

The ovary-mediated FSH attenuation of the LH surge in the rat involves a decreased gonadotroph progesterone receptor (PR) action but not PR expression

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Abstract

Hyperstimulation of ovarian function with human FSH (hFSH) attenuates the preovulatory surge of LH. These experiments aimed at investigating the mechanism of ovarian-mediated FSH suppression of the progesterone (P₄) receptor (PR)-dependent LH surge in the rat. Four-day cycling rats were injected with hFSH, oestradiol benzoate (EB) or vehicle during the dioestrous phase. On pro-oestrus, their pituitaries were studied for PR mRNA and protein expression. Additionally, pro-oestrous pituitaries were incubated in the presence of oestradiol-17β (E₂), and primed with P₄ and LH-releasing hormone (LHRH), with or without the antiprogestin RU486. After 1 h of incubation, pituitaries were either challenged or not challenged with LHRH. Measured basal and LHRH-stimulated LH secretions and LHRH self-priming were compared with those exhibited by incubated pituitaries on day 4 from ovariectomized

(OVX) rats in metoestrus (day 2) injected with hFSH and/or EB on days 2 and 3. The results showed that: i) hFSH lowered the spontaneous LH surge without affecting basal LH and E₂ levels, gonadotroph PR-A/PR-B mRNA ratio or immunohistochemical protein expression; ii) incubated pro-oestrous pituitaries from hFSH-treated rats did not respond to P₄ or LHRH, and lacked E₂-augmenting and LHRH self-priming effects and iii) OVX reversed the inhibitory effects of hFSH on LH secretion. It is concluded that under the influence of hFSH, the ovaries produce a non-steroidal factor which suppresses all PR-dependent events of the LH surge elicited by E₂. The action of such a factor seemed to be due to a blockade of gonadotroph PR action rather than to an inhibition of PR expression.

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Introduction

Geiger *et al.* (1980) showed that treatment with follicle-stimulating hormone (FSH) at the beginning of a 4-day oestrous cycle in the rat causes a reduction of the spontaneous preovulatory luteinizing hormone (LH) release in the afternoon of pro-oestrus. The major and most relevant endocrine action of FSH on ovarian granulosa cells is the induction of specific genes (*P450SCC* and *P450AROM*) necessary for oestradiol-17β (E₂) biosynthesis (reviewed in Richards 1993). Besides this steroidogenic activity, a significant number of FSH-dependent non-steroidal substances of ovarian origin with regulating activities on the synthesis and release of gonadotrophins (LH and FSH) have recently been identified. Inhibin, activin and follistatin are relatively stronger modulators of FSH secretion (Farnworth *et al.* 1988, de Jong 1988, Ying 1988), whereas gonadotrophin surge-inhibiting or -attenuating factor (GnSI/AF, also named attenuin; Balen 1996, Fowler & Templeton 1996, Fowler *et al.* 2003) and oestrogen-antagonizing

factor (Schuiling *et al.* 1987,1999) are relatively stronger suppressors of the preovulatory LH surge.

In the rat, the preovulatory LH surge is strictly dependent on the ability of the pro-oestrous ovarian E₂ surge to increase gonadotroph responsiveness to the hypothalamic decapeptide LH-releasing hormone (LHRH; Fink 1988). The spontaneous preovulatory LH surge in pro-oestrous afternoon has two components: 1) E₂-augmenting LHRH-induced LH secretion and 2) LHRH self-priming (Fink 1995, Schuiling *et al.* 1999). The latter is the phenomenon whereby a subsequent LHRH pulse stimulates a larger LH pulse from the pituitary than the preceding LHRH pulse. The pituitary thus becomes primed by newly synthesized priming proteins (Fink 1995). Both secretory components of LH surge depend on E₂-induced upregulation of progesterone (P₄) receptor (PR) in the gonadotroph (Krey *et al.* 1973, Turgeon & Waring 1994, 2006, Chappell *et al.* 1999, Szabo *et al.* 2000, Bellido *et al.* 2003), the unique pituitary cell type that expresses PR (Fox *et al.* 1990, Turgeon & Waring 2000, Bellido *et al.* 2003). The pharmacological blockade of PR with

antiprogesterins blocks both components of the LH surge in cycling rats (Sánchez-Criado *et al.* 1990, 2000, 2004). Thus, it is widely accepted that PR activation is an obligatory step for E₂-increased pituitary response to LHRH (Chappell *et al.* 1999) and LHRH self-priming (Turgeon & Waring 1994). These and other (Turgeon & Waring 1990, 1991) observations explicitly demonstrate that the presence of the neuroendocrine integrator PR (Levine *et al.* 2001) is an absolute requirement for the transmission of E₂-induced signals leading to LH surge. Accumulated evidence indicates that gonadotroph PR can be activated by the cognate ligand (Rao & Mahesh 1986) and, in a ligand-independent manner (Blaustein 2004), by LHRH intracellular signalling pathways (Turgeon & Waring 1994, Fink 1995, Garrido-Gracia *et al.* 2006).

The present study examined the effects of human FSH (hFSH) treatment on gonadotroph PR mRNA and protein expression and PR action (E₂-augmenting LHRH-induced LH secretion and LHRH self-priming) in the cyclic rat. In addition, the involvement of the ovary in the hFSH-suppressed LH secretion was evaluated using ovariectomized (OVX) rats in metoestrus.

Materials and Methods

Animals, general conditions and surgery

Adult female Wistar rats weighing 190–210 g were housed under a 14 h light:10 h darkness cycle (light on at 0500 h) and 22 ± 2 °C room temperature, with *ad libitum* access to rat chow and tap water. Rats were included in the experiments after showing at least three consecutive 4-day oestrous cycles (oestrus, metoestrus, dioestrus and pro-oestrus). Vaginal smears were taken 7 days/week, and the day of vaginal oestrus was considered arbitrarily as day 1. Bilateral OVX was performed under light ether anaesthesia at 0900 h on day 2. All experimental protocols were approved by the Ethical Committee of the University of Córdoba, and experiments were performed in accordance with rules on laboratory animal care and international law on animal experimentation.

Drugs and treatments

Purified hFSH (Gonal-f; Serono, Aubonne, Switzerland) was dissolved at a concentration of 50 IU/ml saline. Rats were s.c. injected at 1400 h on day 2 and at 0900 h on day 3 with 10 IU of hFSH. Oestradiol benzoate (EB; Sigma Chemical Co.) was injected s.c. at the physiological doses of 10 µg/0.2 ml oil (Schuiling *et al.* 1987) at 0900 h on day 3. Control rats were given 0.2 ml oil/saline mixture.

Effect of hFSH and EB on serum concentrations of E₂ and LH and ovulation rate

The aim of this first experiment was to determine serum concentrations of E₂ and LH and the ovulation rate in cyclic rats

injected with hFSH or EB. At 0900 h on pro-oestrus, eight rats per group (vehicle-, hFSH- and EB-injected rats) were lightly etherized and less than 0.4 ml blood was obtained by direct jugular venipuncture. Again, at 1900 h (the zenith, of the LH surge in our colony; Sánchez-Criado *et al.* 1990), another blood sample was obtained. Blood was allowed to clot, centrifuged at 4 °C and the serum stored at –20 °C until quantified for E₂ and LH by specific RIAs. At 0900 h of the next day (day of oestrus), the oviduct was examined for the presence of eggs, which were counted under light microscopy.

Effect of hFSH and EB on PR expression

In the second experiment, PR mRNA and protein levels were determined in pituitaries from vehicle-, hFSH- and EB-injected rats. At 0900 h on pro-oestrus, rats from each of the three groups were decapitated and their anterior pituitaries collected. Pituitaries from OVX rats on day 2 (metoestrus) and killed on day 4 were used as controls. Pituitaries from each of the four groups (OVX rats and vehicle-, hFSH- or EB-injected cyclic rats), were assigned to either RT-PCR (*n*=9) or immunohistochemistry analysis (*n*=3).

Effect of hFSH and EB on in vitro LH pituitary responsiveness

In the third experiment, we investigated the effect of hFSH and EB treatments on LH pituitary responsiveness in intact cycling rats. Control injections consisted of 0.2 ml oil/saline mixture. At 0900 h on pro-oestrus, 20 rats per group were decapitated and the anterior pituitary glands removed and divided in halves for the incubation study.

In the fourth experiment, we evaluated *in vitro* the involvement of the ovary in the inhibitory effects of hFSH on LH secretion. Day 2 OVX rats were injected with hFSH on days 2 and 3 and/or with EB on day 3. Control injections consisted of 0.2 ml oil/saline mixture. At 0900 h on day 4, rats were decapitated and the anterior pituitary glands were removed and divided in halves for the incubation study. All pituitaries except those from OVX rats injected with vehicle were incubated with E₂.

Hemipituitary incubation protocol

Incubation of pituitaries was carried out as described previously (Bellido *et al.* 2003). Briefly, halves of anterior pituitaries were incubated at 37 °C with constant shaking (60 cycles/min) in an atmosphere of 95% O₂ and 5% CO₂. Each vial contained 1 ml Dulbecco's modified Eagle's medium, without L-glutamine and phenol red, containing glucose (4.5 g/l), 10⁻⁸ M E₂ (Sigma) and BSA (0.1% w/v), pH 7.4. After 1 h of pre-incubation, hemipituitaries were incubated for 75 min. During the 1-h pre-incubation period, no test substances were added except for RU486 (Fig. 1). During the first 60 min of the incubation period, fresh medium containing priming agents was added (Fig. 1). Test substances included: medium alone, 10⁻⁸ M LHRH (Peninsula 7201, Peninsula Lab. Inc., Merseyside, UK),

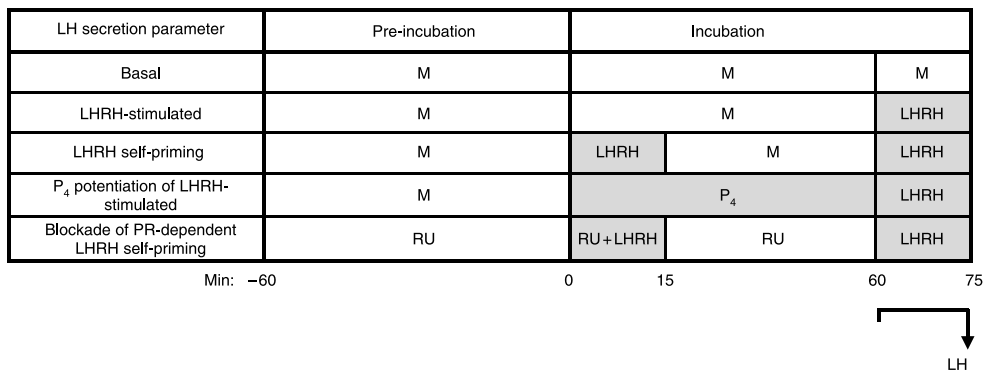


Figure 1 Diagram showing the hemipituitary incubation protocol used in each of the six groups of rats: intact cycling rats injected with i) hFSH (10 IU at 1400 h on metoestrus and at 0900 h on dioestrus), ii) EB (10 µg/0.2 ml oil) at 0900 h on dioestrus or iii) vehicle and ovariectomized rats at 0900 h on day 2 (metoestrus) and injected with iv) EB + hFSH, v) EB alone or vi) vehicle on days 2 and/or 3. After 1 h of pre-incubation, hemipituitaries were incubated for 75-min period. Media accumulation of LH during the last 15 min of the incubation period (arrow) was used to determine: basal and LHRH-stimulated LH secretion, LHRH self-priming, progesterone (P₄) potentiation of LHRH-stimulated LH secretion and antiprogestin RU486 (RU) effects on LHRH self-priming. LHRH = 10⁻⁸ M LH-releasing hormone, P₄ = 10⁻⁷ M progesterone and RU = 10 nM of the antiprogestin at the receptor RU486.

100 nM P₄ (Sigma) and LHRH + 10 nM antiprogestin RU486 (Exelgyn, Paris, France). The first LHRH challenge lasted 15 min (Fig. 1); after, fresh medium without LHRH was added. Finally, during the last 15 min of the incubation period fresh medium containing LHRH was added. Afterwards, medium was removed for the determination of LH concentration and pituitary responsiveness (Fig. 1). Doses of hormones and drugs used and timing of treatments derive from previous papers (Denner *et al.* 1990, Turgeon & Waring 1991, Sánchez-Criado *et al.* 2004, 2006).

RIA of LH and E₂

Concentrations of LH in serum and incubation media were measured in duplicate by RIA using a double antibody method with kits supplied by the NIH (Bethesda, MD, USA) and a previously described microassay method (Sánchez-Criado *et al.* 1990). Rat LH-I-10 was labelled with ¹²⁵I by the chloramine T method. All serum and media samples were assayed in the same assay. Intra-assay coefficient of variation was 8%, and assay sensitivity was 3.75 pg/tube. LH concentrations were expressed as ng/ml serum of the reference preparation LH-rat-RP-3. Serum E₂ concentrations were determined in duplicate using a commercially obtained kit (Diagnosis Products Corporation, Los Angeles, CA, USA). The sensitivity of the assay was 1 pg/tube, and the intra-assay coefficient of variation was 7%. E₂ concentrations were expressed as pmol/l serum.

PR-AB and PR-B mRNA analysis by semi-quantitative RT-PCR in rat anterior pituitary

Anterior pituitaries were immediately frozen in liquid nitrogen and stored at -80 °C until used for RNA analysis. RT-PCR, optimized for semiquantitative detection, was used

to analyse relative expression levels of PR-AB and PR-B mRNA in pituitaries from experimental groups. For comparative purposes, pituitary PR-AB and PR-B mRNA expression on day 4 in OVX rats on day 2 (metoestrus) was also monitored. Total RNA was isolated from pituitary samples using the single-step, acid guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski & Sacchi 1987). Analysis of total PR-AB mRNA expression was carried out using a primer pair flanking a 326 bp coding area common to both PR-A and PR-B isoforms (Szabo *et al.* 2000, Bellido *et al.* 2003), while assessment of PR-B mRNA levels was performed using a primer combination that enables amplification of a 221 bp fragment from the PR unique to the functional B isoform (Tena-Sempere *et al.* 2004). In addition, to provide an appropriate internal control, parallel amplification of a 249 bp of the S11 ribosomal protein mRNA was carried out for each sample. For amplification of the targets, RT-PCR was run in two separate steps. In addition, to enable appropriate amplification of specific signals and S11 ribosomal protein, transcripts were carried out in separate reactions with different number of cycles but using similar amounts of the corresponding cDNA templates, generated in single RT reactions, as previously described (Tena-Sempere *et al.* 2004). PCRs consisted of a first denaturing cycle at 97 °C for 5 min, followed by a variable number of amplification cycles defined by denaturation at 96 °C for 30 s, annealing for 30 s and extension at 72 °C for 1 min. A final extension cycle of 72 °C for 15 min was included. Annealing temperatures were adjusted for each target: 55 °C for PR-AB, 56 °C for PR-B and 58 °C for RP-S11. These cycling conditions had previously been optimized to ensure amplification of each transcript in the exponential phase PCR (Bellido *et al.* 2003, Tena-Sempere *et al.* 2004). Semiquantitative data from RNA assays were expressed as mean ± s.e.m.

from at least three independent determinations within each experimental group. In all assays, liquid controls and reactions without RT were included, yielding negative amplification (data not shown).

Immunoreactivity of PR protein in the rat gonadotroph

The immunohistochemical study was performed on dewaxed and rehydrated 3 µm thick tissue sections of formalin-fixed, paraffin-embedded tissue samples. The commercial mouse monoclonal anti-human PR antibody clone PR10A9, raised against the recombinant hormone-binding domain of human PR located on the C-terminal domain (Kastner *et al.* 1990) of PR (Immunotech, Marseille, France), diluted 1:15 000, and the avidin–biotin–peroxidase complex technique (Vector, Burlingame, CA, USA) were used as previously described (Sánchez-Criado *et al.* 2004). Accordingly, the antibody recognized both A and B isoforms of PR. Tissue sections from similarly processed samples of rat uterus and human breast carcinoma were used as positive controls. The specificity of the PR antibody was shown by the lack of staining after pre-incubation of tissue sections of rat uterus and pituitaries from OVX rats treated with EB with 10^{-9} , 10^{-7} and 10^{-5} M of the cognate ligand for 1 h at 37 °C. The reaction was blocked completely at 10^{-5} M, a concentration much higher than any physiological title of the hormone. Substitution of the specific primary antibody by mouse ascitic fluid (Sigma) at the same dilution as the specific primary antibody in tissue sections of the cases under study was used as negative control. Nuclear counterstaining was performed with Mayer's haematoxylin in all cases. The number of cells immunoreactive to PR antibody was counted in 15 high power fields (×40) per pituitary tissue section (five sections from each of the three rats per group) and expressed as number of positive nuclei/field.

Statistical analysis

Statistical analysis was performed by ANOVA to test for significant differences among groups. When significant differences existed, ANOVA was followed by the Student–Newman–Keuls multiple range test to compare means. Significance was considered at the 0.05 level.

Results

Effects of hFSH and EB on vaginal cytology and uterine ballooning

On pro-oestrus in intact cycling rats injected with vehicle, hFSH or EB, and on day 4 in day 2 OVX rats injected with EB, with or without hFSH, vaginal smears displayed, predominantly, nucleated epithelial cells characteristic of vaginal pro-oestrus. The uterus was ballooned in all rats except for OVX rats injected with vehicle. Uterine ballooning was more evident in EB- than in vehicle- or hFSH-injected rats.

Effects of hFSH and EB on serum concentrations of E₂ and LH during pro-oestrus and on the number of ova/ovulating rat on oestrus

In the morning of pro-oestrus, no differences in serum LH concentrations (0.6 ± 0.1 ng/ml) were found among groups of intact cycling rats, whereas serum E₂ concentrations were significantly increased in both hFSH- and EB-treated rats (Fig. 2A). In the afternoon of pro-oestrus, coinciding with the peak of the LH surge, serum LH concentrations increased in EB treated, but decreased in hFSH-treated intact cycling rats (Fig. 2B). No differences were noted in the number of ova shed in the oviducts between vehicle- and EB-treated rats. However, the number of ova shed in the morning of oestrus was increased in hFSH-treated rats (Fig. 2C).

Effects of hFSH and EB on pituitary PR-AB and PR-B mRNA and gonadotroph PR protein expression in pro-oestrus

Compared with the levels measured in pituitaries from OVX rats, both PR-AB and PR-B mRNA levels were significantly increased in pituitaries from cyclic rats injected with vehicle, hFSH and EB (Fig. 3). Moreover, the levels of both PR-AB and PR-B mRNA were significantly reduced, by 37.4 and 34.8% respectively, in pituitaries from hFSH-injected cyclic rats with respect to the mRNA levels seen in pituitaries from vehicle-injected pro-oestrous rats (Fig. 3). This similar percentage reduction of PR-AB and PR-B mRNA directly implies that the PR-A/PR-B transcripts ratio did not change after hFSH treatment. No differences were found between vehicle- and hFSH-injected rats either in the intensity of staining or the number of cells immunoreactive to PR antibody in the nuclei of anterior pituitary cells (Fig. 4). It is worth noting the close correlation ($r=0.955$) observed between E₂ levels (Fig. 2A) and the number of pituitary PR-positive cells (Fig. 4) in rats injected with vehicle or treated with hFSH or EB.

Pro-oestrous pituitary responsiveness to LHRH in cycling rats treated with hFSH or EB and primed in vitro with LHRH, P₄ and LHRH+ RU486: effect of OVX on metoestrus

Pituitaries from pro-oestrous cycling rats injected with vehicle exhibited: increased LH secretion in response to LHRH; LHRH self-priming that was significantly reduced by addition of RU486 to the media and P₄ potentiation of LHRH-stimulated LH secretion (Fig. 5). All these responses were significantly ($P<0.05$) augmented in pituitaries from pro-oestrous cycling rats injected with EB. In sharp contrast, pituitaries from hFSH-injected rats did not respond to LHRH, and did not exhibit LHRH self-priming or P₄-potentiated LHRH-stimulated LH secretion. No differences in basal LH secretion among pituitaries from vehicle-, hFSH- or EB-treated rats were noted (Fig. 5).

In comparison with pituitary responsiveness from day 2 OVX rats injected with vehicle (Fig. 6 A), pituitaries from EB-treated OVX rats showed on day 4 LHRH-stimulated LH secretion,

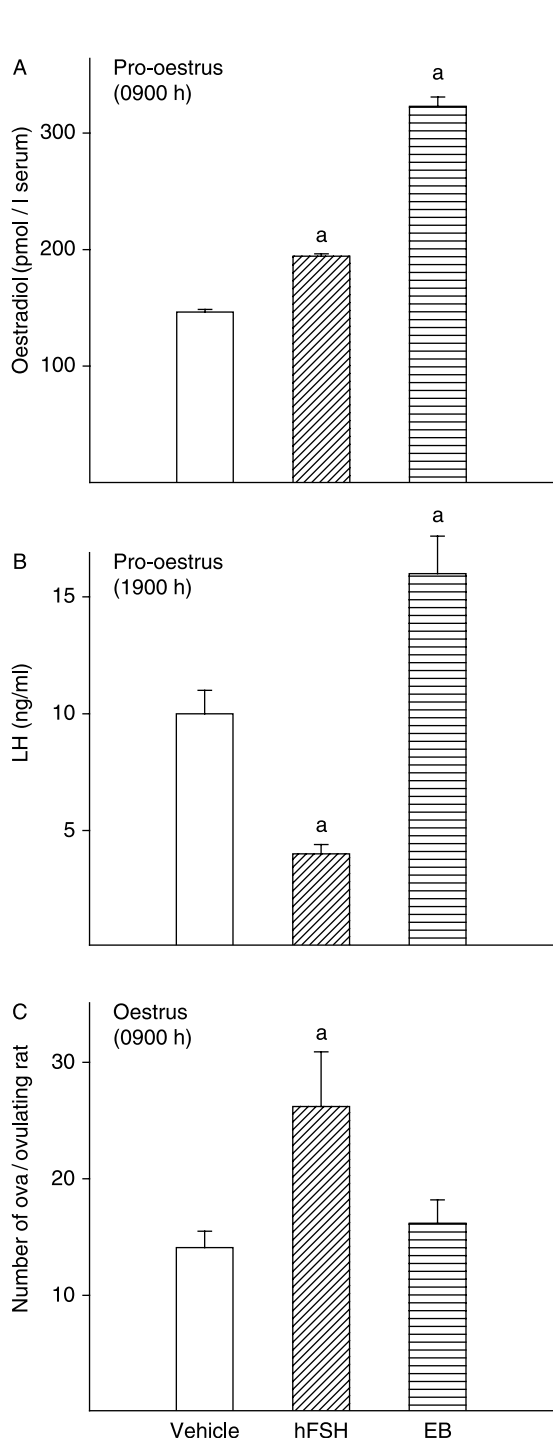


Figure 2 Serum levels of oestradiol-17 β and LH at (A) 0900 and (B) 1900 h on pro-oestrus respectively, and ovulation rate at (C) 0900 h of the next day (oestrus) in intact cycling rats injected with vehicle, hFSH or EB (see legend of Fig. 1 for details of treatment). Values are given as mean \pm s.e.m. of eight rats per group. ^a $P < 0.05$ versus vehicle-injected rats. ANOVA and Student–Newman–Keuls multiple range test. The morning concentrations of LH (0.6 ± 0.1 ng/ml) are not shown.

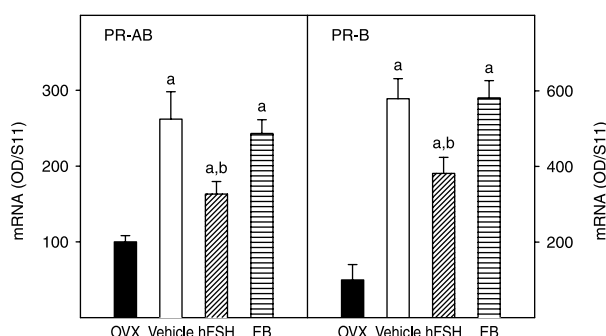
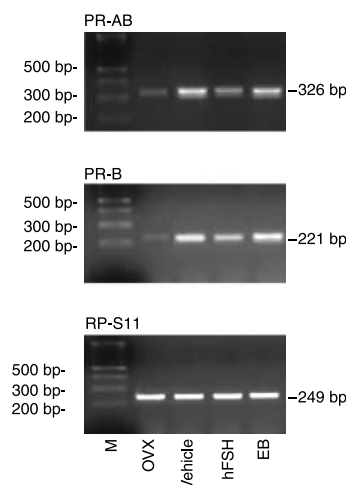


Figure 3 Expression of total progesterone receptor (PR)-AB and PR-B mRNA in pituitaries from intact cycling rats on pro-oestrus injected with vehicle, hFSH or EB (see legend of Fig. 1 for details of treatment). For comparative purposes, pituitary PR mRNA levels at 0900 h on day 4 in rats ovariectomized (OVX) at 0900 h on day 2 (metoestrus) are also shown. Upper panel: representative ethidium-stained gel electrophoresis of PR-AB, PR-B and RP-S11 cDNA fragments amplified by semiquantitative RT-PCR from total pituitary RNA in the experimental groups. Lower panel: semi-quantitative data on the steady-state levels of total PR-AB and PR-B mRNA in experimental groups. Relative expression levels were obtained, in each sample, by normalization of absolute optical densities (ODs) of the specific target to that of RP-S11 signal. Expression levels of PR-AB and PR-B transcripts in OVX rats were taken as 100%, and the other values were normalized accordingly. Values are given as mean \pm s.e.m. of nine independent determinations. ^a $P < 0.05$ versus OVX and ^b $P < 0.05$ versus vehicle. ANOVA and Student–Newman–Keuls multiple range test.

LHRH self-priming, P₄ potentiation of LHRH-stimulated LH secretion and RU486 blockade of LHRH self-priming. These effects of EB in OVX rats, which did not differ from those of intact rats in pro-oestrus (Fig. 5), were not affected by hFSH treatment (Fig. 6).

Discussion

The present results first show that ovarian stimulation with hFSH during the dioestrous phase in the cyclic rat decreased

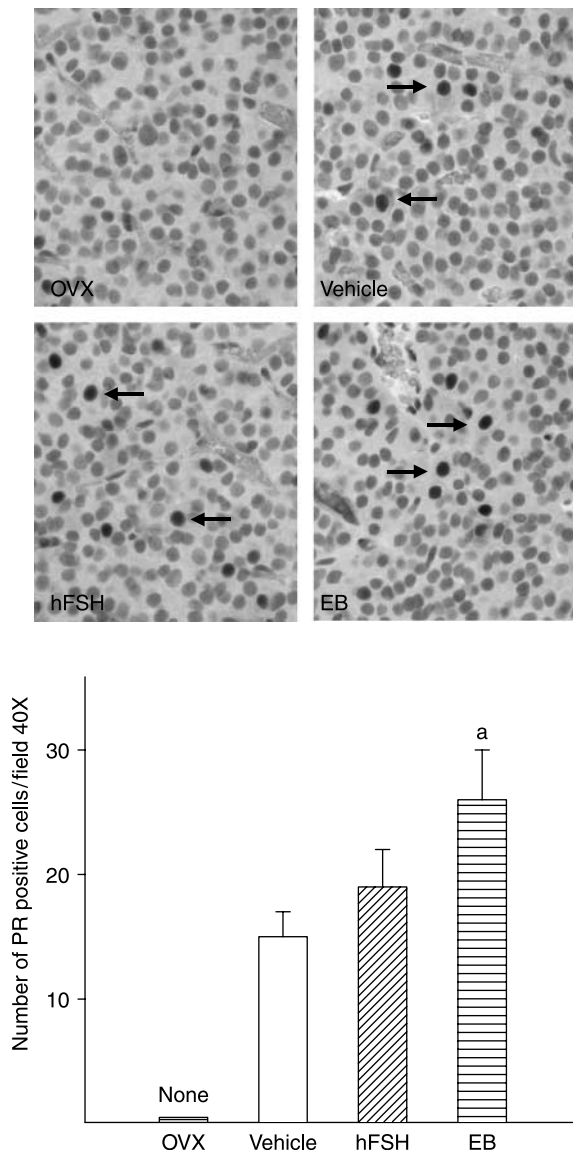


Figure 4 Immunocytochemistry of progesterone receptor (PR) in the anterior pituitaries of intact cycling rats on pro-oestrus injected with vehicle, hFSH or EB (see legend of Fig. 1 for details of treatment). For comparative purposes, pituitaries on day 4 from rats ovariectomized (OVX) on day 2 (metoestrus) are also shown. Numerous immunoreactive nuclei with black or medium grey colour (arrows) are seen in gonadotrophs from intact rats in pro-oestrus. No immunoreactive nuclei were appreciated in OVX rats. ABC technique, nuclei counterstained with Mayer's haematoxylin, $\times 40$. The lower panel shows the number of PR-positive cells (20 fields of about 15 gonadotrophs per rat). Values are the mean \pm S.E.M. of four rats per group. ^a $P < 0.05$ versus oil. ANOVA and Student–Newman–Keuls multiple range test.

serum LH concentration in pro-oestrous afternoon, a basic finding wholly in agreement with previously reported data (Geiger *et al.* 1980, de Koning *et al.* 1987, Culler 1992, Schuiling *et al.* 1999); secondly, that hFSH stimulated the production

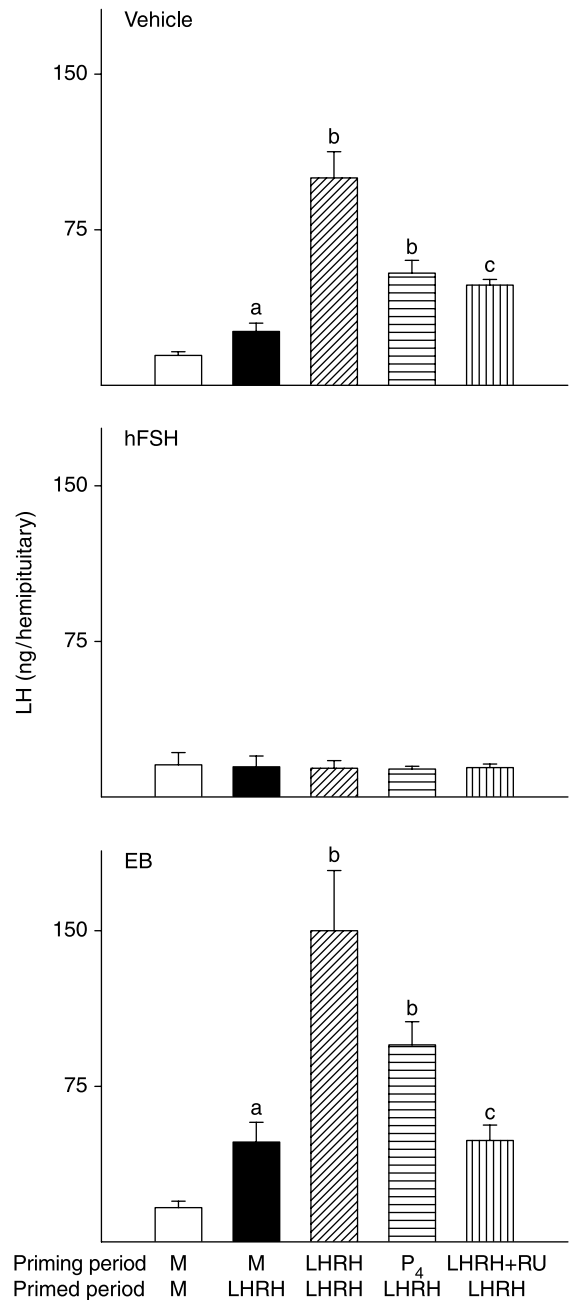


Figure 5 Effects of hFSH and EB treatments during dioestrous phase (see legend of Fig. 1 for details of treatment) on basal (open bars), LHRH-stimulated LH secretion (filled bars) and on LHRH self-priming (hatched bars) in incubated hemipituitaries from pro-oestrous rats. The potentiation effect of progesterone (P₄) during the priming period on LHRH-stimulated LH secretion and the LHRH self-priming blockade by RU486 (RU) during pre-incubation and priming periods are included. ^a $P < 0.05$ versus basal LH secretion; ^b $P < 0.05$ versus LHRH-stimulated LH secretion and ^c $P < 0.05$ versus LHRH self-priming. Values are the mean \pm S.E.M. of eight hemipituitaries. ANOVA and Student–Newman–Keuls multiple range test.

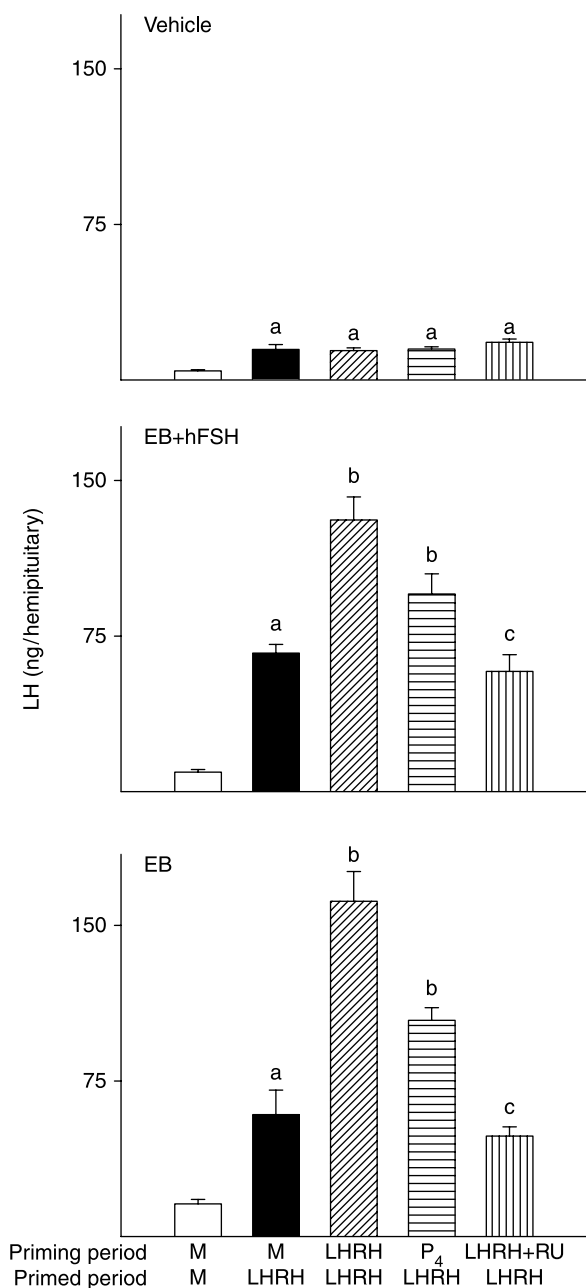


Figure 6 Effects of hFSH and/or EB treatment on days 2 and 3 on basal (open bars) and LHRH-stimulated LH secretion (filled bars) and on LHRH self-priming (hatched bars) in incubated pituitaries from day 2 (metoestrus) OVX rats on day 4. The potentiation effect of progesterone (P₄) during the priming period on LHRH-stimulated LH secretion and the effect of RU486 (RU) during pre-incubation and priming periods on LHRH self-priming on pituitaries collected on day 4 from OVX rats are included. ^aP<0.05 versus basal LH secretion; ^bP<0.05 versus LHRH-stimulated LH secretion and ^cP<0.05 versus LHRH self-priming. Values are the mean ± S.E.M. of eight hemipituitaries. ANOVA and Student–Newman–Keuls multiple range test.

of some ovarian factor(s) other than E₂ that reduced the preovulatory LH surge, but not basal LH secretion; and thirdly, that the ovary-mediated hFSH inhibition of LH surge was exerted on gonadotroph PR action.

Increases in both serum E₂ levels in the morning of pro-oestrus and ovulation rate the following day were observed in hFSH-treated cycling rats. This hFSH-stimulated ovarian activity was associated, however, with a decrease in LH secretion in the afternoon of pro-oestrus. Geiger *et al.* (1980) proposed the existence of an inappropriate feedback of the increased E₂ levels on the LH surge as the mechanism underlying the inhibitory effect of FSH. Nevertheless, administration of EB on dioestrus to intact cyclic rats, a treatment that increases E₂ serum concentrations in the morning of pro-oestrus without ovarian stimulatory activity, did not reduce serum LH concentrations in pro-oestrous afternoon. By contrast, physiological doses (Schuiling *et al.* 1987) of injected EB acted in a very convincing positive feedback manner (Fink 1988) resulting in an increased magnitude of the LH surge. Also, the observed inhibitory effect of hFSH on *in vitro* LH secretion from incubated pituitaries was absent in pituitaries from OVX rats. All these facts demonstrate conclusively that hFSH stimulates the production of some factor(s) of ovarian origin other than E₂, and that these hFSH-dependent ovarian factor(s) inhibit the preovulatory LH surge. A drop in circulating levels of such FSH-dependent ovarian peptides with LH surge-inhibitory bioactivity has been adduced as playing a critical role in switching from negative to positive the action of E₂ on LH secretion (Balen & Jacobs 1991, Fowler & Templeton 1996, Fowler *et al.* 2003).

The positive feedback effect of E₂ at the pituitary level includes: 1) induction of PR expression in gonadotrophs (Fox *et al.* 1990, Sánchez-Criado *et al.* 2004, present results), 2) increase of gonadotroph responsiveness to LHRH (Aiyer & Fink 1974, present results) and 3) induction of gonadotrophin-releasing hormone self-priming (Turgeon & Waring 1994, present results). These E₂ effects trigger, in the obligatory presence of gonadotroph PR (Chappell *et al.* 1999), the preovulatory LH surge (Fink 1988). E₂-dependent PR can be activated by the cognate ligand P₄ from ovarian granulosa cells during pro-oestrous afternoon and evening (Rao & Mahesh 1986) or, in a ligand-independent manner (Blaustein 2004), by intracellular signals of LHRH:cAMP and/or protein kinase C (PKC) (Turgeon & Waring 1994, Fink 1995, Garrido-Gracia *et al.* 2006) shortly after the noon in pro-oestrus (Fink 1988). All these facts, and the present *in vitro* observations of the inhibitory effect of RU486 on LHRH self-priming, demonstrated that the presence of PR and its activation are absolute requirements for the transmission of E₂-induced signals leading to the LH surge. Treatment with FSH reduced only the magnitude of the LH surge with no effect on basal LH secretion (Geiger *et al.* 1980, present experiments), and it would appear that FSH may well decrease the preovulatory LH release by affecting PR expression and/or action (Garrido-Gracia *et al.* 2007).

Unexpectedly, an inhibitory effect of hFSH on pituitary PR-A and PR-B transcripts was unmistakably proved in these

experiments. There is currently no satisfactory explanation for this effect of hFSH, which merits further independent investigation. However, the results clearly indicate that the inhibitory influence of this hFSH-dependent ovarian factor on LH secretion takes place at the gonadotroph PR post-translational level rather than at E₂-dependent (Kastner *et al.* 1990) PR mRNA transcription or PR protein translation levels. This is because no effects of hFSH on PR-A/PR-B mRNA ratio or total immunoreactive PR protein were noted. In addition, hFSH treatment did not affect, either *in vivo* or *in vitro*, basal LH secretion, a finding also in agreement with the PR-independent character of the negative E₂ feedback on LH secretion (Chappell *et al.* 1999, Garrido-Gracia *et al.* 2007). In full agreement with others (Manni *et al.* 1981), a positive translational effect of the E₂ background on PR protein was evidenced. This, as well as the effects of hFSH on PR transcripts, could easily be due to the differential time-course expression and/or stability of PR mRNA and protein (Lee Kraus & Katzenellenbogen 1993).

Although the FSH-dependent ovarian hormones inhibin (Culler 1992, Byrne *et al.* 1995) and follistatin (Wang *et al.* 1990) have been postulated to have GnSI/A bioactivity, the majority of evidence support the view that the inhibitory effect of exogenous FSH on the LH surge is mainly due to the activity of the putative GnSI/AF (de Koning *et al.* 1987, Balen & Jacobs 1991, Balen 1996, Byrne *et al.* 1996, Fowler & Templeton 1996, de Koning *et al.* 2001, Fowler *et al.* 2003). GnSI/AF is a non-steroidal, non-inhibin ovarian hormone of ~60–70 kDa (Fowler *et al.* 2003) secreted by granulosa cells in response to FSH with the specific bioactivity of inhibiting LHRH self-priming (Balen 1996, Fowler & Templeton 1996, Fowler *et al.* 2003). This action of GnSI/AF keeps LH concentrations low despite the positive feedback effect of E₂ at the pituitary level during the late follicular phase (de Koning 1995). In addition, an FSH-dependent ovarian non-steroidal oestrogen-antagonizing factor, different from GnSI/AF, has been postulated to specifically suppress the sensitizing effect of E₂ on LHRH-stimulated LH secretion (Schuiling *et al.* 1999). The results of the present experiments demonstrate that hFSH treatment, like RU486 (Rao & Mahesh 1986, Sánchez-Criado *et al.* 1990), reduced both PR-dependent E₂ actions and, accordingly, attenuated the PR-dependent (Chappell *et al.* 1999) LH surge.

Irrespective of the nature of the ovarian factor involved in the FSH suppression of preovulatory LH secretion, it acts on gonadotroph membrane receptors different from those of LHRH (Fowler & Templeton 1996). This implies the existence of an intracellular functional antagonism between GnSI/AF and LHRH (de Koning 1995). Though we cannot rule out an effect through other pathways, present results have shown that the action of the putative ovarian GnSI/AF must have suppressive effects on gonadotroph PR activation through interference with the actions of P₄ and the second messenger pathway of LHRH in the gonadotroph. In perfused rat hemipituitaries, steroid-free bovine follicular fluid, which possesses GnSI/AF bioactivity, antagonizes the priming action of 8-bromo-cAMP and phorbol 12-myristate

13-acetate (PMA) on LH secretion (Tijssen *et al.* 1997). Also, it appears that GnSI/AF from follicular fluid of women undergoing superovulation treatments suppresses the potentiating effect of E₂, P₄ and PMA on LHRH-stimulated LH secretion (Fowler & Templeton 1996). These findings indicate that GnSI/AF has widespread suppressive effects on the second messenger pathway transducing the LHRH signal within the gonadotroph. In the present experiments, neither P₄ nor LHRH potentiated LHRH effect on LH secretion in FSH-treated cycling rats, even in the presence of both E₂ and E₂-dependent PR. This ovary-mediated effect of hFSH could be pointing to an inefficient response of gonadotroph PR to physiological phosphorylation/activation induced by P₄ and PKA or PKC (Denner *et al.* 1990, Zhang *et al.* 1994), which are ligand-dependent and -independent activators of PR respectively. It has been found that E₂ inhibits PR action and LHRH self-priming by activating membrane ER α of the gonadotroph both *in vitro* (Sánchez-Criado *et al.* 2005) and *in vivo* (Garrido-Gracia *et al.* 2007). The inhibitory action of activated membrane ER α on LH secretion is reverted by inhibition of intracellular phosphatases and mimicked by the stimulation of protein phosphatases (Sánchez-Criado *et al.* 2006). Similarly, intracellular signals of the obligatory activation of gonadotroph membrane receptors with GnSI/AF may have blocked both P₄-dependent and ligand-independent phosphorylation/activation of E₂-induced expression of PR in gonadotrophs. Whether or not the inhibitory action of FSH on LH surge is the result of an imbalance of phosphorylase/phosphatase activities on the phosphoprotein transcription factor PR (Evans 1988, Mangelsdorf *et al.* 1995) in the gonadotroph is currently under investigation.

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