Effects of ovarian hyperstimulation and isolated preovulatory follicles on LH responses to GnRH in rats*

N. J. Busbridge, D. M. Buckley, M. Cornish and S. A. Whitehead

Department of Physiology, St Georges Hospital Medical School, Cranmer Terrace, Tooting, London SW17 0RE, U.K.

Summary. Adult rats were pretreated with a 3-day regimen of human menopausal gonadotrophin (hMG), PMSG, human FSH or hCG and experiments were carried out on the day of pro-oestrus. Treatment with hMG and hFSH induced a significant increase in the number of preovulatory follicles on the day of pro-oestrus and this was correlated with increased circulating concentrations of oestradiol. There was a parallel increase in the self-priming effect of GnRH, as observed from the biphasic LH response to a continuous GnRH challenge. PMSG treatment did not stimulate increased numbers of maturing follicles and was less effective in raising circulating oestrogen concentrations compared with hMG and hFSH. However, pituitary responsiveness was much higher after PMSG treatment and the biphasic response to continuous perfusion with GnRH was absent; LH release was high from the initiation of the stimulus. hCG alone failed to stimulate follicular maturation but enhanced pituitary LH responses. Hemi-pituitary glands perfused in the presence of isolated preovulatory follicles also showed augmented biphasic LH responses to GnRH compared with control hemi-pituitary glands. The apparent dissociation which can occur between follicular maturation, circulating oestrogen concentrations and pituitary responsiveness to GnRH supports the idea of non-steroidal ovarian factors modulating LH release.

Keywords: luteinizing hormone; gonadotrophin releasing hormone; ovaries; follicles; feedback

Introduction

There is accumulating evidence that non-steroidal ovarian factors released from maturing Graafian follicles exert direct feedback effects on LH secretion from the pituitary gland. During the oestrous cycle in rats (de Koning et al., 1980) and the menstrual cycle of monkeys (Littman & Hodgen, 1984), ovarian factors are involved in maintaining pituitaries in a state of low responsiveness to gonadotrophin-releasing hormone (GnRH). However, GnRH stimulation of sufficient strength and duration, as occurs during the pro-oestrous LH surge, causes a transition to a high responsive state and the pituitary gland then shows an augmented release of LH in response to GnRH. This is the classical self-priming effect of GnRH which is dependent upon protein synthesis (Pickering & Fink, 1976; de Koning et al., 1980).

Experiments by de Koning et al. (1987) indicate that a liver-labile ovarian factor(s), which is neither oestradiol nor inhibin, maintains the rat pituitary gland in a state of low responsiveness to GnRH. For example, after long-term ovariectomy, self-priming effects disappear and maximal LH responses are seen from the beginning of a GnRH challenge. Similar studies with humans (Messinis & Templeton, 1986) and monkeys (Littman & Hodgen, 1984) suggest that FSH stimulates the formation of ovarian factors which inhibit GnRH-induced LH secretion. Hence, stimulation of multiple follicular development with human menopausal gonadotrophin or FSH alone can prevent the spontaneous preovulatory surge of LH.

*Reprint requests to: Dr S. A. Whitehead.
Based on these data, we investigated whether there was any direct correlation between pituitary responsiveness to GnRH and the population of developing follicles in the ovary. Hyperstimulation of rat ovaries was induced with pregnant mares' serum gonadotrophin (PMSG), human chorionic gonadotrophin (hCG), human menopausal gonadotrophin (hMG) or human follicle-stimulating hormone (hFSH). Subsequently the self-priming effects of GnRH were assessed from the biphasic LH response observed during continuous perfusion of the pituitary gland with GnRH (de Koning et al., 1980). These responses were correlated with the number and size of developing ovarian follicles. In parallel, the direct effects of ovarian follicles on GnRH-stimulated LH release were assessed by perfusing pituitary glands in the presence of isolated preovulatory follicles.

Materials and Methods

Animals and treatments. Adult female Porton–Wistar rats were maintained at a constant temperature of 22°C and a controlled lighting regimen with lights on between 06:00 and 18:00 h. They had free access to food and water. Daily vaginal smears were taken and only rats showing regular 4-day oestrous cycles were used for the experiments. All hormone treatments were given by a daily subcutaneous injection between 09:00 and 09:30 h on the days of oestrus and dioestrus 1 and 2, and the animals were used for experimentation on the day of pro-oestrus. Those animals that did not display a pro-oestrous smear and typical ballooning of the uteri were excluded from the analysis of the results.

Groups of rats received the following hormone treatments every day for 3 days; (1) 5 i.u. PMSG, (Sigma Ltd, Poole, Dorset, U.K.), (2) 5 i.u. hCG (Sigma), (3) 5 i.u. hMG, (Serono Laboratories (U.K.) Ltd, Welwyn Garden City, Herts, U.K.), (4) 2.5 i.u. hFSH (Serono Laboratories). Gonadotrophins were dissolved or diluted in saline (0.9% NaCl) and controls received an equivalent volume of diluent alone.

Pituitary perfusions. Animals were perfused from 10:00 and 10:30 h, by stunning and decapitation, and trunk blood was collected. Each pituitary gland was dissected out, cut into quarters and placed in perifusion chambers of 200 µl volume which have been described in detail previously (Carter & Whitehead, 1981). In any one experiment, controls were always run in parallel with experimental groups and thus two separate control groups are shown in the results. The glands were perfused at a rate of 0.15 ml/min with Krebs’–Ringer–bicarbonate (KRB) containing 0.1% bovine serum albumin and 1% glucose. The perfusate was constantly gassed with 95% O2/5% CO2 and maintained at a temperature of 37°C.

After a 2-h stabilization period, the first 10-min perfusate fraction was collected before the glands were perfused with KRB containing 50 ng synthetic GnRH/ml (Cambridge Research Biochemical, Cambridge, U.K.). In one series of experiments 0.184 µm-cycloheximide (Sigma) was also added to the perfusing medium to confirm that the second phase of increased LH release (the primed response) was protein synthesis-dependent. Fractions were collected for a further 2 h. All serum perfusate samples were stored at −20°C until assayed.

In a final series of experiments, semi-pituitary glands from untreated pro-oestrous rats were co-incubated with isolated ovarian follicles. The ovaries were placed in warm KRB and, under a dissecting microscope, 4–5 large Graafian follicles from each ovary were rapidly dissected and placed in a perifusion chamber together with the hemi-pituitary gland. The other semi-pituitary gland served as a control and was perfused in the presence of an equal volume of muscle fragments. For these experiments perfusate samples were collected every 10 min.

At the end of each experiment the follicles were fixed in Bouin’s solution. Using the same techniques as for the ovarian histology (see below), follicles were then examined microscopically to determine whether they had undergone any atresia during the experimental period.

Ovarian histology. After the pituitaries had been removed, the ovaries were rapidly dissected out, weighed and placed in Bouin’s fixative for 18 h. Following standardized dehydration, clearing and embedding in paraffin wax, complete 10 µm serial sections of one randomly chosen ovary from each rat were cut and stained with haematoxylin and eosin.

Follicular volumes were measured using the method of Welschen (1973). Briefly, this involved measuring two diameters of the follicle at right angles to each other in the section in which the nucleolus was found. Volumes were then calculated from the mean of these two measurements and classified in accordance with the classes shown in Fig. 4. Only follicles with a volume between 250 × 10⁻³ µm³ and 499 × 10⁻³ µm³ (360–455 µm mean diameter) were included in the analysis, and follicles ≥ 500 × 10⁻³ µm³ are considered to be preovulatory follicles (Welschen & Rutte, 1971). Cyst-like, thin-walled follicles or follicles containing pycnotic granulosa cells, nude oocytes or oocytes without a nucleus were considered atretic and not included in the analysis.

Assays and statistical analyses. Perfusate and serum LH concentrations were measured by radioimmunoassay using the procedure outlined by NIADDK and are expressed in terms of ng rat LH RP2/ml. Inter- and intra-assay variations were 9.5% and 8.3% respectively and the assay sensitivity was 0.6 ng LH/ml.

Serum concentrations of oestradiol-17β were measured using a ¹²⁵I direct radioimmunoassay kit (Steranti Research Ltd, St Albans, U.K.) with a sensitivity of 10 pg/ml (37 pmol/l). Cross-reactivity of the antiserum with oestrone and oestriol was 2% and inter- and intra-assay coefficients of variation were 12% and 5% respectively.
All results are expressed as mean (±s.e.m.). LH responses to a continuous GnRH challenge are expressed as cumulative LH release with basal LH secretion being subtracted from each subsequent observation. Statistical comparisons of these responses were made with a one-way analysis of variance followed by Gabriel’s test (Kendall & Stuart, 1968) which is suitable for groups of unequal sizes. When only two groups were being compared an unpaired Student’s t test was used. The Wilcoxon two-sample rank sum test was used for statistical analysis of the histological data.

Results

Gonadotrophin treatment did not overtly alter the normal oestrous cycle, and on the expected day of pro-oestrus over 90% of all rats showed characteristic vaginal smears and ballooning of the uteri. The number of preovulatory follicles \( (\geq 500 \times 10^5 \, \mu m^3) \) was also subsequently measured, and this indicated that ovulation had not been advanced by the gonadotrophin treatment (Table 1).

Table 1. The effects of 3-day treatments with PMSG, hMG, hCG or hFSH on circulating concentrations of oestradiol and LH and the corresponding numbers of preovulatory follicles in rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Serum oestradiol (pg/ml)</th>
<th>Serum LH (ng LH-RP2/ml)</th>
<th>No. of preovulatory follicles ( (500 \times 10^5 , \mu m^3) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>25.9 ± 7.8 (8)</td>
<td>0.64 ± 0.16 (8)</td>
<td>9.5 ± 1.0 (4)</td>
</tr>
<tr>
<td>PMSG</td>
<td>54.3 ± 4.9 (9)*</td>
<td>1.06 ± 0.16 (7)</td>
<td>9.0 ± 1.4 (4)</td>
</tr>
<tr>
<td>hMG</td>
<td>79.2 ± 9.0 (5)*</td>
<td>0.70 ± 0.09 (8)</td>
<td>27.0 ± 2.5 (4)</td>
</tr>
<tr>
<td>hCG</td>
<td>15.7 ± 4.2 (7)</td>
<td>1.34 ± 0.38 (7)</td>
<td>7.0 ± 0.2 (4)</td>
</tr>
<tr>
<td>hFSH</td>
<td>62.3 ± 7.8 (5)*</td>
<td>0.61 ± 0.14 (7)</td>
<td>19.0 ± 2.0 (4)</td>
</tr>
</tbody>
</table>

Values are mean ± s.e.m. for the number of observations in parentheses.  

* \( P < 0.005 \) compared with value for controls and hCG treatment (Gabriel’s multiple-comparison test).  

† \( P < 0.05 \) compared with value for PMSG treatment (Gabriel’s test).  

‡ \( P < 0.05 \) compared with value for controls (Wilcoxon test).  

Continuous perfusion of pituitary glands with 50 ng GnRH/ml produced the typical biphasic LH response. During the first hour LH release was low (unprimed response) after which a higher rate of secretion was observed (primed response). Figure 1 shows that this primed response was blocked by the protein synthesis inhibitor, cycloheximide. The pituitary glands from hMG-treated animals also showed a biphasic LH response to GnRH, but the cumulative rate of LH secretion during the second phase of the response was significantly higher than in the control (Fig. 2a). In contrast, the pituitary glands obtained from rats treated with PMSG showed high levels of LH secretion from the start of the GnRH stimulus, the cumulative LH release being consistently and significantly higher than from pituitary glands of saline-treated controls (Fig. 2a). The pituitary glands from rats treated with hFSH alone or hCG showed a similar biphasic response to controls, but in both groups cumulative LH secretion was enhanced. Cumulative LH secretion was significantly higher in the hCG-treatment group compared with controls only, but when a multicomparison test was used there were no statistically significant differences (Fig. 2b).

However, the circulating oestradiol concentrations and number of preovulatory follicles after gonadotrophin treatment did not correlate with the pituitary LH responses to GnRH (Table 1). Treatment with hMG induced maturation of the largest number of preovulatory follicles and the highest concentration of serum oestradiol. On the other hand, PMSG treatment did not increase the mean number of preovulatory follicles while the serum oestradiol concentrations were significantly higher compared with controls but significantly lower than in the hMG-treated group. hFSH alone induced a significant increase in the number of preovulatory follicles and, similar to the hMG treatment, a proportional and significant increase in circulating oestradiol concentrations. After
hCG treatment both mean follicular counts and circulating oestradiol concentrations were reduced, but this reduction was not significantly different from controls.

Figure 3 shows the effects of gonadotrophin treatment on the differential counts of follicular volume. hMG induced a significant increase in the number of large follicles (1000 × 10^5 µm^3) compared with controls, and similar but insignificant trends were observed after the PMSG, hFSH and hCG treatments. The number of smaller (stage 4) follicles was significantly reduced after PMSG treatment compared with controls.

Finally, pituitary glands from untreated, pro-oestrous rats which were perfused in the presence of 8–10 isolated follicles showed significant increases in both the unprimed and primed LH responses to GnRH compared with control pituitaries incubated with fragments of muscle tissue (Fig. 4). Mean oestrogen concentrations measured in the pituitary/follicle perfusates at the start of the experiment were 26.0 ± 4.2 pg/ml compared with undetectable levels in pituitary perfusates without follicles. The histological appearance of the follicles did not indicate any appreciable atresia during the course of the experiment when compared with follicles which had been fixed immediately after dissection, nor was there any evidence of oocyte maturation.

**Discussion**

In the rat, recruitment of preantral follicles for ovulation during the next cycle appears to take place in the early hours of oestrus (Hirshfield, 1982). It is believed that this recruitment is due to the prolonged pro-oestrous portion of the FSH surge (Hirshfield & Midgley, 1978; Hoak & Schwartz, 1980). However, Matsuzono et al. (1986) have suggested that this FSH surge is not required and that basal FSH secretion is sufficient to initiate follicular maturation.
Fig. 2. Cumulative LH release from pituitary glands of pro-oestrous rats perfused with Krebs'-Ringer-bicarbonate containing 50 ng GnRH/ml. Pituitary glands were obtained from rats treated daily between oestrus and dioestrus day 2 with (a) 5 i.u. PMSG (▼), 5 i.u. hMG (■) or saline (●), and (b) with 5 i.u. hCG (▲), 2.5 i.u. hFSH (■) or saline (●). Values are mean ± s.e.m. for the no. of observations indicated in parentheses. *P < 0.05 compared with corresponding control value (a, Gabriel’s test; b, Student’s t test).

After recruitment of an oversized cohort of follicles, a wave of atresia, between oestrus and dioestrus day 1, reduces the population of large follicles to an approximate number for ovulation (Hirschfield, 1982). In the rat, this atresia may be triggered by the declining concentrations of FSH (Hirschfield, 1986) although in monkeys oestradiol appears to play an important role in regulating follicular atresia (Dierschke et al., 1985; Zeleznik et al., 1985).

LH and FSH have different actions on the maturing follicle and the effects of exogenous gonadotrophins vary according to the stage of the oestrous cycle at which they are administered (Welschen, 1973). Since different gonadotrophin preparations were being compared in these experiments, a simple and consistent regimen of daily administration of gonadotrophins was adopted at doses that did not overtly disrupt ovarian cyclicity or induce abnormal follicular atresia.

In immature rats, doses of 5–8 i.u. PMSG/rat produce a pattern of circulating steroid concentrations similar to that seen during the adult oestrous cycle, and induce an LH surge which generates a normal number of ovulatory follicles (Wilson et al., 1974; Parker et al., 1976). In the adult, hypophysectomized rat higher doses of PMSG are required (12–16 i.u./rat) to sustain follicular maturation comparable to that observed in the intact female (Welschen, 1973). Thus, despite the
presence of endogenous gonadotrophins, 5 i.u. PMSG was found to be insufficient to induce an increased number of preovulatory follicles.

The in-vitro primed pituitary responses to a continuous GnRH challenge after hMG and hFSH treatment could be generally correlated with the number of preovulatory follicles and the related changes in oestrogen secretion observed in vivo. Since oestrogen augments the GnRH-stimulated biosynthesis and release of LH (Liu & Jackson, 1977; Waring & Turgeon, 1980; Ramsey et al., 1987) it is likely that the prior exposure of pituitary glands to increased concentrations of oestrogen enhanced the in-vitro LH responses to GnRH after hFSH and hMG treatment. That hMG induced higher concentrations of oestrogen than hFSH is likely to be due to the LH component of hMG stimulating steroidogenesis. These results are in agreement with those of Martikainen et al. (1986), who showed that hMG treatment during the follicular phase of the human menstrual cycle significantly increased androgen production compared with hFSH treatment, and that this could account for the higher observed output of oestrogens. However, hCG alone did not increase oestrogen secretion (there was no recruitment or follicular maturation in the absence of FSH activity), although mean pituitary responses were higher than control values and those observed after hFSH treatment.

The LH responses observed after PMSG treatment are more difficult to interpret. The biphasic response was absent, LH release being high from the start of the GnRH stimulus. Furthermore, circulating oestrogen concentrations were lower than those measured after hFSH or hMG treatment, yet LH responses were higher. In immature rats, PMSG treatment enhanced the oestrogen and androgen response of the ovaries to hCG, but inhibited the progesterone response (Aguado & Ojeda, 1985). If a similar action occurs in adult animals, the consequent reduction in progesterone secretion might promote the sensitizing effects of oestradiol on GnRH-stimulated LH release. Alternatively, the lack of correlation between circulating concentrations of oestradiol and pituitary responses in both hCG- and PMSG-treated rats may suggest that there is a factor released by maturing follicles which attenuates the feedback effects of oestrogen.

Ovarian hyperstimulation did not provide clear-cut evidence for a non-steroidal factor which directly maintains the pituitary gland in a state of low responsiveness (de Koning et al., 1987) and inhibits the preovulatory LH surge (Schenken & Hodgen, 1983). However, Littman & Hodgen
Fig. 4. The effects of isolated preovulatory follicles on the cumulative release of LH from the hemi-pituitary glands of pro-oestrous rats perfused Krebs'–Ringer–bicarbonate containing 50 ng GnRH/ml. One half of the gland was perfused in the presence of 8–10 isolated ovarian follicles (■), while the corresponding hemi-pituitary gland was perfused with a similar volume of muscle fragments (○). Values are mean ± s.e.m. for 8 observations. *P < 0.05, compared with corresponding control value in the absence of ovarian follicles (t test).

(1984) have suggested that the postulated surge-inhibiting factor has a short circulating half-life (of ~90 min). We therefore investigated the LH response to GnRH when pituitary glands of pro-oestrous rats were perfused in the presence of isolated preovulatory follicles. The enhanced LH responses to GnRH presumably reflected the action of oestrogen released from the follicles into the perfusate, although Batra & Miller (1986) have reported that a pig follicular fluid preparation sensitizes cultures of sheep pituitary cells to GnRH. In contrast, bovine follicular fluid administered to ewes had a suppressive effect on the LH responses to ovariectomy and to GnRH analogue treatment (Martin et al., 1986).

If indeed there are non-steroidal ovarian factors which maintain the gonadotroph in a state of low responsiveness to GnRH, they are not overtly manifest when the pituitary glands are perfused in the presence of isolated ovarian follicles. However, the results from the ovarian hyperstimulation experiments, showing a lack of correlation between in-vivo concentrations of oestradiol, the number of preovulatory follicles and pituitary LH responses, are suggestive of follicular factors, other than oestradiol, controlling LH secretion.
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References


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