Action of recombinant follicle stimulating hormone in superfused human granulosa cells in vitro

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The aim of this work was to compare the action of recombinant follicle stimulating hormone (rFSH) and urinary FSH (uFSH). Moreover we aimed to compare the secretory efficiency of continuous versus pulsatile stimulation by rFSH in superfused human luteal cells. Progesterone concentration was measured in culture medium by radioimmunoassay. Action of rFSH and uFSH was compared in static cultures of human granulosa cells at doses of 0.001–10 IU/ml. The secretory efficiency of both rFSH and uFSH was found to be similar in a defined range of concentrations (0.001–0.3 IU/ml). At concentrations of 1 and 10 IU/ml, the action of uFSH was significantly more potent than rFSH, up to 139% (**P < 0.01**) and 133% (**P < 0.01**) respectively. A concentration of 0.3 IU/ml of rFSH was most potent in static cultures, and evoked progesterone release up to 80 mg/ml. For a stimulation period of up to 4 h, the action of rFSH and uFSH in human granulosa cells was time-dependent and differences between them were not significant. Irregularities were observed at >4 h stimulation time. In another experiment, in superfused human granulosa cells, we showed that the stimulatory effectiveness of pulsatile rFSH administration (time interval 60 min, application time 10 min) was greater for progesterone release (3973 ng of progesterone/1 IU rFSH) than was continuous administration (848 ng of progesterone/1 IU rFSH). In conclusion, the secretory action of rFSH is similar to that of uFSH for defined times and doses. Moreover, pulsatile rFSH administration is more efficient at stimulating the release of progesterone than continuous administration.

Key words: recombinant FSH/human granulosa cells/progesterone

Introduction

Gonadotrophin-releasing hormone (GnRH) is released by the hypothalamus in a pulsatile way (Belhetz et al., 1978). As a consequence, the gonadotrophin luteinizing hormone (LH) and follicle stimulating hormone (FSH) are also secreted in the pituitary in a pulsatile manner and vary significantly during the menstrual cycle (Veldhuis et al., 1984). The importance of pulsatile delivery of LH to the ovary for steroidogenesis has been shown by several authors (Peluso and Steger, 1987; Weiss et al., 1989). Other reports (Fillicori et al., 1984; Veldhuis et al., 1988) did not confirm such a role, at least in the early luteal phase.

The aim of this work was to compare the actions of recombinant follicle stimulating hormone (rFSH) (Loumaye et al., 1995) and urinary FSH (uFSH). This is the first report of the action of rFSH on progesterone release in human granulosa cells. In addition, we aimed to compare the secretory efficiency of continuous versus pulsatile stimulation by rFSH in superfused human luteal cells, thereby studying the physiological significance of its pulsatile action. We decided to choose a method of superfusion of cultured granulosa cells attached to sephadex beads. This system is especially suitable as it allows pulsatile administration of ligand, similar to the pulsatile secretion of FSH from the pituitary. The advantage of this method is that it allows the direct observation of the secretory reaction of the cells; and to illustrate the differences in dynamism and in dimension of hormone secretion that depend on the time interval between two stimulations. In a second experiment, we used static cultures of human granulosa cells attached to the bottom of dishes in 24-well plates to investigate the influence of concentration and time of application on rFSH and uFSH. This method is simple, has a low frequency of errors and is suitable for answering our questions.

Materials and methods

Cell culture

Human granulosa cells were obtained from in-vitro fertilization (IVF). Pergonal (Serono, Freiberg, Germany) was used in a step-down protocol starting with 3 ampoules per day for 3 days followed by 2 ampoules per day until ovulation induction. There was an average number of follicles per patient of 7.26 (number of patients = 74). The diameter of the follicles was 15–31 mm and the number of cells per follicle 13.8×10^4 (+ 2.05×10^4); number of cells per patient 100.2×10^4 (+ 14.88×10^4).

The samples were washed free of erythrocytes with Dulbecco's phosphate-buffered saline (PBS) + Ca^2+ /Mg^2+ isotonic solution, layered onto Percoll 60% (Pharmacia, Freiburg, Germany) and centrifuged at 300 g for 20 min at room temperature. This sedimented >90% erythrocytes, leaving a band of granulosa cells which was removed with a sterile pipette and washed twice for 15 min at 100 g
with PBS containing 20 mM HEPES (both: Biochrom, Berlin, Germany). For static culture experiments, cells were plated in 24-well plates, 10,000 cells per well in 1 ml of medium per well and incubated for 10 days before the experiments were carried out. This time interval was chosen because our earlier work, in static cultures showed that ligand-induced secretory reactivity changes during cultivation and is best on the 10th day of culture (T. Rabe et al. unpublished). The medium was changed every 3–5 days. Cultured cells were stimulated for 24 h by rFSH or uFSH at several concentrations: 0.001, 0.01, 0.03, 0.1, 1, or 10 IU/ml. In another experiment, cultured cells were stimulated by rFSH or uFSH at a concentration of 1 IU/ml for durations of 1, 2, 4, 8, 12, or 24 h. Samples were collected and assayed for progesterone by enzyme immunological test kits (Boehringer, Mannheim, Germany). Each result was the mean of five samples ± SD.

### Superfusion

Cells were maintained on Cytodex beads (Pharmacia) with a cell/bead ratio of 20:1 in medium 199 + 0.01% L-glutamine + 10% fetal calf serum (FCS) + 100 nM androstenedione + penicillin/streptomycin/amphotericin B (all from Biochrom) in 10 cm Petri dishes and incubated at 37°C in 5% CO₂:95% air (3×10⁶ cells per dish). After 3 days of culture, the superfusion experiments were carried out. We chose this time interval because our earlier studies indicated that secretory activity in superfused granulosa cells is at its best on the third day of culture (T. Rabe et al., unpublished). Cells attached to Cytodex beads were gently transferred with a pipette to the superfusion columns (7 mm diameter; 100 mm long) (BioRad, Munich, Germany), 3×10⁶ cells per column. Effective volume of the column was 2 ml, the flow rate of the superfusion medium was 0.5 ml/min. Cells were superfused with the same medium as before for 2 h before being stimulated by the addition of rFSH 0.1, 1 or 10 IU/ml (Serono) or by human chorionic gonadotrophin (HCG) (Sigma, Munich, Germany). Agents were added once for 10 min only, or for 10 min during each 60 min, or 30 min, or continuously. Fractions were collected every 5 min and assayed for progesterone by enzyme-immunological test kits (Boehringer). Each superfusion was repeated three times. Student's t-test was used for statistical analysis.

### Results

The secretory efficiency of both rFSH and uFSH in static cultures was found to be similar for concentrations between

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**Figure 1.** Dose-dependent action of recombinant follicle stimulating hormone (rFSH) and urinary FSH in static cultures of human granulosa cells. Cell concentration was 10,000/ml; stimulation time was 24 h. Each value shown is the mean of five samples ± SD. *Significantly different (P < 0.01).

**Figure 2.** Time-dependent action of recombinant follicle stimulating hormone (rFSH) and urinary FSH (1 IU/ml) in static cultures of human granulosa cells. Cell concentration was 10,000/ml. Each value shown is the mean of five samples ± SD. *Significantly different (P < 0.01).

**Figure 3.** The effect of recombinant follicle stimulating hormone (rFSH) (0.1, 1 or 10 IU/ml) and human chorionic gonadotrophin (HCG) (1 IU/ml) in 10 min pulses on superfused human granulosa cells.
Action of recombinant FSH in granulosa cells

If the period of stimulation lasted up to 4 h, the action of rFSH and uFSH in human granulosa cells was time-dependent and differences between the two agents were not significant (Figure 2). However, when the stimulation period was >4 h, we observed irregularities: at 8 h uFSH stimulated significantly less progesterone release (76%, P < 0.01) than rFSH; at 12 h stimulation there were no distinct differences; but at 24 h stimulation FSH stimulated significantly less progesterone secretion than uFSH (77%, P < 0.01). Moreover, when the stimulation time was >4 h, there was no direct time-dependence of progesterone release observed for either rFSH or uFSH. Intra- and interassay coefficients of variation were 2.4 and 6.1% for static culture experiments.

In another experiment in superfused human granulosa cells (Table I), we showed that the stimulatory effect of pulsatile rFSH administration (time interval 60 min, application time 10 min) was greater in relation to progesterone release (3973 ng progesterone/1 IU rFSH) than was continuous rFSH administration (848 ng of progesterone/1 IU rFSH).

The administration of rFSH to human granulosa cells in a superfusion system caused an increase of progesterone secretion up to 290% within 20 min (Figure 3). This effect was dose-dependent. Maximum stimulation occurred after 10 IU rFSH/ml, a slightly weaker effect was observed after 1 IU rFSH/ml, and no stimulation was produced by 0.1 IU rFSH/ml. The progesterone surge occurred after 10 min and reached its maximum after 20–30 min. The concentration dropped to baseline within the next 20–30 min. For comparison, we induced the secretion of progesterone by chorionic gonadotrophin which evoked 471% stimulation (Figure 3) and maximum release was achieved within 50 min, after which it slowly declined to the base value within 4 h.

Figure 4 shows the alteration in progesterone secretion in response to stimulations of 10 min duration every 30 min, or every 60 min or continuous rFSH stimulation at a concentration of 1 IU rFSH/ml. When cells were stimulated every 60 min, the highest secretory efficiency was achieved, i.e. 3973 ng progesterone per 1 IU of rFSH (Table I). Stimulation by rFSH every 30 min yielded 2416 ng progesterone per 1 IU of rFSH, while by continuous stimulation, 848 ng progesterone were released per 1 IU of rFSH. Intra- and interassay coefficients of variation were 1.8 and 7.6% for superfusion experiments.

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Table I. Comparison of secretory efficiency of continuous versus pulsatile stimulation by recombinant follicle stimulating hormone (rFSH) in superfused human luteal granulosa cells. Each value in column II represents the mean of three superfusions calculated as area under the curve. In column III the value indicating the amount of progesterone gained was corrected to 1 IU of rFSH administered.

<table>
<thead>
<tr>
<th>I. Total dose of rFSH (IU) administered</th>
<th>II. Total progesterone (ng) gained</th>
<th>III. Stimulatory effectiveness. Proportion of gained progesterone (ng) per 1 IU rFSH</th>
</tr>
</thead>
<tbody>
<tr>
<td>rFSH every 60 min</td>
<td>5</td>
<td>19868</td>
</tr>
<tr>
<td>rFSH every 30 min</td>
<td>10</td>
<td>24168</td>
</tr>
<tr>
<td>rFSH continuously</td>
<td>30</td>
<td>25458</td>
</tr>
</tbody>
</table>

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(0.001–0.3 IU/ml respectively (Figure 1). At concentrations of 1 and 10 IU/ml, the action of uFSH was significantly more potent than rFSH, up to 139% (P < 0.01) and 133% (P < 0.01) respectively. Concentration of 0.3 IU/ml of rFSH was most potent in static cultures and evoked progesterone release up to 80 ng/ml.

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Discussion

In this study we found that the secretory action of rFSH is similar to that of uFSH in defined time and dose ranges. Moreover, we have shown that pulsatile FSH administration is a more efficient stimulus for the release of progesterone. This is the first report on the action of FSH on progesterone release in human granulosa cells.

Earlier work by ourselves (Rabe et al., 1995) and other authors (Weiss et al., 1989; Bodis et al., 1993) confirmed that the superfused granulosa cell system is suitable for studying the dynamics of steroid secretion. The pulsatile periodic changes of the hormone concentration in human serum has been described by Veldhuis et al. (1984). Several authors have observed that each pulse of LH follows a progesterone peak and have suggested that the LH secretory pattern plays a role in steroid secretion (Yen et al., 1972). In the present study, a pulsatile mode of administration of FSH was applied which mimics physiological action. The results confirmed the physiological significance of pulsatile action of FSH, showing that this is the most efficient way in which to stimulate the secretory function of granulosa cells. The present results show that secretory efficiency of both rFSH and uFSH is similar at concentrations of 0.001-0.3 IU/ml. The reason for the more potent action of uFSH than rFSH at concentrations of 1 and 10 IU/ml is unclear. It is possible that rFSH activates inhibitory feedback mechanisms more effectively. Irregularities observed at > 4 h stimulation when investigating the time dependence of progesterone release can also be explained by feedback mechanisms. At this time interval, inhibitory feedback mechanisms probably play a role in secretory proceedings. The weaker secretory effect observed with the continuous administration of rFSH is likely to have been caused by desensitization. Our results suggest that such a reduced reactivity follows each stimulation. This desensitization seems to be of limited duration, so a pulse of FSH administration triggers the secretion of progesterone more effectively when the recovery time between each stimulation is longer. In conclusion, the secretory action of FSH is similar to that of uFSH in defined time and dose ranges. Moreover, we have shown that pulsatile administration of FSH is the most efficient manner in which to stimulate the secretory function of the granulosa cells.

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References


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