Characterization of a factor from human ovarian follicular fluid which stimulates Leydig cell testosterone production

S. A. Khan, P. Hallin1,2, J. Bartlett, Ch. De Geyter and E. Nieschlag

Max Planck Clinical Research Unit for Reproductive Medicine, Institute of Reproductive Medicine of the University of Münster, FRG and Department of Zoophysiology, University of Uppsala, Sweden

Abstract. A factor from human ovarian follicular fluid (hFF) has been characterized which stimulates testosterone production of human, rat, mouse and hamster Leydig cells. hFF was obtained from women participating in an in vitro fertilization programme. Basal and hCG stimulated testosterone production of rat interstitial cells, and Percoll purified Leydig cells were significantly stimulated by hFF. The steroidogenic response of the cells was 3–5 fold higher than that obtained after stimulation with maximal doses of hCG. A serum pool from the same patients was found to be about 30 times less potent than hFF in stimulating steroidogenesis. The stimulatory activity was retained after precipitation with ammonium sulphate and dialysis. Precipitation with ethanol, ether and acetone resulted in a partial loss of activity, whereas extraction with charcoal or heating at 100°C for 10 min resulted in significant loss of activity. When hFF was fractionated by gel chromatography, the stimulatory activity was eluted in a molecular weight region between 30 and 50 kD. The stimulatory factor was further purified by chromatofocusing and was eluted as a homogeneous peak with an isoelectric point between 8.8 and 9.5. The SDS-PAGE analysis of these fractions, however, revealed that the active substance was not homogeneous. The purified factor was immunologically distinct from hCG and hLH. These studies demonstrate for the first time the presence of a factor in the hFF which may potentiate the action of LH in the ovary.

In addition to pituitary gonadotropins, local factors regulate testicular and ovarian function (Sharpe 1986; Hsueh 1986; Tonetta & di Zerega 1986). Among these are factors which either inhibit or enhance the action of pituitary gonadotropins in both gonads according to local requirements (Reichert & Abou-Issa 1977; Sharpe 1983; Sharpe & Cooper 1984; di Zerega et al. 1983; Syed et al. 1985; Risbridger et al. 1987; Verhoeven & Cailleau 1987; Krishnan et al. 1986; Rommerts et al. 1986).

Ovarian follicular fluid represents a rich source of such peptides, and several factors which inhibit the binding of both FSH (Reichert & Abou-Issa 1977) and LH (Yang et al. 1979) to their respective receptors, or enhance their action (Sluss et al. 1987; Risbridger et al. 1987) have been identified in bovine, ovine and porcine ovaries. Rat ovarian extracts have also been shown to contain at least two such factors: one of these factors inhibited the binding of LH to its ovarian receptors and consequently blocked progesterone production, whereas another factor stimulated in vitro testosterone production by minced rat testes (Yang et al. 1979). A factor which stimulates Leydig cell steroidogenesis has also been found in bovine follicular fluid (Risbridger et al. 1987).

Human follicular fluid obtained from pre-ovulatory follicles has recently been shown to contain

1 Present address: Kabi Vitrum AB, Peptide hormones, Stockholm, Sweden.

2 Present address: Kabi Vitrum AB, Peptide hormones, Stockholm, Sweden.
much higher biologically active LH (40-fold) than immunoreactive LH (Cha et al. 1986). A similar increase in biologically active LH (up to 50-fold) was also observed during the peri-ovulatory period in female rhesus monkeys (Marut et al. 1981). As bioactive LH in these studies was determined by an in vitro bioassay based on testosterone production by isolated mouse interstitial cells, the high biological activity could have been due to the presence of an additional ovarian peptide with Leydig cell stimulatory activity. The present study was performed to characterize the factors present in human follicular fluid which may influence testosterone production of Leydig cells in vitro.

Materials and Methods

Chemicals

All the tissue culture media, phosphate buffered saline (Dulbecco’s formula) and L-glutamine were purchased from Flow Laboratories, Irvine, Scotland. Bovine serum albumin (BSA), collagenase (Type I) and dibutyryl adenosine 3′-5′-cyclic monophosphate (db cAMP) were obtained from Sigma Chemicals, München, FRG. Ultra-gel Aca 54 for gel filtration was from LKB (München, FRG) and polybuffer 96 for chromatofocusing from Pharmacia Fine Chemicals (Freiburg i. Br., FRG). A highly purified preparation of hCG (Bahl hCG 11.1.82) was a gift from Dr O. P. Bahl, Buffalo, New York, USA, through the courtesy of the World Health Organization’s Special Programme of Research in Human Reproduction, Geneva, Switzerland. An antiserum to hCG (R 13, Pool D) was provided by Dr G. Bialy, NICHD, Bethesda, MD.

Incubation medium

All incubations of interstitial cells or purified Leydig cells were carried out in a 1:1 mixture of HAM’s F-12 and Dulbecco’s modified Eagle’s minimum essential medium (DMEM) containing 20 mmol L-glutamine and 0.2% BSA.

Human ovarian follicular fluid

Ovarian follicular fluid (hFF) was obtained from women participating in an in vitro fertilization programme of the University of Münster. All women had been treated with human menopausal gonadotropins (hMG, 150 IU daily for 5 days) and hCG (10 000 IU) for induction of follicular maturation and ovulation, respectively. Follicles were penetrated 34 to 36 h after hCG administration for collection of oocytes. Follicular fluid (1–3 ml) was aspirated, centrifuged at 1500 × g for 10 min to remove cellular components and stored at −80°C until analysed. A blood sample was also taken at the time of puncture. The sera from different patients were pooled and used in parallel with the hFF.

Assay of interstitial cell regulatory factors

Interstitial cells from adult (60–70 days old) rat testes were prepared as described by Syed et al. (1985). Briefly, the decapsulated testes were incubated in medium containing 0.25 g/l collagenase for 10 min at 32°C. The interstitial cells were washed and pre-incubated for 1 h at 32°C. After centrifugation, the cell pellet was resuspended in fresh incubation medium and 200 µl cell suspension (approximately 30 000 viable cells) was incubated for 3 h with or without the test materials. Testosterone in the incubation medium was determined by radioimmunoassay. Diluted antiserum (final dilution 1:45 000) and [3H]-testosterone (approximately 10 000 cpm) were added to the incubation media and incubated at 4°C overnight. The antibody bound and free fractions were separated using dextran coated charcoal (see Wickings et al. 1979 for further details).

For some experiments, rat interstitial cells were fur-
Ther purified on Percoll gradients to obtain pure and intact Leydig cells according to the procedure described by Bartlett & Sharpe (1987). The Leydig cells (approximately 30,000) were then incubated with the test materials as described above. Interstitial cells from human testicular tissue were isolated according to the method employed by Verhoeven & Cailleau (1987) and were incubated with hCG and hFF as described above.

The effects of hFF on in vitro testosterone production of interstitial cells obtained from mouse or Djungarian hamster testes were also studied. The interstitial cells were prepared and incubated by the same procedures as described for rats. The test materials were analysed at 9 dose levels and the results were expressed as means of triplicate determinations, unless otherwise stated.

**RIA of hCG and hLH**

Immunoreactive hCG was determined by a highly specific immunoassay using a monoclonal antibody to hCG which does not cross-react with LH (MAIA Clone: Serono Diagnostica, Freiburg i. Br., FRG). hLH was determined by a specific RIA as described by Bartfai et al. (1979).

**Treatment of human follicular fluid**

**Dialysis.** Pooled hFF was dialysed against phosphate buffered saline (PBS, 0.05 mol/l, pH 7.4) for 48 h at 4°C using dialysis membranes (Sigma Chemicals) with a molecular weight retention limit of 10 kD.

**Heating.** The pooled hFF was placed in a water bath at 60°C or 100°C for 10 min. After heating, the fluids were cooled to 4°C and centrifuged at 1500 × g for 10 min. The supernatants were diluted with incubation medium and used in the bioassay.

**Ammonium sulphate precipitation.** hFF proteins were precipitated with 80% ammonium sulphate overnight at 4°C. The precipitates were washed with the same concentration of ammonium sulphate, dissolved in PBS and dialysed for 48 h as described above.

**Charcoal extraction.** Individual samples or the pooled hFF were incubated with dextran coated charcoal (1 g/l) for 15 min at 4°C (Sharpe & Cooper 1984). After centrifugation at 1500 × g for 10 min, the supernatant dilutions were added to interstitial cells.

**Treatment with organic solvents.** Ethanol or acetone (2 × 5.0 ml) were added to a portion (0.5 ml) of dialysed hFF and thoroughly mixed. The precipitates obtained after centrifugation at 1500 × g were redissolved in the incubation medium. hFF (0.5 ml) was also extracted.

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**Fig. 2.**

Effects of human follicular fluid on testosterone production of rat interstitial cells when incubated alone or in the presence of either half-maximal dose of hCG or an antiserum against hCG (1:1000). Left panel shows the testosterone produced by control cells (C) or in the presence of the maximal dose of hCG (1 μg) added either alone or in combination with an antiserum against hCG (1:1000). Each point represents a mean of triplicate determinations. • hFF; ○ hFF + hCG (0.25 ng); ■ hFF + anti-hCG.
with 2 × 50 ml diethylether. The redissolved proteins or aqueous phase after ether extraction were analysed for their action on rat interstitial cells.

**Gel chromatography.** Follicular fluid proteins obtained after ammonium sulphate precipitation and dialysis were fractionated on an Ultrogel (LKB) AcA 54 column (dimensions 1.6 × 90 cm) at 4°C. The elution was performed at a speed of 6 ml/h using Tris-HCl (0.1 mol/l, pH 7.4) buffer. Optical density was monitored at 280 nm. Two-ml fractions of the eluate were collected and analysed for their effect on basal and hCG stimulated testosterone production of rat interstitial cells.

**Chromatofocusing.** The individual fractions from AcA 54 chromatography which stimulated testosterone production of interstitial cells were pooled, concentrated, dialysed and further purified by chromatofocusing using a fast protein liquid chromatography (FPLC) system (Pharmacia) on a 'mono P' (HR 5/20, Pharmacia) column. Ethanolamine-HCl (0.025 mol/l, pH 9.0) was used as starting buffer. The chromatofocusing was performed in a linear pH gradient generated by 10% polybuffer 96 (pH 6.0) which was used as eluent. The separations were performed in the pH range between 6.0 and 9.0, unless otherwise stated. The column was eluted at a speed of 1 ml/min and 1.0-ml fractions were collected. Optical density was measured at 280 nm and pH was monitored by a flow cell pH electrode during elution. The proteins retained in the column were eluted by 1 mol/l NaCl. The individual fractions were filtered through small columns of Sephadex G-25 (PD 10, Pharmacia) using PBS (with 0.1% BSA) as an eluent before they were analysed for their influence on rat interstitial cells.

**SDS-PAGE.** The biologically active fractions obtained after chromatofocusing were subjected to SDS-PAGE

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**Fig. 4a—c.**

Influence of hFF on testosterone production of human (a), mouse (b) and hamster (c) interstitial cells. Each point represents mean of triplicate determinations.
both in reducing and non-reducing conditions using 'Phast System' (Pharmacia Fine Chemicals) according to the instructions of the manufacturers. One microlitre sample was purified on 'homogeneous 20' gels for 100 volthours using PhastGel SDS buffer strips. The gels were stained by silver staining.

Protein estimation. The protein content of different purified fractions was determined by the method of Bradford (1976).

Results

Effects of hFF on interstitial cells

Several doses of pooled human follicular fluid and pooled serum and hCG were incubated with rat interstitial cell preparations and the testosterone (T) produced during the 3-h incubation period was determined by RIA. The results are shown in Fig. 1. Follicular fluids used at very low doses (5 µl or less) caused a significant dose-dependent increase in T production. The stimulation was much higher (3–5-fold) than that obtained with the maximal dose of hCG. A pool of serum from the patients also stimulated T production of rat interstitial cells. However, hFF was found to be about 30-fold more potent than the serum. When incubated in the presence of half the maximal dose of hCG (0.25 ng), hFF further enhanced the hCG-stimulated T production (Fig. 2). The stimulation of T production by hFF was still evident after incubation with an anti-hCG serum which neutralized the biological activity of the maximum (1 µg) concentration of hCG (Fig. 2).

When Percoll purified Leydig cells were used instead of interstitial cells, hFF stimulated both

Fig. 5a.

Influence of dialysis, ammonium sulphate precipitation and heating on the interstitial cell stimulatory activity of hFF. Testosterone produced in the absence (C) and presence of hCG (1 ng) is also shown. Data shown as mean ± SD of triplicate determinations.

Fig. 5b.

Dialysed human follicular fluid was treated with ether, ethanol or acetone to precipitate proteins. The influence of redissolved proteins (acetone, ethanol) or aqueous phase (ether) on testosterone production of rat interstitial cells is shown in this figure.
AcA 54 gel chromatography profile of interstitial cell stimulatory activity. 10.0 ml of dialysed hFF (0.4 g protein) was applied on the column (1.6 x 90 cm) and 2.0-ml fractions were collected. The interstitial cell stimulatory activity was determined in 50-µl (approximately 100 µg protein) aliquots of individual fractions. $V_o =$ void volume, $V_t =$ total volume, BSA (mol weight 66 kD), ovalb (ovalbumin, mol weight 45 kD), myoglobin (mol weight 19 kD) and cyto C (cytochrome C, mol weight 12 kD) were used as molecular weight markers.

Profile of interstitial cell stimulatory activity, immunoreactive hCG and hLH in the pools of 5 fractions obtained after AcA 54 gel chromatography of dialysed human follicular fluid. Testosterone produced by unstimulated interstitial cells (C) and in the presence of hCG (1 ng) is shown in the left panel. — Interstitial cell bioassay; *** hCG-RIA; ... LH-RIA.
basal and hCG-stimulated T production as shown in Fig. 3. As both the interstitial cells and purified Leydig cells showed a similar response to this stimulation, only interstitial cells were employed in further experiments.

To study the species specificity, the effect of hFF on T production of human, mouse and hamster interstitial cells was also studied. As shown in Fig. 4, hFF stimulated in vitro T production of interstitial cells from the three species in a manner similar to that seen with the rat cells.

**Effects of various treatments on hFF stimulatory activity**

In order to elucidate the nature of interstitial cell stimulatory activity (ICSA), pooled hFF was dialysed, heated, precipitated with ammonium sulphate and/or extracted with either charcoal or organic solvents before being added to the interstitial cell preparations.

The effects of one common dose of hFF on T production after various treatments are shown in Fig. 5a and b. When the hFF was dialysed against PBS for 48 h, the stimulatory activity was retained, suggesting that the active substance(s) had a molecular weight higher than 10 kD. Similar results were obtained after precipitation of hFF proteins with ammonium sulphate followed by dialysis. The T content of the dialysed hFF was undetectable (<25 pg). Heating of the hFF at 100°C for 10 min, resulted in a significant (>90%) loss of activity.

The precipitates obtained after acetone and ethanol treatment and the aqueous phase after ether extraction of hFF retained the ICSA, although there was a variable decrease in potency. Charcoal extraction of the hFF, on the other hand, resulted in a complete loss of stimulatory activity.

To rule out the possibility that some component(s) of hFF may directly influence the estimation of T by RIA, the incubation media were either heated at 100°C for 15 min or extracted with diethylether prior to T RIA. The T content in untreated and treated incubation media was similar, suggesting that the effects of hFF were not due to its direct influence on T estimation (data not shown).

**Gel chromatography of ICSA**

The hFF proteins, after ammonium sulphate precipitation and dialysis, were fractionated on an Ultrogel AcA 54 column. The effects of individual fractions on T production by rat interstitial cells are shown in Fig. 6a, indicating that the stimulatory activity was eluted in a molecular weight region between 30 and 50 kD.

Pools of 5 fractions were also analysed for hCG and LH by specific immunoassays along with the
Fig. 8.
A narrow pH range (8.0 to 9.5) chromatofocusing profile of interstitial cell stimulatory activity.
For further details see legend to Fig. 7.

Fig. 9.
SDS-PAGE analysis of interstitial cell stimulatory fractions obtained after chromatofocusing (Fig. 8). The electrophoresis was performed in both non-reducing (A) and reducing (B) conditions. α-lactalbumin (14.4 kD), soybean trypsin inhibitor (20.1 kD), carbonic anhydrase (30 kD), ovalbumin (43 kD), bovine serum albumin (67 kD), and phosphorylase b (94 kD) were used as molecular weight markers.
ICSA. Fig. 6b shows that both immunoreactive hCG and hLH were eluted in the same region where most of the ICSA was found. The levels of these hormones were, however, very low compared with ICSA.

Chromatofocusing of ICSA

Pooled fractions from AcA 54 chromatography containing ICSA were further purified by chromatofocusing in a pH range between 6.0 and 9.0. The profile of ICSA is shown in Fig. 7. The activity was found in two regions. A high peak of activity was eluted in the alkaline pH (> 9.0) region, whereas another less marked peak was found in the acidic region (< 6.0). The latter activity could have been due to hCG.

The activity found in the alkaline region was further purified by chromatofocusing in a narrow pH range (8.0 to 9.5). The activity was eluted as a single peak with an isoelectric point (pI) between 8.8 and 9.5 (Fig. 8). As the pI of these fractions was very close to that of human LH (Robertson & Diczfalusy 1977), individual fractions were also analysed for immunoreactive LH. No immunoreactivity was found in any of these fractions.

SDS-PAGE of ICSA

Fig. 9 shows the SDS-PAGE profile of ICSA after the second chromatofocusing purification. The material was not homogeneous after staining with the silver stain and revealed the presence of high molecular weight protein bands. There was, however, a band seen in the molecular weight region between 20 and 30 kD which could represent ICSA.

Discussion

The present study demonstrates for the first time the presence of a potent stimulator of Leydig cell steroidogenesis in human ovarian follicular fluid. This substantiates previous reports on the presence of similar factors in the rat ovary (Yang et al. 1979) and in bovine follicular fluid (Risbridger et al. 1987). Factors which enhance basal and LH/hCG stimulated T production of Leydig cells have previously been shown also to be present in human, rat and pig testes (Verhoeven & Cailleau 1987; Sharpe & Cooper 1984; Benahmed et al. 1985). The magnitude of the stimulation observed in the present study was similar to that obtained with a factor present in rat testicular interstitial fluid (Sharpe & Cooper 1984). However, the hFF factor differs from the testicular factor in two respects. Firstly, in contrast to the testicular factor which enhances hCG-stimulated T production and has only marginal effects on basal T production, the hFF factor stimulated both basal and hCG-stimulated androgenesis. Secondly, whereas the testicular factor is resistant to charcoal treatment, the activity of the ovarian factor was completely lost after charcoal extraction of the follicular fluid; in this respect it is also different from a similar factor present in bovine follicular fluid (Risbridger et al. 1987). A similar loss of LH bioactivity after charcoal treatment of hFF has previously been reported (Cha et al. 1986). Moreover, the ovarian factor stimulated T production of interstitial cells of three other species (mouse, hamster and man) suggesting lack of species specificity of this factor. This is in contrast to the stimulatory action of GnRH-like factors which cause an increase in T production of rat Leydig cells but have no effects on mouse Leydig cells (Hunter et al. 1982). The ovarian factor also seems to be different from a factor found in the human testis which stimulates Leydig cell steroidogenesis (Verhoeven & Cailleau 1987). The testicular factor seems to be of smaller molecular size (> 30 kD) than the ovarian factor (30–50 kD).

The present study, however, failed to demonstrate the presence of an LH receptor binding inhibitor previously shown to be present in ovine, porcine and rat ovaries (Yang et al. 1979). This is in accordance with the observation by these authors that this inhibitor did not influence the LH binding to its testicular receptors, whereas it blocked LH binding in the ovary. The authors suggested that LH receptors in the rat ovary and testis may have different physicochemical characteristics.

As the ovarian follicular fluid contains various steroids and steroid precursors which may be converted to androgens by Leydig cells and as the extraction of hFF with charcoal resulted in a loss of the stimulatory activity, various procedures were used in the present study to rule out the possible influence of steroids or steroid precursors on the stimulation of T production. Results obtained after several such treatments (dialysis, extraction with organic solvents and ammonium ...
sulphate precipitation), however, indicated that the stimulatory activity was associated with a macromolecule (mol wt > 10 kDa). This was also supported by the loss of activity after heating of hFF at 100°C. The insolubility of this factor in organic solvents also speaks against the steroidal nature of the active substance.

Gel chromatography of hFF proteins established that the ICSA was indeed associated with a 30–50 kDa macromolecule. As the molecular weight of the active substance is close to that of hLH (32 kDa) and hCG (45 kDa), it is logical to suspect that its bioactivity could be due to the presence of these hormones in hFF. Further purification of ICSA by chromatofocusing, however, showed it to be completely different from hCG in its pl value. Moreover, the ICSA was devoid of any hLH or hCG immunoreactivity.

Both LH and hCG stimulate Leydig cell steroidogenesis after binding to a common membrane receptor. As the hFF factor further enhanced T production of cells which had been maximally stimulated by hCG, it can be assumed that ICSA stimulates Leydig cell steroidogenesis by a mechanism which is distinct from that utilized by LH/hCG. Our preliminary results also showed this factor to stimulate steroidogenesis in the presence of maximal stimulatory doses of cAMP and cholera toxin (Khan et al., unpublished data), suggesting that the stimulator may act at a step beyond the production of cAMP. Response of rat Leydig cells to a factor present in testicular fluid has also been shown to depend upon a mechanism which is distinct from that used by LH/hCG or by GnRH (Rommerts et al. 1986). Further studies are underway to find out the mechanism of action of ICSA (in hFF) on Leydig cell steroidogenesis.

The physiological relevance of these findings is not yet clear since all hFF samples used in this study were obtained from patients participating in an in vitro fertilization programme following ovarian stimulation. Studies are underway to investigate the levels of the hFF factor(s) in normal cycles to determine whether the potency of this factor may be related to follicular size or maturity. A sudden rise of LH bioactivity in peri-ovulatory follicles as reported previously in women and rhesus monkeys (Cha et al. 1986; Marut et al. 1981), suggests that this factor may be secreted by the mature follicles and may play a significant role in ovarian function.

On the basis of the evidence obtained in the present study, it is suggested that the human ovarian follicular fluid contains a macromolecular factor which stimulates steroidogenesis of Leydig cells. The active substance is distinct from hLH and hCG and does not seem to influence the Leydig cell function through the LH/hCG receptors.

Dedication

This paper is dedicated to Professor Egon Diczfalusy, the great teacher of Reproductive Endocrinology.

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Prof Dr E. Nieschlag,
Max Planck Clinical Research Unit
for Reproductive Medicine,
Steinfurter Strasse 107,
D-4400 Münster,
FRG.