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Ovarian stimulation with FSH reduces phosphorylation of gonadotrope progesterone receptor and LH secretion in the rat

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Abstract

Administration of human FSH (hFSH) to cyclic rats during the dioestrous phase attenuates progesterone receptor (PR)-dependent events of the preovulatory LH surge in pro-oestrus. The increased bioactivity of the putative ovarian gonadotropin surge inhibiting/attenuating factor induced by hFSH treatment is not associated with a decrease in PR protein expression, and the possibility of its association at a PR posttranslational effect has been raised. The present experiments aimed to analyse PR phosphorylation status in the gonadotrope of rats with impaired LH secretion induced by *in vivo* hFSH injection. Two experimental approaches were used. First, incubated pro-oestrous pituitaries from hFSH-injected cycling and oestrogen-treated ovariectomized (OVX) rats were used to analyze the effect of calyculin, an inhibitor of intracellular phosphatases, on PR-dependent LH release, which was measured in the incubation medium by RIA. Second, pituitaries taken from hFSH-injected intact cycling and OVX rats and later incubated with P or GNRH1 were used to assess the phosphorylation rate of gonadotrope. The latter was analysed in formalin-fixed, paraffin-embedded tissue sections by immunohistochemistry using a MAB that recognizes the phosphorylated (p) form of PR at Ser294. Calyculin reduced the ovary-mediated inhibition of hFSH in GNRH1-stimulated LH secretion. In addition, the immunohistochemical expression of pSer294 PR was significantly reduced after ovarian stimulation with hFSH in pituitaries from pro-oestrous rats incubated with P or GNRH1. Altogether, these results suggested that the ovarian-dependent inhibitory effect of FSH injection on the preovulatory LH secretion in the rat may involve an increase in dephosphorylation of PR.
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Introduction

In the rat, exogenous FSH increases and/or prolongs the bioactivity (secretion and/or action) of putative gonadotropin surge inhibiting/attenuating factor (GnSI/AF; de Koning 1995, Fowler *et al*. 2003, Messinis 2006), a non-steroidal and non-inhibin ovarian factor (Fowler *et al*. 1993, 2003, Byrne *et al*. 1995). GnSI/AF negatively regulates GNRH1-stimulated LH secretion (Tijssen *et al*. 1997) and GNRH1 self-priming (Koppenaal *et al*. 1991, Fink 1995). These two events depend on gonadotrope progesterone receptor (PR; Chappell *et al*. 1999, Gordon *et al*. 2008).

The mechanism of action of GnSI/AF is largely unknown (Fowler & Templeton 1996, Messinis 2006), although some data are available. Thus, GnSI/AF neutralizes downstream actions of second messengers involved in GNRH1 self-priming (Fowler & Templeton 1996, Tijssen *et al*. 1997), and this action is associated with a blockade of gonadotrope PR action (Byrne *et al*. 1996) rather than with an inhibition of PR expression (Gordon *et al*. 2008). The findings reported by Gordon *et al*. (2008) also suggest that the inhibitory influence of GnSI/AF on preovulatory LH secretion is exerted at gonadotrope PR posttranslational level. Phosphorylation is a posttranslational modification known to modulate the activity of the hormone-activated transcription factor PR (Denner *et al*. 1990, Faus & Haendler 2006), which appears to occur at serine (Ser) residues exclusively (Beck *et al*. 1992, Takimoto & Horwitz 1993).

Former studies from our group had shown that membrane oestrogen receptor-initiated intracellular signalling enhances gonadotrope protein phosphatase activity and results in the inhibition of GNRH1 self-priming (Sánchez-Criado *et al*. 2006, Garrido-Gracia *et al*. 2007). The aim of the present study was to analyse PR phosphorylation status in the gonadotrope of rats with impaired LH secretion induced by GnSI/AF. For this purpose, the biological activity of endogenous GnSI/AF was first increased in cycling rats by the administration of FSH (Geiger *et al*. 1980, Koppenaal *et al*. 1991, Culler 1992). Then, pro-oestrous pituitaries from these rats were used to analyse the following: first, the *in vitro* effect of calyculin, a potent inhibitor of intracellular phosphatases (Condrescu *et al*. 1999), on GNRH1-stimulated LH

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secretion and GNRH1 self-priming, and second, the *in vitro* effect of ligand-dependent and -independent activators of PR on the immunohistochemical (IHC) expression of PR phosphorylated at the Ser residues level.

Results

Effects of human FSH (hFSH) treatment on vaginal cytology, uterine ballooning and serum 17β-oestradiol (E_2) concentration in intact cycling and OVX+oestradiol benzoate (EB) rats

Details of the incubation protocols used are given in the Materials and Methods and summarized Fig. 1. On day 4, vaginal smears from intact cycling and OVX+EB rats treated or not with hFSH displayed nuclear epithelial/cornified cells characteristic of vaginal pro-oestrus. Also, uteri were ballooned to a similar extent in all four groups of rats. However, differences in E_2 serum concentrations were noted. Whereas treatment with hFSH increased significantly serum E_2 levels in intact cycling rats, treatment with hFSH did not increase serum E_2 concentrations in OVX+EB rats (Fig. 2).

Effects of hFSH treatment on GNRH1-, phorbol 12-myristate 13-acetate (PMA)-, forskolin (FSK)-, calyculin A- and ionomycin-induced LH secretion in intact cycling and OVX+EB rat pituitaries

Pituitaries from pro-oestrous cycling rats injected with saline exhibited increased LH secretion in response to GNRH1; GNRH1 self-priming; and PMA, FSK and calyculin potentiation of GNRH1-stimulated LH secretion (Fig. 3). In contrast, pituitaries from intact cycling rats treated with hFSH did not display a significant increase in LH secretion in response to GNRH1, PMA and FSK with respect to LH secretion from pituitaries incubated with medium alone. Calyculin at the dose of 10^{-7} M significantly increased GNRH1 pituitary responsiveness in pituitaries from hFSH-treated intact cycling rats (Fig. 3). Moreover, this increasing effect of calyculin on GNRH1-stimulated LH secretion was demonstrated to be dose-dependent (Fig. 4). The addition of the Ca^{++} ionophore ionomycin to the incubation medium resulted in a massive release of LH in pituitaries from cyclic rats injected with saline but not in pituitaries from cyclic rats injected with hFSH. No differences were found in LH secretion between calyculin+GNRH1 and ionomycin in intact cycling rats treated or not with hFSH. No differences for any LH secretion parameter were noted on day 4 between pituitaries from OVX+EB rats whether or not treated with hFSH (Fig. 3).

Effect of hFSH treatment on pituitary LH content in intact cycling and OVX+EB rats

Treatment with hFSH decreased pro-oestrous pituitary LH content in intact cycling rats. This inhibitory effect of hFSH on pituitary LH content was not observed in OVX+EB rats on day 4 (Fig. 5).

Effect of FSH treatment on pituitary PR10A9 expression in intact cycling and OVX+EB rats

No significant effect of hFSH was found in the number of PR10A9-positive cells in either intact cyclic or OVX+EB rats (Figs 2 and 6A). The number of PR10A9-positive

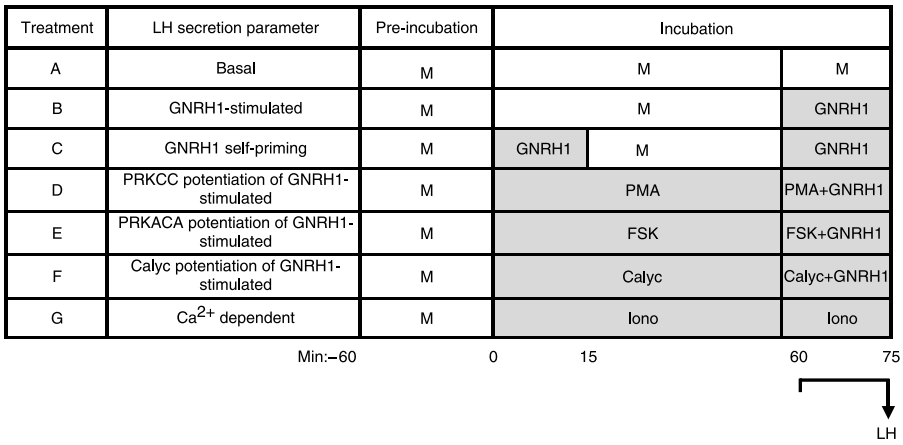

Figure 1 Diagram showing the pituitary incubation protocol used in each of the four groups of rats: intact cycling+saline or hFSH, ovariectomized (OVX)+oestradiol benzoate (EB)+saline or hFSH (see the Materials and Methods section for details of treatments). After 1 h of pre-incubation with medium (M) alone, hemipituitaries from the four groups of rats were incubated for a 75-min period. Medium accumulation of LH during the last 15 min of the 75-min incubation period (arrow) was used to determine (A) basal LH secretion, (B) GNRH1-stimulated LH secretion, (C) GNRH1 self-priming, (D) PRKCC potentiation of GNRH1-stimulated LH secretion, (E) PRKACA potentiation of GNRH1-stimulated LH secretion, (F) calyculin A (calyc) effect on GNRH1-stimulated LH secretion and (G) Ca^{++}-dependent LH secretion. GNRH1=10^{-8} M LH-releasing hormone; PMA=10^{-7} M of the PRKCC activator phorbol 12-myristate 13-acetate; FSK=5×10^{-5} M of the activator of adenylyl cyclase forskolin; calyc=10^{-7} M of the inhibitor of intracellular phosphatases calyculin A; iono=5×10^{-5} M of the Ca^{++} ionophore ionomycin.

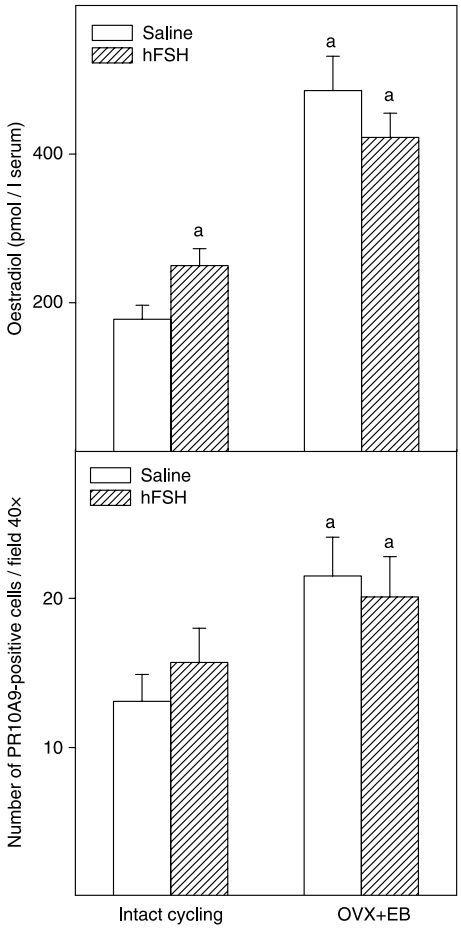

Figure 2 Effect of hFSH treatment on serum 17β-oestradiol (E_2) concentrations (pmol/l serum) and the number of pituitary PR10A9-positive cells/field 40× at 0900 h on day 4 (pro-oestrus in intact cycling rats) and in OVX rats injected with EB (see the Materials and Methods section for details of treatments). Values are means±S.E.M. of eight rats. $^{a}P<0.05$ versus intact cyclic rats+saline (ANOVA and Student–Newman–Keuls multiple range test).

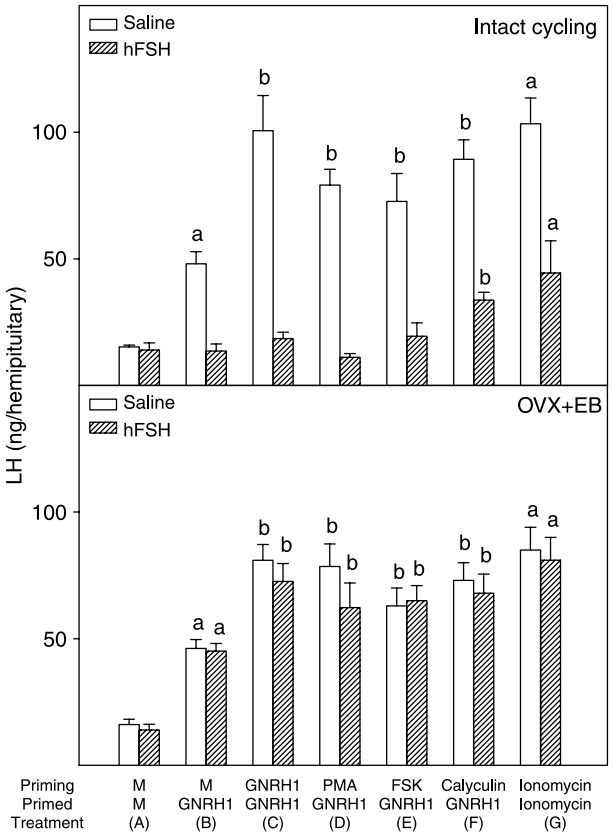

Figure 3 Effect of hFSH treatment on LH release to the medium (ng/hemipituitary) during the last 15 min of incubation by pituitaries from: intact cycling or OVX rats injected with EB (see the legend of Fig. 1 for details of treatments). Values are mean±S.E.M. of eight hemipituitaries. $^{a}P<0.05$ versus pituitaries incubated with medium (M) alone, and $^{b}P<0.05$ versus GNRH1-stimulated LH secretion ANOVA and Student–Newman–Keuls multiple range test. Priming and primed: test substances during the first 60 min or the last 15 min of the incubation period respectively.

pituitary cells in OVX+EB rats was significantly higher than that in intact cycling rats (Fig. 2). A high correlation ($r=0.987$; $y=0.01x+8.91$) was found between serum E_2 concentrations and the number of PR10A9-positive pituitary cells.

Pituitary pSer294 expression in pro-oestrous cycling rats

Immunoreactive products to pSer294 MAB, diluted 1:300, were observed in the nuclei of some pituitary cells (Fig. 6). The number of pSer294-positive cells was higher in pituitaries incubated for 1 or 2 h either with P or GNRH1 than in those incubated with medium alone (Figs 6B and 7). This effect, which was more evident in pituitaries incubated with the cognate ligand (the effect of P was about threefold higher than that of GNRH1), was demonstrated to be dose dependent (Fig. 7).

Effect of hFSH treatment on P- and GNRH1-induced pSer294 expression in intact cycling and in OVX+EB rat pituitaries

The number of cells expressing pSer294 was significantly reduced in pituitaries from hFSH-treated cycling rats incubated either with P or GNRH1. In contrast, the number of gonadotropes expressing pSer294 was not affected by hFSH in OVX+EB rats injected regardless of incubation conditions (Fig. 8).

Discussion

The results of the present study showed that the putative ovarian GnSI/AF action on PR-dependent LH secretion in pro-oestrous rats was associated with an increase in gonadotrope PR dephosphorylation. In physiological conditions, the bioactivity of GnSI/AF is expressed in dioestrus (Tijssen *et al.* 1997), but it is prolonged up to pro-oestrus under the influence of exogenous FSH

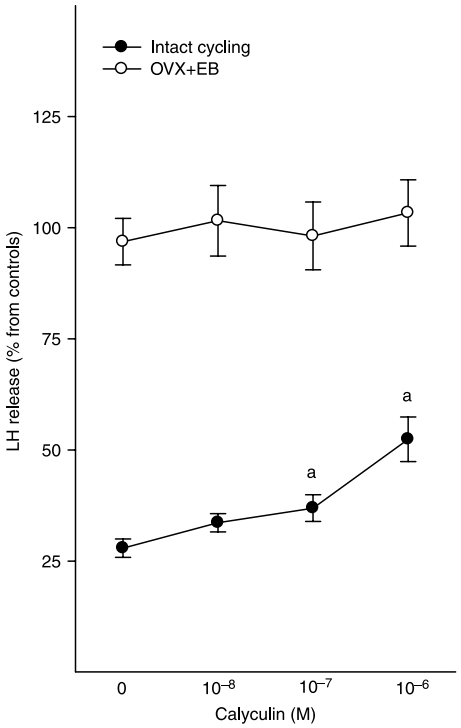

Figure 4 Dose-dependent sensitization effect of calyculin to GNRH1 in pituitaries from intact cycling or OVX + EB rats injected with hFSH (see the Materials and Methods section and legend of Figs 1 and 3 for details of treatments). Values were expressed as the percentage of LH release from controls (pituitary LH release from intact cyclic or OVX rats injected with saline). Values are mean ± S.E.M. of eight hemipituitaries. ANOVA and Student–Newman–Keuls multiple range test. [a]$P<0.05$ versus LH release without calyculin.

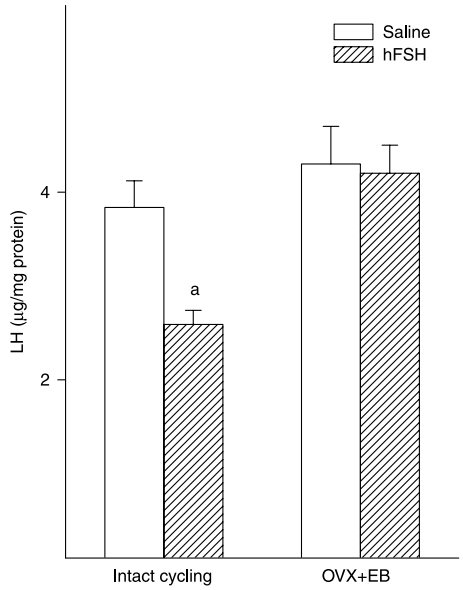

Figure 5 Pituitary LH content (µg/mg protein) on day 4 in intact cycling and OVX + EB rats injected with hFSH or saline (see the Materials and Methods section and legend of Fig. 1 for details of treatments). Values are mean ± S.E.M. of eight pituitaries. ANOVA and Student–Newman–Keuls multiple range test. [a]$P<0.05$ versus intact cycling.

(present results). These facts are in agreement with previous results showing that the decrease in serum FSH concentration during dioestrus reduces and/or shortens GnSI/AF bioactivity and advances the preovulatory LH release in cyclic rats by 1 day (Tebar *et al.* 1998). Overall, the present results suggested that PR dephosphorylation was involved in the ovary-mediated FSH inhibitory effect on GNRH1-stimulated LH secretion (Tijssen *et al.* 1997).

The inhibitor of intracellular phosphatases calyculin sensitized only in part the incubated pituitaries from intact cycling pro-oestrous rats injected with hFSH to the releasing action of GNRH1. In contrast, calyculin fully primed the incubated pituitaries collected from saline-injected intact cycling pro-oestrous rats, as well as those collected from OVX + EB rats, to the releasing action of GNRH1 regardless of whether they were treated or not with hFSH. Pituitaries collected from pro-oestrous rats treated with hFSH had low pituitary responsiveness to well-known secretagogue and priming phosphorylating agents (GNRH1, PRKCC and PRKACA). This was also observed in a previous report from our laboratory in which the potentiating effect of the cognate phosphorylating ligand P on GNRH1-stimulated LH secretion was found to be absent in hFSH-treated cyclic rats (Gordon *et al.* 2008). OVX reversed all inhibitory actions of hFSH on gonadotrope function (Gordon *et al.* 2008, present results). This effect of OVX indicated that the inhibitory effects of hFSH on LH secretion were mediated by an ovarian factor (de Koning *et al.* 1987, Balen 1996) that was found to be non-steroidal in nature (Fowler *et al.* 2003, Messinis 2006). As calyculin inhibits phosphatase-induced dephosphorylation, these findings seemed to indicate that the putative GnSI/AF produces an imbalance of phosphorylase/phosphatase activities on gonadotrope PR, limiting the magnitude of the PR-dependent LH secretion. In addition, lower LH synthesis may also exist in intact cycling rats injected with hFSH. This is because treatment with hFSH reduced pituitary LH content in intact cycling rats but not in OVX rats. Furthermore, the Ca^{++} ionophore ionomycin induced, as expected, a vast LH exocytosis in pituitaries from all experimental groups, except for pituitaries from intact cycling rats given hFSH, in which LH exocytosis was only modest.

In rats injected with hFSH, non-steroidal ovarian substances inhibin (Ying 1988) and GnSI/AF (Messinis 2006) are ovarian factors probably involved in the attenuation of the preovulatory LH surge (Culler 1992). However, accumulated evidence indicates that GnSI/AF is different from inhibin in many respects (Scott & Burger 1981, de Koning *et al.* 1987, Balen *et al.* 1995, Fowler *et al.* 2003). In this regard, the findings by Ishigame *et al.* (2004) are of interest because they show attenuated LH surges in rats after ovarian stimulation with FSH in the absence of bioactive inhibin. As a matter of fact, the negative feedback of ovarian inhibin on FSH release is via interaction with a specific receptor at the gonadotrope membrane

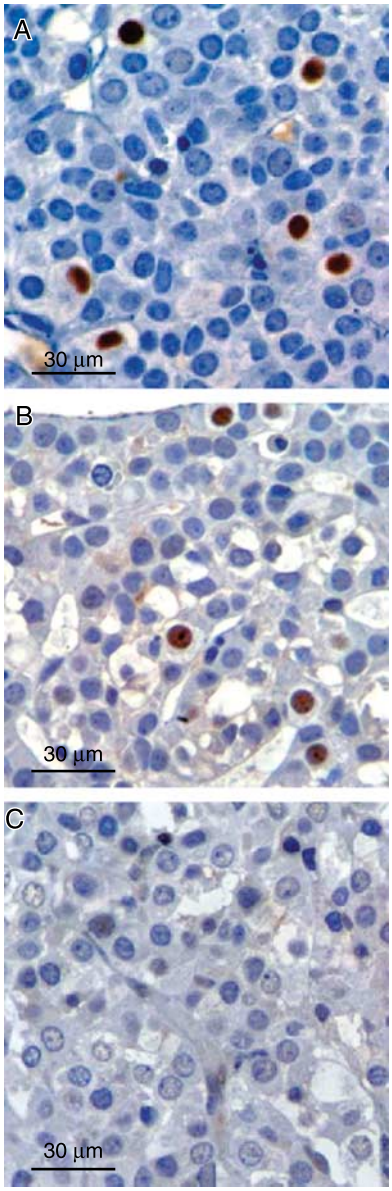

Figure 6 Immunohistochemical (IHC) expression of progesterone receptors (PR) and phosphorylated PR in anterior pituitary cells. (A) Immunoreactive products to PR10A9 antibody are seen in the nuclei of some cells from pituitaries incubated with medium alone. (B) Also, immunoreactive products to pSer294 antibody are observed in the nuclei of cells from pituitaries incubated with 10^{-6} M progesterone over two hours, (C) but not with medium alone. Note that the number of phosphorylated PR is lower (B) than the number of PR (A). See the lower panel of Fig. 2 for the total number of E_2-dependent pituitary PR10A9-positive cells and Fig. 8 for ligand-dependent and -independent pituitary pSer294-positive cells in the four groups of rats. ABC method.

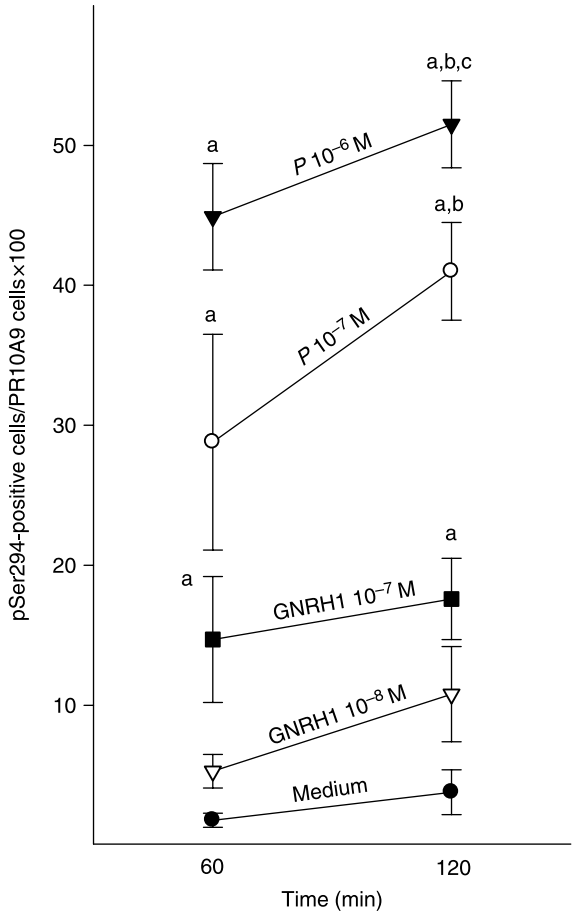

Figure 7 Ratio of pSer294/PR10A9-positive cells in pro-oestrous pituitaries from cycling rats after incubation over 60 or 120 min with medium alone or with different doses of progesterone (P) or GNRH1. See the Materials and Methods section for details. Values are mean ± S.E.M. of three pituitaries. ANOVA and Student–Newman–Keuls multiple range test. $^{a}P<0.05$ versus medium, $^{b}P<0.05$ versus GNRH1, and $^{c}P<0.05$ versus $P\ 10^{-7}$ M.

(Robertson *et al.* 2000), and involves inhibition of both secretion and synthesis of FSH (Scott & Burger 1981, Attardi *et al.* 1991). Similarly, the GnSI/AF acting on specific gonadotrope membrane receptors different from GNRH1 receptors (Fowler & Templeton 1996) reduced preovulatory LH secretion and pituitary LH synthesis.

The balance between the activities of protein kinases and phosphatases (Weigel & Moore 2007) is the most common posttranslational processing event of PR (Faus & Haendler 2006). Thus, phosphorylation seemed to be involved in PR-regulated gene transcription (Denner *et al.* 1990, Moore *et al.* 2007). Studies on PR (Beck *et al.* 1992, 1996) indicate that only Ser residues are phosphorylated (Takimoto & Horwitz 1993), and that Ser294 phosphorylation is strongly hormone dependent (Moore *et al.* 2007). The cognate agonist and the intracellular messengers of GNRH1 (Denner *et al.* 1990, Bai *et al.* 1997) phosphorylate Ser294 and induce PR-mediated transcription (Zhang *et al.* 1994), expression of PR-dependent GNRH1 self-priming proteins (Fink 1995, Tijssen *et al.* 1997), preovulatory LH secretion (Fink 1988; Fig. 9) and PR down-regulation (Lange *et al.* 2000). We have standardized the IHC method for the detection of PR with a phosphorylated Ser residue at position 294 in rat pituitaries. The antibody pSer294 recognizes the immunogen corresponding to

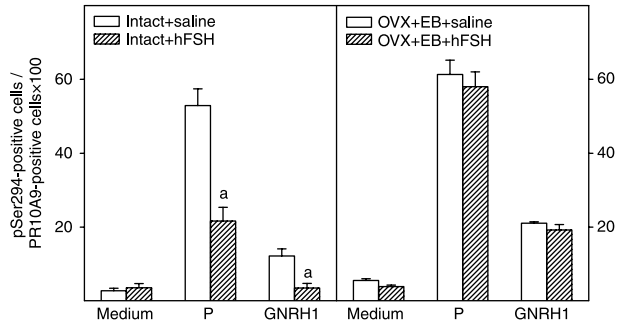

Figure 8 Effect hFSH treatment (see the Materials and Methods section and the legend of Fig. 1 for details of treatment) in intact cycling or OVX rats injected with EB in the ratio of pSer294/PR10A9-positive cells in pituitaries incubated for 2 h with medium alone or 10^{-6} M progesterone (P) or 10^{-7} M GNRH1. See the legend of Fig. 7 and the Materials and Methods section for details of positive cells counting. ANOVA and Student–Newman–Keuls multiple range test. $^{a}P<0.05$ intact cycling rats injected with saline.

amino acid residues 288–300 from human PR with Ser294 being phosphorylated, P(288)MAPGRS(p)PLATTV(300) (Clemm *et al.* 2007). PR alignment revealed an almost full-sequence homology of amino acid residues between the human immunogen and rat PR: P(286)-VAPGRSPLATTV(298). The effect of different phosphorylating agents on incubated pituitaries was expressed as the percentage of PR (PR10A9)-positive pituitary cells expressing pSer294 immunoreactive products. In the rat, gonadotropes are the only pituitary cell type expressing PR (Fox *et al.* 1990, Turgeon & Waring 2000, Sánchez-Criado *et al.* 2004), and the present experiments showed a high correlation between E_2 serum levels and the number of pituitary PR10A9 immunoreactive cells. Our results also showed that gonadotropes exhibited a low PR phosphorylation rate (pSer294 expression) in the absence of P or GNRH1 in the incubation medium, while both P and GNRH1 induced pSer294 immunoreactivity in a dose- and time-dependent manner. This ligand-dependent and -independent phosphorylation of gonadotrope PR was significantly reduced in pituitaries from rats undergoing the ovary-mediated inhibitory action of hFSH. Similarly, it has been shown that treatment with alkaline phosphatases inhibits PR phosphorylation in T47D cells (Takimoto & Horwitz 1993). Finally, PR is expressed as two isoforms: PR-A and PR-B (Kastner *et al.* 1990). While PR-B is the stronger transcriptional activator isoform of PR, PR-A is a modulatory isoform of PR-B action (Kazmi *et al.* 1993). As the antibody pSer294 has preferential affinity for PR-B (Moore *et al.* 2007, Weigel & Moore 2007), results of the present study suggest that the deficient phosphorylation/activation of PR-B isoform could be a probable causative factor of the ovary-mediated hFSH-induced attenuation of LH secretion (Fig. 9).

In conclusion, the results suggested that the inhibitory effect of the FSH-dependent putative ovarian bioactive GnSI/AF on the preovulatory LH secretion in the rat may be due to dephosphorylation of gonadotrope PR.

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 (lights on at 0500 h) and 22±2 °C room temperature, with access to rat chow and tap water *ad libitum*. Rats were included in the experiments after showing at least three consecutive 4-day regular oestrous cycles. Vaginal smears were taken 6 days/week and the day of vaginal oestrus was arbitrarily considered as day 1. Bilateral ovariectomy (OVX) was performed under light ether anaesthesia at 0900 h on day 2 of the cycle. Rats were decapitated at 0900 h on day 4; their

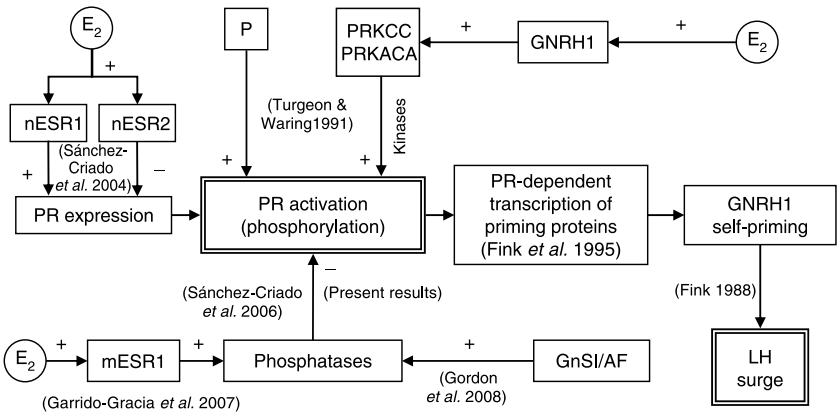

Figure 9 Schematic showing the proposed interrelationship of the cellular and molecular components that may be involved in the regulation of progesterone receptor (PR) activity and LH release. At the pituitary level, E_2 activates nuclear (n) ESR1 and nESR2 which, in addition to many other priming effects, induces PR expression. These PR become activated/phosphorylated by P and/or the E_2-dependent GNRH1-intracellular messengers PRKCC and PRKACA. Also at the pituitary level, E_2 activates gonadotrope membrane (m) ESR1, which increases intracellular phosphatases activity. The latter can also be activated by GnSI/AF (present paper). The balance between intracellular kinases and phosphatases activities elicits PR-dependent transcription of priming proteins, GNRH1 self-priming and LH surge.

pituitary glands removed, separated from the neural lobe, divided in halves and assigned to incubation, pituitary LH content or IHC studies. 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.

Treatment with hFSH

Recombinant hFSH (Gonal-f; Serone, Aubonne, Switzerland) was dissolved at a concentration of 50 IU/ml saline and 10 IU hFSH was given s.c. at 1400 h on day 2 and at 0900 h on day 3 (Gordon *et al.* 2008). This dose of hFSH derives from the results of previous experiments (Gordon *et al.* 2008). Non-stimulated controls were given 0.2 ml saline. Groups of rats treated with hFSH included intact cycling rats (intact cycling + hFSH) and ovariectomized (OVX) rats treated with EB (OVX + EB + hFSH). OVX rats were given 5 and 10 μg EB/0.2 ml oil at 1400 h on day 2 and at 0900 h on day 3 respectively (Schuiling *et al.* 1999).

Rat pituitary incubation studies

General incubation protocol of rat pituitaries

Incubation of pituitaries was carried out, with minor modifications, as described previously (Gordon *et al.* 2008). Briefly, hemipituitaries were divided into quarters (Knox & Schwartz 1993) and incubated at 37 °C with constant shaking (60 cycles/min) in an atmosphere of 95% O_2 and 5% CO_2. Each incubation vial contained 250 μl Dulbecco's modified medium without L-glutamine and phenol red and containing glucose (4.5 g/l), 10^{-8} M E_2 (Sigma Chemical Co.) and BSA (0.1% w/v, pH 7.4). After 1-h pre-incubation without test substances, hemipituitaries were incubated for 75 min with different test substances (Fig. 1). In the last 15 min, the 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, Gordon *et al.* 2008).

Effects of hFSH treatment on GNRH1-, PMA-, FSK-, calyculin A- and ionomycin-induced LH secretion in intact cycling and OVX + EB rat pituitaries

Hemipituitaries of the four groups of animals (intact cycling + hFSH, intact cycling + saline, OVX + EB + hFSH and OVX + EB + saline) were incubated (see general incubation protocol) with different test substances (Fig. 1). Test substances included were: 10^{-8} M GNRH1 (Peninsula 7201, Peninsula Lab. Inc., Merseyside, UK), 10^{-7} M of the PRKCC activator PMA (Sigma), 5×10^{-5} M of the activator of adenylyl cyclase FSK (Sigma) and 10^{-7} M calyculin A (Sigma), an inhibitor of intracellular phosphatases (Marantz *et al.* 1995, Condrescu *et al.* 1999). Whereas PMA, FSK and calyculin A were present during the entire 75-min incubation period, GNRH1 challenge was present during the first 15 min only (Fig. 1). Except for hemipituitaries incubated with medium alone and ionomycin, fresh medium containing the corresponding test substance (medium, PMA, FSK or calyculin) plus GNRH1 was added during the last 15-min period. Finally, to evaluate the secretory capacity of the gonadotropes, hemipituitaries from intact cycling + saline, intact cycling + hFSH, OVX + EB + saline and OVX + EB + hFSH rats were incubated with 5×10^{-5} M of the ionophore ionomycin (Sigma) during the 75-min incubation period. Additional hemipituitaries from the four experimental groups of rats were incubated in the presence of increasing doses of calyculin (0, 10^{-8}, 10^{-7} and 10^{-6} M) during the 75-min incubation period and challenged with GNRH1 during the last 15 min of incubation. Values in this last experiment were expressed as the percentage of LH release from controls (pituitary LH release from cyclic or OVX + EB rats injected with saline).

Concentrations of LH in incubation media were measured in duplicate by RIA using a double-antibody method with kits supplied by NIH (Bethesda, MD, USA) and a previously described microassay method (Sánchez-Criado *et al.* 1990, Garrido-Gracia *et al.* 2007). Rat LH-I-10 was labelled with ^{125}I by the chloramine-T method (Greenwood *et al.* 1963). All media samples were assayed in the same test. The intra-assay coefficients of variation were 8%. Assay sensitivity was 3.75 pg/tube. Results are expressed as ng/hemipituitary of the RP LH-rat-RP-3.

Effect of hFSH treatment on anterior pituitary LH content and E_2 serum levels

Anterior pituitaries from the four groups of animals were obtained at 0900 h on day 4 to evaluate the LH content by RIA and the protein content by the Bradford method (Bradford 1976) using the Quick Start Bradford protein assay (Bio-Rad Laboratories, Inc). Trunk blood was obtained from these rats, allowed to clot, centrifuged and the serum stored at −20 °C until quantified for E_2. Pituitary LH content was measured in duplicate by the RIA method described above. Results are expressed as μg/mg pituitary protein.

Serum E_2 concentrations were determined in duplicate using a commercially obtained kit (Abbott Diagnostic Division). The sensitivity of the assay was 10 pg/ml, and the intra-assay coefficient of variation was 7%. E_2 concentrations were expressed as pmol/l serum.

IHC studies of the PR phosphorylation status in rat pituitaries

IHC expression of PR in gonadotropes

The commercial mouse monoclonal anti-human PR antibody (PR10A9), raised against the recombinant hormone-binding domain of human PR located in the C-terminal domain (Kastner *et al.* 1990) of PR (Immunotech, Marseille, France), diluted 1:15 000, was analysed using the avidin–biotin–peroxidase complex (ABC) IHC method as described elsewhere (Sánchez-Criado *et al.* 2004, 2006, Gordon *et al.* 2008). The number of cells immunoreactive to PR10A9 antibody was determined by counting all positive nuclei in 8–15 fields at a magnification of 40× (high-power field, HPF) per tissue section and expressed as the number of positive nuclei/HPF. Four consecutive tissue sections were immunostained and counted per pituitary.

IHC expression of phosphorylated PR (phospho-PR-pSer294) in gonadotropes

The commercial mouse monoclonal phospho-PR (pSer294) antibody clone 608, raised against the immunogen P(288)MAPGRS(p)PLATTV(300) located in the N-terminal domain (Affinity BioReagents, Golden, CO, USA), diluted 1:300, was analysed using the ABC IHC technique (Vectastain, ABC kit Elite, Vector Corporation, Burlingame, CA, USA). This method was standardized in formalin-fixed, paraffin-embedded pituitary tissue sections from rats in pro-oestrus under phosphorylating conditions. For this purpose, three pituitaries/group from pro-oestrous cycling rats were incubated over 1 or 2 h with medium alone or with 10^{-7} and 10^{-6} M P (ligand-dependent activator of PR) or 10^{-8} and 10^{-7} M GNRH1 (ligand-independent activator of PR). Three additional pituitaries were incubated with medium alone as PR phosphorylation negative controls. Three different dilutions of pSer294 antibody were assayed, 1:100, 1:300 and 1:500, and the final working dilution of 1:300 was selected on the basis of the quality and specificity of immunostaining (Fig. 6). Briefly, dewaxed and rehydrated 3 μm thick tissue sections were subjected to high-temperature antigen retrieval by incubation with 0.01 M citrate buffer (pH 6.0) at 95 °C for 8 min in a decloaking chamber (Biocare Medical, Concord, CA, USA). All further IHC staining procedures were according to the test kit instructions. Tissue sections were counterstained with Mayer's hematoxylin. The substitution of specific primary antibody with non-immune mouse IgG_1 (Affinity Bioreagents) in tissue sections of the pituitaries under study were used as negative control in every assay.

Effect of hFSH treatment on the IHC expression of phosphorylated PR (phospho-PR-pSer294) in gonadotropes

Three pituitaries from each of the four experimental groups of rats were incubated for 2 h with 10^{-6} M P, 10^{-7} M GNRH1 or medium alone. Concentrations and incubation times of P and GNRH1 were selected on the basis of the IHQ standardization protocol results.

The number of cells immunoreactive to pSer294 antibody was determined similarly than PR10A9 antibody. The number of PR phosphorylated at pSer294 was expressed as the number of phosphorylated PR at Ser294 (pSer294-positive cells)/number of PR-positive gonadotropes (PR10A9-positive cells)×100. Eight consecutive tissue sections were immunostained and counted per pituitary (even for PR10A9 and odd for pSer294).

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 the means. Significance was considered at the 0.05 level.

Declaration of interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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