Gonadotrophic regulation of prolactin mediated progesterone secretion in vitro

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Abstract. The effects of preincubating rat granulosa cells with FSH, LH, and Prl on subsequent Prl mediated progesterone secretion were investigated. Granulosa cells were isolated from ovarian follicles 50 h after injection of 5 IU PMSG and were then plated on poly-L-lysine coated coverslips in serum supplemented medium. Cells were preincubated for 24 h in the absence of hormones (control) or with the addition of either 0.25, 2.5, 25 ng/ml rat FSH or rat LH, or 1 µg/ml rat Prl. Following the preincubation period, cells were maintained for an additional 6 or 8 days in the presence or absence of 1 µg/ml Prl. When cells were preincubated with FSH or LH, only the two higher concentrations (2.5 and 25 ng/ml) stimulated significantly more progesterone secretion than control cultures during the 24 h preincubation period. For each series of preincubations, cells cultured for 6 or 8 days in the presence of Prl secreted significantly more progesterone at each day of culture than cells cultured without Prl. Cells preincubated and cultured with Prl secreted only 3–7-fold more progesterone than cells preincubated in control medium and then cultured with Prl. Preincubation with FSH or LH promoted a 20–45-fold increase in Prl mediated progesterone secretion compared to control preincubation cultures that also subsequently were cultured with Prl. The magnitude of Prl mediated progesterone secretion observed through 6 days of culturing was dose dependent on the preincubation concentration of FSH or LH. The establishment of an in vitro model system in which gonadotrophins enhance the responsiveness of granulosa cells to Prl in serum supplemented medium provides the opportunity for study of the regulatory mechanisms involved with the induction and maintenance of such responsiveness.

It is well accepted that Prl is the principal luteotrophic hormone responsible for maintenance of luteal function during the first week of pregnancy in the rat (Smith 1980). However, the exact role played by steroid and gonadotrophic hormones in luteal cell development and subsequent responsiveness to Prl remains unclear. In vivo, FSH and LH stimulate an increase in the number of Prl receptors on rat granulosa (Richards et al. 1976) and luteal cells (Richards & Williams 1976) respectively. Furthermore, FSH is capable of increasing Prl receptors on granulosa cells isolated from hypophysectomized rats and cultured in serum-free medium (Wang et al. 1979; Navickis et al. 1982).

The objective of the present study was to investigate LH and FSH regulation of luteinizing rat granulosa cell responsiveness to Prl stimulation for 6 or 8 days by employing a modified in vitro model system developed previously by our laboratory (Crisp & Denys 1975; Crisp 1977). This system allows us to examine and compare the effects of gonadotrophic hormones on subsequent responsiveness to Prl by granulosa cells from intact animals that are maintained in serum supplemented media.

Materials and Methods

Animals and cultures procedures

Sexually immature Sprague-Dawley female rats (27 days old) were injected sc at 12.00 h with 5 IU pregnant mare serum gonadotrophin (PMSG; Calbiochem-Behring). The rats were killed by cervical dislocation 50 h after

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PMSG injection, prior to the pre-ovulatory gonadotrophin surge (Hillensjö et al. 1974; Wilson et al. 1974). The ovaries were removed and granulosa cells were isolated from the largest follicles (approx. 800 µm in diameter, 6–8 per ovary). Cell viability was determined by trypan blue exclusion. Within each individual experiment, equal numbers of viable cells (2.8–4.1 x 10⁵ cells/ml) were aliquoted to each culture well.

Culture medium consisted of 85% Medium 199 (GIBCO) supplemented with 15% human male serum, penicillin (50 IU/ml) and streptomycin (50 µg/ml). Human male serum has been shown to be a suitable supplement to cultured rat granulosa cells (Crisp 1977). Cells were cultured upon poly-L-lysine (Sigma) coated 15 mm round glass coverslips within plastic multiwells (Falcon).

Granulosa cells were preincubated for 24 h in the absence of hormones (control medium) or in the presence of LH, FSH, or Prl. Following preincubation, cultures were rinsed thoroughly with Medium 199 with 0.1% bsa. Cells were then cultured with either control medium or 1.0 µg Prl/ml medium. This concentration of Prl is effective in promoting and maintaining rat granulosa cells in a fully luteinized state (Crisp 1977). Cultures were maintained for an additional 6 or 8 days following preincubation. Media were changed every 48 h and stored frozen until assayed for progesterone by RIA as previously described (Crisp 1977). Days in culture represent the time following the 24 h preincubation period.

A dose response study using 3 concentrations of Prl (0.01, 0.1, and 1 µg/ml) was performed to further confirm that enhanced progesterone secretion observed after 6 days in culture was attributable to Prl and not to the small contaminating fractions of FSH or LH contained within the Prl preparation used in these studies.

**Hormones**

Rat Prl (NIAMDD-rPrl-B2, biological potency 20 IU/mg, < 0.05 x NIH-FSH-S1 and < 0.001 x NIH-LH-S1), rat FSH (NIH-FSH-I3, 150 x NIH-FSH-S1, < 0.002 x NIH-LH-S1), and rat LH (NIH-LH-I4, 1 x NIH-LH-S1, < 0.04 x NIH-FSH-S1) were obtained from Dr. A. Parlow and the NIAMDD.

**Statistical procedures**

Progesterone concentrations in culture media were expressed as mean ± SEM (ng/ml) for replicate cultures and were analyzed statistically by analysis of variance and Student-Newman-Keuls tests (Zar 1974). Differences between means were considered significant if P < 0.05.

**Results**

Granulosa cells preincubated with 25 ng/ml FSH or LH secreted more progesterone than cells preincubated in control medium alone (Table 1). Cells preincubated with Prl secreted significantly (P < 0.025) lower amounts of progesterone than FSH or LH stimulated cultures.

Preincubation for 24 h with FSH or LH (25 ng/ml) dramatically increased the responsiveness of granulosa cells to Prl during the remaining 8 days of culture compared to preincubation in the absence of hormones (Fig. 1). For each series of preincubations, cells cultured for 8 days in the presence of Prl secreted significantly more progesterone at each day of culture than cells cultured without Prl. Cells cultured without Prl secreted maximum amounts of progesterone after 2 days of culture and progressively lower amounts thereafter, whereas progesterone secretion by Prl stimulated cultures reached a maximum after 6 days. Cells that were preincubated with LH secreted the greatest amounts of progesterone in response to Prl. Progesterone production by cells that were both preincubated and subsequently cultured with Prl was 70 to 90% less than progesterone secreted by LH preincubated cells in response to Prl stimulation.

Results from the dose response study indicate that cells cultured with 0.01 µg/ml Prl secreted 6-fold more progesterone than cells maintained in the absence of Prl for 6 days of culture (Table 2). In addition, while both 0.1 and 1 µg/ml Prl promoted significantly (P < 0.05) more progesterone secretion compared to 0.01 µg/ml Prl, no significant differences in progesterone secretion were observed between the two higher concentrations of Prl.

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**Table 1.**

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Progesterone (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>49 ± 2</td>
</tr>
<tr>
<td>FSH</td>
<td>303 ± 19**</td>
</tr>
<tr>
<td>LH</td>
<td>336 ± 30**</td>
</tr>
<tr>
<td>Prl</td>
<td>126 ± 6*</td>
</tr>
</tbody>
</table>

Statistical differences vs control are indicated by asterisks: *P < 0.05, **P < 0.01. Prl vs LH or FSH, P < 0.025.
Effects of 24 h preincubation hormones on the ability of granulosa cells to secrete progesterone during 8 additional days in culture. All cultures then were maintained in the absence of additional hormones (control, ■) or with 1 µg/ml PRL (□). Note that some cultures maintained in control medium for 8 days secreted levels of progesterone that were too low (< 30 ng/ml) to be illustrated. Each column and vertical line represents mean ± SEM progesterone concentration (ng/ml). n = 5.

Once it was established that preincubation with either FSH or LH increased subsequent PRL mediated progesterone secretion, an attempt was made to determine if the magnitude of PRL mediated progesterone secretion was dose dependent upon the concentration of the preincubation hormone. During the 24 h preincubation period, only cells preincubated with the two higher concentrations (2.5 and 25 ng/ml) of FSH or LH secreted significantly more (P < 0.025 for 2.5 ng/ml vs control; P < 0.01 for 25 ng/ml vs control) progesterone than cells preincubated in medium alone (Table 3). Progesterone levels were the

### Table 2.

Effects of 3 concentrations of PRL (0.01, 0.1, and 1 µg/ml) on progesterone secretion during 6 days of culture. Control cultures were cultured in the absence of PRL. 

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Progesterone (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>224 ± 12</td>
</tr>
<tr>
<td>0.01 µg PRL</td>
<td>1320 ± 56*</td>
</tr>
<tr>
<td>0.1 µg PRL</td>
<td>1785 ± 37*</td>
</tr>
<tr>
<td>1 µg PRL</td>
<td>1920 ± 29*</td>
</tr>
</tbody>
</table>

Statistical differences vs control are indicated by asterisks, P < 0.01. 0.01 µg vs 0.1 or 1 µg PRL, P < 0.05. 0.1 µg vs 1 µg PRL, P > 0.05.
Table 3.
Effects of 3 concentrations (0.25, 2.5 and 25 ng/ml) of LH and FSH on progesterone secretion by rat granulosa cells during a 24 h incubation. Control cultures were incubated in the absence of hormones. Mean ± SEM, n = 10.

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Progesterone (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>51 ± 4</td>
</tr>
<tr>
<td>0.25 ng LH</td>
<td>50 ± 2</td>
</tr>
<tr>
<td>2.5 ng LH</td>
<td>167 ± 11*</td>
</tr>
<tr>
<td>25 ng LH</td>
<td>203 ± 11**</td>
</tr>
<tr>
<td>0.25 ng FSH</td>
<td>52 ± 2</td>
</tr>
<tr>
<td>2.5 ng FSH</td>
<td>116 ± 7*</td>
</tr>
<tr>
<td>25 ng FSH</td>
<td>175 ± 6**</td>
</tr>
</tbody>
</table>

Statistical differences vs control are indicated by asterisks: *P < 0.025, **P < 0.01.

Highest when cells were exposed to 25 ng/ml of either LH or FSH.

Cells preincubated with the lowest concentration of FSH or LH, along with cells preincubated in the absence of hormones, secreted similar amounts of Prl mediated progesterone (Fig. 2). Preincubation with 2.5 ng/ml LH or FSH stimulated up to 7-fold more Prl mediated progesterone secretion than preincubation with no hormones. Cells preincubated with 2.5 ng/ml LH, compared to cells preincubated with 2.5 ng/ml FSH, secreted more progesterone at days 2 and 4, and at each day when the preincubation concentration was 25 ng/ml. Increasing the preincubation concentration of both LH and FSH from 2.5 to 25 ng/ml resulted in significantly more progesterone secretion in response to Prl stimulation.

Fig. 2.
Effects of 24 h preincubation with gonadotrophins on the ability of granulosa cells to secrete Prl mediated progesterone during 6 additional days in culture. Cells were preincubated with 3 concentrations of LH and FSH as indicated. All cultures were then maintained solely in the presence of 1 μg/ml Prl. Each column and vertical line represents mean ± SEM progesterone concentration (ng/ml). n = 5.
Discussion

These experiments demonstrate that FSH and LH promote enhanced progesterone responsiveness to subsequent Prl stimulation by luteinizing rat granulosa cells maintained in serum supplemented medium for 6 or 8 days. Furthermore, the magnitude of Prl mediated progesterone secretion was dependent on the concentration of FSH or LH to which the cells were initially exposed. This study additionally demonstrates that cells collected prior to the PMSG induced gonadotrophin surge and then exposed to gonadotrophins in vitro respond to Prl in a similar manner as cells harvested following their in vivo exposure to gonadotrophins (Crisp 1977; Centola 1979).

The results presented here are in slight contrast to those of Wang et al. (1979) in which a progressive increase in Prl mediated progesterone secretion was not observed following FSH priming, whereas in the present study Prl progressively increased progesterone secretion through 6 days of culture. This difference may be due to the use of serum supplemented media in the present study since granulosa cells cultured in the absence of serum may not have sufficient substrate for extended periods of Prl mediated progesterone secretion due to their dependence on lipoprotein transported cholesterol as their major substrate for progesterone synthesis (Christie et al. 1979; Schuler et al. 1979). Alternatively, the possibility that certain serum components promote an increase in Prl’s effect on progesterone biosynthetic and catabolic enzymes (Jones & Hsueh 1981; Jones et al. 1983) must be considered.

It should be noted that the differences in levels of progesterone observed in Table 1 and Fig. 1 compared to those found in Table 3 and Fig. 2 are attributed to these data originating from separate experiments in which different concentrations of cells were initially plated in culture. Using our culture system, we have documented that there is a strong correlation between number of granulosa cells in culture and the amount of progesterone secreted (Alexander & Crisp 1981).

The magnitude of Prl mediated progesterone secretion was considerably less when cells were preincubated with Prl than when preincubated with either LH or FSH. If Prl is able to induce its own receptor sites, as it has been shown to do in the liver (Costlow et al. 1975; Posner et al. 1975) and mammary gland (Djiane & Durand 1977), then one would expect that cells preincubated in Prl would have exhibited a much greater response to Prl during additional culturing. This did not occur. A possible explanation for this may be that granulosa cells must be exposed first to adequate levels of LH or FSH prior to autoregulation, if any, by Prl of its receptor. It is possible that the progressive increases in Prl mediated progesterone secretion observed through day 6 is due in part to Prl increasing its own receptor activity.

Only Prl or placental lactogen, and not FSH or LH, is capable of promoting the sustained progesterone response through 6 or 8 days by cells cultured following in vivo (Crisp & Denys 1975; Crisp 1977) or in vitro (Alexander, unpublished observations) exposure to gonadotrophins. The Prl dose response data of this study extends these previous observations. The lowest concentration of Prl used (0.01 µg/ml) was capable of stimulating progesterone secretion 6-fold more than cells maintained without Prl. At this low concentration, contaminating FSH (<0.01 mIU) and LH (<0.006 mIU) are negligible, since they are much less than the hormonal activities present in 0.25 ng FSH or LH, a concentration that was ineffective in stimulating progesterone secretion.

It seems likely that both FSH and LH promote enhanced Prl mediated progesterone secretion by the well known increase in cAMP, which increases Prl receptor sites (Katikineni & Davies 1980; Knecht et al. 1981). Since steroids have been shown to modulate the actions of Prl (Veldhuis et al. 1981), progesterone secretion during the preincubation period may contribute to subsequent responsiveness to Prl. Examination into the preincubation mechanisms that result in increased responsiveness to Prl is the focus of ongoing studies.

Acknowledgments

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