

Given the pivotal role of FSH in the induction of granulosa cell receptors for luteotropic and lactogenic ligands,[3,7] this finding strongly suggests that the acquisition of Sm-C/IGF-I responsiveness may be part and parcel of granulosa cell ontogeny. Accordingly, gonadotropins may condition the cell to respond optimally to Sm-C/IGF-I, thereby conferring selective advantage upon follicles so endowed.

The above notwithstanding, little is known at this time regarding possible alteration(s) in ovarian Sm-C/IGF-I reception in the course of ontogeny, follicular maturation, and luteinization. In support of such possibility, we have recently observed significant developmental control of ovarian Sm-C/IGF-I reception during the infantile and prepubertal periods. At the cellular level, several central issues remain unresolved including the role of the Type II IGF receptor in Sm-C/IGF-I hormonal action[2] and the molecular mechanism(s) underlying the heteroregulation of Type I IGF receptors.

Ovarian Sm-C/IGF-I Reception: Mid-Infantile to Mid-Prepubertal Period

In the course of studies on the ontogeny of ovarian Sm-C/IGF-I reception, rats were sacrificed at weekly intervals on post-partum days 14 (mid-infantile period), 21 (early prepubertal period), and 28 (mid-prepubertal period). Following sacrifice, the ovaries from each age group (n = 28, 10, and 10, respectively) were removed, pooled, and their Sm-C/IGF-I binding determined (Fig. 5). Specific Sm-C/IGF-I binding proved highest on post-partum day 14 situated in the midst of the infantile period (*i.e.* the period spanning days 7-21 of

life). Significantly, however, this was followed by a time-dependent decline in ovarian Sm-C/IGF-I reception coinciding with the early (day 21) and mid- (day 28) prepubertal period. Similar results were obtained when the data was normalized per mg ovarian weight or per mg ovarian protein. As such, the pattern of ovarian Sm-C/IGF-I reception appears to closely follow that of the circulating levels of iFSH (Fig. 5) as described by Ojeda and Ramirez.[46] Most importantly, it would appear that the day 14 high observed for ovarian Sm-C/IGF-I reception follows in short sequence the day 12 peak in the circulating levels of iFSH. Similarly, ovarian Sm-C/IGF-I reception appears to decline as the circulating levels of iFSH diminish markedly to reach low values at the end of the prepubertal period. Given the ability of FSH to upregulate granulosa cell Sm-C/IGF-I binding,[7] it is tempting to speculate that the ontogeny of the ovarian Sm-C/IGF-I receptor during the first month of life may be coupled, if only in part, to FSH release. Although these findings need not be equated with a role for intraovarian Sm-C/IGF-I in the infantile period, they nevertheless suggest that ovarian Sm-C/IGF-I reception is well poised to play such a role at this critical point in life. Indeed, it is during this time that more follicles start to grow (and at a faster rate) than at any time later,[45] a situation in which the proliferative potential of Sm-C/IGF-I[16] could be readily applied.

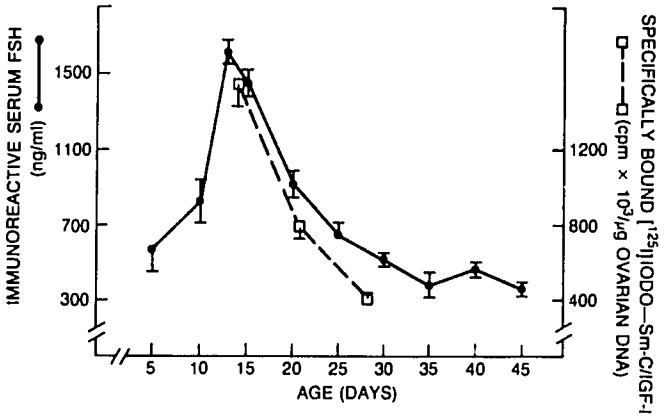


Figure 5: Ovarian Sm-C/IGF-I reception: Mid-infantile to mid-prepubertal period.

THE GRANULOSA CELL AS A SITE OF Sm-C/IGF-I ACTION

Studies carried out in the last several years have clearly established the granulosa cell as a site of Sm-C/IGF-I action.[1,4,6,10,11,13,18,23,54,55,56,57,58,60] Sm-C/IGF-I action at the level of the rat,[1,5,6,8,11,12,18,23,54] but not porcine[54,55,57,58] granulosa cell appears largely (but not exclusively) contingent upon its ability to synergize with pituitary gonadotropins (Fig. 6). These effects are unaccounted for by enhanced cellular viability, plating efficiency, or DNA synthesis.[12] Thus, this ability of Sm-C/IGF-I to augment differentiated phenotypic expression of the developing granulosa cell may be distinct from its well established growth

promoting property[12] and was thus considered a novel biologic effect of this polypeptide. In this connection, we have been able to show that Sm-C/IGF-I is capable of augmenting FSH-supported (but not basal) progesterone[5,6,12] and estrogen[1] biosynthesis as well as the FSH-mediated acquisition of LH receptors.[11] More recently, Sm-C/IGF-I was also found to augment basal as well as FSH-supported proteoglycan biosynthesis.[8] In this respect, Sm-C/IGF-I appeared to exert its classic "sulfation factor" activity at the level of the granulosa cell, the very chondrotropic effect which led to its discovery. Fractionation of the major extracellular proteoglycan species revealed FSH to favor the exclusive production of dermatan sulfate, whereas Sm-C/IGF-I supported the simultaneous biosynthesis of both heparan and dermatan sulfate. These findings suggest that Sm-C/IGF-I may effect marked quantitative as well as qualitative alterations in proteoglycan economy. Given the possible role of proteoglycans in follicular antrum formation and follicular atresia,[20] these findings raised the possibility that Sm-C/IGF-I of granulosa cell origin may partake in the growth as well as demise of the developing ovarian follicle.

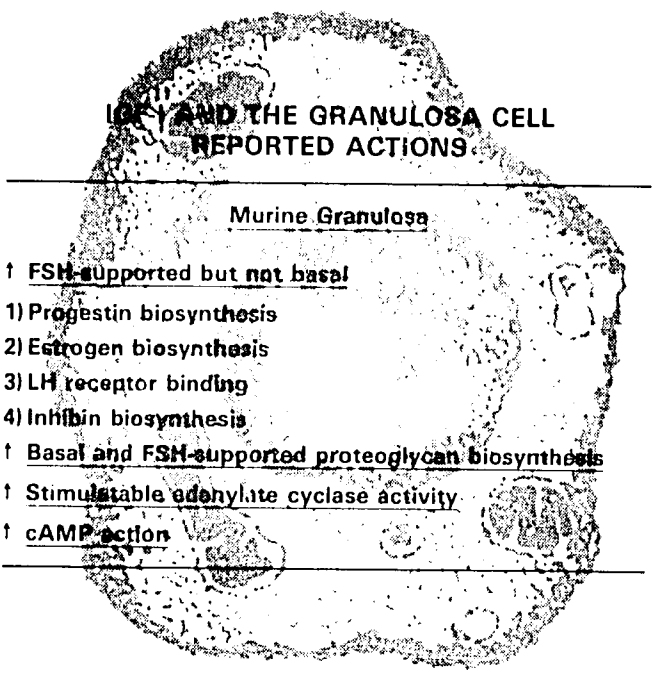


Figure 6: Reported actions of Sm-C/IGF-I in reference to the rat granulosa cell.

Specifically, Sm-C/IGF-I proved capable of amplifying the FSH transduction sequence at a multiple cellular site(s) both proximal[4,16,13,23] and distal[4] to cAMP generation (Fig. 7). Although without effect on cAMP breakdown,[4] Sm-C/IGF-I appeared to exert a direct stimulatory effect at the level of the adenylate cyclase complex in the face of unaltered FSH

receptor content.[4] However, additional studies will be required to distinguish between direct effect(s) of this peptide on the stimulatory ($G_S = N_S$) or inhibitory ($G_i = N_i$) regulatory membrane proteins, the catalytic (C) cyclase protein, or combinations thereof. In addition, Sm-C/IGF-I was observed to exert a potent stimulatory effect on cAMP action as reflected in cAMP-supported progesterone biosynthesis.[4] However, additional studies will be required to determine which of the post cAMP events may be involved. The above notwithstanding, there is reason to believe that the ability of Sm-C/IGF-I to enhance FSH hormonal action may not be limited to perturbation of intracellular signaling but may also involve enhanced intercellular communication *e.g.* granulosa cell clumping and intercellular gap junction formation.[42]

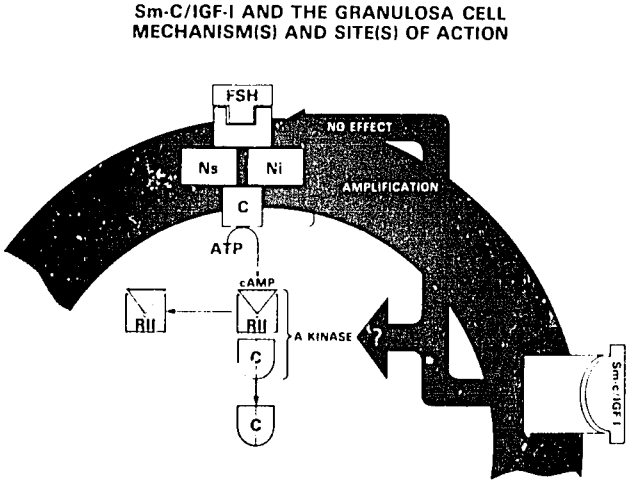


Figure 7: Sm-C/IGF-I and the rat granulosa cell: Mechanism(s) and site(s) of action.

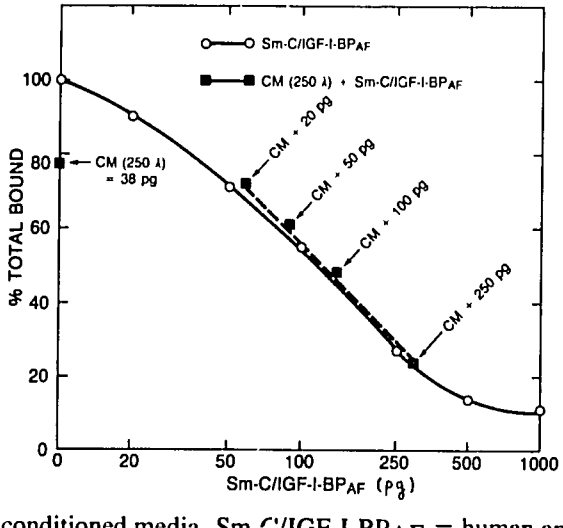


Figure 8: CM = conditioned media. Sm-C/IGF-I-BP_{AF} = human amniotic fluid Sm-C/IGF-I binding protein standard.

SUMMARY

We have recently observed that the theca-interstitial, like the granulosa cell, may be a site of Sm-C/IGF-I reception and action and that physiological concentrations of Sm-C/IGF-I may also participate in the regulation of ovarian androgen biosynthesis.[35] As such, these observations are in keeping with the view that Sm-C/IGF-I of granulosa cell origin may not only play an autocrine role but may also serve as one of several signals through which the granulosa cell may communicate in a paracrine fashion with the adjacent theca-interstitial cell compartment. In doing so, the granulosa cell may exert some control over its own destiny by enhancing ovarian androgen provision to suit its aromatizing capabilities and the estrogen requirements of the developing follicle as a whole. This line of reasoning introduces a level of complexity not previously envisioned, implicating Sm-C/IGF-I in the orchestration of the coupling of androgen to estrogen biosynthesis thereby promoting coordinated follicular development. Thus, Sm-C/IGF-I may subserve at least three central functions:

1. Amplification of gonadotropin hormonal action—A key requirement given the exponential nature of follicular development.
2. Integration of follicular development—An essential facet concerned with the coordination of granulosa-theca cooperation.
3. Selection of dominant follicle(s)—A speculative proposition assuming timely and selective activation of the Sm-C/IGF-I system in "chosen" follicles.

Aside from its possible role(s) in the course of established follicular cycles, Sm-C/IGF-I (and/or IGF-II) may also participate in the very formation of the follicular apparatus during the late fetal/early neonatal period. Although the ovary is gonadotropin-independent at that time,[45] we have previously shown[20,35] that Sm-C/IGF-I (and/or IGF-II) may well interact with VIPergic input now implicated in the morphodifferentiation of the follicular apparatus.[14,25,29,30] Similarly, Sm-C/IGF-I may be concerned with the promotion of juvenile and early pubertal follicular development, a time period characterized by low, ever declining gonadotropin (FSH) levels,[27,46,47] but rising titers of serum growth hormone.[28,44] At the clinical level, ovarian Sm-C/IGF-I may have a bearing on the puberty promoting effect of growth hormone. Indeed, an association appears to exist between isolated growth hormone deficiency and delayed puberty in both rodents[15,17,26] and human[41,53] subjects, a process reversed by systemic growth hormone replacement therapy.[33,53] Given that ovarian Sm-C/IGF-I[23] and its receptor[9] may be growth hormone-dependent, it is tempting to speculate that the ability of growth hormone to accelerate pubertal maturation may be due, at least in part, to the promotion of ovarian Sm-C/IGF-I production and reception with the consequent local potentiation of gonadotropin action.

REFERENCES

1. ADASHI EY, RESNICK CE, BRODIE AMH, SVOBODA ME, VAN WYK JJ: Somatomedin-C mediated potentiation of follicle stimulating hormone-induced aromatase activity of cultured rat granulosa cells. Endocrinology 117:2313, 1985
2. ADASHI EY, RESNICK CE, D'ERCOLE AJ, SVOBODA ME, VAN WYK JJ: Insulin-like growth factors as intraovarian regulators of granulosa cell growth and function. Endocr Rev 6:400-420, 1985

3. ADASHI EY, RESNICK CE, HERNANDEZ ER, SVOBODA ME, VAN WYK JJ: Characterization and regulation of a specific cell membrane receptor for somatomedin-C/insulin-like growth factor I in cultured rat granulosa cells. Endocrinology (in press) 1987
4. ADASHI EY, RESNICK CE, MAY JV, KNECHT M, SVOBODA ME, VAN WYK JJ: Somatamedin-C/insulin-like growth factor I as an amplifier of follicle-stimulating hormone action: Studies on mechanism(s) and site(s) of action in cultured rat granulosa cells. Endocrinology (submitted; see appendix) 1987
5. ADASHI EY, RESNICK CE, SVOBODA ME, VAN WYK JJ: A novel role for somatomedin-C in the cytodifferentiation of the ovarian granulosa cell. Endocrinology 115:1227, 1984
6. ADASHI EY, RESNICK CE, SVOBODA ME, VAN WYK JJ: A role for somatomedin-C in the differentiation of the immature rat granulosa cell. In: TOFT DC, RYAN RJ (eds): Proceedings of the Fifth Ovarian Workshop, p 227. Ovarian Workshops, Champaign, 1985
7. ADASHI EY, RESNICK CE, SVOBODA ME, VAN WYK JJ: Follicle-stimulating hormone enhances somatomedin-C binding to cultured rat granulosa cells: Evidence for cAMP-dependence. J Biol Chem 261:3923, 1986
8. ADASHI EY, RESNICK CE, SVOBODA ME, VAN WYK JJ, HASCALL VC, YANAGISHITA M: Independent and synergistic actions of somatomedin-C in the stimulation of proteoglycan biosynthesis by cultured rat granulosa cells. Endocrinology 118:456, 1986
9. ADASHI EY, RESNICK CE, SVOBODA ME, VAN WYK JJ: *In vivo* regulation of granulosa cell somatomedin-C/insulin-like growth factor I receptors. Endocrinology (submitted) 1987
10. ADASHI EY, RESNICK CE, SVOBODA ME, VAN WYK, JJ: Somatomedin-C an amplifier of follicle-stimulating hormone action: Enhanced accumulation of adenosine 3',5'-cyclic monophosphate. Endocrinology 118:149, 1986
11. ADASHI EY, RESNICK CE, SVOBODA ME, VAN WYK JJ: Somatomedin-C enhances induction of luteinizing hormone receptors by follicle-stimulating hormone in cultured rat granulosa cells. Endocrinology 116:2369, 1985
12. ADASHI EY, RESNICK CE, SVOBODA ME, VAN WYK, JJ: Somatomedin-C synergizes with follicle-stimulating hormone in the acquisition of progesterone biosynthetic capacity by cultured rat granulosa cells. Endocrinology 116:2135, 1985
13. ADASHI EY, RESNICK CE, SVOBODA ME, VAN WYK JJ: Synergistic interactions of Somatomedin-C with adenosine 3',5'-cyclic monophosphate-dependent granulosa cell agonists. Biol Reprod 34:81, 1986
14. AHMED CE, DEES WL, OJEDA SR: The immature rat ovary is innervated by vasoactive intestinal peptide (VIP)-containing fibers and responds to VIP with steroid secretion. Endocrinology 118:1682, 1986
15. ADVIS JP, SMITH WHITE S, OJEDA SR: Activation of growth hormone short loop negative feedback delays puberty in the female rat. Endocrinology 108:1343, 1981
16. BARANAO JLS, HAMMOND JM: Comparative effects of insulin and insulin-like growth factors on DNA synthesis and differentiation of porcine granulosa cells. Biochem Biophys Res Commun 124:484, 1984
17. BARTKE A: Histology of the anterior hypophysis, thyroid and gonads of two types of dwarf mice. Anat Rec 149:225, 1978.
18. BICSAK TA, TUCKER EM, CAPPEL S, VAUGHAN J, RIVIER J, VALE W, HSUEH AJW: Hormonal regulation of granulosa cell inhibin biosynthesis. Endocrinology 119:2711, 1986
19. BELL GI, STEMPIEN MM, FONG NM, RALL LB: Sequence of liver cDNAs encoding two different mouse insulin-like growth factor I precursors. Nucleic Acids Research, 14:7873, 1986
20. BELLIN ME, AX RL: Chondroitin sulfate: An indicator of atresia in bovine follicles. Endocrinology 114:428, 1984
21. CASELLA SJ, SMITH EP, VAN WYK JJ, JOSEPH DR, HYNES MA, HOYT EC, LUND PK: Isolation of rat testis cDNAs encoding a somatomedin-C/insulin-like growth factor I precursor. Nucleic Acids Research (in press)
22. DAVOREN JB, HSUEH AJW, LI CH: Somatomedin-C augments FSH-induced differentiation of cultured rat granulosa cells. Am J Physiol 249:E26, 1985
23. DAVOREN JB, HSUEH AJW: Growth hormone increases ovarian levels of immunoreactive somatomedin-C/insulin-like growth factor I *in vivo*. Endocrinology 118:888, 1986
24. DAVOREN JB, KASSON BG, LI CH, HSUEH AJW: Specific insulin-like growth factor (IGF) I- and II-binding sites on rat granulosa cells: Relation to IGF action. Endocrinology 119:2155, 1986
25. DEES WL, AHMED CE, OJEDA SR: Substance P and vasoactive intestinal peptide-containing fibers reach the ovary by independent routes. Endocrinology 119:638, 1986

26. DEREVIERS MM, VIGUIER-MARTINEZ MC, MARIANA JC: FSH, LH and prolactin levels, ovarian follicular development and ovarian responsiveness to FSH in the Snell drawf mouse. Acta Endocrinol 106:121, 1984
27. DOHLER KD, WUTTKE W: Changes with age in levels of serum gonadotropins, prolactin, and gonadal steroids in prepubertal male and female rats. Endo 97:898, 1975
28. EDEN S: Age- and sex-related differences in episodic growth hormone secretion in the rat. Endocrinology 105:555, 1979
29. GEORGE FW, OJEDA SR: Vasoactive intestinal peptide enhances aromatase activity in the neonatal rat ovary before development of primary follicles or FSH responsiveness. Proc Natl Acad Sci USA (in press), 1987
30. GOZES I, TSAFRIRI A: Detection of vasoactive intestinal peptide-encoding messenger ribonucleic acid in the rat ovaries. Endocrinology 119:2606, 1986
31. HAMMOND JM, BARANAO JLS, SKALERIS D, KNIGHT AB, ROMANUS JA, RECHLER MM: Production of insulin-like growth factors by ovarian granulosa cells. Endocrinology 117:2553, 1985
32. HAMMOND JM: Peptide regulators in the ovarian follicle. Aust J Biol Sci 34:491, 1981
33. HAMMER RE, PALMITER RD, BRINSTER RL: Partial correction of murine hereditary growth disorder by germ-line incorporation of a new gene. Nature 311:65, 1984
34. HERNANDEZ ER, HOYT E, VAN WYK JJ, ADASHI EY: The somatomedin-C/insulin-like growth factor I (Sm-C/IGF-I) gene is expressed in the rat ovary. Presented at the 69th Annual Meeting of the Endocrine Society, Indianapolis, IN (Abstract #821), 1987
35. HERNANDEZ ER, RESNICK CE, SVOBODA ME, VAN WYK JJ, PAYNE DW, ADASHI EY: Somatomedin-C/insulin-like growth factor I (Sm-C/IGF-I) as an enhancer of androgen biosynthesis by cultured rat ovarian cells. Endocrinology (submitted; see appendix), 1987
36. HSU C-J, HAMMOND JM: Gonadotropins and estradiol stimulate immunoreactive insulin-like growth factor-I production by porcine granulosa cells *in vitro*. Endocrinology 120:198, 1987
37. HSUEH AJW, ADASHI EY, JONES PBC, WELCH JR, TH: Hormonal regulation of cultured ovarian granulosa cells. Endocr Rev 5:76, 1984
38. HYNES MA, VAN WYK JJ, BROOKS PJ, D'ERCOLE AJ, JANSEN M, LUND PK: Growth hormone dependence of somatomedin-C/insulin-like growth factor I and insulin-like growth factor-II messenger ribonucleic acids. Mol Endocrinol 1:233, 1987
39. LEUNG PCK, ARMSTRONG DT: Interactions of steroids and gonadotropins in the control of steroidogenesis in the ovarian follicle. Ann Rev Physiol 42:71, 1980
40. LUND PK, MOATS-STAATS BM, SIMMONS JG, HOYT E, D'ERCOLE AJ, MARTIN F, VAN WYK JJ: Nulceotide sequence analysis of cDNA encoding human ubiquitin reveals that ubiquitin is synthesized as a precursor. J Biol Chem. 260:7609, 1985
41. MAGNER JA, ROGOL AD, GORDON P: Reversible growth hormone deficiency and delayed puberty triggered by a stressful experience in a young adult. Am J Med 76:737, 1984
42. MAY JV, SCHOMBERG, DW, GORDON S, AMSTERDAM A: Synergistic effect of insulin and follicle-stimulating hormone on biochemical and morphological differentiation of porcine granulosa cells. Biol Reprod 32:(Suppl) 53, 1985
43. MURPHY LJ, BELL GI, FRIESEN HG: Tissue distribution of insulin-like growth factor I and II messenger ribonucleic acid in the adult rat. Endocrinology 120:1279, 1987
44. OJEDA SR, JAMESON HE: Developmental patterns of plasma and pituitary growth hormone (GH) in the female rat. Endocrinology 100:881, 1977
45. OJEDA SR, ANDREWS WW, ADVIS JP, SMITH-WHITE S: Recent advances in the endocrinology of puberty. Endocr Rev 1:228, 1980
46. OJEDA SR, RAMIREZ VD: Plasma level of LH and FSH in maturing rats: Response to hemigonadectomy. Endocrinology 90:446, 1972
47. OJEDA SR, WHEATON JE, JAMESON HE, MCCANN SM: The onset of puberty in the female rat: Changes in plasma prolactin, gonadotropins, luteinizing hormone-releasing hormone (LHRH), and hypothalamic LHRH content. Endo 98:630, 1976
48. RAMASHARMA K, LI CH: Human pituitary and placental hormones control human insulin-like growth factor II secretion in human granulosa cells. Proc Natl Acad Sci USA 84:2643, 1987
49. RICHARDS JS: Hormonal control of ovarian follicular development. A 1978 perspective. Recent Prog Horm Res 35:343, 1979

50. ROBERTS CT, LASKY SR, LOWE WL, SEAMAN W, LEROITH D: Molecular cloning of rat insulin-like growth factor I complementary deoxyribonucleic acids: Differential messenger ribonucleic acid processing and regulation by growth hormone in extrahepatic tissues. Mol Endocrinol 1:243, 1987
51. ROTWEIN P, POLLOCK KM, DIDIER DK, KRIVI GG: Organization and Sequence of the human insulin-like growth factor I. Alternative RNA processing produces two insulin-like growth factor I precursor peptides. J Biol Chem 261:4828, 1986
52. ROTWEIN P: Two insulin-like growth factor I messenger RNAs are expressed in human liver. Proc Natl Acad Sci USA 83:77, 1986
53. SHEIKHOLISLAM BM, STEMPFEL RS: Hereditary isolated somatotropin deficiency: Effects of human growth hormone administration. Ped 49:362, 1972
54. VAN WYK JJ, RUSSELL WE, UNDERWOOD LE, SVOBODA ME, GILLESPIE GY, PLEDGER WJ, ADASHI EY, BALK SD: Action of somatomedins on cell growth: Effect of selective neutralization of somatomedin-C (insulin-like growth factor I) with a monoclonal antibody. In RAITI S, TOLMAN RA (eds): Human Growth Hormone, p 585, Plenum Publishing Corporation, 1986
55. VELDHUIS JD, DEMERS LM, GARMEY J, JUCHTER D, AZIMI P: A role for somatomedin-C as a differentiating hormone and amplifier of hormone action on ovarian cells: Studies with synthetically pure human somatomedin C and swine granulosa cells. Biochem Biophys Res Commun 130:234, 1985
56. VELDHUIS JD, FURLANETTO RW, JUCHTER D, GARMEY J, VELDHUIS P: Trophic actions of human somatomedin-C/insulin-like growth factor I on ovarian cells: *In vitro* studies with swine granulosa cells. Endocrinology 116:1235, 1985
57. VELDHUIS JD, RODGERS RJ, DEE A, SIMPSON ER: The insulin-like growth factor, somatomedin-C, induces the synthesis of cholesterol side-chain cleavage cytochrome P-450 and adrenodoxin in ovarian cells. J Biol Chem 261:2499, 1986
58. VELDHUIS JD, RODGERS RJ, FURLANETTO RW, AZIMI P, JUCHTER D, GARMEY J: Synergistic actions of estradiol and the insulin-like growth factor somatomedin-C on swine ovarian (granulosa) cells. Endocrinology 119:530, 1986
59. VOUTILAINEN R, MILLER WL: Coordinate tropic hormone regulation of mRNAs for insulin-like growth factor II and the cholesterol side-chain-cleavage enzyme, P450ssc, in human steroidogenic tissues. Proc Natl Acad Sci USA 84:1590, 1987
60. ZHIWEN Z, CARSON RS, HERINGTON AC, LEE VWK, BURGER HG: Follicle-stimulating hormone and somatomedin-C stimulate inhibin production by rat granulosa cells *in vitro*. Endocrinology 120:1633, 1987

DISCUSSION

CHAIRPERSON — G. VIRGINIA UPTON, Ph.D.

DR. PETRAGLIA: Have you any evidence of ovarian vein secretion of IGFI in women? Secondly, what are the endocrine effects, or the possible endocrine effects, of IGFI on steroid production in the human ovary?

DR. ADASHI: With respect to the first question, we have not investigated that, but we are in the process of doing so. Dr. Robert Williams, here in Norfolk at the Eastern Virginia Medical School, has been sampling some subhuman primates for us.

We do not have any information in the human. What we would, hopefully, like to demonstrate is some gradient whereby the ovarian vein concentration of IGF-I would be greater than that in the periphery.

With respect to the effects of IGF-I on steroidogenesis, there have generally been positive, up regulatory effects; never by themselves, at least not in the Murine model, but usually in conjunction with FSH or LH. In the porcine model, as a rule, IGF-I has been active on its own, although it did tend to synergize with gonadotropins as well.

DR. SCHOMBERG: In the follicular fluid samples which you examined for the somatomedin-C binding protein, which was a very nice finding, did you look for IGF-I in the same samples? Were they positive?

DR. ADASHI: Yes, they were positive, but we have never really been able to demonstrate IGF-I levels in follicular fluid, per se, that were in significant excess relative to peripheral levels. That, actually, has been published in one of our reviews. So, to answer your question, we have merely confirmed that, yes, there is IGF-I in human follicular fluid, but the levels are not impressive.

The binding protein levels are rather high and are comparable, in fact, to what we observed in first and second trimester pregnancy in amniotic fluid; those levels are quite high. As you can imagine, those samples were used in the initial purification of this binding protein.

DR. FORD: Will you comment on the specificity of the binding protein for IGF-I in relationship to IGF-II?

DR. ADASHI: Yes. It binds both IGF-I and IGF-II with a tremendously high affinity of 10-9 to 10-10M, but it is unable to bind insulin, a fact which really helps substantially in studies that attempt to define the presence of this binding protein on the surface of cells, which is one of the next things we are going to do.

DR. BURGER: I wanted to ask you to what extent you believe that the actions of the gonadotropins might be mediated by IGF-I? Do you think you can explain most or all of the effects, for example, of FSH by a local induction of IGF-I, and it being a kind of second messenger for FSH? Do you have any thoughts about its role with LH action as well?

DR. ADASHI: I doubt that IGF-I mediates gonadotropin action. Perhaps it is possible to a small degree. I believe your co-workers have, in fact, presented some evidence to the effect that PMSG action can be somewhat neutralized by monoclonal antibodies to IGF-I. It is true that approximately 40 or 50 percent of growth hormone action in the appropriate experimental model can be neutralized by monoclonal antibodies to IGF-I. I do not believe FSH action is likely to utilize IGF-I production in a significant way.

DR. PETRAGLIA: Are there any local interrelationships with growth hormone itself and IGF-I? I am reminded that individuals with isolated growth hormone deficiency seem not to enter puberty even into their twenties.

DR. ADASHI: The information that is available suggests the following: That growth hormone does stimulate ovarian IGF-I gene expression and translation, and that it has a marginal effect on enhancing IGF-I reception, above and beyond what FSH is capable of doing. Thus, growth hormone has a tropic effect on IGF-I at the level of the ovary. I do believe it has some bearing on the clinical problem that you mentioned, although that clinical problem tends to resolve itself spontaneously as these people ultimately enter puberty. But we believe that this is probably due, in part, to the fact that other factors regulate ovarian

IGF-I gene expression including FSH, which ultimately rises in these individuals with delayed puberty.

I would like to draw your attention to earlier observations by the late Dr. Vande Wiele who, in the late sixties, attempted to induce ovulation with exogenous gonadotropins in hypophysectomized women, and found that the requirements for gonadotropins were much higher in hypophysectomized individuals as opposed to intact individuals. He did, in fact, attempt to treat some of these women with growth hormone, only to find that the gonadotropin requirements had been substantially reduced. Of course, from our biased point of view, we would like to interpret this to mean that growth hormone stimulated ovarian IGF-I generation locally allowing it to synergize with gonadotropins and, hence, increase the inefficacy.

DR. FORD: Does IGF-I have a role in the testis?

DR. ADASHI: Yes. I would like to say it has a potential role, and this is the same statement I made with respect to the ovary. There is a large body of literature emanating largely from France, which has already demonstrated clearly that the testis, i.e. multiple compartments of the testis are, in fact, targets of IGF-I action. I do not recall specifically whether any IGF-I production has, in fact, been demonstrated, but multiple IGF-I actions have been shown including the promotion of LH-stimulated testosterone production.

DR. BURGER: Have you any views on the possible role of IGF-I, or IGF-I deficiency, in the pathogenesis of polycystic ovary syndrome.

DR. ADASHI: Not really. I think, if anything, the situation there is such that the IGF-I receptors may subserve the relative hyperinsulinemia in some of the patients you encounter, and, in fact, may be part of the hyperandrogenic picture of these patients. Thus, I do not think IGF-I deficiency would account for it, but I would be very interested in hearing how you developed that concept.

DR. BURGER: My question is really related to the fact that I have heard some anecdotal suggestions that growth hormone therapy also improves the ovulation induction response of patients with polycystic ovarian disease, and it was thought that the lack of growth factor in the ovary may in some way be related to the failure of gonadotropin induction.

*DR. ADASHI:*That is very interesting. I was not aware of that.

DR. PETRAGLIA: Regarding the growth hormone or gonadotropin regulation of IGF-I in pubertal maturation, have you any evidence that IGF-I is expressed from the first year of life, or following puberty? Do you have any plasma levels showing any possible participation of IGF-I in reaching puberty?

DR. ADASHI: The circulating levels of IGF I have been characterized in the human, but they do not necessarily reflect ovarian secretion. They probably reflect, for the most part,

hepatic production. We do not have any evidence that pertains, specifically, to the ovary in the human. However, in the rat, we are in the process of following the ontogeny of IGF-I from birth to day 40, attempting to carefully study IGF-I gene expression and reception in the ovary as the animal matures.

24

Growth Factors and Reproduction

DAVID W. SCHOMBERG

Few recent discoveries in basic biological research have been as dramatic as those linking certain growth factors or their receptors, oncogenes, and cancer. Some of these have involved cells of the reproductive system. Less dramatic, but no less important, are the discoveries relating how growth factors regulate growth and differentiation in normal cells. Some of this work also involves cells of the reproductive system. Since the study of growth factors in reproduction is truly in its infancy, a selective, but broadly based review of this topic is still a manageable task. In this chapter I will attempt to emphasize those studies which have demonstrated either production of growth factors or responsiveness to growth factors by cells of the reproductive system, including those of the early embryo.

GENE EXPRESSION AND/OR PRODUCTION OF GROWTH FACTORS BY NORMAL OR NEOPLASTIC CELLS OF THE REPRODUCTIVE SYSTEM

Table 1 contains a listing of the cell or tissue types and associated growth factors for which either mRNA expression and/or the secreted gene product have been identified.

Since the subject of growth factor production by cells of the reproductive system is in its infancy, much remains to be established with respect to definitive demonstrations of cellular production. Collectively, several caveats should be kept in mind. Validated radioimmunoassays (RIAs) for several of the factors are not yet available. RIAs may also be complicated by the simultaneous presence of growth factor binding proteins.[11] Demonstration of mRNA expression at the cellular level may not necessarily equate with secretion of the factor. At this point in time there are only a small number of studies which demonstrate conclusively that growth factor production at the transcriptional or secretory levels is hormonally regulated. Accurate identity of cellular origin is also very important. In a recent study, Han *et al.* made the surprising observation by *in situ* hybridization histochemistry that IGF-I and IGF-II messenger RNAs were localized to perisinusoidal connective tissue or mesenchymal cells rather than the hepatocytes of the fetal liver.[34] This issue becomes very important from a practical or strategical perspective in terms of whether or not the specific growth factor-producing reproductive target cells of interest can be influenced by the pharmaceutical approaches available. Another important consideration is that cell-specific production of a given growth factor might be developmentally dependent as has been shown for PDGF in the cytotrophoblast.[31] Furthermore, production may also be influenced by the developmental program of neighboring cells. The granulosa-theca com-

TABLE 1
GROWTH FACTOR GENE EXPRESSION OR GROWTH FACTOR PRODUCTION BY CELLS OF THE REPRODUCTIVE SYSTEM

Growth Factor or Oncogene	Cell or Tissue of Origin	Reference
IGF-I	ovary, uterus, Sertoli cell, placenta	35,64,79,21
IGF-II	granulosa cell, placenta	21,83
TGF-α	theca, decidua, pituitary, mammary	53,33,52,57
TGF-β-type 1	ovary, bone	37,73,70
TGF-β-type 2	MCF-7 cells, prostatic adenocarcinoma	49,58
EGF	ovarian follicle, uterus	40,18
FGF	granulosa cells	66
PDGF	cytotrophoblast	31
HER-2/neu	breast	76
IGF-like	breast, granulosa cell	40,17
EGF-like	breast	17
TGF-β-like	Sertoli cell	39
TGF-β-like	Sertoli cell	63
Seminiferous growth factor	Sertoli cell	22

partments of the ovarian follicle could perhaps prove to be such an example for a differential pattern of growth factor production seems to be emerging in this tissue.[73,74] It is also important to keep in mind the well-known facts that: 1) Production of compounds by malignant versus normal cells or cells maintained *in vitro* versus *in vivo* is not always the same. 2) Production during culture by normal as well as malignant cells may vary qualitatively. It will be extremely interesting to know the detailed amino acid sequence of the EGF-like and IGF-like compounds produced by cultured breast cancer cells and whether subtle structure-function differences exist between these compounds and the more abundant fac-

tors. Similarly, the HER—2/neu oncogene, overpressed in breast cancer, is closely related to, but not identical with, the erb B (EGF receptor-like) oncogene. Structure-function comparisons between these genes and gene products might prove extremely useful.

RESPONSIVENESS TO GROWTH FACTORS BY NORMAL OR NEOPLASTIC CELLS OF THE REPRODUCTIVE SYSTEM

Table 2 summarizes a variety of responses to growth factors by cells of the reproductive system.

TABLE 2

RESPONSES TO GROWTH FACTORS BY CELLS OF THE REPRODUCTIVE SYSTEM

Growth Factor	Cell Type	Response	Reference
IGF-I, IGF-II, TGF-β, EGF	granulosa cell	enhanced steroidogenesis	81,4,1,2, 6,82,19,48
EGF, FGF, EGF plus TGF-β	granulosa cell	attenuated steroidogenesis	42,43,69,8
IGF-1	granulosa cell	enhanced proteoglycan production,	5
		inhibin production	84
IGF-1	cytotrophoblast	modulated steroidogenesis	65
IGF-1	human ovary	receptor binding	9
IGF-1	mammary epithelial	enhanced casein gene expression; glucose and a-lactalbumin transport activity	67
IGF-1, TGF-β	granulosa	enhanced LH receptor induction	19,3
IGF-1, EGF	theca	androgen secretion	36,20
EGF, FGF, EGF plus TGF-β	granulosa	attenuated LH receptor induction	62,50,23, 51
TGF-β	granulosa	variable 3H-thymidine incorporation	1,60,61
TGF-β, TGF-α	bone	development, resorption	16,44,45

TGF-β	malignant breast, prostate	decreased mitotic activity	49
TGF-β	normal mammary	decreased mitotic activity	72
TGF-β	anterior pituitary	increased FSH secretion	55
TGF-β	Leydig cell	attenuated steroidogenesis	54
EGF, FGF, IGFs	granulosa	increased mitotic activity	60,26,28, 29,32
EGF, PDGF, TGF-β	testis peri-tubular	increased ornithine decarboxylase (ODC) activity	77
EGF, TGF-β	Sertoli-spermatogenic	increased ODC activity	77
PDGF	Sertoli-spermatogenic	decreased ODC activity	77
EGF	ovary *in vivo*	variable mitotic activity	56,27,68,71
EGF	malignant breast	variable binding	14
EGF	malignant pituitary	decreased growth hormone	47
EGF	granulosa	increased FSH receptor binding	59
EGF	testis	spermatogenesis	80
EGF	malignant Leydig	increased steroidogenesis	7
EGF	prostate	EGF receptors	78
EGF, TGF-β	oocyte	enhanced maturation	15,24
EGF	uterus	receptor binding	10
FGF	endothelial cells	increased cell growth	30
FGF	*Xenopus* embryo	maturational alterations	75

The large number of documented responses by ovarian and testicular cells indicates potentially important roles for growth factors in intragonadal regulation which remain to be defined. Similarly intensive investigation of other cell types may reveal many more actions of growth factors in nongonadal systems as well. These observations pose several important questions for further research. Are these factors mediating secondarily all of the actions of the classical reproductive hormones? If not, how do actions of growth factors and those of the classical reproductive hormones interact to regulate growth and/or differentiation in reproductive-hormone-responsive cells? Are the actions of the growth factors in reproductive-hormone-target cells different from their actions in other cells of the body? If the cellular actions of growth factors are basically the same in non-reproductive as well as reproductive-hormone-responsive tissues, how are the signalling processes uniquely constructed in the responsive cells?

At a more integrative level, one might ask whether certain growth factors might prove to be some of the gonadal "organizers" or "integrators" postulated to exist by earlier workers. The topic of thecal cell ontogeny has been recently reviewed by Hirshfield[38] in a broader context but is germane to this concept. The demonstration that FGF might be one of the "vegetalizing factors" acting on the early *Xenopus* embryo is also particularly germane.[75] Moreover, certain growth factors might be considered as candidates to help mediate stromal-epithelial interactions in reproductive tissues.[12,13] Lastly, growth factors might help to integrate responses between systems as exemplified by the intriguing observations that interleukin 2 attenuates granulosa cell progesterone production and that trophoblastic cells are capable of producing interferon-like compounds.[25,46]

Within the context of this meeting, the challenge will be to establish whether the information forthcoming about this class of nonsteroidal regulators can be used to discover new pathways to the control of reproductive function.

ACKNOWLEDGMENTS

I wish to acknowledge the expert secretarial assistance of Ms. Lynn Honeycutt. Supported in part by Grants HD 11827 and 21271 from the NICHD, NIH.

REFERENCES

1. ADASHI EY, RESNICK CE: Antagonistic interactions of transforming growth factors in the regulation of granulosa cell differentiation. Endocrinology 119:1879, 1986
2. ADASHI EY, RESNICK CE, D'ERCOLE AJ, SVOBODA ME, VAN WYK JJ: Insulin-like growth factors as intraovarian regulators of granulosa cell growth and function. Endocr Rev 6:400, 1985
3. ADASHI EY, RESNICK CE, SVOBODA ME, VAN WYK JJ: Somatomedin-C enhances induction of luteinizing hormone receptors by follicle-stimulating hormone in cultured rat granulosa cells. Endocrinology 116:2369, 1985
4. ADASHI EY, RESNICK CE, SVOBODA ME, VAN WYK JJ: Somatomedin-C synergizes with follicle-stimulating hormone in the acquisition of progestin biosynthetic capacity by cultured rat granulosa cells. Endocrinology 116:2135, 1985
5. ADASHI EY, RESNICK CE, SVOBODA ME, VAN WYK JJ, HASCALL VC, YANAGISHITA M: Independent and synergistic actions of somatomedin-C in the stimulation of proteoglycan biosynthesis by cultured granulosa cells. Endocrinology 118:456, 1986
6. ADASHI EY, RESNICK CE, SVOBODA ME, VAN WYK JJ: Somatomedin-C as an amplifier of follicle-stimulating hormone action: enhanced accumulation of adenosine 3'5'-monophosphate. Endocrinology 118:149, 1986

7. ASCOLIM N, EUFFA J, SEGALOFF DL: Epidermal growth factor activates steroid biosynthesis in cultured Leydig tumor cells without affecting the levels of cAMP and potentiates the activation of steroid biosynthesis by choriogonadotropin and cAMP. J Biol Chem 262:9196, 1987
8. BAIRD A, ESCH F, MORMEDE P, UENO N, LING N, BOHLEN P, YING SY, WEHRENBERG WB, GUILLEMIN R: Molecular characterization of fibroblast growth factor: distribution and biological activities in various tissues. Recent Prog Horm Res 42:143, 1986
9. BALBONI GC, ORLANDO C, VANELLI GB, SERIO M, BARNI T: Transferrin and somatomedin-C receptors in the human ovarian follicles. Fertil Steril 48:796, 1987
10. CHEGINI N, RAO CH V, WAKIM N, SANFILIPPO J: Binding of 125I-epidermal growth factors in human uterus. Cell Tissue Res 246:543, 1986
11. CLEMMONS DR, HAN VKM, ELGIN RG, D'ERCOLE AJ: Alterations in the synthesis of a fibroblast surface associated 35K protein modulates the binding of somatomedin-C/insulin-like growth factors I. Mol Endocrinology 1:339, 1987
12. COOKE PS, YOUNG PF, CUNHA GR: A new model system for studying androgen-induced growth and morphogenesis *in vitro*: The bulbourethral gland. Endocrinology 121:2161, 1987
13. COOKE PS, UCHIMA FD-A, FUGI DK, BERN HA, CUNHA GR: Restoration of normal morphology and estrogen responsiveness in cultured vaginal and uterine epithelia transplanted with stroma. Proc Natl Acad Sci USA 83:2109, 1986
14. DAVIDSON NE, GELMANN EP, LIPPMAN ME, DICKSON RB: Epidermal growth factor receptor gene expression in estrogen receptor-positive and negative human breast cancer cell lines. Mol Endocrinology 1:216, 1987
15. DEKEL N, SHERIZLY I: Epidermal growth factor induces maturation of rat follicle-enclosed oocytes. Endocrinology 116:406, 1985
16. CENTRELLA M, MASSAGUE J, CANALIS E: Human platelet-derived transforming growth factor-β stimulates parameters of bone growth in fetal rat calvariae. Endocrinology 119:2306, 1986
17. DICKSON RB, MCMANAWAY ME, LIPPMAN ME: Estrogen-induced factors of breast cancer cells partially replace estrogen to promote tumor growth. Science 232:540, 1986
18. DI AUGUSTINE RP, LANNON DE, MCLACHLAN JA: Sex steroid hormones rapidly increase uterine epidermal growth factor (EGF). Program of the 67th Annual Meeting of the Endocrine Society, Baltimore, MD, p. 269 (Abstract), 1985
19. DODSON WC, SCHOMBERG DW: The effect of transforming growth factor-β on follicle-stimulating hormone-induced differentiation of cultured rat granulosa cells. Endocrinology 120:512, 1987
20. ERICKSON GF, CASE E: Epidermal growth factor antagonizes ovarian theca-interstitial cytodifferentiation. Mol Cell Endocrinol 31:71, 1985
21. FANT M, MUNRO H, MOSES AL: An autocrine paracrine role for insulin-like growth factors in the regulation of human placental growth. J Clin Endocrinol Metab 63:499, 1986
22. FEIG L, KLAGSBRUN GT, BELLVE AR: Mitogenic polypeptide from the mammalian seminiferous epithelium: biochemical characterization and partial purification. J Cell Biol 97:1435, 1983
23. FENG P, CATT KJ, KNECHT M: Transforming growth factor-β regulates the inhibitory actions of epidermal growth factor during granulosa cell differentiation. J Biol Chem 261:14167, 1986
24. FENG P, EVANS J, KNECHT M: Transforming growth factor-β stimulates meiotic maturation of the rat oocyte. Endocrinology 122:181, 1988
25. FUKUOKA M, MORI T, TAII S, YASUDA K: Interleukin-1 inhibits luteinization of porcine granulosa cells in culture. Endocrinology 122:367, 1988
26. GOSPODAROWICZ D, BIALECKI H: The effects of the epidermal and fibroblast growth factors on the replicative lifespan of bovine granulosa cells in culture. Endocrinology 103:854, 1978
27. GOSPODAROWICZ D, MESCHER AL, BIRDWELL CR: Control of cellular proliferation by the fibroblast and epidermal growth factors. In: Third Decennial Review Conference: Cell, Tissue and Organ Culture. National Cancer Institute Monograph 48: Bethesda USPHS, NIH, p. 109, 1978
28. GOSPODAROWICZ D, BIALECKI H: Fibroblast and epidermal growth factors are mitogenic agents for cultured granulosa cells of rodent, porcine and human origin. Endocrinology 104:744, 1979
29. GOSPODAROWICZ D, ILL CR, BIRDWELL CR: Effects of fibroblast and epidermal growth factors on ovarian cell proliferation *in vitro*. I. Characterization of the response of granulosa cells to FGF and EGF. Endocrinology 100:1108, 1979

30. GOSPODAROWICZ D, MASSOGLIA S, CHENG J, JUJII DK: Effect of fibroblast growth factor and lipoproteins on the proliferation of endothelial cells derived from bovine adrenal cortex, brain cortex and corpus luteum capillaries. J Cell Physiol 127:121, 1986
31. GOUSTIN AS, BETSHOLTZ C, PFEIFER-OHLSSON S, PERSSON H, RYDNERT J, BYWATER M, HOLMGREN G, HELDIN CH, WESTERMARK B, OHLSSON R: Coexpression of the sis and myc protooncogenes in developing human placenta suggests autocrine control of trophoblastic growth. Cell 41:301, 1985
32. HAMMOND JM, YOSHIDA K, VEI DHUIS JD, RECHLER MM, KNIGHT AB: In GREENWALD GS, TERRANOVA PF (eds): *Factors Regulating Ovarian Function*. Raven Press, New York, p 197, 1983
33. HAN VKM, HUNTER S III, PRATT RM, ZENDEGUI JG, LEE DC: Expression of rat transforming growth factor α mRNA during development occurs predominantly in the maternal decidua. Mol Cell Biol 7:2335, 1987
34. HAN VKM, D'ERCOLE AJ, LUND PK: Cellular localization of somatomedin (insulin-like growth factor) messenger RNA in the human fetus. Science 236:193, 1987
35. HERNANDEZ ER, HOYT E, VAN WYK JJ, ADASHI EY: The Somatomedin-C/insulin-like growth factor-I (Sm-C/IGF-I) gene is expressed in the rat ovary. Program of the 69th annual meeting of the Endocrine Society, Abstract 821, 1987
36. HERNANDEZ ER, RESNICK CE, SVOBODA ME, ADASHI EY: The ovarian theca-interstitial cell as a site of somatomedin-C insulin-like growth factor I reception and action. Program of the 69th Annual Meeting of the Endocrine Society, Abstract 152, 1987
37. HERNANDEZ ER, TWARDZIK DR, PURCHIO A, ADASHI EY: Gonadotropin-dependent ovarian transforming growth factor-β gene expression. Biol Reprod (Suppl 1), 36:58, 1987.
38. HIRSHFIELD AN: Thecal ontogeny: A critical evaluation of established theories. In PARVINEN M, (ed): Factors Regulating Ovarian Function, Raven Press (in press)
39. HOLMES SD, SPOTTS G, SMITH RG: Rat Sertoli cells secrete a growth factor that blocks epidermal growth factor (EGF) binding to its receptor. J Biol Chem 261:4076, 1986
40. HSU CJ, HAMMOND JM: Gonadotropins and estradiol stimulate immunoreactive insulin-like growth factor-I production by porcine granulosa cells *in vitro*. Endocrinology 120:198, 1987
41. HSU CJ, HOLMES D, HAMMOND JM: Ovarian epidermal growth factor-like activity. Concentrations in porcine follicular fluid during follicular enlargement. Biochem Biophys Chem Commun 147:242, 1987
42. HSUEH AJW, ADASHI EY, JONES PBC, WELSH TH Jr: Hormonal regulation of the differentiation of cultured ovarian granulosa cells. Endocrine Revs 5:76, 1984
43. HSUEH AJW, WELSH TH Jr, JONES PBC: Inhibition of ovarian and testicular steroidogenesis by EGF. Endocrinology 108:2002, 1981
44. IBBOTSON KJ, D'SOUZA SM, KG KW, OSBORNE CK, NIALL M, MARTIN TJ, MUNDY GR: Tumor-derived growth factor increases bone resorption in a tumor associated with humoral hypercalcemia of malignancy. Science 221:1292, 1983
45. IBBOTSON KJ, D'SOUZA SM, TWARDZIK DR, HARGREAVES WR, TODARO GJ, MUNDY GR: Stimulation of bone resorption *in vitro* by synthetic transforming growth factor α. Science 228:1007, 1985
46. IMAKAWA K, ANTHONY RV, MAROTTI KR, POLITES HG, ROBERTS RM: Is ovine trophoblast-1 related to the interferons? Biol Reprod 36 (Suppl 1): Abstract 11, 1987
47. JOHNSON LH, BAXTER JD, VLODAVSKY I, GOSPODAROWICZ D: Epidermal growth factor and expression of specific genes: Effects on cultured rat pituitary cells are dissociable from the mitogenic response. Proc Natl Acad Sci (USA) 77:394, 1980
48. JONES PBC, WELSH TH Jr, HSUEH AJW: Regulation of ovarian progestin production by epidermal growth factor in cultured rat granulosa cells. J Biol Chem 257:11268, 1982
49. KNABBE C, LIPPMAN ME, WAKEFIELD LM, FLANDERS KC, KASID A, DERYNCK R, DICKSON RB: Evidence that transforming growth factor-β is a hormonally regulated negative growth factor in human breast cancer cells. Cell 48:417, 1987
50. KNECHT M, CATT KJ: Modulation of cAMP-mediated differentiation in ovarian cells by epidermal growth factor and platelet-derived growth factor. J Biol Chem 258:2789, 1983
51. KNECHT M, FENG P, CATT KJ: Bifunctional role of transforming growth factor-β during granulosa cell development. Endocrinology 120:1243, 1987
52. KOBRIN MS, ASA SL, SAMSOODAR J, KUDLOW SE: Alpha-transforming growth factor in the bovine anterior pituitary gland: secretion by dispersed cells and immunohistochemical localization. Endocrinology 121:1412, 1987

53. KUDLOW JE, KOBRIN MS, PURCHIO AF, TWARDZIK DR, HERNANDEZ ER, ASA SL, ADASHI EY: Ovarian transforming growth factor-α gene expression: Immunohistochemical localization to the theca-interstitial cells. Endocrinology 121:1577, 1987
54. LIN T, BLAISDELL J, HASKELL JF: Transforming growth factor-β inhibits leydig cell steroidogenesis in primary culture. Biochem Biophys Res Commun 146:387, 1987
55. LING S-Y, BECKER A, BAIRD A, LING N, UENO N, ESCH F, GUILLEMIN R: Type β transforming growth factor (TGF-β) is a potent stimulator of the basal secretion of follicle-stimulating hormone (FSH) in a pituitary monolayer system. Biochem Biophys Res Commun 135:950, 1986
56. LINTERN-MOORE S, MOORE GPM, PANARETTO BA, ROBERTSON D: Follicular development in the neonatal mouse ovary: effect of epidermal growth factor. Acta Endocrinol 96:123, 1981
57. LIU SC, SANFILIPPO B, PEROTEAU I, DERYNCK R, SALAMON DS, KIDWELL WR: Expression of transforming growth factor α (TGF-α) in differentiated rat mammary tumors: Estrogen induction of TGF α production. Mol Endocrinology 1:683, 1987
58. MARGUARDT H, LIOUBIN MN, IKEDA T: Complete amino acid sequence of human transforming growth factor type B2. J Biol Chem 262:12127, 1987
59. MAY JV, BUCK PA, SCHOMBERG DW: Epidermal growth factor enhances (125I) iodo-follicle-stimulating hormone binding by cultured porcine granulosa cells. Endocrinology 120:2413, 1987
60. MAY JV, SCHOMBERG DW: Synergistic action of EGF, TGF-β, and Sm-C on initiation of DNA synthesis in porcine granulosa cells maintained in serum-free medium. Program of the 69th annual meeting of the Endocrine Society, Abstract 309, 1987
61. MONDSCHEIN JS, CANNING SF, HAMMOND JM: Growth Factors (GFs) modulate insulin-like growth factor (IGF) secretion and 3H-Thymidine incorporation in cultured porcine granulosa cells (GCs). Biol Reprod 36 (Suppl. 1):36, 1987
62. MONDSCHEIN JS, SCHOMBERG DW: Growth factors modulate gonadotropin receptor induction in granulosa cell cultures. Science 211:1179, 1981
63. MORERA AM, BENHAMED M, COCHET C, CHAUVIN MA, CHAMBAZ E: A β-TGF-like as a possible intratesticular modulator of steroidogenesis, 9th Testis Workshop, Vanderbilt University, Nashville, p 78 (Abstract), 1986
64. MURPHY LJ, MURPHY LC, FRIESEN HG: Estrogen induces insulin-like growth factor-I expression in rat uterus. Mol Endocrinology. 1:445, 1987
65. NESTLER JE: Modulation of aromatase and P450 cholesterol side-chain cleavage enzyme activities of human placental cytotrophoblasts by insulin and insulin-like growth factor I. Endocrinology 121:1845, 1987
66. NEUFELD G, FERRARA N, SCHQUEIGERER L, MITCHELL R, GOSPODAROWICZ D: Bovine granulosa cells produce fibroblast growth factor. Endocrinology 121:597, 1987
67. PROSSER CG, SANKARAN L, HENNIGHAUSEN L, TOPPER YJ: Comparison of the roles of insulin and insulin-like growth factor I in casein gene expression and in the development of α-lactalbumin and glucose transport activities in the mouse mammary epithelial cell. Endocrinology 120:1411, 1987
68. RADFORD HM, PANARETTO BA, AVENELL JA, TURNBULL KE: Effect of mouse epidermal growth factor on plasma concentrations of FSH, LH, and progesterone and on oestrus, ovulation and ovulation rate in merino ewes. J Reprod Fert 80:383, 1987
69. SCHOMBERG DW, MAY JV, MONDSCHEIN JS: Interaction between hormones and growth factors in the regulation of granulosa cell differentiation *in vitro*. J Steroid Biochem 19:291, 1983
70. SEYEDIN SM, SEGARINI PR, ROSEN DM, THOMPSON AY, BENTZ H, GRAYCAR J: Cartilage-inducing factor-β is a unique protein structurally and functionally related to transforming growth factor-β. J Biol Chem 262:1946, 1987
71. SHAW G, JORGENSON GI, TWEENDALE R, TENNISON M, WATTERS MJ: Effect of epidermal growth factor on reproductive function of ewes. J Endocrinol 107:429, 1985
72. SILBERSTEIN GB, DANIEL GW: Reversible inhibition of mammary gland growth by transforming growth factor-β. Science 237:291, 1987
77. SKINNER MK, KESKI-OJA J, OSTEEN KG, MOSES HL: Ovarian thecal cells produce transforming growth factor-β which can regulate granulosa cell growth. Endocrinology 121:786, 1987
74. SKINNER MK, LOBB D, DORRINGTON JH: Ovarian thecal/interstitial cells produce an epidermal growth factor-like substance. Endocrinology 121:1892, 1987

75. SLACK MW, DARLINGTON BG, HEATH JK, GODOAVE SF: Mesoderm induction in early Xenopus embryos by heparin-binding growth factors. Nature 326:197, 1987
76. SLAMON DJ, CLARK GM, WONG SG, LEVIN WJ, ULLRICH A, MCGUIRE WL: Human breast cancer: Correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science 235:177, 1987
77. SMITH FF, TRES LL, KIERSZENBAUM AL: Ornithine decarboxylase activity during rat spermatogenesis *in vivo* and *in vitro*: Selective effect of hormones and growth factors. J Cell Physiol 133:305, 1987
78. TRAISH AM, WOTIZ HH: Prostatic epidermal growth factor receptors and their regulation by androgens. Endocrinology 121:1461, 1987
79. TRES LL, SMITH EP, VAN WYK JJ, KIERSZENBAUM AL: Immunoreactive sites and accumulation of somatomedin-C in rat sertoli-spermatogenic cell co-cultures. Exp Cell Res 162:33, 1986
80. TSUTSUMI O, KURACHI H, OKA T: A physiological role of epidermal growth factor in male reproductive function. Science 233:975, 1986
81. VELDHUIS JD, FURLANETTO RW, JUCHTER D, GARMEY J, VELDHUIS P: Trophic actions of human somatomedin C/insulin-like growth factor I on ovarian cells: *in vitro* studies with swine granulosa cells. Endocrinology 116:1235, 1985
82. VELDHUIS JD, RODGERS RJ, FURLANETTO RW, AZIMI P, JUCHTER D, GARMEY J: Synergistic actions of estradiol and the insulin-like growth factor somatomedin-C on swine ovarian (granulosa) cells. Endocrinology 19:530, 1986
83. VOUTILAINEN R, MILLER WL: Coordinate trophic regulation of mRNAs for insulin-like growth factor II and the cholesterol side-chain-cleavage enzyme, P450scc, in human steroidogenic tissue. Proc Natl Acad Sci 84:1590, 1987
84. ZHIWEN Z, CARSON RS, HERINGTON AC, LEE VWK, BURGER HG: Follicle-stimulating hormone and somatomedin-C stimulate inhibin production by rat granulosa cells *in vitro*. Endocrinology 120:1633, 1987

DISCUSSION

CHAIRPERSON: VIRGINIA C. UPTON, Ph.D.

DR. BIALY: I think you very wisely put your finger on the difficulties that exist in translating certain things observed *in vitro* to the whole animal.

DR. DONAHOE: We have clearly shown steroid modulation of growth inhibitors as well, and I am sure that the bifunctional aspect fits TGF beta. With Mullerian Inhibiting Substance, there is clear evidence that estrogen inhibits and testosterone enhances the activity of this in so many of the systems that we have investigated.

DR. SCHOMBERG: Yes, I think it will be fascinating to look at the interactions between growth factors and what could be called the classical reproductive hormones, e.g., estrogen, testosterone and other steroids, or to look at the interactions between growth factors and hormones which act via cyclic AMP-dependent processes.

DR. DONAHOE: One of the exciting actions is going to be that the steroids presumably are acting at the nucleus and the growth factor is acting at the cell membrane, and how that interaction is going to take place. And, it also may very well be that some interactions occur at the cell surface, in the case of steroids, as Clara Siego has been saying for years, and as has been demonstrated with progesterone and oocytes.

25

Prorenin and Reproductive Function

JEAN E. SEALEY
JOSEPH ITSKOVITZ

The classical view of the renin-angiotensin system is that of a hormonal system involved with the control of blood pressure, body fluid volumes and electrolyte metabolism.[1-6] The system consists of a cascade of reactions resulting in the formation of the biologically active octapeptide, angiotensin II (Fig. 1). In the first step, renin, a proteolytic enzyme, synthesized by the kidney and processed therein from its biological precursor prorenin, is secreted into the blood and acts on its substrate angiotensinogen to form the inactive peptide angiotensin I. This decapeptide is acted upon by converting enzyme, which occurs in high concentrations in the pulmonary vascular bed, to form angiotensin II. Angiotensin II has two actions related to the control of blood pressure: it acts on arterioles to cause vasoconstriction and on adrenal glomerulosa cells to stimulate aldosterone synthesis which in turn causes renal sodium retention and potassium secretion.

The renin gene is expressed in a number of endocrine tissues.[7] This presentation focuses on the female reproductive system where its expression appears to have physiological relevance as it responds dynamically to biological stimuli.[28,30] This extra-renal source appears to operate via the biological precursor of renin, prorenin, which was previously thought to be enzymatically inactive.[24]

CHARACTERISTICS OF PRORENIN

Prorenin, like renin, circulates in the blood but at approximately 10 times the level of renin.[24] Prorenin is not converted to renin in the plasma *in vivo*. Rather, the origin of all plasma renin is the kidney; this is shown by the absence of renin in the plasma of individuals who have undergone bilateral nephrectomy. The concurrent fall of plasma prorenin by about 90% shows that the kidney is a major source of plasma prorenin as well. Two pathways have been postulated for the products of renin gene expression in the kidney. One pathway involves packaging of prorenin into secretory granules and subsequent processing into active renin; the other involves release of prorenin, without storage or processing.[9,15]

We first became aware of the existence of prorenin in the early 1970's, when we observed that renin activity increased in plasmas stored in a faulty freezer (for review see reference 24). As there were no changes in either the amounts of angiotensinogen or the kinetics

This work was supported by National Heart Lung and Blood Institute grant HL18323SCR of the National Institutes of Health.

of the reaction, we suggested that cold activates an inactive form of renin, most likely prorenin. Other investigators observed that acidification to pH 3.3 activates inactive renin in amniotic fluid and in plasma. We and others observed that plasma inactive renin increased in parallel with active renin during reduction in sodium intake, diuretic therapy and converting enzyme inhibition. The parallel changes in active renin and prorenin supported the idea that a majority of inactive renin was secreted from the kidney; this was confirmed by the close to 90% fall in plasma prorenin that occurred after bilateral nephrectomy.

Later studies documented that inactive renin is indeed prorenin. Several investigators used synthetic peptides based on the partial amino acid sequence of the prosegment of prorenin to obtain antibodies. These antibodies bind to inactive renin derived from both plasma and renal sources. Both renin and prorenin bind identically to anti-renin antibodies, indicating homology of the two.[3,4,15]

Prorenin can be reversibly or irreversibly activated. It is irreversibly activated by many of the serine proteases, including trypsin. The product of trypsin activation has catalytic

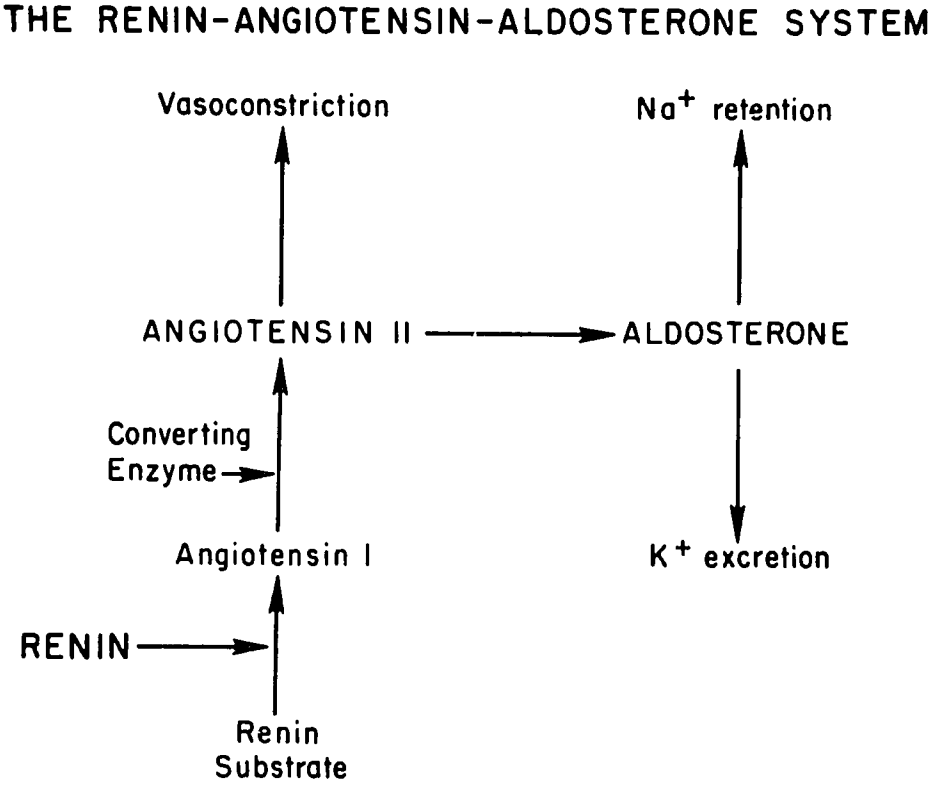


Figure 1. The circulating renin-angiotensin-aldosterone system. All of the active renin in the blood is derived from the kidneys. Prorenin (not shown) is the biosynthetic precursor of renin.

and chromatographic properties similar to that of endogenous renal renin. In fact, trypsin activation is the basis of our assay for total renin. [25]

Prorenin, like pepsinogen with which it has significant homology, can be reversibly activated by acid.[2,6,11,17] Reversibility of acid activation proceeds by first order kinetics, an observation that is not consistent with reassociation of active renin with an inhibitor. Figure 2 shows the results of a study in which we demonstrated reversible acid activation of purified prorenin from human kidney.[2] Dialysis to pH 3.3 resulted in almost complete activation of prorenin. Subsequent incubation at 37 °C in pH 7.4 buffer resulted in essentially complete inactivation, *i.e.*, reversal of activation, within 2 hours. This loss of activity was not due to destruction of renin because renin activity was restored by treatment with trypsin. The data are not shown but activity was also restored after treatment with acid, which further supports complete reversibility of acid activation. Reversible acid activation in this purified preparation probably proceeds via an acid-induced change in the conformation of the prorenin, resulting in a movement of the prosequence away from the molecule and exposure of the active site.

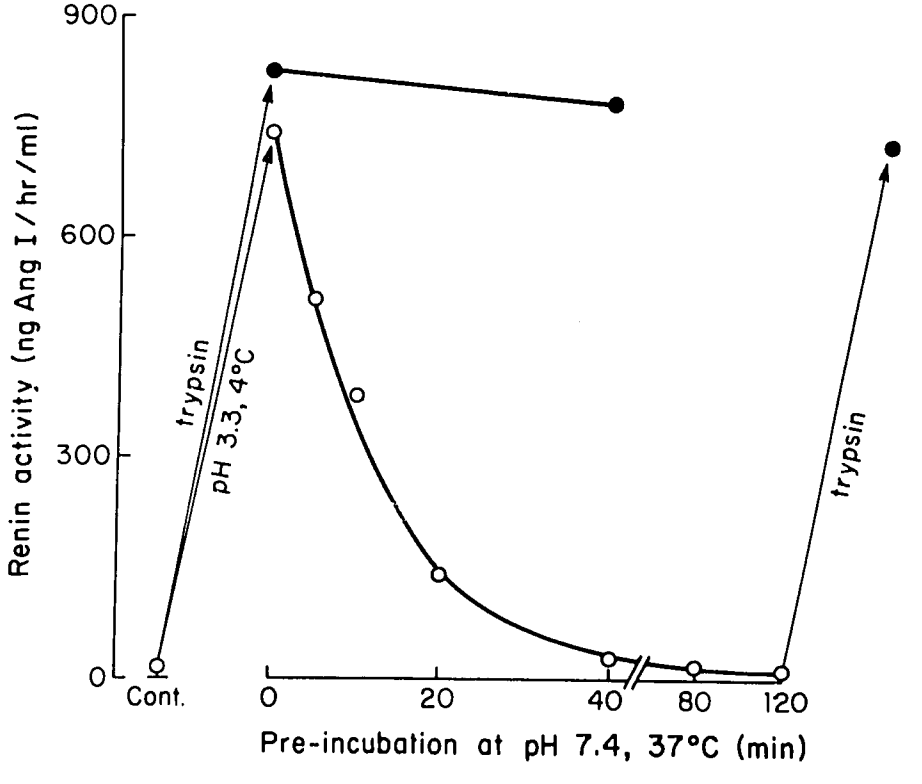
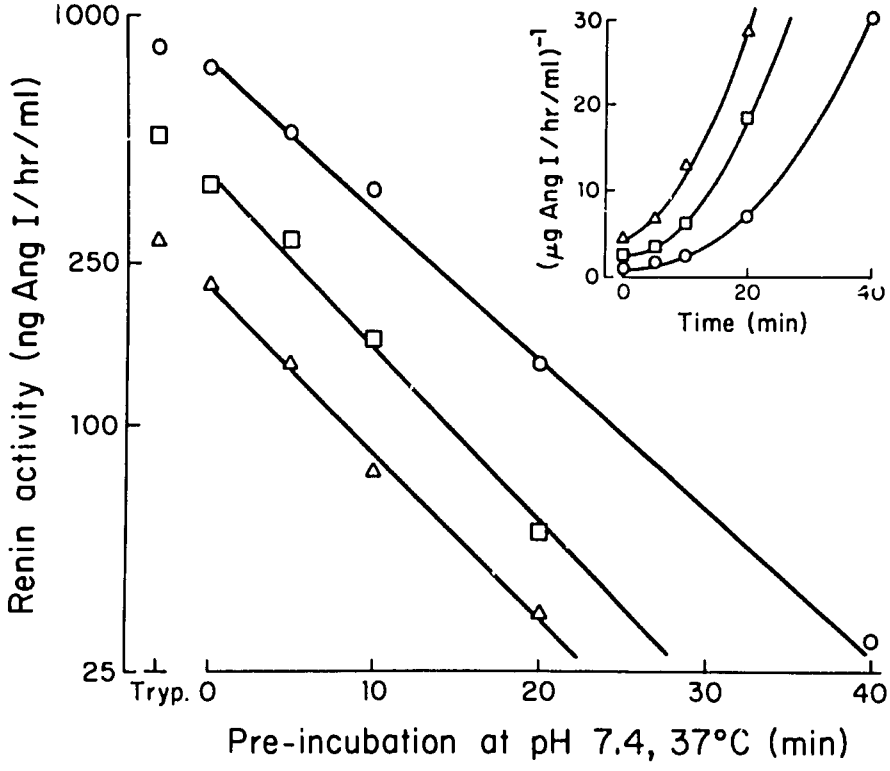


Figure 2. Reversible acid-activation of partially purified prorenin from human kidney.

a) After dialysis to pH 3.3, samples were restored to pH 7.4 and then incubated at 37°C for various times. Immediately thereafter, renin activity was determined. O, pH 3.3 dialysed; •, trypsin-treated. After 120 minutes at 37°C renin activity disappeared, but was recovered after re-acidification (not shown) or incubation with trypsin.



b) Decline in renin activity during incubation of 10 (△), 20 (□) or 40 (O) μl of pH 3.3-dialysed prorenin. The data fit the first-order rate equation -dE/dt = kE (where E represents the remaining enzyme activity). Values obtained for k were 0.095, 0.089 and 0.086 minute^{-1} respectively, with corresponding half times of 7.3, 7.1 and 8.1 minutes. The inset demonstrates that the data do not fit the expression -dE/dt = kE^2, which would be expected for a second-order reaction as would be the case with an enzyme inhibitor complex. Abbreviation: ANG I = angiotensin I.

Irreversible acid activation and irreversible cryoactivation has also been observed in plasma and amniotic fluid. This appears to proceed through inactivation of endogenous serine protease inhibitors, with a concurrent acid- or cold-induced change in prorenin conformation resulting in exposure of the peptide bond between the prosequence and renin, which can then be cleaved by endogenous plasma proteases.[5,23]

The important point, however, is that acid, and perhaps also cold conditions, can cause the prosequence to move away from the active site leading to reversible activation of prorenin. Altogether the data demonstrate that under certain conditions prorenin itself can duplicate the function of renin in the renin angiotensin system. The importance of this will be apparent later.

RENIN IN REPRODUCTIVE FUNCTION

While the kidney is the major source of plasma prorenin, it can arise from other sources. A potential source is the reproductive system. Prorenin is the major form of renin in the placenta; it is synthesized in the chorionic cells and seems to be secreted into amniotic fluid, where it occurs in much higher concentrations than in normal human plasma.[1,20,32] Maternal plasma prorenin increases as much as 10-fold in pregnancy. This increase begins during the first 2 to 4 weeks after conception and is maintained throughout gestation. After parturition, plasma levels of both prorenin and hCG fall, but prorenin levels fall much more slowly, despite having a much shorter halflife. This slow postpartum fall of plasma prorenin indicates that the placenta is not the source of elevated plasma prorenin in pregnancy, but that the origin is maternal.[27]

Since it seemed possible that the rise of the plasma prorenin level during gestation may have been initiated during the previous menstrual cycle, we decided to determine whether changes in prorenin occurred in normal menstrual cycles.

We therefore investigated changes in plasma prorenin and renin during the menstrual cycle in relation to known changes in luteinizing hormone (LH), progesterone, and estradiol.[25] Seven young women, aged from 21 to 28 years, were studied throughout a total of 12 cycles; complete hormonal measurements were made of blood collected 3 times a week, every 2 to 3 days, during 9 apparently normal cycles. Plasma prorenin levels varied predictably throughout the normal menstrual cycle. The baseline during the follicular phase, when an egg is selected for maturation, was stable. In the early luteal phase, near the time of the LH peak, prorenin increased nearly two-fold. Prorenin remained above baseline during the luteal phase and then declined further after the progesterone peak in the mid-luteal phase (Fig. 3).

Changes in plasma renin were much less consistent than the changes in prorenin and were not synchronized with prorenin. Active renin was only significantly elevated during the mid-luteal phase, concurrent with the progesterone peak (Fig. 3). We speculated that two independent factors may be involved with the increase in plasma prorenin during the menstrual cycle; the elevated prorenin in the mid-luteal phase may be related to the diuretic effect of progesterone. While we could not be certain of the source of prorenin associated with the LH surge, we suggested that it was likely due to prorenin synthesis in the ovary by the cells of the maturing follicle in response to LH.

A follow-up study made it possible to more clearly define the temporal relationship between changes in the plasma levels of LH and prorenin at mid-menstrual cycle.[29] Six normal women had blood samples taken every 8 hours during the late follicular phase after their plasma estradiol levels rose above 100 pg/ml. The study was continued for at least 72 hours after the LH surge (documented by a positive urinary LH test). Figure 4 shows the observed hormonal changes. The rise in LH began 16 hours before the rise in prorenin and the peak occurred 8 hours before the peak of prorenin. The prorenin peak lasted about 40 hours, *i.e.* until past the time of ovulation; prorenin levels returned to baseline almost 16 hours later than did LH levels. No change in plasma renin was apparent during the peak of prorenin.

This study clearly shows that the LH ovulatory surge precedes the rise in plasma prorenin in the early luteal phase of the menstrual cycle. This sequence suggests that prorenin is synthesized or secreted in response to the LH surge.

Human hCG has also been shown to stimulate plasma prorenin. Figure 5 shows the results in a woman undergoing ovarian hyperstimulation. She was given Clomid on days 3, 4 and 5 of her menstrual cycle, followed by Pergonal, a combination of LH and follicle stimulating hormone (FSH), on days 6 to 10. Because ovarian stimulated women often do not have a spontaneous LH surge, she was given hCG on day 10 to induce ovulation. Plasma prorenin increased to 1000% above baseline after hCG. Plasma prorenin reached its max-

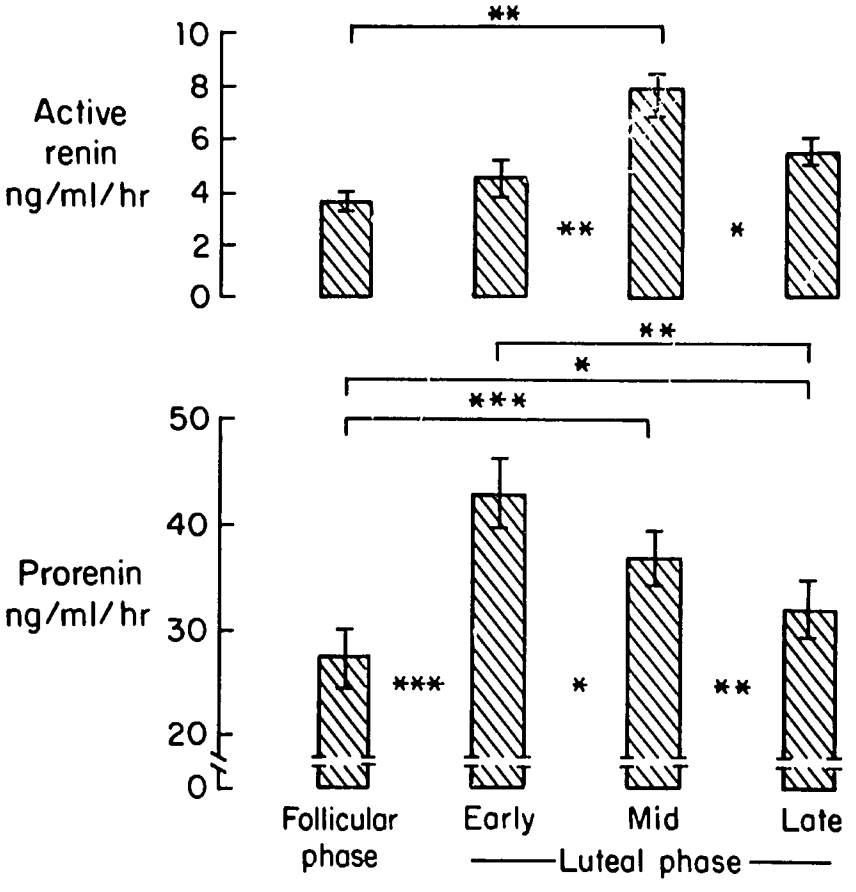


Figure 3. Changes in active renin and prorenin during nine cycles in five normal subjects. Because active renin fluctuated markedly from day to day, data were averaged from each subject during four phases of the cycle. Day zero was taken as the day of the peak of the LH surge; follicular phase, from first day of menstruation to day minus 1; early-luteal phase, days 0 to 4; mid-luteal phase, days 5 to 11; late-luteal phase, day 12 to last day before menstruation. Prorenin increased significantly during the early luteal phase whereas active renin did not increase until the midluteal phase when progesterone was high. The data represent the mean ± SEM of the average renin or prorenin for each period. * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

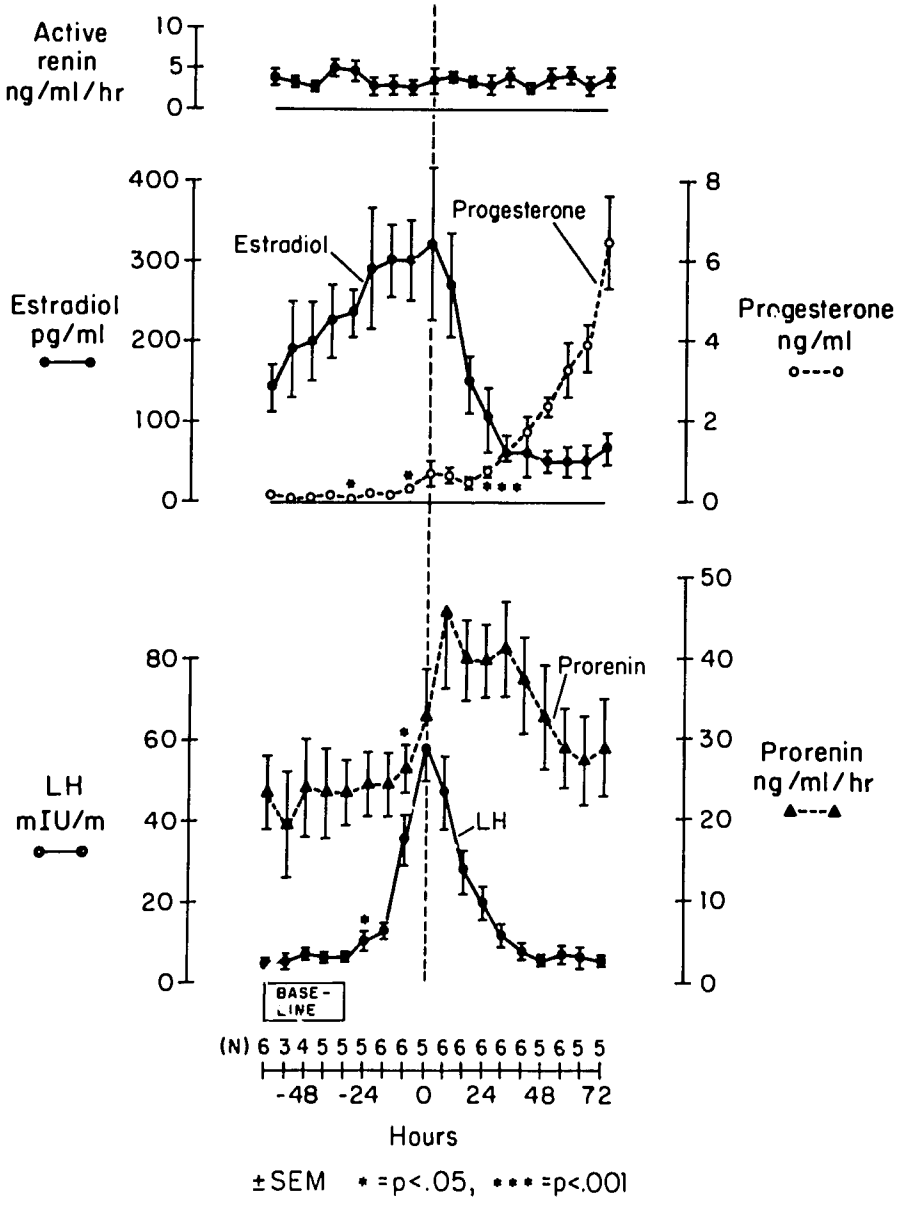


Figure 4. Plasma prorenin, active renin, LH, estradiol and progesterone changes during mid-menstrual cycle in 6 normal subjects. Samples were collected every 8 hours. Plasma LH was increased 24 hours before the LH peak whereas plasma prorenin first increased 16 hours later. Active renin did not change.

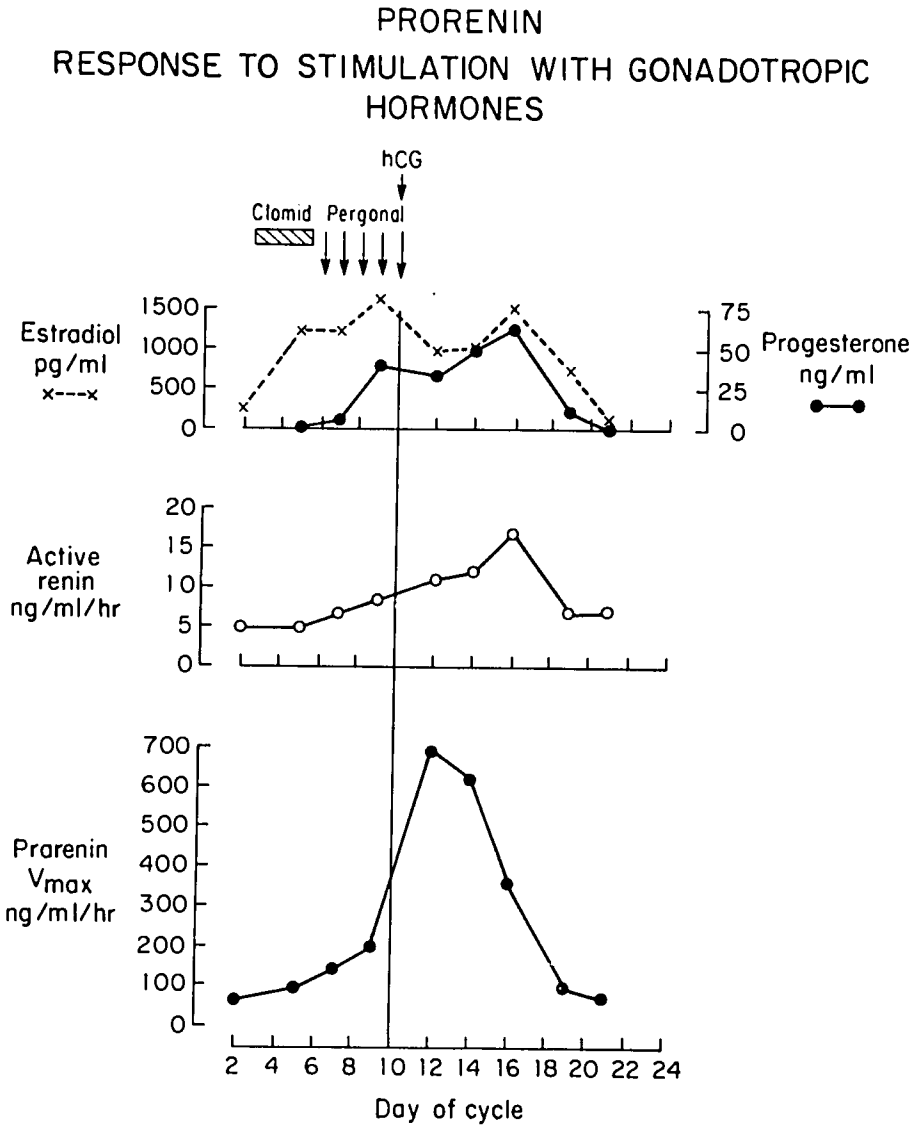


Figure 5. Effects of Clomid (50 mg on days 3 to 5), Pergonal (75 units intramuscularly daily, two times, on days 6 to 10), and hCG (10,000 units on day 10) on plasma prorenin, active renin, estradiol, and progesterone. To estimate changes in prorenin independent of changes in renin substrate concentration, the prorenin concentration at excess substrate concentration (Prorenin V^{max}) was calculated from the plasma prorenin and renin substrate levels with the use of the Michaelis-Menten equation and a Michaelis-Menten coefficient of 2700 ng/ml.

imum level on day 12. Renin also increased in this patient but it was out of phase with the changes in prorenin, reaching its maximum on day 16, concurrent with the progesterone peak. Exogenous hCG evoked a greater prorenin response than any other previously reported stimulus.[26] Subsequently, we found that the plasma prorenin response to hCG is directly related to the number of mature follicles present at the time of aspiration.[13] (See accompanying report by Itskovitz *et al.*[14]).

Altogether, these results support the view that the rise in prorenin after conception is the result of stimulation by hCG and that LH and hCG stimulate prorenin biosynthesis or release from the ovary.

To pursue this idea we measured the prorenin concentration of human ovarian follicular fluid at the time of ovulation.[10] Fluid from 18 different ovarian follicles and peripheral venous blood were collected from 3 women, ages 29 to 32, after ovarian hyperstimulation. All had received Pergonal from day 3 to day 12 or 13 of the cycle; hCG was administered intramuscularly 36 hours before follicular fluid aspiration on day 13 or 14. The results are presented in Table 1.

In previous studies,[25] plasma prorenin during the follicular phase of the cycle averaged 27 ng/ml/hr, about 10 times the active renin concentration. Plasma prorenin concentrations 36 hours after hCG administration were elevated to 188 ± 10 ng/ml/hr. Follicular fluid prorenin was much higher; the average was 2170 ng/ml/hr, with a range from 730 to 5430 ng/ml/hr. Renin substrate was less concentrated in follicular fluid as compared to plasma, but this most likely reflects sample dilution during collection. The higher concentrations

TABLE I

HUMAN OVARIAN FOLLICULAR FLUID PRORENIN, ACTIVE RENIN, RENIN SUBSTRATE, ESTRADIOL AND PROGESTERONE CONCENTRATIONS

	Plasma (mean ± SEM) N=3	Follicular Fluid (FF) (mean ± SEM) N=18	FF/Plasma ratio
Prorenin (ng/ml/hr)	188 ± 10	2170 ± 267	11.5
Active renin (ng/ml/hr)	8.8 ± 3.8	24 ± 3	2.1
% Prorenin	95	99	
Renin substrate (ng/ml)	4043 ± 520	2464 ± 111	0.61
Estradiol (ng/ml)	1.83 ± 1.06	473	259
Progesterone (ng/ml)	3.6 ± 0.85	2329 ± 321	647

of angiotensins I and II (not shown, see reference 10) and of active renin in follicular fluid as compared to plasma may be artefactual because of inadvertent *in vitro* activation of prorenin (see discussion below). Estradiol and progesterone, both synthesized by the cells of the maturing ovary, were respectively, 259 and 647 times higher in follicular fluid than in plasma.

We were not able to find any differences between prorenin from follicular fluid and prorenin from kidney.[10] We compared the immunochemical and biochemical properties of follicular fluid prorenin with those of partially purified renal prorenin and renal renin. Anti-renin antibodies bound follicular fluid and renal prorenins and renin identically. Our anti-propeptide anti-serum also bound both prorenins identically, but failed to bind either active renin or trypsin-activated follicular fluid prorenin. Trypsin-activated prorenin had the same pH optimum as active renin when incubated with purified human angiotensinogen.

The demonstration that prorenin is the predominant form of renin in follicular fluid at the time of ovulation supports the role of the ovary as a major extrarenal source of prorenin. Other unpublished studies in baboons have demonstrated ovarian secretion of prorenin at mid-menstrual cycle.

Altogether, the data suggest that the ovary does not normally synthesize prorenin during the follicular phase of the menstrual cycle. Prorenin begins to be synthesized by the ovary (presumably by the maturing follicle) either just before or at the same time as the LH surge. This rise may be related in part to the rise in estradiol or related steroids. High concentrations of gonadotropins clearly augment prorenin secretion at this time. We do not know if any prorenin is synthesized by the corpus luteum in non-conception, normal menstrual cycles. However, the corpus luteum seems able to respond to gonadotropins since the ovary apparently secretes prorenin as soon as hCG rises following conception and throughout gestation, and prorenin continues to be secreted during the luteal phase of the menstrual cycle in IVF patients who have been given hCG (see accompanying paper by Itskovitz *et al.*[14]). Whether the ovaries are the sole source of the pregnancy-related rise in maternal plasma prorenin throughout gestation remains to be established.

The ovulatory changes in plasma prorenin are not accompanied by any change in active plasma renin. If active renin is formed in the ovary *in vivo*, it does not get into the circulation in appreciable amounts.

ROLE OF PRORENIN IN REPRODUCTIVE FUNCTION

MECHANISM OF ACTION

Several lines of evidence suggest that extra-renal renin systems like that in the ovary operate via prorenin, not renin.[28,30] Arguing against this hypothesis, however, is the unassailable fact that renin has been identified in amniotic fluid[32] and ovarian follicular fluid [8,10] in concentrations higher than in concurrently collected plasma. Renin is also often found in high concentrations in extracts of adrenal, testis, uterus, placenta and brain.[7,19] Nonetheless, when we examined venous plasma from testis[31] and ovary and adrenal (unpublished observations), we found no evidence of renin secretion, only prorenin, suggesting that renin is not secreted from such tissues.

Other physiological evidence suggests that prorenin, not renin, is the functional form of the extra-renal renin system in primates. Thus, only prorenin circulates in the blood

of patients after chronic bilateral nephrectomy.[22,33] Only prorenin increases in plasma during the LH surge of the menstrual cycle.[25,29] After hCG is given to induce ovulation in hyperstimulated women, initially only prorenin increases in plasma, sometimes to levels 30 times above baseline.[13] In the la ter situation renin does increase too, but later when the progesterone level rises high enough to interfere with aldosterone action. In addition, while ovarian prorenin secretion can on occasion increase the blood levels to as high as those seen in patients with a renin secreting tumor, such ovarian hyperstimulated patients do not exhibit hypertension or any physiological evidence of excessive renin production. Also, there is no pathological state of reproductive function attributable to renin excess — yet the ovaries can apparently secrete prorenin at rates much higher than those ever produced by the kidneys.

How, then, can one explain the presence of renin and angiotensins in extracts of extra-renal tissues where the renin gene is expressed? There is no known endogenous renin inhibitor that could effectively inactivate renin once it is formed *in vivo*. We were unable to detect a renin inhibitor in ovarian follicular fluid or in plasma. The most plausible explanation is that the renin that is detected in amniotic fluid, in ovarian follicular fluid and in extra-renal tissue extracts is formed *in vitro* and that once active renin is formed, angiotensin II is also formed *in vitro*. Biochemical studies suggest that this is possible. As discussed above, prorenin can be cryoactivated.[24] This appears to involve a conformational change in the prorenin molecule as well as proteolytic cleavage of all or part of the prosegment. All samples assayed for renin or prorenin are cooled to at least 4 °C. These conditions favor the activation of prorenin. In plasma where the renin:prorenin ratio is about 1:9, cryoactivation of 1% of the prorenin could pass unnoticed. But, the prorenin concentration in ovarian follicular fluid is about 300 times higher than the active renin concentration of plasma.[10] Cryoactivation of only 1% of the ovarian follicular fluid prorenin would result in an active renin level that would be much higher than that in the concurrently collected plasma. Thus, one could wrongly come to the conclusion that active renin was normally produced by the ovary. The point is that it is very difficult to tell whether the ovary, or any other tissue that synthesizes prorenin concurrently produces small amounts of active renin. So far, in primates only the kidney has been shown conclusively to secrete active renin; other tissues seem to secrete only prorenin.

Those of us who have tried to purify prorenin from plasma or kidney extracts, or who have produced it by recombinant DNA technology, are well aware how difficult it is to keep prorenin catalytically inactive *in vitro*. A product that is completely inactive one day has some catalytic activity the next, and slowly develops more and more activity as time passes. It is our current opinion that prorenin is only completely inactive under physiological conditions of temperature.

If prorenin is catalytically inactive *in vivo*, how does it function? Wherever the renin gene is expressed, including the ovary, angiotensin receptors have been identified.[7,12] This suggests that prorenin functions to produce angiotensin, and supports the view that prorenin can become catalytically active *in vivo*. The rest of the evidence suggests that this activity is tightly controlled. It is possible but unlikely that sufficient cooling could occur in the testis to result in the development of prorenin activity, but no such situation is likely to occur in the ovaries. We have therefore developed the hypothesis that there may be a prorenin receptor that binds the prosequence of prorenin and causes the prosequence to

emerge from the active site, thus rendering prorenin catalytically active only for as long as binding continues.[28,30] In this hypothesis, the activity of the extrarenal prorenin system would be regulated by the presence or absence of prorenin receptors (Fig. 6a).

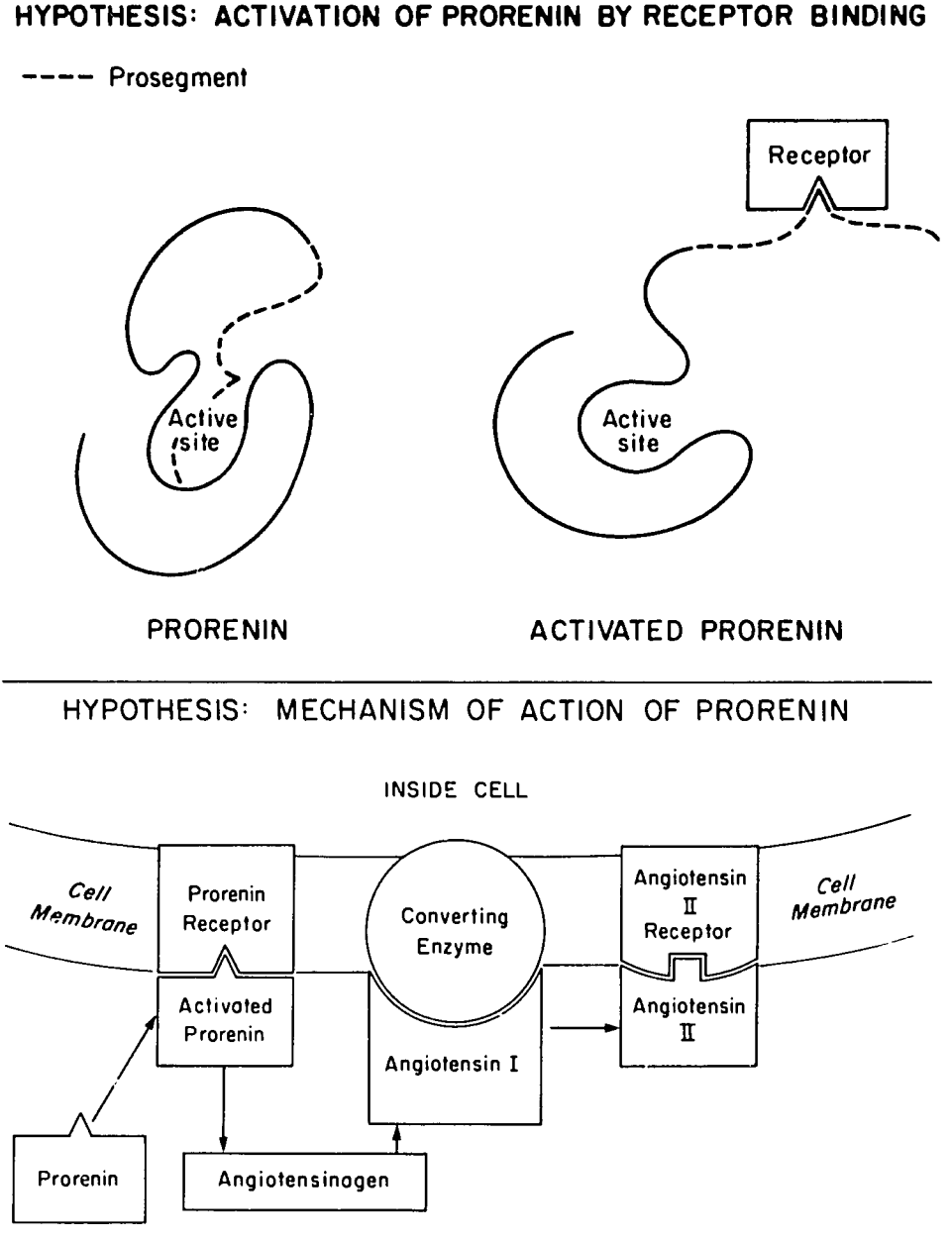


Figure 6. Hypothesis

(a) Prorenin develops intrinsic catalytic activity following binding to a receptor.

(b) Prorenin functions as a locally active form of the renin-angiotensin system.

The existence of such prorenin receptors remains to be established. For such a system to function the putative prorenin receptors should be located near converting enzyme, angiotensinogen and angiotensin II receptors. Converting enzyme and angiotensinogen are widely distributed and, as noted earlier, angiotensin receptors are present in the ovary and elsewhere where the renin gene is expressed (Fig. 6b). Thus the stage is set for the demonstration of a locally active renin-angiotensin system whose function is regulated by changes in prorenin concentration and prorenin and angiotensin receptors. Future studies should define how prorenin acts in reproductive function without interfering with the effects of the circulating renin system on the regulation of fluid and electrolyte homeostasis and blood pressure.

PHYSIOLOGICAL EFFECTS OF THE OVARIAN PRORENIN-ANGIOTENSIN SYSTEM

At this time we do not know the physiological role(s) of the extrarenal prorenin-angiotensin systems. A report that angiotensin II stimulates estrogen production in rat ovarian granulosa cells in culture remains to be confirmed.[21] The presence of prorenin in tissues that synthesize steroids, *e.g.* ovary, testis, adrenal, placenta, makes attractive the hypothesis that prorenin has an effect on steroid biosynthesis. Nonetheless, the physiological clues about its role do not clearly point to steroid biosynthesis. Thus, ovarian prorenin reaches peak concentrations just after the LH surge, a time when estrogen biosynthesis is markedly reduced and progesterone synthesis is only beginning to increase. In the menstrual cycle when estrogen biosynthesis is at peak levels, i.e. just before the LH surge, very little prorenin is found in ovarian follicular fluid. When plasma progesterone levels are at their peak, plasma prorenin is only slightly higher than baseline — and that could be explained by the diuretic effect of progesterone action stimulating the renal secretion of prorenin. In the placenta the major site of prorenin biosynthesis is the chorion laeve[1,20] which is not considered to be an important site of steroid biosynthesis. In this regard, however, in a recent study from our group, Lenz observed a significant relationship between prorenin and progesterone concentrations in extracts from placentae that were delivered close to term.[18] These relationships are intriguing and studies to dissect out their meaning are on the frontiers of renin research.

Besides steroid biosynthesis, angiotensin II could participate in reproductive function as a direct vasoconstrictor agent, or even as a vasodilator agent via its ability to stimulate vasodilator prostaglandin production. It is also reported to induce neovascularization and other reports suggest that it is a mitogenic factor. We have been intrigued by the association of prorenin with fluid accumulation and transfer, for example in the kidney (glomerular filtration), chorion laeve (amniotic fluid), and ovary (follicular fluid). Clearly many possibilities exist and it is likely that physiological clues derived from studies of the biological responsiveness of ovarian, testicular, placental and uterine prorenin will lead to the appropriate studies which will define its role. The work described by Dr. Itskovitz in the following chapter[14] has already begun to form the basis for such studies.

SUMMARY

The classical concept of the renin-angiotensin system is that of a circulating hormonal system that participates in blood pressure and fluid and electrolyte homeostasis. In this report we see the renin system through a new and different window, that of prorenin, and in a new and different role, that of reproductive function. The ovary is a recently identified source of prorenin. Ovarian secretion of prorenin changes dynamically in response to biological stimuli, namely a change in plasma gonadotropin levels. Prorenin is secreted by the ovary for 3 days each month, at the time of ovulation. Following ovarian hyperstimulation, ovarian secretion of prorenin can raise the blood level of prorenin to 1000 times that of renin without any apparent derangement in blood pressure and electrolyte homeostasis. We hypothesize that prorenin can mimic the function of renin, *i.e.* it can cleave angiotensin I from angiotensinogen, but only after binding to a specific prorenin receptor. We suggest that changes in prorenin concentration, and in prorenin and angiotensin receptor number, regulate the function of a localized prorenin-angiotensin system. Identification of the physiologic role of prorenin in reproductive function is the focus of current research.

REFERENCES

1. ACKER GM, GALEN FX, DEVAUX C, FOOTE S, PAPERNIK E, PESTY A, MENARD J, CORVOL P: Human chorionic cells in primary culture: a model for renin biosynthesis. J Clin Endocrinol Metab 55:902, 1982
2. ATLAS SA, HESSON TE, SEALEY JE, LARAGH JH: Reversible acid-activation of inactive renins: Evidence favoring a unimolecular reaction. Clin Sci 63:167s, 1982
3. ATLAS SA, CHRISTOFALO P, HESSON T, SEALEY JE, FRITZ LC: Immunological evidence that inactive renin is prorenin. Biochem Biophys Res Commun 132:1038, 1985
4. BOUHNIK J, FEHRENTZ JA, GALEN FX, SEYER R, EVIN G, CASTRO B, MENARD J, CORVOL P: Immunological identification of both plasma and human renal inactive renin as prorenin. J Clin Endocrinol Metab 60:399, 1985
5. DERKX FHM, BOUMA BN, SCHALEKAMP MPA, SCHALEKAMP MADH: An intrinsic factor XII-prekallikrein dependent pathway activates the human plasma renin-angiotensin system. Nature 280:315, 1979
6. DERKX GH, SCHALEKAMP MPA, SCHALEKAMP MADH: Two-step proreninrenin conversion: isolation of an intermediary form of activated prorenin. J Biol Chem 262:2472, 1987
7. DESCHEPPER CF, GANONG WF: Renin and angiotensin in endocrine glands. In MARTINI L, GANONG WF (eds): Frontiers in Neuroendocrinology, Vol 10, p 79. New York, Raven Press, 1988
8. FERNANDEZ LA, TARLATZIS BC, RZASA PJ, CARIDE VJ, LAUFER N, NEGRO-VILAR AF, DE CHERNEY AH, NAFTOLIN F: Renin-like activity in ovarian follicular fluid. Fertil Steril 44:219, 1985
9. GALEN FX, DEVAUX C, HOUOT AM, MENARD J, CORVOL P, CORVOL MT, GUBLER MC, MOUNIER F, CAMILLER JP: Renin biosynthesis by human tumoral juxtaglomerular cells: evidences for a renin precursor. J Clin Invest 73:1144, 1984
10. GLORIOSO N, ATLAS SA, LARAGH JH, JEWELEWICZ R, SEALEY JE: Prorenin in high concentrations in human ovarian follicular fluid. Science 233:1422, 1986
11. HSUEH WA, CARLSON EJ, ISRAEL-HAGMAN M: Mechanism of acid-activation of renin: role of kallikrein in renin activation. Hypertension 3 (Suppl 1): 22, 1981
12. HUSAIN A, BUMPUS RM, DE SILBA P, SPETH RC: Localization of angiotensin II receptors in ovarian follicles and the identification of angiotensin II in rat ovaries. Proc Natl Acad Sci USA 84:2489, 1987
13. ITSKOVITZ J, SEALEY JE, GLORIOSO N, ROSENWAKS Z: Plasma prorenin response to hCG in ovarian hyperstimulated women: correlation with the number of ovarian follicles and steroid hormone concentrations. Proc Natl Acad Sci USA 84:7285, 1987

14. ITSKOVITZ J, SEALEY JE: Ovarian prorenin in normal pregnancy and in women undergoing ovarian stimulation with gonadotropins. In HODGEN GD, ROSENWAKS Z, SPIELER JM (eds): Nonsteroidal Gonadol Factors: Physiological Roles and Possibilities in Contraceptive Development, Proceedings of the CONRAD First International Workshop, Norfolk, chp 26, 1988
15. KIM S-J, HIROSE S, MIYAZAKI H, UENO N, HIGASHIMORI K, MORINAGA S, KIMURA T, SAKAKIBARA S, MURAKAMI K: Identification of plasma inactive renin as prorenin with a site directed antibody. Biochem Biophys Res Commun 126:641, 1985
16. LARAGH JH, SEALEY JE: The renin-angiotensin-aldosterone hormonal system and regulation of sodium, potassium, and blo pressure homeostasis. In ORLOFF J, BERLINER RW (eds): Handbook of Physiology: Renal Physiology, p 831. American Physiological Society, Maryland, Waverly Press, 1973
17. LECKIE BJ, McGHEE NK: Reversible activation-inactivation of renin in human plasma. Nature 288:702, 1980
18. LENZ T, SEALEY JE, TAUFIELD PA, JAMES GD, LARAGH JH: Higher prorenin, progesterone and angiotensinogen levels in human placentas from vaginal deliveries compared to cesarian section. J Clin Endocrinol Metab, submitted.
19. NARUSE M, TAKII Y, INAGAMI T: Renin exists in high concentration in the adrenal gland of the rat. Biochem Res 2:583, 1981
20. POISNER AM, WOOD GW, POISNER R, INAGAMI T: Localization of renin in trophoblasts in human chorion laeve at term pregnancy. Endocrinology 109:1150, 1981
21. PUCELL AG, BUMPUS FM, HUSAIN A: Rat ovarian angiotensin II receptors: Characterization and coupling to estrogen secretion. J Biol Chem 262:7076, 1987
22. SEALEY JE, WHITE RP, LARAGH JH, RUBIN AL: Plasma prorenin and renin in anephric patients. Circ Res 41 (Suppl 2):17, 1977
23. SEALEY JE, ATLAS SA, LARAGH JH, SILVERBERG M, KAPLAN AP: Initiation of plasma prorenin activation by Hageman factor-dependent conversion of plasma prekallikrein to kallikrein. Proc Natl Acad Sci USA 76:5914, 1979
24. SEALEY JE, ATLAS SA, LARAGH JH: Prorenin and other large molecular weight forms of renin. Endocr Rev 1:365, 1980
25. SEALEY JE, ATLAS SA, GLORIOSO N, MANAPAT H, LARAGH JH: Cyclical secretion of prorenin during the menstrual cycle: Synchronization with luteinizing hormone and progesterone. Proc Natl Acad Sci USA 82:8705, 1985
26. SEALEY JE, GLORIOSO N, TOTH A, ATLAS SA, LARAGH JH: Stimulation of plasma prorenin by gonadotropic hormones. (Letter) Am J Obstet Gynecol 153:596, 1985
27. SEALEY JE, McCORD D, TAUFIELD PA, ALES KA, DRUZIN ML, ATLAS SA, LARAGH JH: Plasma prorenin in first trimester pregnancy: Relationship to changes in human chorionic gonadotropin. Am J Obstet Gynecol 153:514, 1985
28. SEALEY JE, GLORIOSO N, ITSKOVITZ J, LARAGH JH: Prorenin as a reproductive hormone: A new form of the renin system. Am J Med 81:1041, 1986
29. SEALEY JE, CHOLST I, GLORIOSO N, TROFFA C, WEINTRAUB ID, JAMES G, LARAGH JH: Sequential changes in plasma luteinizing hormone and plasma prorenin during the menstrual cycle. J Clin Endocrinol Metab 63:1, 1987
30. SEALEY JE, GLORIOSO N, ITSKOVITZ J, ATLAS SA, PITARRESI TM, PREIBISZ JJ, TROFFA C, LARAGH JH: Ovarian prorenin. Clin Exp Hypertens A9 (8/9):1435, 1987
31. SEALEY JE, GOLDSTEIN M, PITARRESI T, KUDLAK TT, GLORIOSO N, LARAGH JH: Prorenin secretion from human testis: no evidence for secretion of active renin or angiotensinogen. J Clin Endocrinol Metab 66:5 (in press), 1988
32. SKINNER SL, CRAN EJ, GIBSON R, TAYLOR R, WALTERS WAW, CATT KJ: Angiotensins I and II, active and inactive renin, renin substrate, renin activity, and angiotensinase in human liquor amnii and plasma. Am J Obstet Gynecol 121:626, 1975
33. WEINBERGER MH, WADE MB, AOI W, USA T, DENTINO M, LUFT F, GRIM CE: An extrarenal source of "renin-like" activity in anephric man. Circ Res 40 (Suppl 1):1, 1977

DISCUSSION:

CHAIRPERSON: G. VIRGINIA UPTON, Ph.D.

DR. HSUEH: Would you comment on any data from your group or any other group on the function of this system? Is there really a physiological role?

DR. SEALEY: We do not know the physiological role. There is one publication which needs to be confirmed suggesting that ovarian renin may be involved in estrogen biosynthesis.

Renin is expressed in the adrenal, testes, ovary, and placenta. Wherever the renin gene is expressed you often find steroid biosynthesis. It is interesting to speculate that prorenin might be involved in steroid biosynthesis. This would not be surprising given that angiotensin II is able to stimulate aldosterone biosynthesis. This stimulation occurs, in part, at a very early step in the biosynthetic pathway suggesting that it need not be specific for aldosterone. But so far, the evidence as to what ovarian prorenin does is not available. One major problem in resolving this is that we do not have a good non-primate animal model to study prorenin.

DR. HSUEH: How about localization studies? Is there any difference between growing follicles and atretic follicles to suggest a function?

DR. SEALEY: Dr. Itskovitz will address that question in his presentation.

DR. KHAN: Referring to the question of Dr. Hsueh, you said renin was produced in the testes and that prorenin might be involved. Where is it localized in the testes?

DR. SEALEY: There is evidence from studies in rats and mice that renin is present in Leydig cells. Angiotensin II and angiotensin receptors have also been demonstrated in Leydig cells. But in this regard there is also negative evidence in the human. We have not been able to extract renin from the testes, and others have been unable to identify it in human testes using *in situ* hybridization and immunohystochemical techniques. There may be major species differences.

DR. LEAL: What about the half-life between prorenin and renin? I wonder if you never detect renin because the half-life may be too short?

DR. SEALEY: The half-life of active renin is between fifteen and thirty minutes, and the half-life of prorenin is probably between thirty minutes and two hours, so there is no major difference between the two.

26

Ovarian Prorenin in Women Undergoing Ovarian Stimulation with Gonadotropins and During Pregnancy

JOSEPH ITSKOVITZ
JEAN E. SEALEY

The results from the studies summarized in Chapter 25[20] clearly indicate that prorenin, the enzymatically inactive precursor of renin, is synthesized and secreted by the human preovulatory ovarian follicle in the middle of the menstrual cycle.[14,18] Ovarian stimulation with gonadotropins, which results in the development of multiple follicles, is associated with remarkable stimulation of prorenin secretion from the ovary.[8,15] Pregnancy is also associated with a marked rise in plasma prorenin levels soon after the time of implantation and this rise appears to be of ovarian origin.[16,17]

In this chapter, we have summarized our studies in which we investigated the dynamics of prorenin secretion under these two circumstances by examining the time-course of plasma prorenin (and active renin) response to FSH/LH and hCG in patients undergoing ovarian stimulation for *in vitro* fertilization (IVF), and in women who conceived following embryo transfer. Finally, preliminary results from studies of follicular fluid prorenin levels in relation to follicular development, oocyte maturation and follicular fluid steroid hormone concentrations are also reported.

PLASMA PRORENIN IN OVARIAN-STIMULATED WOMEN

To test the hypothesis that prorenin is synthesized and secreted from the maturing follicle and the corpus luteum, we examined whether ovarian-stimulated women (who have multiple follicles and corpora lutea) have changes in plasma prorenin that are related to the number of ovarian follicles.[8] In 12 patients undergoing ovarian stimulation with gonadotropins for IVF we measured blood levels of prorenin and active renin during the follicular and the luteal phases. Because serum samples were frozen and thawed several times for routine hormonal measurements, which caused cryoactivation of prorenin and falsely high active renin levels, only total renin (prorenin and active renin) is reported in this group of patients. However, active renin comprised <20% of the total at all times. Total renin remained relatively unchanged during FSH/LH administration in the follicular phase, until the day of hCG injection (Fig. 1). A significant 2-fold increase was observed on the day of hCG injection, before hCG was given. This rise occurred whether the patients had been stimulated with FSH alone or with a combination of FSH and LH, suggesting that this

rise is not due to exogenously administered LH and therefore may suggest that prorenin is secreted by the preovulatory follicle before the LH surge. Presumably, the preovulatory follicle normally secretes small amounts of prorenin before the LH surge that are undetectable in the peripheral circulation in the nonstimulated cycle. When multiple follicles develop, as occurs in a stimulated cycle, it is possible that the secretion of prorenin increases enough to be detectable in the blood before hCG injection.

Following hCG injection (day 0) total renin increased markedly. The levels achieved were much higher than those that occur normally during the spontaneous LH surge of a natural cycle.[14,18] The more sustained rise in total renin is consistent with the longer half-life of hCG compared to LH. It reached a peak, 9-fold of baseline level on day 4-6 and returned to baseline on day 12-14. In four pregnant women total renin increased again after day 10 and reached a second peak thereafter (see below).

The height of the circulating total renin response to hCG on day 2 (the day of egg retrieval) was directly related to the number of mature follicles ($r=0.93$, $P<0.001$) suggesting that prorenin is produced and secreted by the mature ovarian follicles. The highly significant correlation between blood total renin levels during the luteal phase and the number of follicles ($r=0.89$, $p<0.001$) suggests that prorenin is secreted by the corpora lutea that were

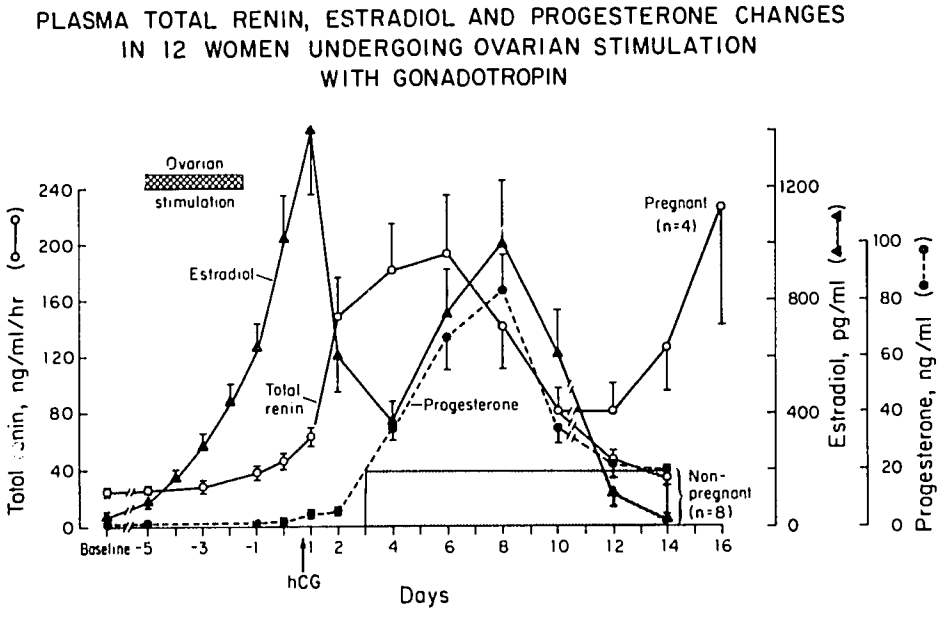


Figure 1: Serum total renin, estradiol, and progesterone (mean ± SEM) in 12 ovarian-stimulated patients. hCG was injected on day 0 and follicles were aspirated on day 2. The shaded area represents the estimated level of serum progesterone (based on the level of progesterone on day 14 in the nonpregnant patients) caused by the injection of progesterone, given from day 3 until day 14 (From Itskovitz *et al.*[8]).

formed from the follicles. Figure 2 illustrates the spectrum of responses in serum total renin in representative individual subjects with a low, intermediate, or high number of follicles.

The rising levels of prorenin in the face of falling levels of hCG during the early and mid-luteal phase and the precipitous rise of prorenin very early in pregnancy demonstrate the inherent sensitivity of the corpus luteum to produce prorenin in response to hCG throughout the luteal phase. However, it is less clear whether the corpus luteum secretes prorenin during the luteal phase of the normal menstrual cycle. Only a small rise of prorenin occurs at the mid-luteal phase of the menstrual cycle and this peak is also associated with a significant increase of active renin at the time when plasma progesterone concentration is high.[14] It is possible that the mid-luteal rise in plasma prorenin and active renin is of renal origin and is secondary to the natriuretic effect of progesterone. Nevertheless, the initial increase in prorenin following the LH surge and after hCG administration is clearly not associated with a rise in active renin.[8,14,18] Thus, in the ovary in contrast to the kidney, prorenin is secreted independently of active renin.

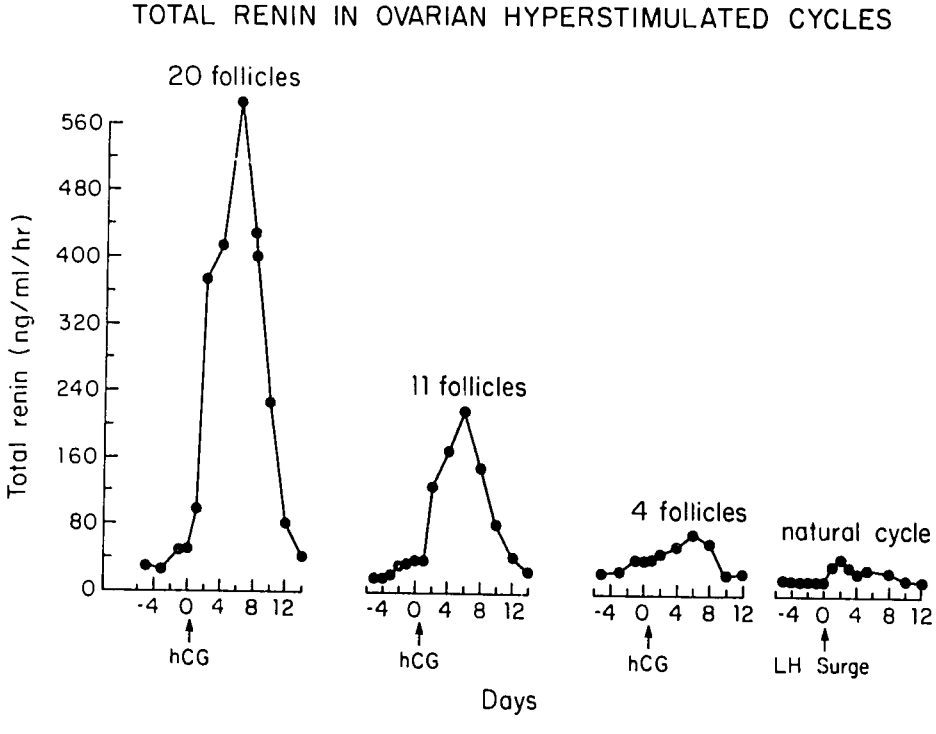


Figure 2: Total serum renin levels throughout stimulated cycles of three patients with different numbers of ovarian follicles. Shown for comparison are the changes in total renin throughout a natural cycle (From Itskovitz *et al.*[8]).

To further characterize the time-course of prorenin secretion from the ovary in response to LH stimulation, we studied a group of ovarian-stimulated patients in which instead of hCG we used a GnRH analog to induce LH surge and oocyte maturation.[9] Administration of Buserelin 500 μg (12 hours apart) resulted in a significant rise of serum LH within 1 hour, which was maintained for about 24 hours. Consistent with the observation made in the normal menstrual cycle, plasma prorenin increased within 8 to 12 hours, and reached a peak 24 to 36 hours following the injection of the GnRH analog. It fell close to baseline within 3 to 4 days in patients who disclosed no corpus luteum function but was above baseline for a longer period of time in the patients in whom corpus luteum activity was detected. The height of LH response varied between patients but no relationship between the magnitude of LH peak and prorenin response was apparent. However, the highest prorenin levels were observed in patients who had the greatest number of follicles.

Taken together these results suggest that gonadotropins stimulate ovarian prorenin release and that the ovarian renin system seems to function differently from the renal renin system in that the rate of change of ovarian prorenin is faster, being measured in terms of hours, rather than days.[12] Furthermore, the ovaries appear to secrete prorenin at rates much higher than those ever produced by the kidneys.

PRORENIN IN HUMAN OVARIAN FOLLICULAR FLUID

Prorenin is the predominant form of ovarian renin and is present in very high concentrations in follicular fluid at the time of ovulation.[3,5] This ovarian prorenin disclosed immunochemical and biochemical properties that were identical to purified renal prorenin.[5] However, its relationships to follicular development and oocyte maturation are unknown. We therefore measured prorenin concentrations in follicular fluids obtained from patients undergoing ovarian stimulation for IVF and correlated these levels with the morphology of the oocyte-cumulus complex and the granulosa cells 36 hours following hCG injection (unpublished observations). In follicles containing apparently healthy mature oocytes (metaphase II, fertilized and cleaved) prorenin averaged 2617 ng/ml/hr but it was only 1/3 of this level in follicles containing typical immature eggs (germinal vesicle stage, compact cumulus, immature granulosa cells). Intermediate levels of prorenin (1951 ng/ml/hr) were detected in follicles containing immature eggs (germinal vesicle stage) that were associated with expanded cumulus and matured granulosa cells. Prorenin levels were lowest in follicular fluids from immature follicles aspirated during the mid-follicular phase of the normal menstrual cycle, and were close to plasma prorenin levels. It is unclear at precisely what time prorenin begins to be synthesized by the developing follicle but it seems to be either just before or at the time of the LH surge. Whether ovarian prorenin has any role in follicular development and oocyte maturation remains to be determined.

Whereas LH and hCG stimulate ovarian prorenin synthesis or secretion, ovarian steroids may also be involved. Regulation of renin gene expression and biosynthesis by testosterone has been described in the mouse submandibular gland.[7,11,23] Examination of the relationship between follicular fluid prorenin and testosterone levels in mature as well as in immature follicles revealed a highly significant correlation (unpublished observations), suggesting a possible role for testosterone in stimulating prorenin biosynthesis in the human ovary.

PLASMA PRORENIN DURING EARLY PREGNANCY

Plasma prorenin rapidly increases to about 10 times the level in nonpregnant women and remains high throughout the course of pregnancy.[6,13,16] Prorenin is synthesized in the chorionic cells[1,10,22] and is secreted into the amniotic fluid where it occurs in high concentrations.[21] However, maternal plasma prorenin attains its maximal levels within four weeks after conception when placental mass is small, suggesting that the source of elevated plasma prorenin is maternal. Plasma prorenin begins to rise on days 8 to 12 after embryo transfer[8,17] at the time when endogenous hCG from the implanting embryo is detectable in the blood (Fig. 3). The lack of prorenin rise in pregnant patients with ovarian failure who conceived after the transfer of a donated egg[4,17] clearly indicates that the secretion of prorenin during early pregnancy is of ovarian origin (Fig. 3), although the uteroplacental unit may also contribute to the elevated plasma prorenin levels later in pregnancy.[2]

The early rise in prorenin following conception indicates that the corpus luteum may be very sensitive to hCG at this time since increased secretion of prorenin occurs when

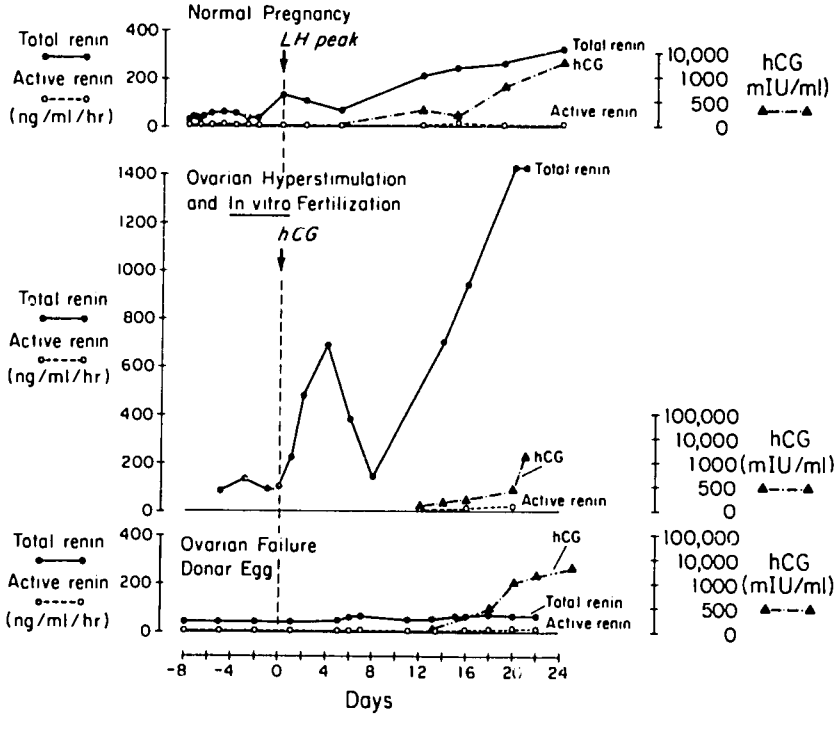


Figure 3: Plasma total renin, active renin, and hCG measurements during early pregnancy in normal pregnancy (upper panel), in ovarian-stimulated patient who conceived following IVF and embryo transfer (middle panel), and in patient with ovarian failure who conceived after induction of an artificial cycle and IVF of a donated oocyte (lower panel).

hCG levels are only moderately elevated and the maximal level of prorenin in the blood is achieved much earlier than the peak of plasma hCG.

Although the time-course of the plasma prorenin rise in patients who conceived after ovarian stimulation for IVF and in normal pregnancy is similar, the magnitude of the changes is quite different (Fig. 3). As mentioned earlier, the rise of plasma prorenin at the luteal phase in response to hCG in ovarian-stimulated women is related to the number of follicles.[8] Thus, the higher levels of prorenin detected in early pregnancy in women who conceived after ovarian stimulation may be due to the existence of multiple corpora lutea during early pregnancy in these patients.

Although the human ovary seems to synthesize and secrete prorenin at a rate that might exceed the amount secreted by the kidney, its physiological role remains speculative at the present time. However, given so many biological effects of angiotensin II (See Chapter 25),[20] the potential exists that the ovarian prorenin-angiotensin system is involved in several reproductive functions such as follicular development, oocyte maturation and extrusion, and corpus luteum function. This system, which operates differently and independently from renal renin, seems to be regulated by changes in prorenin concentration and putative prorenin receptors located near angiotensin receptors.[19]

ACKNOWLEDGMENTS

The collaboration of Dr. S. Iwamura, the National Institute of Animal Health; Dr. F. Gotoh, Kagoshima University; and Dr. Y. Mori, Tokyo University of Agriculture and Technology, is gratefully appreciated. We wish to thank Dr. A. F. Parlow and the National Pituitary and Hormone Distribution Program, NIDDK, NIH, for rat and human gonadotropin RIA kits; Dr. D. J. Bolt, Animal Science Institute, for the bovine FSH RIA kit; Dr. S. Kanematsu, Iwate University, for anti-bovine LH and anti-porcine LH; and Mr. T. Hanafa, Dr. K. Taya and S. Sasamoto, Tokyo University of Agriculture and Technology, for anti-bovine inhibin.

REFERENCES

1. ACKER GM, GALEN FX, DEVAUX C, FOOTE S, PAPERNIK E, PESTY A, MENARD J, CORVOL P: Human chorionic cells in primary culture: A model for renin biosynthesis. J Clin Endocrinol Metab 55:902, 1982
2. BRAR HS, DO TS, TAM HB, VALENZUELA GJ, MURRAY RD, LONGO LD, YONEKURA ML, HSUEH WA: Uteroplacental unit as a source of elevated circulating prorenin levels in normal pregnancy. Am J Obstet Gynecol 155:1223, 1986
3. DERKX FHM, ALBERDA AT, ZEILMAKER GH, SCHALEKAMP MADH: High concentrations of immunoreactive renin, prorenin and enzymatically-active renin in human ovarian follicular fluid. Br J Obstet Gynaecol 94:4, 1987
4. DERKX FHM, ALBERDA AT, DE JONG FH, ZEILMAKER FH, MAKOVITZ JW, SCHALEKAMP MADH: Source of plasma prorenin in early and late pregnancy: Observations in patient with primary ovarian failure. J Clin Endocrinol Metab 65:349, 1987
5. GLORIOSO N, ATLAS SA, LARAGH JH, JEWELEWICZ R, SEALEY JE: Prorenin in high concentrations in human ovarian follicular fluid. Science 233:1422, 1986
6. HSUEH WA, LUETCHER JA, CARLSON EJ, GRISLIS G, FRAZE E, McHARQUE A: Changes in active and inactive renin throughout pregnancy. J Clin Endocrinol Metab 54:1010, 1982
7. INGELFINGER J, PRATT RE, ELLISON KE, DZAU VJ: Regulation of mouse tissue renin expression during ontogeny (abstract). Clin Res 2:362A, 1985

8. ITSKOVITZ J, SEALEY JE, GLORIOSO N, ROSENWAKS Z: Plasma prorenin response to human chorionic gonadotropin in ovarian-hyperstimulated women: Correlation with the number of ovarian follicles and steroid hormone concentrations. Proc Natl Acad Sci USA 84:7285, 1987
9. ITSKOVITZ J, BOLDES R, BARLEV A, ERLIK Y, KAHANA L, BRANDES JM: The induction of LH surge and oocyte maturation by GnRH analogue (Buserelin) in women undergoing ovarian stimulation for *in vitro* fertilization (abstract). International Symposium on GnRH Analogues in Cancer and Human Reproduction. Geneva, Switzerland, 18-21 February, 1988
10. POISNER AM, WOOD GW, POISNER R: Release of inactive renin from human fetal membranes and isolated trophoblasts. Clin Exp Hypertens 11/12:2007, 1982
11. PRATT RE, DZAU VJ, OUELLETTE AJ: Influence of androgen on translatable renin mRNA in the mouse submandibular gland. Hypertension 6:605, 1984
12. SEALEY JE, ATLAS SA, LARAGH JH: Prorenin and other large molecular weight forms of renin. Endocrine Reviews 1:365, 1980
13. SEALEY JE, WILSON M, MORGANTI AA, ZERVOUDAKIS I, LARAGH JH: Changes in active and inactive renin throughout normal pregnancy. Clin Exper Hypertens 4(A):2373, 1982
14. SEALEY JE, ATLAS SA, GLORIOSO N, MANAPAT H, LARAGH JH: Cyclical secretion of prorenin during the menstrual cycle: Synchronization with luteinizing hormone and progesterone. Proc Natl Acad Sci USA 82:8705, 1985
15. SEALEY JE, GLORIOSO N, TOTH A, ATLAS SA, LARAGH JH: Stimulation of plasma prorenin by gonadotropic hormones. (Letter) Am J Obstet Gynecol 153:596, 1985
16. SEALEY JE, McCORD D, TAUFIELD PA, ALES KA, DRUZIN ML, ATLAS SA, LARAGH JH: Plasma prorenin in first trimester pregnancy: Relationship to changes in human chorionic gonadotropin. Am J Obstet Gynecol 153:514, 1985
17. SEALEY JE, GLORIOSO N, ITSKOVITZ J, TROFFA C, CHOLST I, ROSENWAKS Z: Plasma prorenin during early pregnancy: Ovarian secretion under gonadotropin control? J Hypertns 4 (suppl 5):592, 1986
18. SEALEY JE, CHOLST I, GLORIOSO N, TROFFA C, WEINTRAUB ID, JAMES G, LARAGH JH: Sequential changes in plasma luteinizing hormone and plasma prorenin during the menstrual cycle. J Clin Endocrinol Metab 65:1, 1987
19. SEALEY JE, GLORIOSO N, ITSKOVITZ J, ATLAS SA, PITARRESI TM, PREIBISZ JJ, TROFFA C, LARAGH JH: Ovarian prorenin. Clin Exp Hypert A9 (8/9):1435, 1987
20. SEALEY JE, ITSKOVITZ J: Prorenin and reproductive function. In HODGEN GD, ROSENWAKS Z, SPIELER JM (eds): Nonsteroidal Gonadal Factors: Physiological Roles and Possibilities in Contraceptive Development. Chapter 25, Proceedings of CONRAD First International Workshop, Norfolk, January 6-8, 1988
21. SKINNER SL, CRAN EJ, GIBSON R, TAYLOR R, WALTERS WAW, CATT KJ: Angiotensin I and II, active and inactive renin, renin substrate, renin activity, and angiotensinase in human liquor amnii and plasma. Am J Obstet Gynecol 121:636, 1975
22. SYMONDS EM, STANLEY MA, SKINNER SL: Production of renin by *in vitro* cultures of human chorion and uterine muscle. Nature 217:1152, 1968
23. WILSON CM, ERDOS EG, DUNN JF, WILSON JD: Genetic control of renin activity in the submaxillary gland of the mouse. Proc Natl Acad Sci USA 74:1185, 1977

DISCUSSION

CHAIRPERSON: G. VIRGINIA UPTON, Ph.D.

DR. PETRAGLIA: This question is for Dr. Sealey. You measured prorenin, which is a precursor molecule that has no known biological activity. Then you showed that active renin does not change in any case. You have found the changes in prorenin. Can you speculate on what is happening?

DR. SEALEY: We think there may be a binding protein that recognizes the prosequence of prorenin. Since we know that prorenin can be reversibly activated, we are speculating

that once prorenin binds this protein it becomes active, but only as long as it is bound. If it dissociates, or if this protein is membrane bound, it could be internalized, resulting in a locally active renin-angiotensin system modulated by changes in prorenin. Prorenin does have intrinsic activity even with the prosequence attached. You can expose it by acidification or expose to cold temperature. We are now proposing you can expose it by binding.

DR. PETRAGLIA: How can you explain the fact that the changes of angiotensin II during pregnancy are completely opposite to the changes you have shown in prorenin?

DR. SEALEY: We think that ovarian prorenin, although part of the renin-angiotensin system, acts independently.

QUESTION: Does an angiotensin converting enzyme inhibitor, such as captopril, have an impact on female reproduction?

DR. ITSKOVITZ: No. To the best of our knowledge, and from the limited information available, there is no effect of captopril on female reproduction.

DR. UPTON: I would like to know what the concentration gradient of prorenin is between the ovarian vein and the peripheral circulation. Have you ever measured the difference simultaneously?

DR. ITSKOVITZ: We were able to measure prorenin levels across the ovary in a patient who conceived after *in vitro* fertilization and was operated on at 5 1/2 weeks of gestation, because of tubal pregnancy. There was about a 25% positive venous-arterial difference indicating that the ovary produces and secretes prorenin. We were in some way disappointed by the observation of only 25%, but one should take into account that there may be other possible sources (e.g., the uterus) which may contribute to the elevated levels of plasma prorenin during early pregnancy. The data from the baboon studies shown by Dr. Sealey clearly demonstrate a positive venous-arterial difference of prorenin across the ovary.

DR. SCHENKEN: Dr. Sealey, in your baboon studies, did you look at the prorenin concentration in the ovarian venous serum during hyperstimulated cycles only or also during spontaneous cycles?

DR. SEALEY: We looked at both cases, I only showed the data from the ovarian hyperstimulation cycles.

DR. SCHENKEN: Could you tell us how the levels changed during the spontaneous menstrual cycle?

DR. SEALEY: I cannot tell you how the ovarian venous level changed during the cycle. We only measured it at one point, and that was at the time when we expected ovulation, or after we had given hCG. All I can say is that the highest venous-arterial differences

that we observed were after hCG administration; they were much lower in spontaneous cycles.

DR. SCHENKEN: Thank you.

CLOSING PANEL

DR. WAYNE BARDIN
Center for Biomedical Research
The Population Council

It is of note that we have heard five papers on the structure of inhibin and about seven other intergonadal regulators. Still others were left out that could have been spoken about. I think the caveat that I will take away from this meeting is that there is still an enormous amount of work to do.

For example, there are about a hundred Sertoli cell proteins that have not been purified; there are many more proteins in follicular fluid; and there are at least thirty or forty protein products of Leydig cells. We think of Leydig cells as steroid-secreting, but there are also many proteins secreted by this cell. And, possibly also by the theca cells of the ovary. Thus, as we begin to think we understand gonadal regulation, we realize that we have not contemplated fully the task ahead.

I think that in subsequent meetings we will probably hear about the opiate systems. We know that all of the opiate precursors are in the testis and the ovary: POMC, proendorphin and prolinekethelin. The oxytocin and vasopressin molecules are also expressed in ovary and testis. Also, we are seeing the increased importance of oncogenes that were mentioned by several speakers.

DR. SHELDON SEGAL
The Rockefeller Foundation

This is the first international workshop in the CONRAD Program. We should all be pleased about the success of this one and the prospects for a really important series of workshops in the years ahead.

Some of you may know that several years ago the three private foundations based in New York, the Rockefeller, Ford, and Mellon Foundations, decided to sponsor jointly a grants program on selected topics in reproductive biology. We asked an Advisory Group consisting of Griff Ross, Nina Channing and Bob Ryan to advise on what would be some of the important issues of the next decade for which Foundation support might be focused. Their wisdom has been confirmed by the events of these two days. First on their list was the nonsteroidal gonadal factors.

What we heard at this workshop makes it clear that several areas of reproductive biology are coming into focus. The inhibin story, after at least three decades of uncertainty, has reached the stage of maturity, more so from the point of view of our understanding of the chemistry and related phenomena than the physiology. But, it seems to me, this is a prior condition for being able to understand better the normal physiological role of the substance.

Knowledge concerning OMI appears to be lagging behind; there is a need to progress faster on chemical identification and, ultimately, the molecular biology of the substance. This type of advance has been evident with respect to the Mullerian inhibiting substance, a field that has created great interest.

We have seen an important extension of work on the possible use of FSH antibodies for fertility regulation, with the report of Moudgal extending his earlier observations in the male nonhuman primate to the female. That work should be taken as a sign of great en-

couragement to those interested in the possible manipulation of FSH as a route to fertility regulation.

There is a great deal in store for us on growth factors. I have the impression from listening to the papers on growth factors that this, too, is an area that has now become a part of the mainstream of reproductive biology. It is clear that there is a tremendous amount to be learned, but it is also evident that we can no longer simply ignore the possible role of various growth factors in trying to understand the total pattern of reproductive events.

As for the implications of this work for fertility regulation and reproductive health, I think it is too early to predict, but that should not be taken as a discouragement.

I think agencies with an interest in fertility regulation should not be discouraged by the distance there is still to go. It is important to look back and see how far we have come.

DR. ROY HERTZ
George Washington University

The retrospective approach that I am inclined to bring to this discussion goes back to the original concept of inhibin that arose from a clinical observation made by Dr. Perry McCullough. He coined the term following his observation that certain infertile individuals in whom adequate testicular hormonal activity was expressed were found to lack gonadotropin in their urine, as far as could be assessed using the bioassay techniques of the day. Nowadays there seems to be a reemphasis on his original concept through the application of a much more sophisticated approach.

I believe that our discussions have suffered because of the inadequacy of our definitions for the terms employed. Perhaps at the outset this could have been avoided through the provision of definitions specific to distinct entities.

Over the years I have noticed that interest in particular lines of thought has proceeded according to a sort of fashion. An example of this was the tremendous interest in an enzymological approach to the analysis of tissue response. Everyone was working on alkaline phosphatase in the uterus in response to estrogens, and carbonic anhydrase in response to progestins. Prior to this, Dr. Speeman postulated the existence of "organizers" which were substances in one tissue that were capable of altering the genesis and development of adjacent tissues. This was followed by an active interest in dietary trace factors, and the interaction between hormones and vitamins.

Perhaps we should view such trends with some concern in that they might operate against the pursuit of alternate and possibly fruitful approaches.

Finally, I would like to call the attention of this group to the fact that the first nonsteroidal ovarian factor of considerable interest in the study of reproduction, which has not been mentioned during this program, is the peptide relaxin. Relaxin is found in the placenta of women almost as early in pregnancy as is hCG. The level of relaxin increases throughout pregnancy then drops sharply immediately following delivery, signifying an important pregnancy-related or other regulatory function internal to the ovary.

My intention in reactivating interest in this particular peptide comes from having personally had the privilege of assisting Dr. Hisaw in some of his later work on the purification and biological characterization of relaxin. It is a substance which begs a definitive function.

I feel there has been altogether too much emphasis on research at the nonbasic level, even in specific contraceptive development programs. If we pursue fundamental approaches with the kind of knowledge available to us today, we will realize more fruitful results from our efforts. I am prompted to say this because I remember the day when Dr. R.K. Meyer said to Dr. Hisaw: "You know, I kind of think that maybe estrogen given to a rat would suppress pituitary gonadotropin." This formed the basis for the development of the oral contraceptives 25 years later.

JEFFREY SPIELER
Research Division, Office of Population, Agency for International Development

The Agency for International Development (A.I.D.) supports applied research in contraceptive development. Among other things, we are concerned about providing a broad range of improved and totally new contraceptive methods for use in less developed countries. The spin-off of our work is better contraceptive methods for couples in developed countries. To meet this objective A.I.D. primarily supports applied research. With some exceptions, A.I.D. does not support basic research, the latter being supported by agencies like NIH and national medical research councils. A.I.D. uses Foreign Assistance funds and its resources in this area are primarily available for research on short and medium term methods, i.e. methods which are already undergoing clinical assessment or which will be entering human trials within the next several years. However, research on nonsteroidal gonadal factors is one of the exceptions to this general principle. A.I.D. is providing some limited funds to the CONRAD Program and the Population Council to support work on inhibin, GnSIF and OMI.

At this first CONRAD International Workshop we have reviewed the results of some very interesting research in a field that is accelerating at a remarkable pace, primarily owing to the time in history that this work is being conducted. Phenomenal advances in biotechnology during the past several years have permitted progress, that was unthought of a decade ago. And, what actually is in store for us during the next decade is hard for a science administrator, such as myself, to anticipate. Nevertheless, even with a geometric increase in progress, it will probably be some years before the "white powder" has been evaluated, pre-clinical pharmacology and toxicology completed, supply issues resolved and human trials for contraceptive purposes underway in men and women.

For A.I.D., and other agencies supporting applied research in contraceptive development, the nonsteroidal gonadal factors represent the pipeline from which the third generation contraceptives of the year 2000 will emerge. We believe that it is imperative for all investigators, including the chemists and molecular biolologists, to think in terms of new product development and not just basic research and publications. The research scientists must be concerned with how their findings can benefit mankind, and in this field that means, among other things, broadening the range of safe, acceptable and effective methods of contraception in the near future.

DR. ROBIN FOLDESY
Family Health International

Because I believe some among you are not familiar with Family Health International (FHI), and because my comments will be structured around the kinds of activities conducted by FHI, I will begin with a brief description of our organization.

Family Health International is a not-for-profit organization located in Research Triangle Park, North Carolina. It grew out of a program that had its roots in the University of North Carolina almost two decades ago. The program was known then as the International Fertility Research Program.

The CONRAD Program, here at Eastern Virginia Medical School, and FHI are sister organizations funded, in part, by the U.S. Agency for International Development. We are committed to providing contraceptive choices to people around the world. Most of our work at FHI is directed at the needs of the less developed countries, and focuses primarily on Phase III and Phase IV clinical research. Although I have been very impressed with the kind of work presented at this Workshop, from the perspective of FHI my thoughts center around how we begin to move this work towards a product for use in the clinic. Much of the discussion over the past two days has, in fact, been centered on basic research. This is not intended in any way to be a criticism. In fact, Dr. Bardin pointed out to me that TPA emerged from a very basic research program. There are numerous other examples of even more classic pharmacological agents, such as Tagamet, that grew out of basic research programs. However, at some point, the research community needs to think about placing this work within the framework of applied research and how we move from the basic aspects of this field to the development of these compounds as new contraceptive agents. I think Dr. Hodgen may have opened this issue yesterday when he questioned how we are going to get adequate supplies of reagents, how we are going to perform the assays, etc. These are very important questions and the first steps in the process of developing new drugs.

Once we have a supply of compound and standard techniques for measuring it, then there are several activities that can go on in parallel. There need to be further studies on the clinical endocrinology of this compound. Some very exciting results were presented yesterday, and replication and reexamination of some of this work would be very useful. For instance, looking at inhibin in the normal menstrual cycle in greater detail would be useful. Looking at what happens to some of these nonsteroidal factors during pregnancy or after parturition is very important. Also, what are the levels of inhibin during both lactational amenorrhea and reinitiation of the menstrual cycle?

At the same time, we must begin animal pharmacology and toxicology experiments. We must then develop the animal models to test our hypotheses on how these compounds can be used as contraceptives.

In this way, we can establish the efficacy of these compounds, the route of administration, the duration of action, etc. Once we begin to think in this manner, we begin moving toward the development of a contraceptive. A lot of physiological questions remain unanswered and only the lack of compound prevents us from performing the necessary experiments. I believe that this is the key to moving from the basic to the more applied aspects of research on the nonsteroidal gonadal factors.

DR. GABRIEL BIALY
Contraceptive Development Branch
Center for Population Research
National Institute of Child Health and Human Development

Let me touch on some practical matters. I come from NIH, the largest organization in the U.S. supporting basic and applied medical research. What we strive for is a healthy and happy marriage between basic and applied research. One is not better than the other, and I invariably argue that there is need and room for both of them. This meeting has, in a certain way, demonstrated the successful marriage of basic academic science with the participation of industry. This union is healthy because there are many things that industry is able to accomplish that are exceedingly difficult for the academic scientist.

One of the important things we have learned is that sustained progress depends on a variety of tools, one of which is certainly financial. Another tool that propels science forward is the availability of reagents to all parties. However, it is the responsibility of scientists to demonstrate their need for certain compounds, such as inhibin reagents.

I always find it refreshing to be with Roy Hertz because, in a way, we share a common background. We both graduated from the University of Wisconsin and one of the very first projects I was involved in at NIH dealt with the distribution of hCG. Some of you who have used hCG from the NIH most likely did not realize that the prefix code CR stood for Canfield and Ross. Even though Griff Ross is gone, we still use this designation for the new batches.

While we should not be fooled into thinking that the distribution of hCG was cheap, considerable research followed at no cost to the taxpayers. I think we are re-entering that era with some of the gonadal peptides, and I think it is incumbent on all of us to think not just in terms of what the availability of reagents does for me personally but, rather, what it can do for others as well. We need to consider the value of universal availability and the progress it can facilitate. I think the NIH administration showed imagination in its support of reagent distribution. People like Mort Lipsett fully endorsed this practice and the current administration maintains this position.

COMMENT — DR. DO WON HAHN
Ortho Pharmaceutical Corporation

I have been involved in the contraceptive research and development area for about two decades. Each time we start thinking about developing a new contraceptive, the first question we have to ask is why we need another one, especially when you already have a successful product. This is a very difficult question to answer. You have to consider the advantages of a new product in light of all the currently available products.

We now have very successful low-dose oral contraceptives with very few side effects. It is our duty, therefore, to think critically about the need for and advantage of a new product. I would like to call your attention to this type of question when we are thinking about inhibin as a new contraceptive.

I would also like to emphasize the fact that development of a contraceptive takes a long time. As Dr. Bardin mentioned previously, academic communities do not understand what

it takes to develop a product. Ortho just launched a new oral contraceptive a year ago, and it took my colleague, Dr. John McGuire, and myself more than fifteen years of hard, concentrated work to discover the compound. And then it took us another fifteen years to bring that compound to the market. So you see, it is not a simple matter to develop a new product, especially a contraceptive. This applies especially to peptides.

I recall twenty years ago, when I began in contraceptive research. We tried to discover nonsteroidal estrogenic and progestational compounds. We spent a lot of time on this endeavor. We then moved from steroids to prostaglandins. When prostaglandin research began, we thought that prostaglandins would solve our contraceptive problems. That research lasted about ten years. Then, after prostaglandins, we went into a GnRH agonist program. From that we began to understand a little bit about the antagonist. Now we are trying to move into the area of inhibin and other nonsteroidal gonadal peptides.

I think, for example, second generation steroids are now on the market. It is almost thirty years since OOC was first introduced, so in my mind either another prostaglandin or GnRH related product could be on the market even before you leave here.

WORKSHOP PARTICIPANTS

Dr. Yumiko Abe
Gunma University
Gunma, Japan

Dr. Anibal A. Acosta
The Jones Institute
for Reproductive Medicine
Norfolk, Virginia

Dr. Eli Y. Adashi
University of Maryland
School of Medicine
Baltimore, Maryland

Dr. Nancy J. Alexander
The Jones Institute
for Reproductive Medicine
Norfolk, Virginia

Dr. Freedolph D. Anderson
Ortho Pharmaceutical, Inc.
Raritan, New Jersey

Dr. Ted Anderson
The Jones Institute
for Reproductive Medicine
Norfolk, Virginia

Dr. Mason Andrews
The Jones Institute
for Reproductive Medicine
Norfolk, Virginia

Dr. David Archer
The Jones Institute
for Reproductive Medicine
Norfolk, Virginia

Dr. Akira Arimura
U.S.-Japan Biomedical
Research Laboratories
Belle Chasse, Louisiana

Dr. Barbara J. Attardi
Montifiore Hospital
Pittsburg, Pennsylvania

Dr. C. Wayne Bardin
The Population Council
New York, New York

Dr. Gabriel Bialy
National Institute of Child Health
and Human Development
Bethesda, Maryland

Dr. Michael Blank
Hazleton Biotechnologies Company
Vienna, Virginia

Dr. C.Y. Bowers
Tulane University School
of Medicine
New Orleans, Louisiana

Dr. Robert Brzyski
The Jones Institute
for Reproductive Medicine
Norfolk, Virginia

Dr. Henry G. Burger
Prince Henry's Hospital
Melbourne, Australia

Dr. Marino Buscaglia
Salk Institute
La Jolla, California

Dr. C. Yan Cheng
The Population Council
New York, New York

Dr. Beverly Chilton
Anatomy TTUHSC
Lubbock, Texas

Dr. Alan Corbin
Wyeth Laboratories, Inc.
Philadelphia, Pennsylvania

Dr. William F. Crowley
Massachusetts General Hospital
Boston, Massachusetts

Dr. Horacio Croxatto
Centro Nacional de la Familia
Santiago, Chile

Dr. Douglas R. Danforth
The Jones Institute
for Reproductive Medicine
Norfolk, Virginia

Dr. Gere S. diZerega
University of Southern California
School of Medicine
Los Angeles, California

Dr. Patricia K. Donahoe
Massachusetts General Hospital
Boston, Massachusetts

Dr. Laneta Dorflinger
Office of Population
Agency for International Development
Washington, D.C.

Dr. Kathy Droesch
The Jones Institute
for Reproductive Medicine
Norfolk, Virginia

Dr. Charles Faiman
University of Manitoba
Manitoba, Canada

Dr. Jill Flood
The Jones Institute
for Reproductive Medicine
Norfolk, Virginia

Dr. Robin G. Foldesy
Family Health International Research
Triangle Park, North Carolina

Dr. J. Joe Ford
U.S. Department of Agriculture
Clay Center, Nebraska

Dr. Jean L. Fourcroy
Food and Drug Administration
Rockville, Maryland

Mr. David Fulgham
The Jones Institute
for Reproductive Medicine
Norfolk, Virginia

Dr. Henry L. Gabelnick
CONRAD Extramural Programs
Arlington, Virginia

Dr. Josue Garza Flores
Instituto Nacional de la Nutrician
Tlalpan, Mexico

Dr. Arnold L. Goodman
Johns Hopkins University
School of Medicine
Baltimore, Maryland

Dr. Keith Gordon
The Jones Institute
for Reproductive Medicine
Norfolk, Virginia

Dr. Satish Kumar Gupta
The Jones Institute
for Reproductive Medicine
Norfolk, Virginia

Dr. Do Won Hahn
Ortho Pharmaceutical Corporation
Raritan, New Jersey

Dr. Janet Hall
Massachusetts General Hospital
Boston, Massachusetts

Dr. Yoshihisa Hasegawa
Gunma University School of Medicine
Gunma, Japan

Dr. Florence Haseltine
National Institute of Child Health
and Human Development
Bethesda, Maryland

Dr. Milan R. Henzl
Syntex Research Institute of
Clinical Medicine
Palo Alto, California

Dr. Roy Hertz
Emeritus Investigator
National Institute of Child Health
and Human Development
Bethesda, Maryland

Dr. Katherine Hines
Anderson Hospital
and Tumor Institute
Houston, Texas

Dr. Gary D. Hodgen
The Jones Institute
for Reproductive Medicine
Norfolk, Virginia

Dr. Glenn Hofman
The Jones Institute
for Reproductive Medicine
Norfolk, Virginia

Dr. Aaron J.W. Hsueh
University of California
at San Diego
San Diego, California

Dr. James S. Hutchison
Serono Laboratories, Inc.
Randolph, Massachusetts

Dr. Joseph Itskovitz
Rambam Medical Center
Haifa, Israel

Mr. Bruno JeanJean
The Jones Institute
for Reproductive Medicine
Norfolk, Virginia

Dr. Masao Jinno
The Jones Institute
for Reproductive Medicine
Norfolk, Virginia

Dr. Georgeanna S. Jones
The Jones Institute
for Reproductive Medicine
Norfolk, Virginia

Dr. Howard W. Jones, Jr.
The Jones Institute
for Reproductive Medicine
Norfolk, Virginia

Dr. Nathalie Josso
Hopital des Enfants Malades
Paris, France

Dr. Subhash Juneja
Old Dominion University
Norfolk, Virginia

Dr. Marvin J. Karten
National Institute of Child Health
and Human Development
Bethesda, Maryland

Dr. Hugh S. Keeping
Montifiore Hospital
Pittsburgh, Pennsylvania

Dr. Bernard Kerdelhue
Institut National de la Recherche
Agronomique
Jouy-en-Josas, France

Dr. Shafiq A. Khan
Max Planck Society
Munster, F.R. Germany

Dr. Tomoko Kudo
Gunma University
Gunma, Japan

Dr. Marceij Kurpisz
The Jones Institute
for Reproductive Medicine
Norfolk, Virginia

Dr. Pekka Lahteenmaki
University of Western Ontario
London, Ontario, Canada

Dr. Juan Leal
The Jones Institute
for Reproductive Medicine
Norfolk, Virginia

Dr. Nicholas P. Ling
The Salk Institute
La Jolla, California

Mr. Roderick Mackenzie
Gyno Med
Piscataway, New Jersey

Dr. Emiko Makabe
Gunma University
Gunma, Japan

Dr. Paul J. Manberg
Serono Laboratories, Inc.
Randolph, Massachusetts

Dr. Anne Marmenout
Innogenetics
Ghent, Belgium

Dr. Kathryn Martin
Massachusetts General Hospital
Boston, Massachusetts

Dr. Anthony J. Mason
Genentech, Inc.
S. San Francisco, California

Dr. John McGuire
Ortho Pharmaceutical Corporation
Raritan, New Jersey

Dr. Noel McIntosh
University of New Mexico
School of Medicine
Albequerque, New Mexico

Dr. Robert I. McLachlan
Prince Henry's Hospital
Melbourne, Australia

Dr. Helene Meunier
The Salk Institute
La Jolla, California

Dr. William L. Miller
North Carolina State University
Raleigh, North Carolina

Dr. Kaoru Miyamoto
Gunma University School of Medicine
Gunma, Japan

Dr. Atsuro Miyata
U.S.-Japan Biomedical
Research Laboratories
Tulane University
Belle Chasse, Louisiana

Dr. Shin Y. Moon
The Jones Institute
for Reproductive Medicine
Norfolk, Virginia

Dr. N.R. Moudgal
Indian Institute of Science
Bangalore, India

Dr. George W. Mulheron
U.S. Dept. Agriculture
Clay Center, Nebraska

Dr. Kotsuji Mumikazu
Gunma University
Gunma, Japan

Dr. Mary V. Nekola
Tulane Medical Center
New Orleans, Louisiana

Dr. C. Alvin Paulsen
University of Washington
School of Medicine
Seattle, Washington

Dr. Felice Petraglia
University of Modena
Modena, Italy

Dr. Seymour Pomerantz
University of Maryland
School of Medicine
Baltimore, Maryland

Dr. Vanaja V. Ragavan
Food and Drug Administration
Rockville, Maryland

Dr. Madhwa H.G. Raj
Louisiana State University
Medical Center
New Orleans, Louisiana

Dr. Sung I. Roh
The Jones Institute
for Reproductive Medicine
Norfolk, Virginia

Dr. Zev Rosenwaks
The Jones Institute
for Reproductive Medicine
Norfolk, Virginia

Dr. M.R. Sairam
Clinical Research Institute
of Montreal
Montreal, Quebec, Canada

Dr. Nanette Santoro
Massachusetts General Hospital
Boston, Massachusetts

Dr. Robert S. Schenken
University of Texas Health
Science Center
San Antonio, Texas

Dr. Alan L. Schneyer
Massachusetts General Hospital
Boston, Massachusetts

Dr. David W. Schomberg
Duke University Medical Center
Durham, North Carolina

Dr. Richard Scott
The Jones Institute
for Reproductive Medicine
Norfolk, Virginia

Dr. Jean E. Sealey
The New York Hospital —
Cornell Medical Center
New York, New York

Dr. Sheldon J. Segal
The Rockefeller Foundation
New York, New York

Dr. James D. Shelton
Office of Population
Agency for International Development
Washington, D.C.

Dr. Anil R. Sheth
Institute for Research
in Reproduction
Parel
Bombay, India

Ms. Susan Sieg
The Jones Institute
for Reproductive Medicine
Norfolk, Virginia

Dr. Patrick M. Sluss
University of Rochester
Medical Center
Rochester, New York

Mr. Jeffrey Spieler
Office of Population
Agency for International Development
Washington, D.C.

Dr. Nongnuj Tanphaichitr
University of Ottawa
Ottawa Civic Hospital
Ottawa, Canada

Dr. James Toner
The Jones Institute
for Reproductive Medicine
Norfolk, Virginia

Dr. Sharon A. Tonetta
USC Medical Center
Los Angeles, California

Dr. Alexander Tsafriri
The Weizmann Institute of Science
Rehovot, Israel

Dr. Shigeru Ueno
Massachusetts General Hospital
Boston, Massachusetts

Dr. Annemarie Ultee-van Gessel
Organon International, BV
Oss, The Netherlands

Dr. Virginia Upton
Wyeth Laboratories, Inc.
Philadelphia, Pennsylvania

Dr. Hugo Van Heuverswyn
Innogenetics
Ghent, Belgium

Dr. Geoffrey M.H. Waites
World Health Organization
Geneva, Switzerland

Dr. Darrell N. Ward
Anderson Hospital
and Tumor Institute
Houston, Texas

Dr. Randall Whitcomb
Massachusetts General Hospital
Boston, Massachusetts

Dr. Robert F. Williams
The Jones Institute
for Reproductive Medicine
Norfolk, Virginia

Dr. James Willie
Sentara Norfolk General Hospital
Norfolk, Virginia

Dr. Stephen J. Winters
Montefiore Hospital
Pittsburgh, Pennsylvania

Prof. Dr. W. Wuttke
Georg-August-Universitat Gottingen
Gottingen, Germany

Dr. Koji Yoshinaga
National Institute of Child Health
and Human Development
Bethesda, Maryland

Dr. Moshey Zilberstein
National Institutes of Health
Bethesda, Maryland