Understanding the regulation of pituitary progesterone receptor expression and phosphorylation

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Abstract

Administration of human FSH (hFSH) during the diestrus phase in cyclic rats is followed by a reduction in the preovulatory LH surge. This inhibitory action of FSH involves a decrease in the stimulatory effect of gonadotrope progesterone receptor (PR) activation, in a ligand-dependent (progesterone) and -independent (GNRH) manner. PR activation and action are mandatory for LH surge, and are dependent on the phosphorylation of serine (Ser) residues. Together with this post-translational modification, PR is marked for downregulation by proteasome machinery. These experiments used the western blotting technique to measure pituitary expression of PR-A and PR-B isoforms and phosphorylation levels of Ser294 and Ser400 PR-B in rats bearing i) hFSH treatment or ii) PR downregulation. Treatment with hFSH reduced LH secretion and increased that of estradiol in proestrus afternoon. hFSH injections, without altering PR-A and PR-B content or ratio, caused a reduction in phosphorylation of Ser294 and Ser400 but only when pituitaries were previously challenged with progesterone or GNRH for 2 h. However, while pSer294 levels increased after 2 h of pituitary incubation with progesterone or GNRH, those of pSer400 were not modified by these *in vitro* treatments. Finally, progesterone had a biphasic effect: in 2-h incubations increased pituitary PR-A and PR-B content, but after 8 h caused downregulation and altered PR-A:PR-B ratio. The results provide a potential mechanism through which LH levels are decreased by hFSH administration and better understanding of the control of PR expression and phosphorylation in rat pituitaries.

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Introduction

Although the cyclical nature of female reproduction has long been recognized, even now, there are unanswered questions. Crucial and still-unresolved questions are what prompts the start and what causes the end of preovulatory luteinizing hormone (LH) secretion. It has been suggested that gonadotrope progesterone receptor (PR) may play a role in both events. Firstly, administration of human follicle-stimulating hormone (hFSH) to cyclic rats during diestrus attenuates the PR-dependent LH surge in proestrus. Secondly, PR activation by its cognate ligand marks the protein for destruction by the proteasome, causing PR downregulation.

In the rat, gonadotropes are the only pituitary cells expressing this steroid receptor (Turgeon & Waring 2001), and there is considerable evidence to suggest that expression and activation of PR are obligatory steps for gonadotropin secretion. That PR activation is critically important in the hypothalamic–pituitary–ovarian axis is reflected by the observation that the administration of PR antagonists to proestrus or estradiol

(E₂)-treated ovariectomized rats, RU486 and ZK299, respectively, blunted the preovulatory secretion of gonadotropin-releasing hormone (GNRH) and consequently that of LH (Sánchez-Criado *et al.* 1994, Chappell & Levine 2000). Furthermore, the E₂-induced LH secretion was blocked by the same PR antagonists in rat pituitary incubations (Bellido *et al.* 1999, Sánchez-Criado *et al.* 2005). Interestingly, this receptor functions as a critically important component of the GNRH self-priming process (Turgeon & Waring 1994, Chappell *et al.* 1999) and becomes an absolute requirement for the generation of gonadotropin surges as shown in *Pr*-knockout mice (Chappell *et al.* 1999).

PR is a transcriptional factor, expressed as two estrogen-inducible isoforms: PR-A and PR-B (Kastner *et al.* 1990). PR-B differs from PR-A in 164 amino acids, but this difference is enough to confer different functions to each isoform (with exceptions, PR-A is regarded as the modulator of PR-B; Levine *et al.* 2001). Phosphorylation, a post-translational modification known to modulate the activity of PR (Denner *et al.* 1990, Faus & Haendler 2006),

takes place exclusively at serine (Ser) residues, which can be divided into basal and induced phosphorylation sites (Takimoto & Horwitz 1993, Moore *et al.* 2007). PR is phosphorylated by protein kinases, which in turn are activated by progesterone or in the absence of its cognate ligand (ligand-dependent and -independent activation respectively; Bai *et al.* 1997). Previous works have shown how GNRH can activate PR in a ligand-independent manner in gonadotropes transfected with reporter plasmids containing progesterone-responsive elements (Turgeon & Waring 1994). These authors proposed that the cAMP/protein kinase A pathway for GNRH self-priming and progesterone potentiation of GNRH-stimulated LH secretion converge at the PR (Waring & Turgeon 1992, Turgeon & Waring 1994). However, although several growth factors and neurotransmitters have been reported to phosphorylate PR (Bai *et al.* 1997, Pierson-Mullany & Lange 2004, Daniel *et al.* 2007), it is not known whether GNRH activation of PR phosphorylates the receptor in pituitary cells.

Administration of hFSH during the diestrus phase in cyclic rats is followed by a reduction in the preovulatory LH surge. As ovariectomy reverses the latter effect of hFSH injections, a nonsteroidal ovarian FSH-dependent factor has been proposed (the gonadotropin surge-attenuating factor (GnSAF); Fowler *et al.* 2003, Messinis 2006, Gordon *et al.* 2008, 2009a). The mechanism of action through which GnSAF exerts this inhibition may involve an interaction with PR at pituitary level, because it involves a decrease in the stimulatory effect of PR activation in a ligand-dependent (progesterone) and -independent (GNRH) manner (Gordon *et al.* 2008, 2009b).

On the other hand, PR phosphorylation is linked to proteasome-mediated downregulation (Clemm *et al.* 2000, Lange *et al.* 2000). Until recently, phosphorylation of Ser residue 294 was regarded as the signal for proteasome degradation (Lange *et al.* 2000, Khan *et al.* 2011). The downregulation phenomenon may be a physiological mechanism for diminishing proestrus LH secretion and avoiding another surge at estrus by eliminating pituitary PR (Turgeon & Waring 2000). However, while most of the information we have about PR downregulation comes from T47D cells, little is known about ligand-dependent and -independent activation on gonadotrope PR turnover or whether the two isoforms are differently controlled. This study thus sought i) to evaluate whether rats treated with hFSH have a reduction in pituitary PR protein expression and/or phosphorylation, and ii) to determine whether pituitary PR ligand-dependent (progesterone) and -independent (GNRH) exposure could induce a PR downregulation. For that purpose, an optimized western blot technique was used to investigate the following in rat anterior pituitaries: i) total PR-A and PR-B protein levels, ii) the relationship between the two isoforms

(PR-A:PR-B ratio), and iii) PR-B phosphorylation status at basal and induced sites.

Materials and methods

Animals and general conditions

Adult female Wistar rats weighing 190–210 g were housed under a 14 h light:10 h darkness cycle (lights on at 0500 h) at 22 ± 2 °C room temperature, with *ad libitum* access to rat chow and tap water. The rats were included in the experiments after displaying at least three consecutive 4-day estrus cycles. Vaginal smears were taken 6 days/week and the day of vaginal estrus was arbitrarily considered as day 1. 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; Serono) was dissolved at a concentration of 50 IU/ml saline, and 10 IU hFSH was given s.c. at 1400 h on day 2 (metestrus) and another dose of 10 IU hFSH at 0900 h on day 3 (diestrus). The dose rates and administration protocol for hFSH were determined in the light of previous findings (Gordon *et al.* 2008). Nonstimulated controls were given 0.2 ml saline.

In vivo experiments

Firstly, rats were treated *in vivo* with hFSH to study its effects on proestrus E_2 , LH, and progesterone secretions (controls were injected with saline). At 1600 h on proestrus, six rats per group (vehicle- and hFSH-injected rats) were lightly etherized and <0.4 ml blood was obtained by direct jugular venipuncture. Other blood samples were obtained at 1800 h (the high level of the LH surge in this colony; Sánchez-Criado *et al.* 1990) and at 2400 h on proestrus. Blood was allowed to clot, centrifuged at 4 °C, and the serum stored at -20 °C until quantified for E_2 , LH, and progesterone by specific RIAs.

RIA of LH, E_2 , and progesterone

Serum LH concentrations were measured in duplicate by RIA using a double antibody method with a kit supplied by the National Institutes of Health (NIH; Bethesda, MD, USA) and a previously described micro-assay method (Sánchez-Criado *et al.* 1990). Rat LH-I-10 was labeled with 125 I using the chloramine T method. All samples were assayed in the same assay; the intra-assay coefficient of variation (CV) was 8%, and assay sensitivity was 3.75 pg/tube. Results are expressed as ng/ml of the RP LH-rat-RP-3. Serum E_2 and progesterone concentrations were determined in duplicate using commercially obtained kits (Diagnosis Products Corporation, Los Angeles, CA, USA). The sensitivity of the assays was 1 and 10 pg/tube and the intra-assay CV were 7 and 6% respectively. E_2 and progesterone concentrations are expressed as pg/ml and ng/ml serum respectively.

In vitro experiments

General pituitary incubation protocol

Incubation of pituitaries was carried out, with minor modifications, as described previously (Gordon *et al.* 2008). Briefly, pituitaries were incubated at 37 °C with constant shaking (60 cycles/min) in an atmosphere of 95% O₂ and 5% CO₂. Each incubation vial contained 250 µl DMEM without L-glutamine and phenol red and containing glucose (4.5 g/l), 10⁻⁸ M E₂ (Sigma Chemical Co.) and BSA (0.1% w/v, pH 7.4). Pituitaries were stabilized for 1 h of pre-incubation in the latter medium but without test substances (progesterone or GNRH). Before carrying out experiments 'Effects of hFSH on pituitary PR expression and phosphorylation' section and 'Effects of long-term exposure to progesterone or GNRH on pituitary PR expression and phosphorylation' section, an incubation protocol was optimized to study phosphorylated PR levels: pituitaries from proestrus rats were incubated with E₂ and progesterone for 30, 60, or 120 min to determine the optimal timing of PR phosphorylation. As a result, 2 h was taken to be the most favorable incubation period for PR activation (data not shown) and was used thereafter to compare with the effects of hFSH (experiment 'Effects of hFSH on pituitary PR expression and phosphorylation') or long-term incubation with progesterone or GNRH (experiment 'Effects of long-term exposure to progesterone or GNRH on pituitary PR expression and phosphorylation'). This treatment was supported by previous research (Clemm *et al.* 2000, Pierson-Mullany & Lange 2004).

Effects of hFSH on pituitary PR expression and phosphorylation

To determine relative levels of PR protein and phosphorylated PR-B isoform, *in vivo* hFSH- and saline-treated rats (*n*=15) were decapitated at 0900 h on day 4; their pituitary glands were removed, separated from the neural lobe, and incubated for 2 h with 10⁻⁸ M E₂ and i) 10⁻⁶ M progesterone (ligand-dependent activator of PR), ii) 10⁻⁷ M GNRH (ligand-independent activator of PR), or iii) medium alone (*n*=5/group) (for more details, see 'General pituitary incubation protocol' section) (Gordon *et al.* 2009b). At the end of the incubation period, the pituitaries were collected, snap-frozen, and stored at -80 °C until use. Tissues samples were homogenized in RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS and 50 mM Tris-HCl, pH 8.0) with a protease and phosphatase inhibitor cocktail (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Proteins were obtained by centrifugation for 15 min at 17949 *g* at 4 °C, and supernatant was quantified by the Bradford method (Bio-Rad). Equal amount of proteins (100 µg/lane) was separated using SDS-PAGE (7.5%) with a Mini-Protean System (Bio-Rad) and transferred over 1 h to Immobilon-P transfer membranes (Millipore, Bedford, MA, USA). The membranes were blocked for 2 h in 5% nonfat dry milk diluted in 0.05% Tween-20 Tris-buffered saline and incubated overnight with the primary antibodies: rabbit polyclonal antibody against PR (PR C-20: sc-539; Santa Cruz Biotechnology; diluted 1:500), which recognizes both PR isoforms (PR-A and PR-B) in rats; 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 1:5000); and the commercial rabbit polyclonal phospho-PR (pSer400) antibody, raised against the immunogen synthetic phosphopeptide derived from human PR around the phosphorylation site of Ser400 (A-R-SP-P-R) (Abcam, London, UK; diluted 1:5000). The antibodies pSer294 and pSer400 were selected to measure hormone-induced and basal phosphorylation levels of PR-B respectively. Finally, a rabbit MAB against α -tubulin (Abcam; diluted 1:10 000) was used as a loading control. After incubation with primary antibodies, membranes were washed and incubated for 1 h with HRP-coupled secondary antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA; diluted 1:5000). Immunoreactive bands were detected using an ECL system (Immobilon Western, Millipore, Billerica, MA, USA). Quantification of the intensity of western blot signals was carried out using a Java-based image processing and analysis program developed by the NIH (ImageJ) and the values for the specific signal (PR-A, PR-B, pSer294 RP-B, and pSer400 RP-B) were normalized to loading control.

Effects of long-term exposure to progesterone or GNRH on pituitary PR expression and phosphorylation

Recent *in vivo* research has suggested that the proestrus progesterone surge lasts ~8 h in saline-injected rats (Fig. 1); this was therefore the interval used to study PR down-regulation, in a treatment schedule supported by other authors (Turgeon *et al.* 1999, Clemm *et al.* 2000, Turgeon & Waring 2000). To determine the effect of progesterone and GNRH on phosphorylation and downregulation of PR, pituitaries from 30 proestrus rats were incubated for 2 h (short-term group) or 8 h (long-term group) with 10⁻⁸ M E₂ and i) 10⁻⁶ M progesterone, ii) 10⁻⁷ M GNRH, or iii) medium alone (*n*=5/group) (for more details, see 'General pituitary incubation protocol' section). Relative levels of PR protein and phosphorylated PR-B isoform were evaluated using the western blot technique and antibodies, as previously detailed in 'Effects of hFSH on pituitary PR expression and phosphorylation' section.

Statistical analyses

Statistical analysis was performed by two-way ANOVA to test for significant differences among groups. When significant differences existed, ANOVA was followed by the Student-Newman-Keul's multiple range test to compare means. Significance was considered at the 0.05 level. Linear regression was used to evaluate the relationship between PR-A and PR-B protein levels obtained in PR downregulation experiments, as well as between PR-B and phosphorylated residues (pSer294 and pSer400). Linear regression was performed considering the significance of the regression parameters and coefficient of determination (*R*²). Data from all experiments were analyzed by Statistix 9 (Analytical Software, Tallahassee, FL, USA).

Figure 1 Effects of hFSH treatment on serum estradiol 17 β (E_2), LH, and progesterone levels at 1600, 1800, and 2400 h on the day of proestrus. Adult female rats were injected with 0.2 ml saline or 10 IU hFSH during the diestrus phase (1400 h in metestrus and 0900 h in diestrus). Values are means $\pm$ s.e.m. of six rats per group. Differences among groups with different letters are statistically significant ($P < 0.05$; ANOVA followed by Student–Newman–Keul’s multiple range test).

Results

Serum E_2 , LH, and progesterone levels during proestrus afternoon in rats injected with hFSH

Serum LH and progesterone concentrations were at baseline levels at 1600 h, peaked 2 h later (1800 h), and returned to baseline at 2400 h, both in saline- and hFSH-injected rats (Fig. 1). In contrast, serum E_2 levels were high at 1600 and 1800 h, and returned to baseline values at 2400 h. While hFSH treatment increased serum E_2 concentrations at 1800 h, it attenuated LH concentrations at the high of the LH peak (Fig. 1).

Effects of hFSH treatment on PR-A and PR-B isoforms and PR-A:PR-B ratio in pituitaries from proestrus rats after incubation with progesterone, GNRH, or DMEM for 2 h

Pituitaries incubated for 2 h with progesterone showed increased signals of both PR isoforms when compared

with pituitaries incubated with DMEM ($P < 0.05$; Fig. 2, upper panel). The latter effect was not influenced by *in vivo* treatment (saline or hFSH). Medium PR signal levels were found in GNRH groups, but differences with respect to control or progesterone groups were not statistically significant (Fig. 2, upper panel). No differences were observed in the PR-A:PR-B ratio between rats treated with saline or hFSH and their respective incubation sub-groups (Fig. 2, lower panel).

Figure 2 Effects of hFSH treatment on PR isoform protein levels and PR-A:PR-B ratio. Adult female rats were injected with saline or hFSH during the diestrus phase and killed on proestrus morning. After 1 h of pre-incubation with the medium containing only 10^{-8} M E_2 , pituitaries were incubated for 2 h with E_2 and i) 10^{-6} M progesterone, ii) 10^{-7} M GNRH, or iii) medium. The upper panel is a representative western blot of PR-A, PR-B, and tubulin. Lower panels are densitometric analyses of PR-A, PR-B, and the PR-A:PR-B ratio. Values are means $\pm$ s.e.m. of five rats per group. Differences among groups with different letters are statistically significant ($P < 0.05$; ANOVA followed by Student–Newman–Keul’s multiple range test).

Effects of hFSH treatment on phosphorylated Ser294 and Ser400 residues of PR-B in pituitaries from proestrus rats after incubation with progesterone, GNRH, or DMEM for 2 h

In saline groups, incubation with progesterone or GNRH prompted higher levels of Ser294 phosphorylation than DMEM ($P<0.05$; Fig. 3, upper panel). Conversely, Ser400 phosphorylation levels in saline groups showed no change when pituitaries were incubated either with progesterone or GNRH (Fig. 3, lower panel). Pituitaries from hFSH-treated rats showed significantly decreased rates of phosphorylation in both Ser294 and Ser400 after incubation for 2 h with progesterone or GNRH ($P<0.05$;

Figure 3 Effects of hFSH treatment on phospho-Ser294 and phospho-Ser400 PR-B protein levels. Adult female rats were injected with saline or hFSH during the diestrus phase and killed on proestrus morning. After 1 h of pre-incubation with medium containing only 10^{-8} M E_2 , pituitaries were incubated for 2 h with E_2 and i) 10^{-6} M progesterone, ii) 10^{-7} M GNRH, or iii) medium. Upper panels are representative western blots of phospho-Ser294, phospho-Ser400, and tubulin. Lower panels are densitometric analyses of phospho-Ser294 and phospho-Ser400 PR-B. Values are means $\pm$ S.E.M. of five rats per group. Differences among groups with different letters are statistically significant ($P<0.05$; ANOVA followed by Student–Newman–Keuls multiple range test).

Fig. 3, upper and lower panels). However, the dephosphorylation effect of hFSH on pSer400 varied when ligand-dependent and -independent activation were compared, being more intense in the latter ($P<0.05$; Fig. 3, lower panel). Interestingly, no changes were observed in pSer400 levels between saline- and hFSH-injected rats when pituitaries were incubated with DMEM (Fig. 3, lower panel).

Effects of incubation for 2 or 8 h with progesterone, GNRH, or DMEM on PR-A and PR-B isoforms and PR-A:PR-B ratio in pituitaries from proestrus rats

As in the hFSH experiments, pituitaries exposed for 2 h with progesterone, but not with GNRH, showed increased signals of both PR isoforms levels, in comparison with pituitaries incubated for 2 h with DMEM ($P<0.05$; Fig. 4, upper panel). PR-A and PR-B bands decreased significantly after 8 h of incubation with progesterone or GNRH when compared with their respective 2-h groups ($P<0.05$), but only in the case of PR-B protein levels were lower than controls ($P<0.05$) (Fig. 4, upper panel). Regardless of the incubation time, no differences in PR-A:PR-B ratio were found between DMEM and GNRH groups. However, pituitaries exposed for 8 h with progesterone displayed an increased PR-A:PR-B ratio due to greater destruction of the PR-B isoform ($P<0.05$; Fig. 4, lower panel).

PR-B phosphorylation levels at Ser294 and Ser400 residues in pituitaries from proestrus rats incubated for 2 or 8 h with progesterone, GNRH, or DMEM

While no differences were found between 2 and 8 h DMEM groups at either pSer294 or pSer400 residue, striking differences appeared in those pituitaries incubated with progesterone or GNRH (Fig. 5). When pSer294 levels were measured, the groups of pituitaries incubated for 2 h with progesterone or GNRH exhibited a similar rise in phosphorylation of PR-B ($P<0.05$; Fig. 5, upper panel). In contrast, no differences were found in pSer400 levels among groups incubated with any test substances for 2 h (Fig. 5, lower panel). Regardless of the phosphorylated Ser residue studied for PR-B, the incubation of pituitaries for 8 h with progesterone or GNRH diminished signal levels when compared with their respective short-term incubation groups ($P<0.05$; Fig. 5, upper and lower panels).

General correlation between PR-A and PR-B protein expression in rat pituitaries

On the basis of data obtained in the PR downregulation experiment, a positive linear correlation was observed between protein levels of the two isoforms ($PR-B = 0.2128 + 1.158 \times PR-A$; $R^2 = 0.4816$; $P<0.0001$). Likewise, total PR-B protein levels displayed a linear

Figure 4 Effects of short- and long-term incubation on PR isoform protein levels and the PR-A:PR-B ratio. Cycling adult female rats were killed on proestrus morning. After 1 h of pre-incubation with the medium containing only 10^{-8} M E_2 , pituitaries were incubated for 2 or 8 h with E_2 and i) 10^{-6} M progesterone, ii) 10^{-7} M GNRH, or iii) medium. The upper panel is a representative western blot of PR-A, PR-B, and tubulin. Lower panels are densitometric analyses of PR-A, PR-B, and the PR-A:PR-B ratio. Values are means $\pm$ S.E.M. of five rats per group. Differences among groups with different letters are statistically significant ($P < 0.05$; ANOVA followed by Student–Newman–Keul's multiple range test).

correlation with those of pSer294 ($PR-B = 0.05205 + 0.4879 \times pSer294$; $R^2 = 0.3207$; $P < 0.001$) and pSer400 ($PR-B = 0.8265 \times pSer400$; $R^2 = 0.8783$; $P < 0.0001$). Conversely, no correlation was found between the two Ser residues.

Discussion

The results reported in this study show that administration of hFSH reduces preovulatory LH secretion in proestrus

rats, and that this action might be mediated by lowering ligand-dependent and -independent phosphorylation of PR-B without affecting pituitary protein levels of both PR isoforms. Moreover, whereas incubation of proestrus pituitaries with progesterone or GNRH for 2 h increases in phosphorylated Ser294 levels, long-term (8 h) incubation with both hormones causes ligand-dependent or -independent PR downregulation and, as a consequence, a reduction in phosphorylated PR-B isoform. As far as the authors are aware, this is the first report on the ligand-dependent and -independent phosphorylation of Ser294

Figure 5 Effects of short- and long-term incubation on phospho-Ser294 and phospho-Ser400 PR-B protein levels. Cycling adult female rats were killed on proestrus morning. After 1 h of pre-incubation with the medium containing only 10^{-8} M E_2 , pituitaries were incubated for 2 or 8 h with E_2 and i) 10^{-6} M progesterone, ii) 10^{-7} M GNRH, or iii) medium. Upper panels are representative western blots of phospho-Ser294, phospho-Ser400, and tubulin. Lower panels are densitometric analyses of phospho-Ser294 and phospho-Ser400 PR-B. Values are means $\pm$ S.E.M. of five rats per group. Differences among groups with different letters are statistically significant ($P < 0.05$; ANOVA followed by Student–Newman–Keul's multiple range test).

and Ser400 in PR-B from rat gonadotropes using the western blot technique. The quantification of total and phosphorylated forms of PR-B has allowed us to delve into the mechanisms of action of GnSAF and examine the evidence of PR downregulation in pituitaries from proestrus rats.

It is widely accepted that administration of hFSH to diestrus rats prompts a reduction in LH secretion on proestrus afternoon (Busbridge *et al.* 1988, Gordon *et al.* 2008, 2009b). Depending on previous *in vitro* results by our group (Gordon *et al.* 2009a) and because GnSAF decreases all PR-dependent parameters of LH secretion, it may be postulated that this action is exerted through a modification of PR expression and/or action (Garrido-Gracia *et al.* 2007). Nevertheless, the effect of hFSH treatment on LH preovulatory secretion does not appear to be due either to an increase in the inhibitory isoform (PR-A) or to a decrease in PR-B. Moreover, in this study, no alterations in the PR-A:PR-B ratio are observed between saline- and hFSH-injected rats (Fig. 2, upper and lower panels). Overall, these findings would suggest that GnSAF inhibit LH preovulatory secretion by a mechanism other than alteration in gonadotrope PR protein levels.

PR is a phosphoprotein that contains several phosphorylation sites, Ser residues, which are susceptible to constitutive or ligand-induced modification (Lange 2008). In several reports, this type of post-translational modification has been shown to alter receptor transcriptional activation, stability, localization, and protein complex formation (Lange 2008, Daniel *et al.* 2009, Knutson *et al.* 2012). Previous findings in hFSH-treated rats showed that calyculin (a potent inhibitor of intracellular phosphatases; Condrescu *et al.* 1999) reduced the inhibitory action of hFSH on LH secretion in a dose-dependent manner (Gordon *et al.* 2009b). Consistent with the above, this study found that, while PR protein levels did not change when rats were injected with hFSH, both Ser residues (Ser294 and Ser400) were dephosphorylated (Fig. 3, upper and lower panels). Taken in conjunction, these results suggest that the inhibitory effect of GnSAF on preovulatory LH secretion may involve an imbalance between the activities of protein kinases and phosphatases, resulting in a dephosphorylation of rat pituitary PR. However, given that we administrated hFSH *in vivo*, it is likely that PR dephosphorylation occurs in other brain areas such as hypothalamic regions. In fact, transcription and activation of PR in the hypothalamus are obligatory events in the induction of the GnRH and LH surges in E₂-primed, ovariectomized rats (Chappell & Levine 2000). As kisspeptin neurons of the anteroventral periventricular nucleus expressed PR and project directly to GnRH neurons (Clarkson & Herbison 2006, Herbison 2007), GnSAF could increase its effects on LH secretion by inhibiting the activation of these PR by neuroprogesterone.

One striking finding of this experiments was that Ser400 did not display increased phosphorylation after progesterone or GnRH challenge (Figs 3 and 5, lower panels). We are unable to explain why in T47D cells this Ser residue presented higher phosphorylation levels when challenged with the PR agonist R5020 (Pierson-Mullany & Lange 2004), while progesterone did not do so in rat gonadotropes (present results). In addition to the higher potency of R5020, it is possible that PR from tumoral cells does not behave in the same way as gonadotrope PR to progesterone in intact rats. In fact, no difference was found in PR pSer400 levels as a function of different incubation times with progesterone (30, 60, or 120 min; results not shown).

Protein and mRNA of PR have been shown to vary in different rat brain regions and under distinct hormonal conditions, such as during the estrus cycle or after sexual maturity (Camacho-Arroyo *et al.* 1998, Guerra-Araiza *et al.* 2001). In fact, there are brain areas where PR is induced by E₂, independent of E₂, downregulated by progesterone or not modified by progesterone (Camacho-Arroyo *et al.* 1998, Guerra-Araiza *et al.* 2003). However, although most of the studies about the regulation of PR expression in the brain have been carried out after long-term exposures with progesterone (Camacho-Arroyo *et al.* 1998, Guerra-Araiza *et al.* 2003, Attardi *et al.* 2007), we have used short- and long-term pituitary incubations to evaluate PR protein phosphorylation and turnover. Interestingly, present results showed that gonadotropes challenged with progesterone for 2 h displayed a greater PR-A and PR-B immunoreactivity, which implies a greater protein expression (Figs 2 and 4). It is likely that short-term exposure to progesterone could stimulate translation of already present PR mRNA by activating membrane PRs instead of, destroying it as has been proposed (Turgeon *et al.* 1999). On the other hand, after 8 h of exposure to progesterone or GnRH, pituitaries suffered PR downregulation (Fig. 4), which even prompted a change in the PR-A:PR-B ratio in the case of progesterone (Fig. 4, lower panel). The PR-A:PR-B expression ratio is a key factor in tissue responsiveness to several stimuli; many reports have made an intensive study of this ratio and its link with cancer (Shyamala *et al.* 1998, Mote *et al.* 2002, Khan *et al.* 2011). PR-B protein degrades faster than PR-A (Khan *et al.* 2011), as also observed when pituitaries were incubated with progesterone, changing the PR-A:PR-B ratio. GnRH-induced PR phosphorylation and activation, through second messengers, provoked ligand-independent downregulation but, in contrast to progesterone, GnRH did not modify the PR-A:PR-B ratio (Fig. 4, lower panel). One possible explanation is that the mechanisms controlling PR isoform degradation/stability change as a function of the external stimulus (Khan *et al.* 2011). Like other researchers, we would suggest that while short-term administration of progesterone or GnRH activates PR, long-term exposure causes PR

downregulation (Turgeon *et al.* 1999, Turgeon & Waring 2000, present results). However, pituitaries were continuously incubated with GnRH, which implies physiological differences with GnRH pulses in proestrus afternoon. In this setting, the detailed mechanism underlying the specific PR isoform turnover requires further investigation.

In the context of the mechanisms controlling LH preovulatory secretion, both groups of experiments tempted us to provide a possible hypothesis regarding the optimal timing of the LH surge: firstly, given that GnSAF is produced during diestrus (Tébar *et al.* 1998), this factor might inhibit phosphorylation of gonadotrope PR already present at this period, which would avoid a premature LH surge. Secondly, the LH surge-dependent progesterone rise during proestrus afternoon would phosphorylate PR-B, marking it for PR downregulation through degradation by the 26S proteasome (Lange *et al.* 2000), which would impede another surge of LH during estrus.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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