Cultured human granulosa cells secrete a follicle stimulating hormone receptor-binding inhibitor

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We previously reported that human follicular fluid contained a protein that inhibits binding of 125I-human FSH to its membrane receptor (FSH-BI) and demonstrates FSH-like agonist activity in vitro. The cellular origin of FSH-BI was unknown, although ovarian granulosa cells seemed a likely source. To address this question, human granulosa cells were collected from patients during routine in-vitro fertilization (IVF) or gamete intra-Fallopian transfer (GIFT) procedures. Cells from 98 patients were cultured and then examined for their ability to secrete FSH receptor-binding inhibitory activity into the culture medium. The function of the cultured cells was confirmed by their ability to respond to added FSH with conversion of exogenous androstenedione to oestradiol. Radioceptor assays were performed individually on cell culture medium obtained from granulosa cell cultures from these 98 patients. Cultured granulosa cells, under basal conditions (in the absence of FSH stimulation), secreted significant FSH-BI activity into the culture medium. In order to accumulate enough material for further study, this culture medium was pooled and lyophilized. The lyophilized medium retained FSH-BI activity, and also demonstrated FSH agonist activity in terms of promoting steroidogenesis by cultured rat Sertoli cells. A fraction showing a single component after purification by polyacrylamide gel electrophoresis had an estimated molecular weight of 25 000, and inhibited 125I-FSH binding to receptor by 50% at 2.5 µg/ml. The results indicate that human granulosa cells secrete a protein with FSH-like activity having potential significance as a local regulator of FSH action in the ovary.

Key words: follicle/granulosa/inhibitor/ovary/receptor

Introduction

The production of a single dominant follicle during an average human menstrual cycle may be described as the result of the culmination of three successive stages: recruitment, follicular growth, and selection and maturation of the pre-ovulatory follicle. The success of this process depends in part upon adequate stimulation of the ovary by follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Channing and Tsafirri, 1977; Richards and Rolfe, 1980). The interaction between cells in the ovary provides numerous potential areas of regulation of activity by putative modulators of follicular maturation. Many follicular fluid components have been examined for possible roles in follicular differentiation as well as on the steroidogenic capacity of the follicle (Fauser and Van Heusden, 1997). These include inhibin, insulin-like growth factors, their binding proteins, epidermal and transforming growth factors, gonadotropin releasing hormone, and FSH receptor-binding inhibitors.

Since the initial report on the presence in bovine follicular fluid of an inhibitor of 125I-human FSH binding to receptors on bovine granulosa cells (FSH-BI) (Darga and Reichert, 1978), a number of studies have been directed towards defining its potential significance as a paracrine regulator of follicular development. Most studies have utilized readily available bovine or porcine follicular fluid as the source of the inhibitor (Reichert et al., 1985). With the development and widespread utilization of in-vitro fertilization (IVF) and gamete intra-Fallopian transfer programmes (GIFT), studies utilizing human follicular fluid became feasible. We have previously reported the presence of low and high molecular weight FSH receptor-binding inhibitors in human follicular fluid and demonstrated that human follicular fluid-derived FSH-BI has FSH agonist activity in terms of promoting steroidogenesis by cultured rat Sertoli cells (Lee et al., 1990, 1991). Human follicular fluid FSH-BI is distinct from human FSH on the basis of size, stability to acid treatment (Lee et al., 1991), and amino acid composition (Lee et al., 1993). A synthetic decapeptide, corresponding to a partial amino acid sequence of the latter, specifically inhibited FSH binding to receptor (Lee et al., 1995). Although granulosa cells seemed a likely source of FSH-BI, this was never determined experimentally. We now report studies demonstrating that human granulosa cells secrete an FSH-BI of potential importance as a local regulator of FSH action on the ovary.

Materials and methods

Materials

Bovine calf testes were obtained from a local abattoir and stored at −20°C until used for preparation of FSH receptor-enriched membranes (Dattatreymurty et al., 1986). Na125I (350 mCi/ml) was purchased from New England Nuclear (Boston, MA, USA). Highly purified human FSH (NIH-human FSH-S1, 4990 IU/mg), provided by the National Hormone and Pituitary Program, was iodinated as the radioligand for the FSH radio receptor assay (RRA). Human FSH
Granulosa cell cultures

Human granulosa cells were obtained in the course of follicular aspiration for retrieval of oocytes during routine IVF or GIFT at Albany Medical Center Hospital (Albany, NY, USA). Multiple follicular aspirations for retrieval of oocytes during routine IVF or GIFT at Human granulosa cells were obtained in the course of follicular membrane binding inhibition assays and for stimulation of granulosa cells. C.A. Alouf

pooled aspirates at 700 x g of blood by inspection, and then collected by centrifugation (700 x g) until the cell suspensions were devoid of Dulbecco’s phosphate buffered saline (PBS) (Gibco, Grand Island, NY, USA) containing 0.1% (w/v) bovine serum albumin (BSA) (Sigma, St Louis, MO, USA) until the cell suspensions were devoid of blood by inspection, and then collected by centrifugation (700 g for 5 min at 21°C). The recovered cells were resuspended in PBS containing 0.2% (w/v) ovine testicular hyaluronidase (Sigma) and incubated at 37°C for 15–20 min. The resulting cell suspension was subjected to gentle repeat pipetting through a narrow bore pipette tip to facilitate cell dispersal. The suspension was then washed free of enzyme with culture medium and cells were counted on a haemocytometer. Cell viability was determined by Trypan Blue exclusion and was always >85%. Cells were then plated in Medium 199 (M199) (Gibco) supplemented with 5% (v/v) charcoal-treated horse serum (HS), penicillin (50 IU/ml) and streptomycin (50 µg/ml) (Sigma) on multiwell plastic culture plates (Becton Dickinson Labware, Lincoln Park, NJ, USA) coated with 5 µg/cm² ECL Attachment Factors (Upstate Biological Inc., Lake Placid, New York, USA). Granulosa cells were plated at a density of 2.5 x 10⁴ cells/well and incubated at 37°C in a humidified atmosphere with 5% CO₂ in air. The medium was changed after 24 h.

Treatment

Medium was aspirated from the wells 72 h after plating, and the adhering cells were washed with, and then incubated in, serum free medium. In some cases, human FSH (800 IU/mg) was used for stimulation of the granulosa cells. Androstenedione, 2.5 x 10⁻⁶ M, (Sigma) was added as substrate for steroidogenesis. Forty-eight hours later, spent medium was aspirated and either frozen until thawed for dialysis or dialysed immediately. Medium was dialysed at 4°C for 48 h against deionized water in Spectra/Por 2 (Spectrum Medical Industries Inc., Los Angeles, CA, USA) dialysis membrane tubing (exclusion limit ~12 000–14 000 M). Following dialysis, the medium samples were either tested for FSH-BI activity or lyophilized until utilized for further study. In order to accumulate enough FSH-BI activity for further characterization, incubated cells were allowed to recover in serum supplemented M199 for 48 h, washed free of medium and then incubated again in serum-free M199 for another 48 h. We determined that no significant loss of measured FSH-BI activity occurred during a total of four consecutive incubation cycles. The resulting cell culture medium was collected, dialysed and lyophilized.

Sertoli cell isolation and culture

Primary cultures of Sertoli cells were prepared from testes of 14–16 day old Sprague–Dawley rats, as previously described (Grasso and Reichert, 1989, 1990). FSH agonist activity was defined as the ability of a test substance to stimulate conversion of added androstenedione to oestradiol by cultured Sertoli cells. oFSH (LER 1996-S, 200 IU/mg) was utilized as a control in each assay to monitor the reproducibility of the system to standard agonist stimulus.

Steroid measurement

The concentration of oestrone present in granulosa cell culture medium was measured by radioimmunoassay, utilizing a slight modification of a previously published procedure (Grasso et al., 1995). Briefly, medium was diluted 1:10 in serum free M199. Oestrone standards were dissolved in M199 to concentrations of 20–10 000 pg/250 µl. Antibody against 17β-oestradiol (GDN244), generously provided by Dr Gordon Niswender, Colorado State University, Fort Collins, Colorado, USA, was diluted to 1:30 000 in PBS gel buffer (0.1% (w/v) gelatine in PBS). A volume of 200 µl of antibody was added to either 100 µl of diluted medium aspirates and 150 µl of M199, or 250 µl of oestradiol standards and incubated at room temperature for 1 h. A volume of 50 µl of 3H-oestradiol (Dupont, Wilmington, DE, USA), diluted to 15 000 c.p.m./50 µl PBS gel buffer, was then added and incubated at 37°C for 1 h (final volume = 500 µl). The antibody-bound complexes were precipitated by addition of an equal volume of saturated ammonium sulphate (500 µg/ml) at room temperature. The samples were centrifuged at 2600 g at 4°C for 15 min and 5 ml of scintillation fluid (EconoSafe, Mt Prospect, IL, USA) was added to 0.5 ml of the supernatant. The concentration of free 3H-oestradiol present in the supernatant was determined by use of a standard curve. Intra- and interassay coefficients of variation were <10%.

Radioligand receptor assays (RRA)

The FSH RRA used for FSH binding inhibition studies were performed as previously described (Lee et al., 1990). Results were expressed as percentage binding inhibition by 250 µl of sample.

Acidified acetone treatment

Medium from cultured granulosa cells was treated with acidified acetone according to methods previously described (Lee et al., 1991). In brief, acetone was acidified with 0.2 M acetic acid (3:1 vol/vol) and streptomycin (50 µg/ml) (Sigma) on mutliwell plastic culture plates (Becton Dickinson Labware, Lincoln Park, NJ, USA) coated with 5 µg/cm² ECL Attachment Factors (Upstate Biological Inc., Lake Placid, New York, USA). Samples were reconstituted to original volumes and dialysed or dialysed immediately. Medium was dialysed against deionized water in Spectra/Por 2 (Spectrum Medical Industries Inc., Los Angeles, CA, USA) dialysis membrane tubing (exclusion limit ~12 000–14 000 M). Following dialysis, the medium samples were either tested for FSH-BI activity or lyophilized until utilized for further study. In order to accumulate enough FSH-BI activity for further characterization, incubated cells were allowed to recover in serum supplemented M199 for 48 h, washed free of medium and then incubated again in serum-free M199 for another 48 h. We determined that no significant loss of measured FSH-BI activity occurred during a total of four consecutive incubation cycles. The resulting cell culture medium was collected, dialysed and lyophilized.

Electrophoresis

Slab gels (12% PAGE) were prepared following the protocol previously described (Lee et al., 1993). An aliquot of 200 µg of dialysed, lyophilized granulosa cell culture medium was dissolved in sample buffer containing 39.5 mM Tris base, 64 mM H₂PO₄ and 10% glycerol, pH 6.8 and applied per lane of the stacking gel. In some experiments, several lanes were run simultaneously. After electrophoresis, each lane of the gel was cut into 0.5 cm fractions, eluted overnight at 4°C into RRA assay buffer and tested for FSH-BI activity. Active fractions, run simultaneously in parallel lanes (preparative runs), were eluted in 5 mM Tris–HCl, pH 6.8, pooled and dried on a Speed-Vac concentrator/evaporator, and fractionated on an 8% SDS–PAGE mini-gel (Bio-Rad) in the absence or presence of 10 mM dithiothreitol. Marker proteins of known molecular weight were run simultaneously to allow estimations of molecular weight of fractions having FSH-BI activity. Following electrophoresis, the gel was silver stained, utilizing a previously described method (Wray et al., 1981), and photographed with a Polaroid CU-5 Land Camera.
Human granulosa cell FSH-BI

Figure 1. Follicle stimulating hormone (FSH)-stimulated conversion of androstenedione to oestradiol. The granulosa cells were treated 72 h after plating in the presence of $2.5 \times 10^6$ M androstenedione. Cells cultured in the presence of substrate were stimulated with 0–80 IU/l human FSH. Significant oestradiol accumulation over basal was obtained with 40 and 80 IU/l (*$P < 0.01$). The bars indicate the mean values of duplicate assays of five patients ± SEM.

Protein determination
Protein content of the PAGE purified fraction was determined using fluorescamine (Sigma), as previously described (Udenfriend et al., 1972). In brief, the dialysed PAGE-purified FSH-BI was brought to a volume of 1.5 ml with 0.05 M sodium phosphate buffer, pH 8.0, and 0.5 ml fluorescamine in acetone (15 mg/100 ml) was rapidly added with constant agitation to the buffered protein solution. The samples were then brought up to 4.0 ml with 0.05 M sodium phosphate buffer and examined for fluorescence with a spectrofluorometer (Varian Associates, Inc., Palo Alto, CA, USA), using an excitation wavelength of 390 nm and an emission wavelength of 445 nm. BSA was used to generate a standard curve.

Statistical analysis
Variations in response between test groups were analysed by Dunnett’s multiple range test (oestradiol responses), or Scheffe’s multiple range test (binding inhibition assays), for determination of statistical significance when multiple groups are compared with one another. Differences were considered significant at $P < 0.01$.

Results

Responsiveness of cultured human granulosa cells to FSH
In order to determine their viability and functional competence, cultured human granulosa cells were tested for steroidogenic responsiveness to stimulation by exogenous human FSH. A typical result is shown in Figure 1. As can be seen, there was a significant increase in conversion of androstenedione to oestradiol by human granulosa cells in response to 40 IU/l human FSH. The cultured cells were therefore utilized for subsequent studies on synthesis of FSH-BI.

Evidence for synthesis of FSH-BI by human granulosa cells
Experiments were designed to assess secretion of FSH membrane receptor binding inhibitor by functionally competent cultured human granulosa cells. Granulosa cells were individu-

ally cultured without FSH stimulation and individual medium samples were collected and dialysed prior to assay. Dialysis was necessary to remove low molecular weight $^{125}$I-FSH membrane receptor binding inhibitors previously shown to be present in human follicular fluid, and possibly derived from granulosa cells (Lee et al., 1990). Figure 2 shows that significant but variable levels of FSH-BI activity (range: 10–52%) were detected in cell culture medium (i.e. ‘conditioned medium’). Human granulosa cells from the 98 individual cell cultures were pooled and lyophilized for use in purification of the FSH-BI.

Resistance of granulosa cell derived FSH-BI to inactivation by acid treatment
Since granulosa cells for culture were taken from patients previously exposed to exogenous gonadotrophin treatment, the possibility was considered that the FSH-BI activity of the cultured cell medium was due to granulosa cell associated pituitary FSH. Pituitary FSH activity is lost following exposure to acid (Lee et al., 1991). The sensitivity to acid treatment of FSH-BI activity in conditioned medium was examined and compared to that of human FSH (Figure 3). Figure 3(A, C) shows that FSH-BI activity in medium alone to which had been added 40 IU/l or 80 IU/l of human FSH was markedly reduced following treatment with acidified acetone. In contrast, the FSH-BI activity of medium from cultured granulosa cells was unaffected by similar treatment (Figure 3B, D). This finding is similar to that previously reported for an FSH-BI factor isolated from human follicular fluid (Lee et al., 1991), and suggests that granulosa FSH-BI and human FSH are not identical and that FSH-BI activity in granulosa cell culture medium is not due to pituitary FSH.

Biological characterization of granulosa cell FSH-BI
The pooled, lyophilized medium was extracted with charcoal dextran (c/d) to remove steroid hormone contaminants and then tested for activity in the Sertoli cell culture assay system. The oestradiol content of the c/d extracted material was negligible, as determined by RIA and a cell-free c/d treated

Figure 2. Percentage binding inhibition in the radioreceptor assay (RRA) of 250 µl of control or granulosa cell conditioned medium from the 98 patients studied. Follicle stimulating hormone-binding inhibitor (BI) activity in the conditioned cell culture medium was significantly greater than control ($P < 0.01$).
Figure 3. Percentage binding inhibition in the radioreceptor assay (RRA) of 250 µl of the following native or acidified acetone (pH 3.5) treated samples: (A) 40 IU/l follicle stimulating hormone (FSH) in 250 µl RLA buffer, (B) FSH-stimulated (40 IU/l) medium from granulosa cells treated for 48 h, (C) 80 IU/l in 250 µl RLA buffer, (D) FSH-stimulated (80 IU/l) medium from granulosa cells treated for 48 h. All samples were dialysed prior to assay.

Figure 4. Oestradiol synthesis by Sertoli cell cultures stimulated with dialysed, lyophilized granulosa cell conditioned medium (125, 250 or 500 µg) or human FSH (8 IU/l). Significant stimulation of oestradiol over basal values was obtained in the presence of 250 and 500 µg medium and 8 IU/l human FSH (*P < 0.01). The bars indicate the mean values of duplicate assays of five cultures ±SEM. Medium control did not induce oestradiol synthesis in the cultures (data not shown). Lyophilized medium had agonist activity on Sertoli cells (Figure 4), stimulating oestradiol production in a dose-dependent manner. A dose of 500 µg/ml of dialysed lyophilized granulosa cell medium induced oestradiol production equivalent to that observed with 8 IU/l human FSH (10 ng/ml).

Purification of FSH-BI

FSH-BI in pooled, lyophilized granulosa cell culture medium was purified by PAGE. The lyophilized medium was first fractionated by electrophoresis on 12% PAGE in multiple lanes, run simultaneously. Following electrophoresis, one lane of the gel was cut into 0.5 cm segments, eluted overnight into either RRA assay buffer or 5 mM Tris–HCl, pH 6.8, and the segments tested for FSH-BI activity. A single region of binding inhibitory activity was detected. Corresponding regions of the other lanes were similarly prepared and eluted into 5 mM Tris–HCl, pooled and dried in a Speed-Vac concentrator/evaporator for further processing.

The active, pooled fraction from above was further purified on 8% SDS–PAGE-tricine gels, under non-reducing conditions. After electrophoresis, the gel was again divided into segments and eluted into either membrane assay buffer or 5 mM Tris–HCl, pH 6.8. The fractions eluted into membrane assay buffer were tested as before for binding inhibitory activity. Prior to testing, the fractions were dialysed to remove tricine that co-eluted from the gel, and protein concentration was determined by the fluorescamine method. Figure 5 shows FSH-BI activity of the gel.
of the purified fraction compared to unfractionated medium and a human FSH reference preparation. The ED50 of 2.5 µg of the purified preparation represents a 160-fold increase in specific activity compared to the precursor dialysed, lyophilized powder.

Silver staining of 8% SDS-tricine gel purified FSH-BI detected a single protein band at 25 000 M_r (Figure 6). The apparent molecular weight of FSH-BI was not affected by electrophoresis under reducing conditions (data not shown), suggesting that FSH-BI may not be composed of disulphide-associated subunits.

Discussion

This is the first report that human granulosa cells in culture secrete an FSH-BI. Our previous studies revealed that human follicular fluid contains a protein, distinct from pituitary FSH, with FSH-BI activity. HFF-FSH-BI was purified (Lee et al., 1993), and a portion of its primary amino acid sequence has been determined (Lee et al. 1995). As with the human follicular fluid derived FSH-BI (Lee et al., 1991, 1993), granulosa cell derived FSH-BI demonstrated FSH-like agonist activity, stimulating oestradiol production in Sertoli cell cultures. However, the apparent molecular weight of the granulosa cell FSH-BI (25 000 M_r) is less than that reported for the human follicular fluid FSH-BI (57 000 M_r) (Lee et al., 1993), and structural relatedness between the two has not yet been determined.

We considered the possibility that the observed FSH-BI activity might be due to granulosa cell associated FSH resulting from prior gonadotrophin treatment of donors. Several lines of evidence indicate that this is not the case. First, two independent immunological (chemiluminescent) assays for FSH failed to detect significant amounts of FSH in the cell culture medium (data not shown). Second, since medium used for our current study was derived exclusively from human granulosa cells not exposed to FSH, the only conceivable source of human FSH contamination in our assays would be from the granulosa cells themselves. However, unlike FSH, granulosa cell FSH-BI was resistant to acid inactivation, so this explanation seems unlikely. Furthermore, we are unaware of any reports of human FSH secretion by human granulosa cells. Finally, although additional studies will be required to confirm its chemical homogeneity, initial amino acid compositional analysis of SDS–PAGE purified granulosa cell FSH-BI indicates that it differs significantly from that of human FSH, human LH, and human chorionic gonadotrophin (HCG) (data not shown).

FSH-BI secretion varied between granulosa cell preparations obtained from different patients. The reason for this is not clear, but may in part reflect variability in the degree of luteinization of the cells derived from pre-ovulatory follicles of individual patients, and thereby the activity of these cells in producing various proteins such as FSH-BI. We have not yet been able to correlate IVF outcome with granulosa cell FSH responsiveness. Such a correlation requires a much larger cohort of patients in order to control for variables such as age, sperm count and motility of the male, stimulation protocol utilized for ovulation, etc. Although their provenance was not determined, there have been several reports of biologically active factors present in human follicular fluid. Khan et al. (1990) described a factor in human ovarian follicular fluid which stimulated testosterone production in rat Leydig cells. The same group isolated a protein from human ovarian follicular fluid which stimulated steroidogenesis in rat testicular and adrenal cells, as well as human granulosa cells (Khan et al., 1990). Schneyer et al. (1991) and Lambert-Messier et al. (1994) have proposed that precursors of alpha inhibin present in human follicular fluid may have FSH-BI activity. It has not yet been possible to determine the relatedness of the human granulosa cell FSH-BI to these entities.

The mechanism by which human FSH-BI secretion is regulated is still poorly understood. It would be attractive to postulate that FSH somehow alters human FSH-BI secretion, which in turn has a paracrine effect to help regulate FSH action. Although preliminary studies suggest that exogenous FSH simulated human FSH-BI secretion by cultured granulosa cells, we have as yet been unable to exclude completely the possibility that the added FSH contributed to the detected increase in binding inhibition. The presence of a granulosa cell derived FSH receptor-binding inhibitor in follicular fluids suggests a potential novel mechanism by which granulosa cells regulate follicular maturation and ovulation, and we hope that further studies will facilitate our understanding of the complex hormonal interactions that regulate this process.

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