Ovarian signalling pathways regulated by leptin during the ovulatory process

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Abstract

Leptin, a protein secreted by different tissues, is able to exert both stimulatory and inhibitory effects on the ovulatory process. Thus, we investigated whether these opposite effects involve changes in the ovarian signalling pathways in response to different levels of leptin. To this end, we performed both *in vivo* and *in vitro* assays using immature rats primed with gonadotrophins to induce ovulation. The acute treatment with leptin, which inhibits the ovulatory process, caused a significant decrease in the phosphorylation of both STAT3 and ERK1/2 and a simultaneous increase in suppressors of cytokine signalling 3 (SOCS3) protein. However, daily administration of a low dose of leptin, which induces the ovulatory process, showed increased phosphorylation of both STAT3 and ERK1/2 and a decreased expression of SOCS3 protein. Using ovarian explant cultures, we also found that leptin was able to activate both STAT3 and ERK1/2 at 10 ng/ml but only STAT3 at 300–500 ng/ml. In addition, at 100–300 ng/ml, leptin increased protein but not mRNA expression of SOCS3. The addition of specific inhibitors of JAK/STAT and MAPK signalling pathways suppressed both the increase and the decrease in leptin-induced progesterone secretion. These results indicate that i) different levels of leptin are able to regulate STAT3, ERK1/2 and SOCS3 at both intra- and extra-ovarian level and that ii) the dual action of leptin on steroidogenesis seems to occur, at least in part, through both the ERK and STAT cascades.

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Introduction

Leptin, the obese (OB) gene product, is synthesised and secreted by different types of cells, including adipocytes, and transported via blood to act as a multifunctional hormone in all tissues. It is involved in different physiological processes both in the CNS and in the periphery, including the reproductive system. Leptin acts through its transmembrane receptors, which have been localised mainly in different neuronal populations of the hypothalamus and many peripheral organs, including the reproductive system (Cioffi *et al*. 1996, 1997, Karlsson *et al*. 1997, Nakamura *et al*. 2009). Only the long isoform (OBRb) was initially considered to be a functional receptor because it contains all the intracellular domains required for effective signalling through activation of both the JAK/STAT and MAPK pathways (Tartaglia *et al*. 1995, Bjørbæk *et al*. 1997). However, OBRa, one of the short isoforms, also has different signalling capacities, especially through the MAPK pathway (Bjørbæk *et al*. 1997, Murakami *et al*. 1997, Caüzac *et al*. 2003, Ye *et al*. 2009). As OBRb does not have intrinsic enzymatic activity, its signalling cascade

occurs via JAK2, which undergoes autophosphorylation and simultaneously induces phosphorylation in other tyrosine residues in the cytoplasmic region of the leptin receptor, which, in turn, allows the association of STAT proteins. Then, these proteins are phosphorylated, dissociated from the receptor, and dimerised to translocate to the nucleus to regulate gene transcription. The signalling cascade via JAK/STAT activation is mainly regulated by suppressors of cytokine signalling (SOCS) proteins (Bjørbæk *et al*. 1998, Baskin *et al*. 2000). The SOCS family consists of eight structurally homologous proteins whose expression is induced by exposure to several cytokines and other growth factors. It has been reported that leptin is able to induce the expression of SOCS3 in both the hypothalamus and Chinese hamster ovary (CHO) cells, but not that of SOCS1 or SOCS2. SOCS3 also regulates the signal transduction modulated by leptin (Bjørbæk *et al*. 2000, Laubner *et al*. 2005, Eguchi *et al*. 2007) and has been proposed as a potential mediator of leptin resistance (Bjørbæk *et al*. 1998, 1999). In addition to JAK/STAT, other signalling factors can be activated by leptin receptors. Some studies have shown that leptin can stimulate the activity

of MAPK by either the long or the short isoform, although some authors state that the latter does so to a lesser extent (Bjørbæk *et al.* 1997, 2001, Banks *et al.* 2000). Activation of the MAPK signalling pathway phosphorylates ERK proteins that induce the gene transcription of the specific gene target (Bjørbæk *et al.* 1997, Kim *et al.* 2000).

Administration of leptin to *ob/ob* mice, which lack circulating leptin, causes decreased food intake, body weight loss, increased ovarian weight, increased number of follicles and restoration of fertility (Chehab *et al.* 1996, Cioffi *et al.* 1997, Mounzih *et al.* 1997). Leptin has both inhibitory and stimulatory actions on ovarian function. Regarding its inhibitory actions, i) leptin can directly suppress insulin, insulin-like growth factor 1 (IGF1), transforming growth factor-β and glucocorticoid-induced steroidogenesis of ovarian granulosa cells of rats (Zachow & Magoffin 1997, Barkan *et al.* 1999, Zachow *et al.* 1999) or humans (Agarwal *et al.* 1999); ii) acute administration of leptin to immature gonadotrophin-primed rats inhibits ovulation (Duggal *et al.* 2000, 2002) and iii) leptin inhibits preantral follicles cultured in the presence of follicle-stimulating hormone (FSH; Kikuchi *et al.* 2001). Regarding its stimulatory actions, i) leptin accelerates the onset of puberty in rodents (Ahima *et al.* 1997, Almog *et al.* 2001) and humans (Clément *et al.* 1998, Strobel *et al.* 1998); ii) leptin induces ovulation in gonadotrophin-releasing hormone-deficient mice (Barkan *et al.* 2005) and equine chorionic gonadotrophin (eCG)/human chorionic gonadotrophin (hCG)-primed rats (Roman *et al.* 2005) and iii) leptin enhances meiotic oocyte maturation and oocyte quality through the activation of both JAK2/STAT3 and MEK1/2 pathways in the rabbit oocyte model (Matsuoka *et al.* 1999, Craig *et al.* 2004, Arias-Álvarez *et al.* 2010). Previous *in vivo* studies have shown that an acute treatment with leptin inhibits ovulation (Duggal *et al.* 2000, Ricci *et al.* 2006) but that a daily treatment with a low dose enhances ovulation in comparison with control animals (Almog *et al.* 2001, Barkan *et al.* 2005, Roman *et al.* 2005) and partially prevents the negative effects caused by severe malnutrition (Gruaz *et al.* 1998, Roman *et al.* 2005). Therefore, in this study, we began to assess whether the dual and opposite effects of leptin on the ovulatory process involve changes in the signalling pathways in response to this protein at the ovarian level.

Materials and methods

Animals

Immature female Sprague Dawley rats aged 21 days were purchased from the School of Veterinarian Sciences of the Buenos Aires University, Argentina. All the animals were kept under controlled conditions of light (12 h light:12 h darkness), temperature (22 °C) and humidity, with free access to food and water. In all the experiments, prepubertal rats were i.p. injected

with 15 IU eCG (in 0.10 ml saline) at 0800 h to induce the growth of the first generation of preovulatory follicles and to prevent the confounding effects of the presence of different types of follicles and corpora lutea from previous cycles. After 48 h, the animals were i.p. injected with 15 IU hCG (in 0.10 ml saline) to induce ovulation, which, in this rat strain, usually occurs within 12 h after hCG administration. Animals were handled according to the Guiding Principles for the Care and Use of Research Animals, and all the protocols were approved by the Institutional Committee of the Medicine School of the Buenos Aires University (CICUAL) by Resolution 2079/07.

In vivo *studies*

Rats received one of the following treatments (Fig. 1): i) acute treatment (Ricci *et al.* 2006), which consisted of five i.p. injections of either recombinant rat leptin (5 μg/0.15 ml PBS–BSA) or PBS–BSA alone (control) 1 h before hCG administration and at intervals of 150 min until killing and ii) daily treatment (Roman *et al.* 2005), at 22 days of age, which consisted of an i.p. injection of either recombinant rat leptin (3 μg/0.15 ml PBS–BSA) or PBS–BSA alone (control) per day until killing. After 10 days of treatment, these animals were injected with gonadotrophin as indicated before. In total, these rats received 12 injections of leptin.

Rats (eight to ten per group) were killed by decapitation 10 h after hCG administration in the acute treatment, and 5 h (1300 h) and 10 h (1800 h) after hCG injection in the daily treatment. The timing samples were selected because i) many of the proinflammatory factors that are up-regulated in the ovary during the ovulatory process in response to hCG are at their maximum expressions in control animals and can be significantly modified by leptin (Roman *et al.* 2005, Ricci *et al.* 2006) and ii) 10 h after hCG administration in the acute treatment and 5 h after hCG in the daily treatment represent 1 h after the last leptin administration, a period that is necessary to obtain an adequate signalling response to leptin as demonstrated below. Both treatments attempt to simulate rats exposed to i) leptin levels that induce ovulation (daily treatment; Almog *et al.* 2001, Roman *et al.* 2005) and ii) high levels of leptin (acute treatment), as it occurs in obese rats, that inhibit ovulation (Duggal *et al.* 2000, Ricci *et al.* 2006). In all cases, both ovaries were immediately dissected out, frozen on dry ice and stored at −72 °C until use. Only one ovary from each animal was used in these studies.

In vitro *studies*

Ovarian explant culture

Animals were killed by decapitation 4 h after hCG administration and the ovarian tissues were incubated as described previously (Ricci *et al.* 2006). Briefly, the ovaries were removed and dissected free of fat and bursa and cut into pieces of approximately equal size. After 30-min preincubation, ovarian slices (four slices per ovary per well) were randomly distributed in DMEM/F12 (1:1) medium (Bio-Rad Laboratories) containing 25 mmol/l HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.5 μg/ml fungizone, and 2 mmol/l L-glutamine.
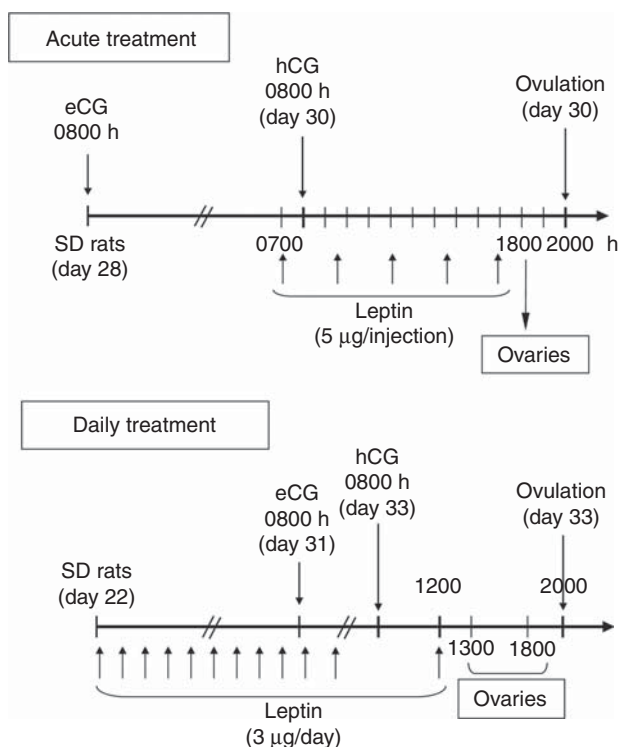
Figure 1 Schematic representation of the leptin treatments performed in our experiments. Acute treatment (upper panel): rats were injected with 15 IU eCG at 28 days of age and 15 IU hCG 48 h later. Both injections were administered at 0800 h. Leptin was administered within before hCG administration and killed through five injections of recombinant rat leptin or vehicle at 150-min intervals (0700, 0930, 1200, 1430 and 1700 h; up arrow). Rats were killed by decapitation at 10 h (1800 h) after hCG administration. In this rat strain, ovulation occurs within 12 h after hCG administration. Daily treatment (bottom panel): at 22 days of age, rats were daily injected with 5 µg leptin or vehicle at 1200 h until killed. In total, the animals received 12 injections. In addition, all rats received 15 IU eCG at 31 days of age and 15 IU hCG 48 h later. Both injections were administered at 0800 h. Rats were killed by decapitation at 5 h (1300 h) and 10 h (1800 h) after hCG administration. In this rat strain, ovulation occurs within 12 h after hCG administration. SD, Sprague Dawley.

Ovarian slices were then incubated at 37 °C in a humidified atmosphere (5% CO_2:95% O_2) i) for 1 and 4 h in the presence or absence of different leptin concentrations (0.3–500 ng/ml) to study the signalling proteins STAT3, ERK1/2 and SOCS3 and ii) for 4 h in the presence or absence of a combination of different leptin concentrations (1–300 ng/ml), a specific MAPK inhibitor PD 98059 (50 µmol/l, Sigma–Aldrich) and a specific JAK2/STAT3 inhibitor AG 490 (25 µmol/l, Sigma–Aldrich) to study progesterone secretion. The range of leptin concentrations was based on previous studies (Duggal *et al.* 2000, 2002, Ricci *et al.* 2006), and in the latter case, we used only those able to alter progesterone secretion, as described previously (Di Yorio *et al.* 2008). After the incubation periods, ovarian tissues and the culture media were recovered and frozen on dry ice and stored at −72 °C until use. Each experiment was repeated at least six times.

RNA isolation and semiquantitative RT-PCR

Total RNA was isolated from the frozen tissues using TRI Reagent (Molecular Research Centre, Cincinnati, OH, USA) according to the manufacturer's instructions. The organic phase of each sample was saved for protein extraction. Total RNA quantitation and purity determination was assessed by spectral absorption (A260/280) prior to RT-PCRs. cDNA was synthesised from 4 µg RNA in 25 µl reaction mixture containing 200 U Moloney murine leukemia virus (Promega), oligo-nucleotides (Random Primers, Invitrogen) and dNTPs (Promega). cDNA was amplified by PCR in a total volume of 25 µl using the primers and the cycles detailed in Table 1. Each reaction also contained 1 U Taq-DNA polymerase (Invitrogen), 0.2 mmol/l of each primer (Invitrogen), 0.2 mmol/l of each dNTP and 1.5 mmol/l $MgCl_2$. The PCR profiles consisted of an initial denaturing step at 94 °C for 5 min and an appropriate number of denaturing cycles at 94 °C for 40 s, annealing at 57 °C for 30 s, extension at 72 °C for 1 min and a final extension step at 72 °C for 5 min. The primer sequence used to amplify SOCS3 was that described previously (Peiser *et al.* 2000; Table 1). In preliminary experiments, the optimum cycle number was determined for each target, so that signals were always in the exponential portion of the amplification curve. An aliquot of each sample of the PCR was electrophoresed in 2% (w/v) agarose gel with subsequent ethidium bromide (10 mg/ml) staining. mRNA bands were visualised and quantified using Image Quant RT ECL (General Electric, Amersham Bioscience Argentina SA) and the freely downloadable ImageJ Software respectively. Data were normalised to β-actin mRNA in each sample, the primer sequence of which was designed by Primer3 Software, as described previously (Rozen & Skaletsky 2000). Negative controls were performed without reverse transcriptase or RNA.

Protein isolation and western blot analysis

Proteins were isolated from the organic phase of RNA isolation according to the manufacturer's instructions. Equal amounts of protein (200 µg) were separated by SDS–PAGE (10% for STAT3 and 15% for ERK1/2 and SOCS3). Proteins were transferred to PVDF membranes (Bio-Rad Laboratories) for 60 min in a cold chamber using a Bio-Rad transblot apparatus. Membranes were first blocked at 4 °C overnight in Tris–HCl:saline (50 mmol/l Tris–HCl:150 mmol/l NaCl, pH 7.5) containing 5% (w/v) non-fat milk powder and then incubated at 4 °C overnight with a specific primary antibody. Rabbit anti-STAT3 (C-20), anti-ERK1/2 (C-16) and anti-phosphorylated ERK1/2 (p-ERK1/2, Thr202/Tyr204) polyclonal antibodies (Santa Cruz Biotechnology), rabbit anti-actin polyclonal antibody (Sigma–Aldrich), goat anti-phosphorylated STAT3 (p-STAT3, Tyr705) and anti-SOCS3 (M-20) polyclonal antibodies (Santa Cruz Biotechnology), each diluted 1/200, were used. Following washing, membranes were treated for 1 h at room temperature with goat anti-rabbit IgG for STAT3, ERK1/2, p-ERK1/2 and β-actin, and bovine anti-goat IgG for p-STAT3 and SOCS3, diluted 1/10 000 as the secondary antibody (Jackson Immuno-Research Lab, Inc., West Grove, PA, USA). Immunoreactive bands were visualised using chemiluminescence detection

Table 1 Primers used for PCR for detection of SOCS3 and actin in ovarian tissue from immature rats primed with eCG/hCG.

Gene	Oligonucleotide sequences	Annealing temperature (°C)	Cycles	Product size (bp)	GenBank
SOCS3	Sense: 5′-CCTTTGAGGTTCAGGAGCAG-3′	57	35	382	NM053565
	Antisense: 5′-GGCTGGATTTTTGTGCTTGT-3′				
β-actin	Sense: 5′-AGCCATGTACGTAGCCATCC-3′	57	35	228	NM031144.2
	Antisense: 5′-CTCTCAGCTGTGGTGGTGAA-3′				

reagents (Sigma–Aldrich) and Image Quant RT ECL (General Electric) and quantified by ImageJ Software. Before reuse, membranes were stripped, blocked and reprobed according to the manufacturer's instructions. Negative controls were carried out by omitting the incubation with the primary antibody and no bands were detected. Molecular weight standards (Kaleidoscope St, Bio-Rad Laboratories) were run under the same conditions to identify protein bands. Phosphorylated proteins were expressed as the ratio of phosphorylated to total protein, and SOCS3 expression was normalised to β-actin protein levels in each sample to avoid procedural variability.

Progesterone RIA

Progesterone was quantified by RIA in the ovarian culture medium as described previously (Ricci *et al.* 2006). Antisera were kindly provided by Dr G D Niswender (Colorado State University, Fort Collins, CO, USA). The sensitivity was 7 pg/ml and the cross-reactivities were 12.0% for 20α-dihydro-progesterone and 1.0% for other steroids. Results are expressed as nanograms per milligram protein. The intra- and interassay coefficients of variation were 7.0 and 8.5% respectively.

Statistical analysis

All data are expressed as means±s.e.m. Statistical analysis for *in vivo* experiments was performed by Student's *t*-test, whereas that for *in vitro* experiments was performed by i) one-way ANOVA with Dunnett's multiple comparison test for mRNA and protein determination and ii) two-way ANOVA with Bonferroni's post-test for progesterone secretion. Differences between groups were considered significant when $P < 0.05$.

Results

Preliminary assays

To study the effect of leptin on the signalling pathways in the ovarian tissue, we first examined the timing and ability of leptin to induce the phosphorylation of STAT3 and ERK1/2 (Fig. 2). We found that leptin increased the phosphorylation of both STAT3 (Fig. 2A) and ERK1/2 (Fig. 2B), with maxima at 60 min. Reduced or no phosphorylation was detected at longer times (data not shown). Thus, the expressions of both phosphorylated and total STAT3 and ERK1/2 proteins were assessed after i) 60 min of the last leptin administration in the *in vivo* assays and ii) a 60-min incubation in the presence of different leptin concentrations in the *in vitro* assays. As leptin mRNA has been found in ovarian cells of many

species (Cioffi *et al.* 1997, Ryan *et al.* 2003), this endogenous protein or some other factors like interleukins, hormones or other cytokines can stimulate phosphorylation of these proteins. Therefore, the phosphorylation detected in the control samples may be either a leptin-independent and/or a leptin-dependent activation.

In vivo *studies*

To study whether the dual and opposite *in vivo* effects of leptin on the ovulatory process involves changes in the transduction signal pathways, rats received either the acute or daily treatment with leptin described in the Materials and methods section, and the phosphorylation of different signalling proteins was analysed in the ovarian tissue. In the acute treatment, phosphorylation of STAT3, expressed as the ratio of phosphorylated to total protein, was significantly lower than that in control animals (-30%, $P < 0.05$; Fig. 3A, left graphic).

Because leptin has been shown to activate other signalling pathways as MAPK in a variety of biological systems, especially in cells that have the short isoform of the leptin receptor (Murakami *et al.* 1997, Banks *et al.* 2000, Bjørbæk *et al.* 2001), we assessed phosphorylation of ERK1/2 by this acute
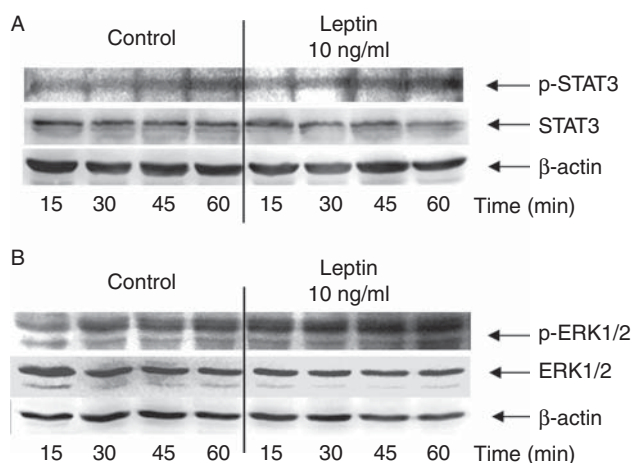
Figure 2 Time course for the *in vitro* phosphorylation of STAT3 (A) and ERK1/2 (B) in ovarian tissue obtained 4 h after hCG administration from immature rats primed with eCG/hCG and incubated for 15, 30, 45 and 60 min in the presence or absence of leptin (10 ng/ml), as described in the Materials and methods section. Phosphorylated proteins were detected by western blot analysis.
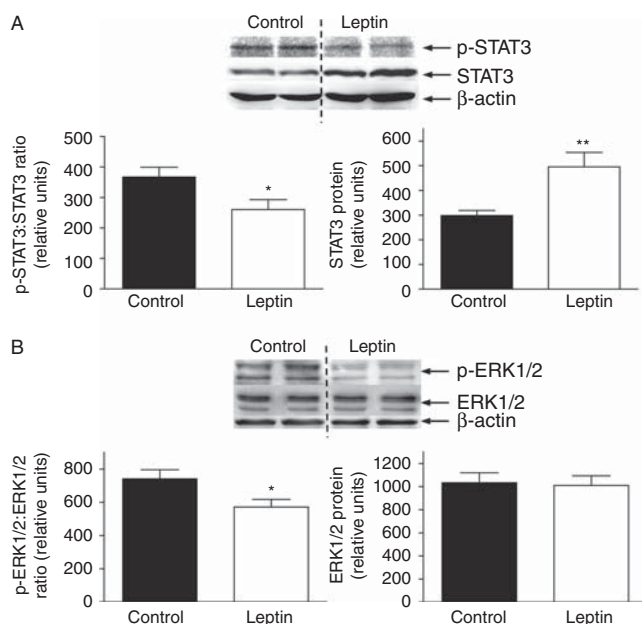
A

B

Figure 3 *In vivo* effect of the acute treatment with leptin on the ovarian expression of STAT3 and ERK1/2. Immature rats were primed with eCG/hCG and treated with vehicle (control) or 5 µg leptin at 1 h before hCG and at 150-min intervals. Animals were killed 10 h after hCG administration, which represents 1 h after the last injection of leptin. (A and B) Representative blots (upper panel) showing the ovarian expression of phosphorylated and total STAT3 (A) and ERK1/2 (B) protein and quantitative analysis (bottom panel) of these proteins expressed as the ratio of phosphorylated to total protein (left graphics) and the total proteins normalised relative to β-actin (right graphics). Data points represent the mean±s.e.m. for eight to ten samples per group. Each sample represents one ovary from each animal with the same treatment ($n=8$–10). *$P<0.05$ and **$P<0.01$ vs controls (Student's *t*-test).

treatment. Phosphorylation of ERK1/2 was slightly but significantly lower than that in control animals (-24%, $P<0.05$; Fig. 3B, left graphic). After the acute treatment with leptin, the expression of total ERK1/2 protein was not changed (Fig. 3B, right graphic), whereas that of total STAT3 protein was significantly increased (56%, $P<0.01$) when compared with control animals (Fig. 3A, right graphic).

In the daily treatment with leptin, when ovarian tissues were obtained 1 h after the last injection of leptin (5 h after hCG), we found increased phosphorylation of both STAT3 ($+25\%$, $P<0.05$) and ERK1/2 ($+44\%$, $P<0.01$) when compared with those obtained in control animals (Fig. 4A and B respectively), but no differences in the expression of total proteins (data not shown). On the other hand, when ovarian tissues were obtained 6 h after the last injection of leptin (10 h after hCG), we found no differences in the phosphorylated and total signalling proteins between leptin-treated and control animals (data not shown).

The levels of expression of both mRNA and protein of SOCS3 were evaluated in the same samples as the other

signalling proteins. In rats that received the acute treatment, the relative expression of SOCS3 protein was significantly higher than in control animals (42%, $P<0.05$), whereas that of mRNA was similar in both groups (Fig. 5). In rats that received the daily treatment, the mRNA and protein expression of SOCS3 in the ovaries obtained 1 h after the last administration of leptin (5 h after hCG) was similar to that in control animals (data not shown). However, in the ovaries obtained 10 h after hCG administration, the expression of SOCS3 protein (45%, $P<0.05$), but not of SOCS3 mRNA, was significantly lower than that in control animals (Fig. 6).
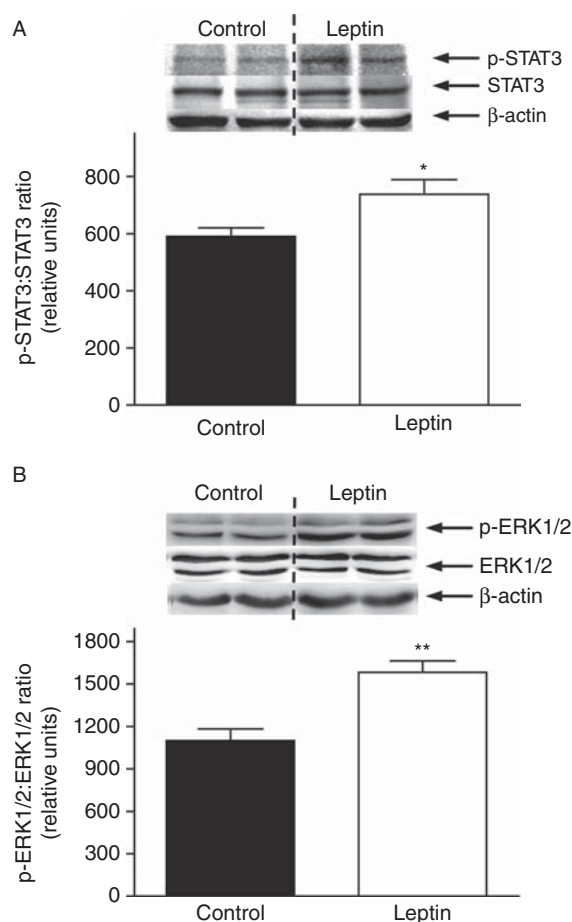
A

B

Figure 4 *In vivo* effect of the daily treatment with leptin (3 µg/day) on the ovarian expression of STAT3 and ERK1/2 in immature rats. On day 22 of age, rats received injections of either vehicle (control) or 3 µg leptin/day. After 10 days of treatment, animals received the eCG/hCG stimulation as indicated in the Materials and methods section. Animals were killed 5 h after hCG administration, which represents 1 h after the last injection of leptin. (A and B) Representative blots (upper panel) showing the ovarian expression of both phosphorylated and total STAT3 (A) and ERK1/2 (B) and quantitative analysis (bottom panel) of these proteins expressed as the ratio of phosphorylated to total protein. Data points represent the mean±s.e.m. for eight to ten samples per group. Each sample represents one ovary from each animal with the same treatment ($n=8$–10). *$P<0.05$ and **$P<0.01$ vs controls (Student's *t*-test).
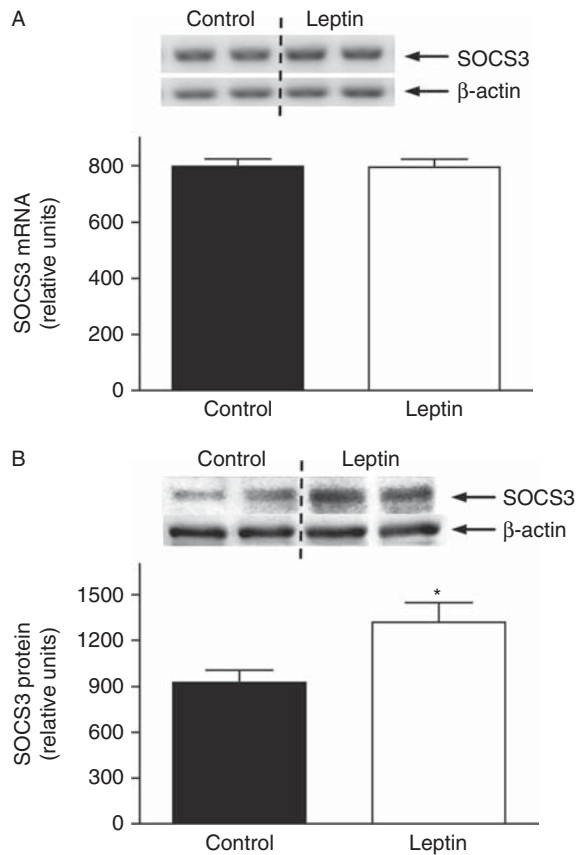
Figure 5 *In vivo* effect of the acute treatment with leptin on the ovarian expression of both SOCS3 mRNA and protein. Immature rats were primed with eCG/hCG and treated with vehicle (control) or 5 µg leptin at 1 h before hCG and 150-min intervals. Animals were killed 10 h after hCG administration, which represents 1 h after the last injection of leptin. (A) Ovarian mRNA expression of SOCS3 and β-actin as transcript control, by RT-PCR (upper panel) and quantitative analysis of mRNA bands (bottom panel). (B) Ovarian expression of SOCS3 protein and β-actin, as protein control, by western blot assay (upper panel) and quantitative analysis of immunoreactive bands (bottom panel). Results are expressed as the mean ± s.e.m. for eight to ten samples per group. Each sample represents one ovary from each animal with the same treatment ($n=8$–10). *$P<0.05$ vs controls (Student's *t*-test).

In vitro *studies*

To study the effect of a narrow range of leptin concentrations on STAT3, ERK1/2 and SOCS3 and to investigate whether the ovary is involved in the changes found in the *in vivo* studies, these signalling proteins were measured in ovarian explant cultures. When the ovarian explants were incubated for 1 h, the presence of leptin caused significant increases in the expression of phosphorylated proteins at different ranges of leptin levels: at 10, 300 and 500 ng/ml for p-STAT3 and at 3–10 ng/ml for p-ERK1/2 (Fig. 7A and B respectively). These increases ranged between 32 and 63% ($P<0.05$), when compared with controls. The expression of total ERK1/2 protein after incubation for 1 h was not changed (Fig. 7B, right graphic), whereas that of total STAT3

protein was significantly increased when the ovarian tissue was exposed to the highest concentrations of leptin (Fig. 7A, right graphic). These increases ranged between 58 and 87% when compared with controls. No phosphorylation of STAT3 or ERK1/2 was detected after incubation for 4 h in the presence of leptin (data not shown). No difference was found in the expression of SOCS3 mRNA and protein after 1 h incubation in the presence of leptin when compared with controls (data not shown). However, when the ovaries were incubated for 4 h, the expression of SOCS3 protein (Fig. 8B), but not of SOCS3 mRNA (Fig. 8A), was significantly increased at high concentrations of leptin.
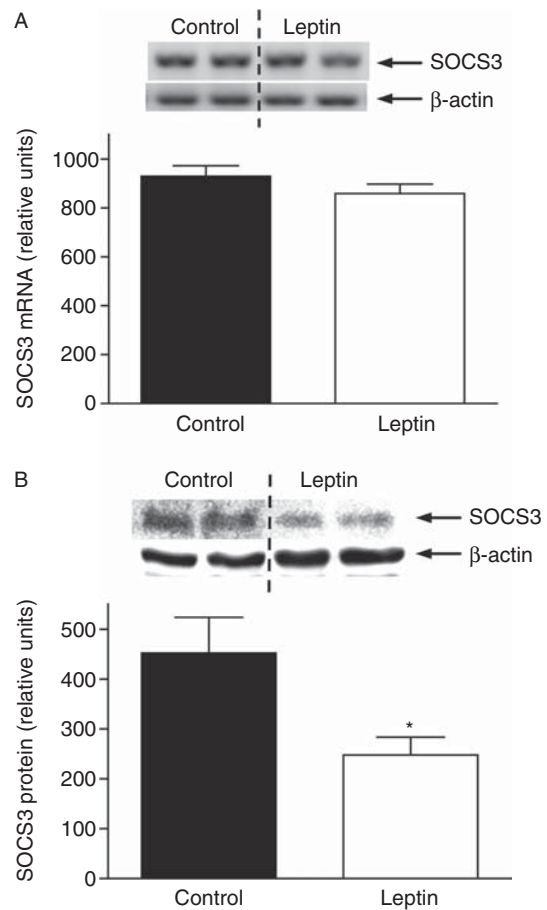

Figure 6 *In vivo* effect of the daily treatment with leptin on the ovarian expression of both SOCS3 mRNA and protein. Immature rats were daily treated with vehicle (control) or 3 µg leptin starting at 22 days of age. After 10 days of treatment, animals were primed with eCG/hCG as indicated in the Materials and methods section. Animals were killed 10 h after hCG administration. (A) Ovarian mRNA expression of SOCS3 and β-actin as transcript control, by RT-PCR (upper panel) and quantitative analysis of mRNA bands (bottom panel). (B) Ovarian expression of SOCS3 protein and β-actin, as protein control, by western blot assay (upper panel) and quantitative analysis of immunoreactive bands (bottom panel). Results are expressed as the mean ± s.e.m. for eight to ten samples per group. Each sample represents one ovary from each animal with the same treatment ($n=8$–10). *$P<0.05$ vs controls (Student's *t*-test).
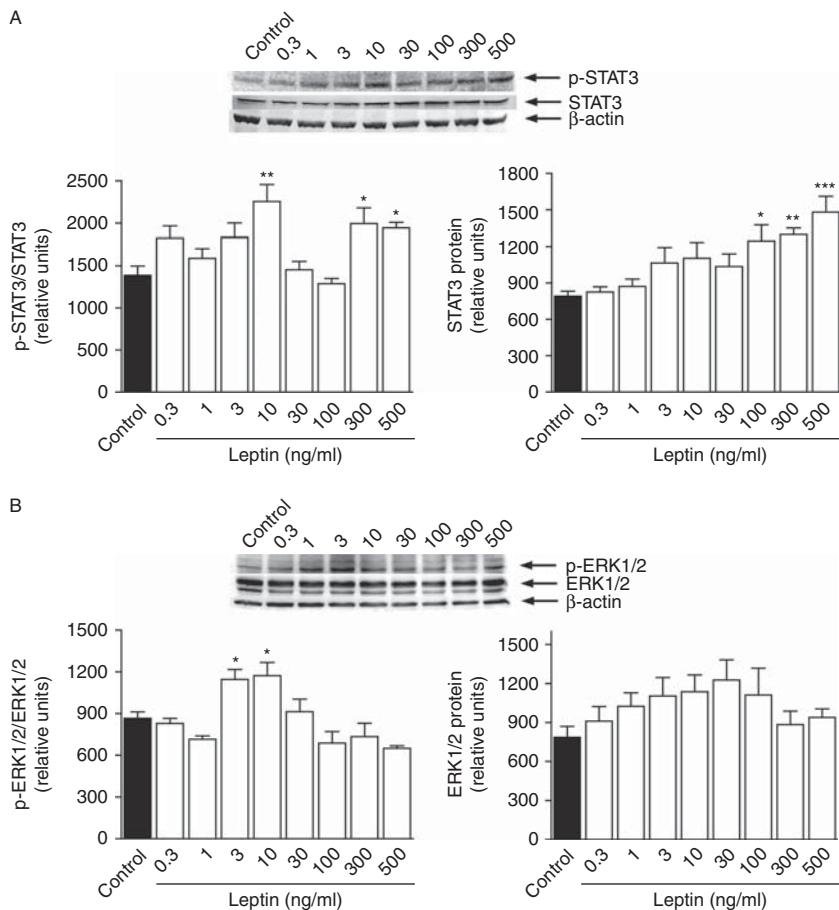
A

B

Figure 7 *In vitro* effect of leptin on the ovarian expression of STAT3 and ERK1/2. Ovarian explants were obtained 4 h after hCG administration from immature rats primed with eCG/hCG and incubated for 1 h either in the presence or in the absence (control) of different concentrations of leptin (0.3–500 ng/ml). (A and B) Representative blots (upper panel) showing the ovarian expression of both phosphorylated and total STAT3 (A) and ERK1/2 (B), and quantitative analysis (bottom panel) of these proteins expressed as the ratio of phosphorylated to total protein (left graphics) and the total proteins normalised relative to β-actin (right graphics). Results are the mean ± s.e.m. of six independent experiments ($n=6$). *$P<0.05$, **$P<0.01$ and ***$P<0.001$ vs controls (one-way ANOVA and Dunnett's multiple comparison test).

These increases represented 83% for the 100 ng/ml concentration ($P<0.01$) and 39% for the 300 ng/ml concentration ($P<0.05$).

Considering i) that the daily treatment with leptin induces (Roman *et al*. 2005) and the acute treatment reduces (Ricci *et al*. 2006) the levels of plasmatic progesterone, ii) that the presence of different levels of leptin causes a biphasic effect on progesterone secretion (Di Yorio *et al*. 2008) and iii) to link the changes found in the signalling pathways to a downstream biological event, we measured the secretion of progesterone in the ovarian incubation medium. Results are summarised in Table 2. The presence of leptin caused a dose-dependent biphasic effect on progesterone secretion, as described previously (Di Yorio *et al*. 2008). Progesterone concentration was significantly increased at 1–10 ng/ml and decreased at 300 ng/ml. The addition of PD 98059, a specific inhibitor of the ERK1/2 pathway, completely suppressed both the increase and the decrease in the leptin-induced progesterone secretion by the ovarian tissue, whereas basal concentrations were not altered. The presence of AG 490, a specific inhibitor of the JAK2/STAT3 pathway, completely reversed the action of leptin on progesterone secretion at 1 and 300 ng/ml leptin ($P<0.01$), but not at 3–10 ng/ml leptin ($P>0.05$),

although the levels of progesterone secretion were not significantly different from those of controls (Table 2).

Discussion

The acute treatment with leptin, which inhibits the ovulatory process, caused a decrease in the phosphorylation of ERK1/2 and an increase in the SOCS3 protein in the ovary. The finding that STAT3 phosphorylation decreased in parallel with an increase in total STAT3 protein in the ovary of rats that received the acute treatment suggests two possibilities: i) the STAT3 cascade is blocked by the increase in SOCS3 caused by the acute treatment with leptin and thus the constitutive protein increases to balance that inhibition or ii) STAT3 phosphorylation seems to be decreased by the increase in the total protein caused by leptin treatment. In either case, it was surprising that the phosphorylation of both STAT3 and ERK1/2 was blocked or reduced when compared with controls. In addition to leptin, other ovarian factors or interleukins, including hormones, can stimulate phosphorylation of these signalling proteins. Therefore, the phosphorylation detected in the control samples could be a leptin-independent activation and, in turn, leptin could be interfering in this activation.
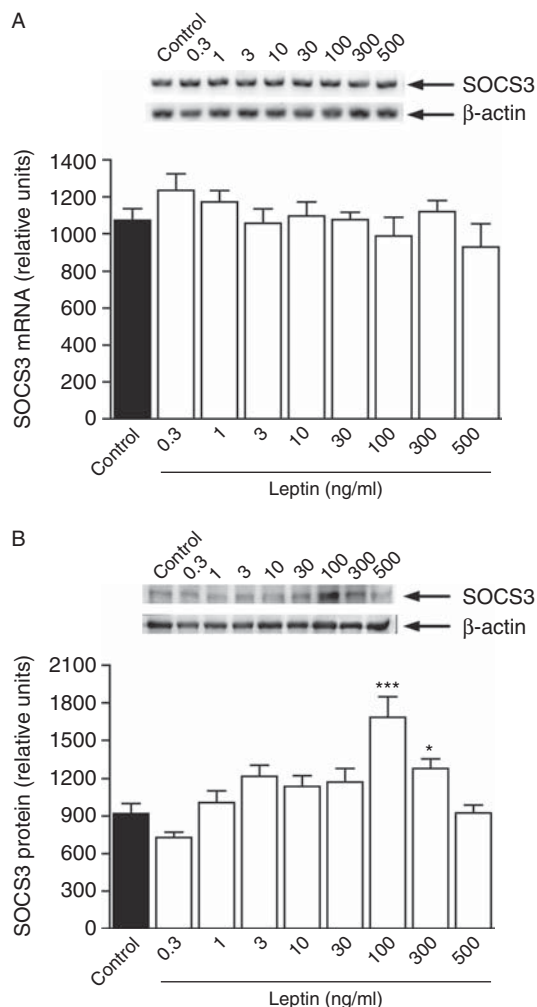
A

B

Figure 8 *In vitro* effect of leptin on the ovarian expression of both SOCS3 mRNA and protein. Ovarian explants were obtained 4 h after hCG administration from immature rats primed with eCG/hCG and incubated for 4 h either in the presence or in the absence (control) of different concentrations of leptin (0.3–500 ng/ml). (A) Ovarian mRNA expression of SOCS3 and β-actin as transcript control, by RT-PCR (upper panel) and quantitative analysis of mRNA bands (bottom panel). (B) Ovarian expression of SOCS3 protein and β-actin, as protein control, by western blot assay (upper panel) and quantitative analysis of protein bands (bottom panel). Results are expressed as the mean ± s.e.m. of six independent experiments ($n=6$). *$P<0.05$ and ***$P<0.001$ vs controls (one-way ANOVA and Dunnett's multiple comparison test).

Another possibility is that the increased levels of SOCS3 caused by the acute treatment may induce leptin resistance at the ovarian level, as suggested in other studies with different systems. Münzberg *et al.* (2004) found that the arcuate nucleus, a specific region within the hypothalamus, is selectively leptin resistant in diet-induced obese mice and suggested that this may be caused by increased levels of SOCS3 in this hypothalamic nucleus. Li *et al.* (2007) suggested that the lower levels of p-STAT3 found in granulosa cells from women with polycystic ovarian syndrome, who exhibit

hyperleptinaemia and high follicular fluid leptin, may be related to ovarian leptin resistance and fecundity in these women. Roy *et al.* (2007) found a clear leptin resistance when the OBRb/STAT3 signalling pathway was completely inhibited when CHO cells stably co-expressing OBRb were subjected to long-term pre-stimulation by leptin and that the overexposure to leptin induces the expression of the negative regulator SOCS3. Rodent and human obesity is characterised by hyper-leptinaemia and by leptin resistance, the latter of which may be caused by a lack of or a decrease in intracellular signals. Based on multiple evidence, it has been suggested that the overreactivity of the SOCS pathway is a potential causal mechanism for leptin-resistant obesity (Bjørbæk 2009). Thus, in this study, it is possible that the acute treatment with leptin caused a leptin resistance state at the ovarian level.

The daily treatment with leptin, which induces the ovulatory process, led to a clear increased phosphory-lation of both STAT3 and ERK1/2 and a decreased expression of SOCS3 protein. A significant body of evidence has demonstrated the involvement of either the JAK/STAT3 or MAPK signalling transduction pathway in different physiological events in the ovarian function, especially in the ovulatory process. The expression of interferon α, a cytokine involved in the differentiation of rat preovulatory granulosa cells and in the expression of other ovulation-related genes, is induced by the presence of luteinising hormone (LH) in rat preovulatory granulosa cells via the ERK1/2 signalling pathway (Lee da *et al.* 2009). In porcine granulosa cells, IGF1 induction of cholesterol side-chain cleavage cytochrome (P450scc) expression is directly and specifically mediated through ERK phosphorylation (Denner *et al.* 2010). Recently, we observed a significant decrease in the ovarian expression of P450scc in parallel with that of ERK1/2 phosphorylation in the same samples of ovarian tissue from rats that received the acute treatment for the present work (Bilbao *et al.* 2013). This result is consistent with the finding that PD 98059, a specific inhibitor of ERK1/2 activation, was able to reverse the action of leptin on progesterone secretion in our *in vitro* studies. It is known that StAR protein (StAR) is involved in the acute regulation of steroidogenesis by facilitating the access of cholesterol to the P450scc action. Some authors have found that the activation of cAMP/protein kinase A (PKA) signalling by gonadotrophins not only induces steroido-genesis but also activates a down-regulation machinery that involves the ERK cascade. Indeed, activation of the ERK cascade downstream of PKA in turn regulates the level of StAR expression, which is probably the key participant in these down-regulation processes (Cameron *et al.* 1996, Seger *et al.* 2001). However, in a more recent study, it has been found that a PKA-dependent phosphorylation of ERK1/2, which in turn interacts with and phosphorylates StAR, is essential to induce steroid biosynthesis (Poderoso *et al.* 2008).

Table 2 Progesterone secretion (ng/mg protein) by ovarian tissue from immature gonadotropin-primed rats in the presence of vehicle (control) and a combination of leptin (0–300 ng/ml) and/or specific inhibitors of kinases: PD 98059 (50 μM) and AG 490 (25 μM) (n=6–8).

	Leptin (ng/ml)				
	0	1	3	10	300
Control	26±2	50±5[†]	43±3[‡]	45±3[‡]	16±2*
PD 98059 (50 μM)	26±3	23±3[‖]	25±3[‖]	30±4[§]	35±3[‖]
AG 490 (25 μM)	31±3	28±2[‖]	36±5	39±4	35±4[‖]

*$P<0.05$, [†]$P<0.01$ and [‡]$P<0.001$ vs leptin 0 ng/ml. [§]$P<0.05$ and [‖]$P<0.01$ vs the respective leptin concentration.

In addition, Fan *et al.* (2009) provided *in vivo* evidence that ERK1/2 is necessary for gonadotrophin-induced oocyte maturation, ovulation and luteinisation by mediating the expression of FSH/LH-target genes in ovarian cells. Taking all these data together and considering i) that the endogenous levels of leptin are drastically decreased (Ryan *et al.* 2003, Ricci *et al.* 2006) in parallel with an increase in plasma progesterone levels after hCG administration in gonadotrophin-stimulated rats as used in the present and previous works (Ricci *et al.* 2006), which could indicate a possible role of leptin in the regulation of progesterone, ii) that the daily treatment with leptin induces and the acute treatment reduces the levels of progesterone (Roman *et al.* 2005, Ricci *et al.* 2006) and iii) that the presence of specific inhibitors of JAK/STAT and MAPK signalling pathways suppressed both the increase and the decrease in the leptin-induced progesterone secretion by the ovarian tissue, it is reasonable to suggest that leptin could be regulating steroidogenesis, at least in part, through both the ERK and STAT cascades, although an action on other signalling pathways cannot be ruled out.

It is generally accepted that SOCS3 is a negative regulator of cytokine signalling via the JAK/STAT pathway activated by the receptor–ligand complex. In this study, the ovarian expression of SOCS3 protein was increased by the acute treatment with leptin and decreased by the daily treatment. These changes occurred without concomitant alterations in *SOCS3* mRNA. While no study has directly shown regulation of ovarian expression of SOCS3 by leptin during the ovulatory process, changes in SOCS3 expression have been observed in the ovary of rodents in response to prolactin-induced signalling during pregnancy and in lactating females (Curlewis *et al.* 2002, Anderson *et al.* 2009). In another study using CHO cells, it has been demonstrated that prolactin and leptin down-regulate their own signalling pathways through the same negative regulator, SOCS3, with no crosstalk between prolactin receptor and OBR (Roy *et al.* 2007). Therefore, to our knowledge, our results are the first to demonstrate that leptin is able to modulate the expression of SOCS3 in parallel with two different signalling cascades in the ovarian tissue during the ovulatory process in the rat, although we cannot state a direct relationship between SOCS3 and both signalling pathways in our biological

models. While both treatments are not comparable, the different effects of leptin on these signalling proteins observed in this work may be in response to different levels of leptin and/or different timings of leptin administration. Therefore, further studies are necessary to clarify this point.

In our *in vitro* experiments, we also found that leptin was able to up-regulate the expression of both STAT3 and ERK1/2 at physiological concentrations and STAT3 at high concentrations (300–500 ng/ml). It is important to point out that the 3–10 ng/ml concentrations used in our *in vitro* studies are of the same order of magnitude as that found in circulation of normally fed rats and between 10 and 30 ng/ml in mildly obese rats (Watanobe & Suda 1999, Almog *et al.* 2001). Thus, 300–500 ng/ml represents the pharmacological concentration of leptin in normally fed female rats, excluding pregnant rats, which are exposed to higher concentrations than non-pregnant ones (García *et al.* 2000). The different responses obtained with these signalling proteins when the gonads are directly exposed to leptin might be related to different action mechanisms. The pattern of responsiveness obtained with p-STAT is i) similar to that observed by Ruiz-Cortés *et al.* (2003) in porcine granulosa cell cultures, where both low (10 ng/ml) and high (1000 ng/ml) leptin levels increase STAT3 phosphorylation and ii) consistent with that found in the expression of OBRb in a previous study using rats treated with gonadotrophins to induce the first ovulation as in the present work (Di Yorio *et al.* 2008). Thus, it is possible that, in the ovarian cells, the STAT3 phosphorylation caused by leptin is an OBRb-activated response. Unlike STAT3, the response profile observed with p-ERK in the present work is similar to that of progesterone measured in the culture medium of ovarian cells as 3–10 ng/ml leptin increases progesterone secretion (Ruiz-Cortés *et al.* 2003, Di Yorio *et al.* 2008) and StAR transcription (Ruiz-Cortés *et al.* 2003). Likewise, in our *in vitro* studies, the expression of SOCS3 was increased at high concentrations but not at low concentrations of leptin. Although it is difficult to compare an *in vivo* effect caused by a repeated or daily systemic exposure, as in our *in vivo* studies, with an effect caused by a tissue directly exposed to effective and known doses, this result seems to be similar to that obtained with the acute treatment and might indicate that high levels of leptin are

able to up-regulate SOCS3 protein expression in ovarian tissue in parallel with a down-regulation of progesterone synthesis, as previously observed (Di Yorio *et al.* 2008). However, further studies are in progress to examine whether other signalling pathways are involved in the leptin action and to evaluate the relationship between these signalling proteins and the ovulatory process.

Although we cannot suggest which isoform mediates the activation of these signalling proteins, the action of leptin in our biological model may be the result of its binding to both receptor isoforms in a dose-dependent manner. Some authors have shown evidence that the short forms of leptin receptor appear to be the dominant forms expressed in different types of cells, including oocytes, and that these isoforms are capable of signalling through the MAPK pathway (Craig *et al.* 2004). Furthermore, it has been recently shown that OBRa is functional and plays a role in the regulation of steroidogenesis in human granulosa cells (Lin *et al.* 2009). However, our results support that the dual leptin action on steroidogenesis seems to be, at least in part, through both ERK and STAT cascades.

In conclusion, our results indicate that leptin is able to regulate STAT3, ERK1/2 and SOCS3 proteins at both intra- and extraovarian level. The leptin action on these signalling pathways could affect, at least in part, some other ovarian events, such as the synthesis of progesterone, which, in turn, could impact on the ovulatory rate as observed previously (Roman *et al.* 2005, Ricci *et al.* 2006).

Declaration of interest

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