Gonadotropic Control of Secretion of Dimeric Inhibins and Activin A by Human Granulosa–Luteal Cells In Vitro

SHANTHI MUTTUKRISHNA,^{1,3} NIGEL GROOME,² and WILLIAM LEDGER¹

Submitted: August 1, 1997

Accepted: September 22, 1997

Purpose: It is well established that human granulosa cells and luteal cells express inhibin/activin subunit protein and secrete immunoreactive inhibin. The gonadotropic control of secretion of different molecular forms of inhibin and activin A by granulosa–luteal cells (G-LCs) was investigated using recently developed specific enzyme immunoassays (EIAs).

Methods: Granulosa–luteal cells obtained at IVF egg pickup were cultured in a serum-free medium at 37°C in a water-saturated incubator with 5% CO₂ for up to 5 days. Experiments with varying concentrations of human FSH, hLH, and hCG were carried out.

Results: FSH raised the secretion of inhibin A and pro- α C-containing inhibins after 24 and 48 hr in culture. Inhibin B was raised after 24 hr and activin A was raised after 48 hr of FSH treatment. LH treatment for 24 hr stimulated inhibin A, inhibin B, pro- α C, and activin A. hCG stimulated G-LC secretion of inhibin A after 48 hr and pro- α C after 24 hr. Paradoxically, inhibin B secretion was inhibited by 1 and 10 ng/ml hCG after 48 hr. Activin A was stimulated by hCG after 24 and 48 hr of incubation. G-LC secretion of estradiol and progesterone was also stimulated significantly by LH and hCG.

Conclusions: Secretion of dimeric inhibins and activin A is controlled differentially by gonadotropins.

KEY WORDS: activin A; granulosa–luteal cells; inhibin A; inhibin B; pro- α C.

INTRODUCTION

Inhibins are dimeric glycoprotein hormones with dissimilar subunits, secreted mainly by the ovaries in

women. Inhibin A contains α - β_A subunits, and inhibin B contains α - β_B subunits. Apart from the dimeric molecules, the ovary also produces the monomeric α subunit in large quantities. The mature α C subunit containing the α N and/or pro region can either combine with the mature β subunit or β subunit precursors, forming pro- α C-containing inhibins, or exist on its own as pro- α C. Activins are homodimers of β subunits. Activin A is a β_A - β_A dimer, activin AB is a β_A - β_B dimer, and activin B is a β_B - β_B dimer. Although these proteins were purified and characterized a decade ago, due to the unavailability of specific assays, little progress has been made in understanding the biology of the different molecular forms of inhibin.

Studies using the inhibin radioimmunoassay (inhibin α subunit-directed RIA) have identified the presence of free inhibin subunits and dimeric inhibins (ir-inhibin) in the ovarian follicular fluid (1–5) and granulosa cell culture supernatant from rat (6) and bovine (7) species. Previous studies in humans have shown the presence of inhibin α - and β -subunit gene expression in the ovary (8, 9). In vitro cell cultures of human granulosa cells (10) and granulosa–luteal cells (11) have also demonstrated secretion of immunoreactive (ir) inhibin by these cells. However, the endocrine mechanisms involved in the production of biologically active dimeric inhibins (A and B) and activin A during follicular and luteal development have not been defined in women. The recent development of specific and sensitive enzyme immunoassays (EIAs) for dimeric inhibin A (12,13), inhibin B (14), pro- α C-containing inhibins (15), and activin A (16) has facilitated the specific measurement of different molecular forms of inhibin during the menstrual cycle in women. During the menstrual cycle, serum inhibin A has a preovulatory peak and a midluteal peak in the circulation, while serum inhibin B levels peak in the early follicular phase (day 5 of the cycle) and just after ovulation. Inhibin B levels remain low during the rest

¹ Nuffield Department of Obstetrics and Gynaecology, University of Oxford, John Radcliffe Hospital, Headington, Oxford OX3 9DU, UK.

² School of Biological and Molecular Sciences, Oxford Brookes University, Oxford, UK.

³ To whom correspondence should be addressed.

of the cycle (14). Activin A levels during the cycle are higher in the early follicular phase, midcycle and late luteal phase (17). These data suggest that inhibin A and activin A are produced by granulosa and luteal cells and inhibin B is produced mainly by granulosa cells. During hyperstimulation for in vitro fertilization (IVF), inhibin A, inhibin B, and activin A levels were increased after recombinant follicle stimulating hormone (FSH) and human chorionic gonadotropin (hCG) (18). The above study shows that ovarian granulosa cell production of inhibins and activin A are altered by gonadotropins in pituitary down-regulated women.

The objective of this study was to investigate the different forms of inhibin and activin A secretion and the gonadotropic control of inhibin and activin A secretion by luteinized granulosa cells cultured in vitro.

MATERIALS AND METHODS

Patients

Patients were treated with gonadotropin releasing hormone (GnRH)-analogue for 3 weeks. Pituitary down-regulated patients were stimulated with a high-purity FSH preparation for 7–10 days and hCG 36 hr prior to egg collection. Follicles 10 mm or greater in diameter were aspirated. Cells obtained from patients treated for male factor or unexplained infertility were used in this study.

GRANULOSA-LUTEAL CELL (G-LC) CULTURE

Cells from individual patients were processed separately under sterile conditions in a laminar-flow cabinet as reported previously (11) with some modifications. In brief, cells obtained from different follicles of a patient were pooled and centrifuged at 800 rpm for 10 min. The supernatant was discarded and the cells were washed (centrifugation and resuspension) with Hanks balanced salt solution (HBSS; Gibco Ltd., Middlesex, UK) containing a 1% (v/v) antibiotic solution (5000 U/ml penicillin–5000 µg/ml streptomycin solution; Sigma Chemicals Co., Poole, Dorset, UK) twice. G-LCs were separated from red blood cells on a 40% Percoll layer. After Percoll separation cells were washed with HBSS and cell aggregates were incubated with 2 ml of HBSS containing 0.1% (w/v) bovine testicular hyaluronidase (Sigma) for approximately 15 min. After washing with HBSS the cell suspension was

mechanically dispersed using sterile Pasteur pipettes to obtain maximum dispersion. The cells were washed again with HBSS, and the pellet was resuspended with 2 ml of culture medium consisting of Medium 199 (M199; Gibco Ltd.) containing 1% (v/v) L-glutamine, 1% (v/v) antibiotic solution (Sigma), and 1% medium supplement solution (0.5 mg/ml insulin, 0.5 mg/ml transferrin, 0.5 µg/ml sodium selenite solution; Sigma) previously dissolved in distilled water and filter sterilized using a 0.2-µm membrane filter (Flow Laboratories Ltd., UK). Cells were counted using the trypan blue exclusion method on a hemocytometer. The mean viability of cells was ~81%. Cells were plated at a concentration of approximately 25,000 live cells/ml/well in sterile 48-well cell culture plates (Flow Laboratories Ltd.) precoated with 10% fetal calf serum (Gibco Ltd.) in Dulbecco's modified Eagle medium (DMEM; Flow Laboratories Ltd.).

Cells were cultured in 1 ml culture medium containing 50 µl/well control medium or the respective concentrations of human FSH (final concentration of 1, 10, and 100 ng/ml), or human luteinizing hormone (LH; final concentration of 5 and 50 ng/ml), or hCG (final concentration of 1, 10, and 100 ng/ml) for either 24 or 48 hr. Plates were incubated in a water-saturated incubator with 5% CO₂ at 37°C throughout the experimental period. Control and all gonadotropin doses were added to quadruplicate wells/patient/culture, and each experiment was repeated a minimum of four times. At the end of the experimental period cell-conditioned culture medium was collected and centrifuged. The supernatant was stored at –20°C for hormone assays.

Preparation of Gonadotropins

Human FSH (100 µg/vial, NIDDK hFSH-AFP-5720D; NIDDK-NIH, Bethesda, MD) was diluted in 50 ml (2 µg/ml) of Hanks containing 0.35% bovine serum albumin (BSA; Sigma) and antibiotic solution. Human LH (400 µg/vial, NIDDK hLH-AFP-4261A; NIDDK-NIH) was diluted in 400 ml Hanks/BSA/antibiotics to give a final concentration of 1 µg/ml (20 × 50 ng/ml). hCG (1 mg/vial, NIDDK hCG-CR127; NIDDK-NIH) was diluted in 500 ml with Hanks buffer to give a 2 µg/ml stock (20 × 100 ng/ml).

Initial experiments were carried out with varying doses of FSH, LH, and hCG. Experimental concentrations of the gonadotropins were determined using the maximal and half-maximal effective doses in the preliminary studies.

Hormone Assays

Inhibin A and Pro- α C EIAs. Concentrations of inhibin A and pro- α C in the cell-conditioned media were measured in duplicate 10- μ l aliquots (48-hr release) or 20- μ l aliquots (24-hr release) using specific EIAs reported elsewhere (Refs. 13 and 15, respectively). The minimum detection limits of the inhibin and pro- α C assays were 2 and 5 ng/L, respectively. The inter- and intraplate variations for the inhibin A EIA were 5.1 and 4.5, respectively. The inter- and intraplate variations for the pro- α C EIA were 9.1 and 6.8%, respectively. Recovery of recombinant inhibin A and native pro- α C standard added to G-LC-conditioned medium was $97.6 \pm 6.2\%$ ($n = 4$) and $96.2 \pm 8.4\%$ ($n = 4$), respectively.

Inhibin B EIA. Cell-conditioned media were assayed in duplicate 50- μ l aliquots for inhibin B using a specific EIA (14) with some modifications. The sensitivity of the assay was 6 pg/ml, and the mean intra- and interplate variations were 7.2 and 11.9, respectively. Under the modified assay conditions, recovery of purified inhibin B (standard preparation) added to different cell culture samples before assay was found to be quantitative ($93.2 \pm 7.9\%$; $n = 4$).

Activin A EIA. Cell-conditioned media were assayed in duplicate 50- μ l aliquots for "total" activin A using the recently reported activin A EIA (16). The sensitivity of the assay was 50 pg/ml, and the mean intra- and interplate variations were less than 10%. Recovery of recombinant activin A added to G-LC-conditioned medium was $95.6 \pm 5.8\%$ ($r = 4$).

FSH, Estradiol, and Progesterone Assays. Serum FSH and concentrations of estradiol and progesterone in the cell culture media were measured using Immulite chemiluminescent assay kits (Diagnostic Products Ltd.). All samples were assayed in the same assay, and the intraassay variation was within 10%.

Statistical Analysis

Cell culture experimental data were normalized (control concentration = 100%), and data were pooled from six patients for the effect of FSH, and four patients for the effect of LH, and four patients for the effect of hCG. Dose-dependent effects of the different gonadotropins were analyzed using one-way analysis of variance (ANOVA) with post hoc tests (Fisher's) to show the statistical difference between different concentrations of a treatment group. Different days' release data are expressed as mean $\pm$ SEM ($n = 4$).

RESULTS

Validation of the Enzyme Immunoassays for Granulosa-Luteal Cell Culture Medium

Serial dilutions of pooled granulosa cell culture medium gave parallel response curves to human recombinant inhibin A (Fig. 1a), human inhibin B (Fig. 1b), human pro- α C (Fig. 1c), and activin A (Fig. 1d) standards. The culture medium blank had an absorbance similar to that of the diluent blank in all three EIAs. Absolute concentrations of hormones secreted during culture are given in Table I.

Function and Viability of Granulosa-Luteal Cells in Culture

Functional viability of the established cell culture system was carried out by quantitating the amount of inhibins, activin A, estradiol, and progesterone secreted by cultured granulosa-luteal cells (cells were pooled from five patients) in a time-dependent manner. Inhibin A ($P < 0.001$), inhibin B ($P < 0.001$), pro- α C ($P < 0.001$), and activin A ($P < 0.01$) secretions by the cells were significantly increased with increasing incubation time (Fig. 2a-d). A significant time-dependent increase in estradiol ($P < 0.01$) and progesterone ($P < 0.01$) production was observed for the first 3 days, and the levels started falling by day 5 in culture, perhaps due to lack of androgen substrate in a serum-free culture. The continuous production of estradiol and progesterone confirms the normal function of luteinized granulosa cells in culture (Fig. 2e).

Effect of Human FSH

Exposure of G-LCs for 24 and 48 hr to increasing concentrations of native human FSH significantly enhanced the secretion of inhibin A [approximately 57%, $P = 0.001$ (24 hr); approximately 33%, $P < 0.001$ (48 hr)] in a dose-dependent manner. Although 1 ng/ml FSH did not have a significant effect, 10 and 100 ng/ml FSH significantly stimulated inhibin A secretion (Fig. 3a).

Inhibin B secretion was significantly increased when the cells were exposed to FSH for 24 hr (approximately 50%, $P = 0.03$). In contrast to the response of inhibin A, G-LCs responded to 1 g/ml (approximately 50%, $P < 0.01$) and 10 ng/ml (approximately 55%, $P < 0.001$) FSH, with no effect in the presence of 100 ng/ml FSH. However, 48-hr incubation of cells with FSH did not significantly alter inhibin B secretion (Fig. 3b).

Fig. 1. Comparison of serial dilution curves of standards (○) and pooled granulosa-luteal cell culture media (●) in the enzyme immunoassays for (a) inhibin A, (b) inhibin B (c) pro- α C, and (d) activin A.

Table 1. Absolute Concentrations of Inhibin A, Inhibin B, Activin A and pro- α C Produced by Untreated Control Luteinized Cells in Culture (25,000 Cells/Well)

Release	Picograms per well			
	Inhibin A	Inhibin B	Activin A	Pro- α C
24 hr	40.15–60.3	20–27.47	163.9–236.9	780.6–854
48 hr	115.1–239.8	25.1–30.34	403.2–983.9	875–1061

G-LC secretion of pro- α C-containing inhibins were significantly increased with increasing FSH concentrations after 24 and 48 hr (approximately 75%, $P = 0.02$; approximately 20%, $P = 0.03$) of incubation. After 24-hr FSH incubation, all three concentrations of FSH enhanced pro- α C production (Fig. 3c).

Fig. 2. Data presented are mean $\pm$ SEM (pg/ml). Daily release of (a) inhibin A, (b) inhibin B, (c) pro- α C, (d) activin A, and (e) estradiol by cultured granulosa-luteal cells.

Fig. 2. Continued.

Activin A secretion by G-LCs was significantly stimulated by FSH after 48 hr (40%; $F < 0.05$). G-LC production of activin A was also stimulated by FSH after 24-hr incubation, although not significantly (Fig. 6a).

Estradiol and progesterone production had a tendency to increase with FSH after 24 and 48 hr of incubation (Table II).

Effect of Human LH

Granulosa-luteal cells cultured for 24 and 48 hr in the presence of increasing concentrations of native human LH showed significantly increased secretion of inhibin A [approximately 53%, $P < 0.001$ (24 hr); approximately 22%, $P < 0.002$ (48 hr)] in a dose-dependent manner. Both 5 and 50 ng/ml LH significantly stimulated inhibin A secretion (Fig. 4a).

Exposure of cells to human LH for 24 hr significantly increased inhibin B secretion (approximately 63%; $P < 0.05$), with no effect after 48 hr of exposure. Similar to the response of inhibin A, both 5 and 50 ng/ml LH significantly stimulated inhibin B secretion (Fig. 4b).

Granulosa-luteal cell secretion of pro- α C-containing inhibins was significantly increased with

Fig. 3. Data presented are the normalized mean $\pm$ SEM ($n = 6$ patients) percentage of control concentrations of (a) inhibin A, (b) inhibin B, and (c) pro- α C produced by granulosa-luteal cells in culture for 24 hr (□) and 48 hr (■) in response to varying concentrations of FSH. P values, one-way ANOVA. Fisher's post hoc tests comparing control versus treatment: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

increasing LH concentrations after 24 and 48 hr (approximately 68%, $P < 0.001$; approximately 21%, $P < 0.05$) of incubation. Both concentrations of LH enhanced pro- α C production but the magnitude of the response was less after 48 hr (Fig. 4c).

LH had a stimulatory effect on activin A after 24 hr (approximately 58%; $P < 0.01$), and 48 hr of incubation did not alter activin A secretion (Fig. 6b).

Estradiol and progesterone secretions by G-LCs were significantly stimulated by LH after 48 hr (approximately 130%, $P < 0.01$, and approximately 170%, $P < 0.01$, respectively) (Table II).

Effect of hCG

Inhibin A secretion by granulosa-luteal cells cultured for 48 hr in the presence of increasing concentrations of native hCG was significantly increased (approximately 49%; $P < 0.001$) in a dose-dependent manner. Incubation of cells with hCG for 24 hr did not alter inhibin A. hCG at 10 and 100 ng/ml significantly stimulated inhibin A secretion (Fig. 5a) after 48 hr.

Table II. Luteinized Granulosa Cell Production of Estradiol (E₂) and Progesterone (P₄) in Response to Varying Concentrations of FSH, LH, and hCG^a

	E ₂		P ₄	
	24 hr	48 hr	24 hr	48 hr
Control	2.01 ± 0.11	3.04 ± 0.31	112 ± 8	229 ± 39.38
FSH				
1 ng/ml	2.24 ± 0.37	2.93 ± 0.31	137.3 ± 15.1	242.2 ± 46.1
10 ng/ml	2.14 ± 0.09	2.82 ± 0.24	134.7 ± 10.3	233.7 ± 35.3
100 ng/ml	2.3 ± 0.075	3.86 ± 0.41	161 ± 17.8*	324 ± 55.6
Control	2.02 ± 0.11	2.85 ± 0.3	110 ± 8.9	232 ± 42.2
LH				
5 ng/ml	2.47 ± 2.1	7.6 ± 1.8***	143 ± 9.5	836 ± 182**
50 ng/ml	2.3 ± 1.44	7.61 ± 1.9***	188.3 ± 52*	763 ± 136***
Control	2.1 ± 0.1	3.24 ± 0.32	111.8 ± 8	226 ± 33.67
hCG				
1 ng/ml	2.78 ± 0.7*	7.51 ± 1.5**	187 ± 4***	908 ± 232***
10 ng/ml	3 ± 0.21**	13.26 ± 3***	189 ± 9***	741 ± 84.8**
100 ng/ml	3.13 ± 0.4**	9.01 ± 0.5**	199 ± 0.5***	804 ± 46.2**

^a Data presented are mean ± SEM concentrations as nM. *P* values, one-way ANOVA. Fisher's post hoc tests comparing control versus treatment. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

Fig. 4. Data presented are the normalized mean ± SEM (*n* = 4 patients) percentage of control concentrations of (a) inhibin A, (b) inhibin B, and (c) pro-αC produced by granulosa-luteal cells in culture for 24 hr (□) and 48 hr (■) in response to varying concentrations of LH. *P* values, one-way ANOVA. Fisher's post hoc tests comparing control versus treatment: **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

Fig. 5. Data presented are the normalized mean ± SEM (*n* = 4 patients) percentage of control concentrations of (a) inhibin A, (b) inhibin B, and (c) pro-αC produced by granulosa-luteal cells cultured for 24 hr (□) and 48 hr (■) in response to varying concentrations of hCG. *P* value, one way-ANOVA. Fisher's post hoc tests comparing control versus treatment: **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

Fig. 6. Data presented are the normalized mean $\pm$ SEM percentage of control concentrations of "total" activin A, produced by granulosa-luteal cells cultured for 24 hr ($\square$) and 48 hr ($\blacksquare$) in response to varying concentrations of (a) FSH ($n = 6$ patients), (b) LH ($n = 4$ patients), and (c) hCG ($n = 4$ patients). P values, one way-ANOVA. Fisher's post hoc tests comparing control versus treatment: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Paradoxically, although inhibin B secretion was not significantly altered in a dose-dependent manner ($P = 0.06$), 1 and 10 ng/ml hCG significantly inhibited the secretion of inhibin B (approximately 29%, $P < 0.05$, and approximately 35%, $P < 0.01$, respectively) by cultured granulosa-luteal cells after 48 hr (Fig. 5b).

Granulosa-luteal secretion of pro- α C-containing inhibins was significantly increased with increasing hCG concentrations after 24 hr (approximately 30%, $P < 0.01$) of incubation. Secretion of pro- α C was not significantly altered in a dose-dependent manner after 48 hr. However, 10 ng/ml hCG significantly stimulated (approximately 30%, $P < 0.01$) pro- α C production by G-LCs after 48 hr (Fig. 5c).

Activin A secretion in the presence of hCG was significantly stimulated after 24 and 48 hr of incubation (approximately 55%, $P < 0.01$, and approximately 40%, $P < 0.05$, respectively) (Fig. 6c).

Estradiol and progesterone secretions were significantly enhanced by hCG (290%, $P < 0.01$; approximately 240%, $P < 0.001$) after 48 hr (Table II).

Relationship of G-LC Production of Inhibins and Follicles

Inhibin A secretion/25,000 cells for 48 hr was positively correlated with the number of cells retrieved ($r = 0.642$, $P < 0.01$) and the percentage viability ($r = 0.49$, $P = 0.022$). The number of cells retrieved was correlated with the number of oocytes ($r = 0.65$, $P < 0.01$) and percentage viability ($r = 0.48$, $P < 0.03$). Age was negatively correlated with viability of G-LCs ($r = -0.42$, $P = 0.05$).

DISCUSSION

This is the first in vitro study to demonstrate gonadotropic control of luteinized granulosa cell secretion of biologically active dimeric inhibins and activin A in women. Secretion of increasing concentrations of estradiol and progesterone with increasing time of incubation confirms the functional viability of the G-LCs in culture. Similarly, G-LC-secreted concentrations of inhibin A, inhibin B, pro- α C-containing inhibins, and activin A also increase with increasing time of incubation. In vitro production of inhibins and activin A by luteinized granulosa cells confirms an ovarian source for circulating concentrations of these proteins during the normal menstrual cycle and hyperstimulated cycles. Furthermore, this study also reconfirms the stimulatory effects of gonadotropins on dimeric inhibins and activin A production in the hyperstimulated women reported previously (18).

This study provides direct evidence for the effect of FSH, LH, and hCG on luteinized GCs. Consistent with published studies, G-LC production of estradiol and progesterone is stimulated by LH and hCG (11, 19). However, FSH did not significantly alter the secretion of estradiol and progesterone in this study. It has been reported previously that FSH significantly stimulates estradiol production by G-LC in the presence of testosterone but does not have an effect on its own (11). In this study, FSH significantly stimulated inhibin A and pro- α C but had a short-term stimulatory effect on inhibin B secretion. The difference in the stimulatory effect of FSH suggests that although G-LCs have FSH receptors, the mechanisms involved in the stimulation of inhibins (A and B) are different.

LH had a short-term stimulatory effect on inhibins and activin A. HCG stimulated inhibin A and activin A secretion, consistent with the reported *in vivo* study (18). Paradoxically, lower doses of hCG (1 and 10 ng/ml) significantly inhibited inhibin B secretion after 2 days. The reason for the opposing effects of hCG on inhibin A and inhibin B is not clear. Further detailed studies need to be carried out in the future.

A previous study conducted with luteinized GCs reported that LH had a stimulatory effect on bioactive inhibin after 4 days and FSH did not have any effect (11). In the above-reported study, an ovine pituitary cell culture system was used to quantitate inhibin bioactivity by measuring FSH inhibition. Although the cell culture samples were steroid stripped, the presence of activin A would have affected the bioassay. The present study provides evidence for the secretion of specific dimeric inhibins and activin A by G-LCs using very specific two-site assays.

Statistical analyses showed that G-LC secretion of inhibin A/25,000 cells/well was positively correlated with the number and viability of cells retrieved per patient, suggesting that inhibin A measurement may be used as a marker of follicle/oocyte quality. The percentage viability of the cells was negatively correlated with age. A recent study reported a significant negative correlation between day 3 serum FSH and inhibin A production by G-LCs and age (20). In this study, day 3 serum FSH levels (10.23 ± 2.6 mIU/ml) of patients did not vary widely as in the above study and there was no significant relationship between day 3 serum FSH and inhibin A ($r = -0.263$) or inhibin B ($r = -0.247$) secreted by G-LCs.

In summary, the data suggest a relationship between the number and viability of cells retrieved and the dimeric inhibin A secretory capacity of granulosa-luteal cells. Further experimental evidence is necessary to confirm this relationship. This study demonstrates that G-LC secretions of inhibin A, inhibin B, pro- α C-containing inhibins, and activin A are controlled differentially by FSH, LH, and hCG.

ACKNOWLEDGMENTS

S.M. thanks Prof. S. G. Hillier (MRC Reproductive Biology Unit, University of Edinburgh) for the advice on setting up the granulosa-luteal cell culture system. The authors thank Judith Asselin and Joan Bellinger (Nuffield Department of Obstetrics and Gynaecology, John Radcliffe Hospital) for carrying out the steroid assays and the embryologists (Oxford IVF Unit) for

helping with granulosa cell collection. We thank NIBSC (Potters Bar, UK) for the human recombinant inhibin A standard and NIDDK-NIH (Bethesda, MD) for the gift of the human FSH, human LH, and hCG used in the experiments.

This research was supported by the Oxford Fertility Unit, Oxford Radcliffe Hospital. S.M. is funded by the Wellcome Trust.

REFERENCES

1. 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* 1986;321:779-782
2. Miyamoto K, Hasegawa Y, Fukuda M, Nomura M, Igarashi M, Kangawa K, Matsuo H: Isolation of porcine follicular fluid inhibin of 32 kdaltons. *Biochem Biophys Res Commun* 1985;129:396-403
3. 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* 1985;126:220-226
4. 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* 1985;82:7217-7221
5. Tsonis CG, Sharpe RM: Dual gonadal control of follicle stimulating hormone. *Nature* 1986;321:724-725
6. Biscak T, Cajander SB, Vale W, Hsueh AJW: Inhibin: Studies of stored and secreted forms by biosynthetic labelling and immuno detection in cultured rat granulosa cells. *Endocrinology* 1988;122:741-748
7. Wrathall JHM, Knight PG: Production of immunoreactive inhibin by bovine granulosa cells in serum free culture: Effects of exogenous steroids and FSH. *Domest Anim Endocrinol* 1993;10:289-304
8. Mason AJ, Niall HD, Seeburgh PH: Structure of human ovarian inhibins. *Biochem Biophys Res Commun* 1986;135:957-964
9. Davis SR, Dench F, Nikolaidis L, Clements JA, Forage RG, Krozowski Z, Burger HG: Inhibin-A subunit gene expression in the ovaries of immature female rats is stimulated by pregnant mare serum gonadotropin. *Biochem Biophys Res Commun* 1986;138:1191-1195
10. Hillier SG, Wickings EJ, Illingworth PI, Yong EL, Reichert LE, Baird DT, McNeilly AS: Control of immunoreactive inhibin production by human granulosa cells. *Clin Endocrinol* 1991;35:71-78
11. Tsonis CG, Hillier SG, Baird DT: production of inhibin bioactivity by human-granulosa-lutein cells: Stimulation by LH and testosterone *in vitro*. *J Endocrinol* 1987;112:R11-R14
12. Groome NP, Illingworth PJ, O'Brien M, Cooke I, Ganesan TS, Baird DT, McNeilly AS: Detection of dimeric inhibin throughout the human menstrual cycle by two-site enzyme immunoassay. *Clin Endocrinol* 1994;40:717-723
13. Muttukrishna S, Fowler PA, Groome NP, Mitchell GG, Robertson WR, Knight PG: Serum concentrations of dimeric inhibin

- during the spontaneous human menstrual cycle and after treatment with exogenous gonadotrophin. *Hum Reprod* 1994;9:1634-1642
14. Groome NP, Illingworth PJ, O'Brien M, Pai R, Rodger FE, Mather J, McNeilly AS: Measurement of dimeric inhibin-B throughout the human menstrual cycle. *J Clin Endocrinol Metab* 1996;81:1401-1405
 15. Groome NP, Illingworth PJ, O'Brien M, Priddle J, Weaver K, McNeilly AS: Quantification of inhibin pro- α C-containing forms in human serum by a new ultrasensitive two-site enzyme-linked immunosorbent assay. *J Clin Endocrinol Metab* 1995;80:2926-2932
 16. Knight PG, Muttukrishna S, Groome NP: Development and application of a two-site enzyme immunoassay for the determination of "total" activin A concentrations in serum and follicular fluid. *J Endocrinol* 1996;148:267-279
 17. Muttukrishna S, Fowler PA, George L, Groome NP, Knight PG: Changes in peripheral serum levels of total activin A during human menstrual cycle and pregnancy. *J Clin Endocrinol Metab* 1996;81:3328-3334
 18. Lockwood GM, Muttukrishna S, Groome NP, Knight PG, Ledger WL: Circulating inhibins and activin A during GnRH-analogue down-regulation and ovarian hyperstimulation with recombinant FSH for in vitro fertilization and embryo transfer. *Clin Endocrinol* 1996;45:741-748
 19. Schipper I, Fauser BCJM, van Gaver EBO, Zarutskie PW, Dahl KD: Development of a human granulosa cell culture model with follicle stimulating hormone responsiveness. *Hum Reprod* 1993;8:1380-1386
 20. Seifer DB, Gardiner AC, Lambert-Messerlian G, Schneyer AL: Differential secretion of dimeric inhibin in cultured luteinized granulosa cells as a function of ovarian reserve. *J Clin Endocrinol Metab* 1996;81:736-739