Leptin modulates the expression of its receptors in the hypothalamic–pituitary–ovarian axis in a differential way

M P Di Yorio[1,2], M G Bilbao[1], M C Pustovrh[1], J P Prestifilippo[1] and A G Faletti[1,2]

[1]Centro de Estudios Farmacológicos y Botánicos (CEFYBO), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET)-Facultad de Medicina, UBA, Paraguay 2155, 16° P, C1121ABG Buenos Aires, Argentina

[2]Dpto. de Química Biológica, Facultad de Ciencias Exactas y Naturales (FCEyN), UBA, Cdad.Universitaria-Pabellón II-4° P, C1428EHA Buenos Aires, Argentina

(Correspondence should be addressed to A G Faletti; Email: agfaletti@yahoo.com.ar)

Abstract

To investigate the expression of leptin receptors (Ob-R) in the rat hypothalamus–pituitary–ovarian axis, immature rats were treated with eCG/hCG and Ob-R expression was evaluated by western blot analysis. The Ob-R expression increased 24 h after eCG administration in all the tissues assayed. In the hypothalamus, these levels immediately decreased to those obtained without treatment. In the pituitary, the Ob-R expression continued to be elevated 48 h after eCG administration, whereas the hCG injection did not modify these levels. Similar results were obtained with the ovarian long isoform. To assess the effect of leptin on its receptors, Ob-R was assessed in hypothalamus, pituitary and ovarian explants cultured in the presence or absence of leptin (0·3–500 ng/ml). In the hypothalamus, we found a biphasic effect: the Ob-R expression was either reduced or increased at low or high concentrations of leptin respectively. LH-releasing hormone secretion increased at 1 ng/ml. In the pituitary, Ob-R increased at 10 or 30 ng/ml of leptin for the long and short isoforms respectively. Leptin also induced an increase in LH release at 30 ng/ml. In the ovarian culture, the presence of leptin produced an increase in Ob-R expression at different ranges of concentrations and a dose-dependent biphasic effect on the progesterone production. In conclusion, all these results clearly suggest that leptin is able to modulate the expression of its own receptors in the reproductive axis in a differential way. Moreover, the positive or negative effect that leptin exerts on the ovulatory process may be dependent on this regulation.

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Introduction

Initially, leptin was known to be a satiety hormone regulating both food intake and energy expenditure, but it is now known that this protein plays an important role in neuroendocrine signalling and reproduction. Leptin is mainly secreted from adipose cells and transported via blood to act in specific central and peripheral systems. Leptin is recognized by the leptin receptor (Ob-R; Tartaglia *et al.* 1995), which is a product of the *db* gene (Chen *et al.* 1996, Chua *et al.* 1996). Six leptin receptor isoforms generated by alternative splicing have been identified to date (Tartaglia 1997, Ahima 2000, Ahima & Flier 2000). These isoforms fall into three categories: a long form (Ob-Rb), which contains all the intracellular motifs required for effective signalling via the JAK-STAT pathway; four truncated forms, namely Ob-Ra, Ob-Rc, Ob-Rd and Ob-Rf, of which Ob-Ra is the most physiologically significant and is considered to act merely as a transporter; and a secreted form (Ob-Re), which lacks both the intracellular and transmembrane domains, and serves as a plasmatic leptin-binding protein (Lee *et al.* 1996, Tartaglia 1997).

The reproductive function is regulated by the interaction of the hypothalamus, the pituitary and the gonads, which form the reproductive axis. The Ob-R has been found all along the hypothalamus–pituitary–gonads (HPG) axis and several groups have demonstrated the presence of Ob-R mRNA in endocrine tissue in the rat. In humans and rodents, different Ob-R isoforms have been found to be widely distributed in many organs, including the hypothalamus (Mercer *et al.* 1996, Hoggard *et al.* 1997, Elmquist *et al.* 1998), the pituitary (Zamorano *et al.* 1997, Jin *et al.* 1999, 2000, Sone *et al.* 2001) and the ovary (Cioffi *et al.* 1996, 1997, Karlsson *et al.* 1997, Zamorano *et al.* 1997, Agarwal *et al.* 1999, Zachow *et al.* 1999, Duggal *et al.* 2002*b*).

Leptin is able to produce both stimulating (Ahima *et al.* 1997, Clément *et al.* 1998, Strobel *et al.* 1998, Almog *et al.* 2001, Barkan *et al.* 2005) and inhibitory (Zachow & Magoffin 1997, Agarwal *et al.* 1999, Barkan *et al.* 1999, Brannian *et al.* 1999, Zachow *et al.* 1999, Duggal *et al.* 2000, 2002*a*) effects on the ovarian function. In previous studies, we found that ovulation is significantly inhibited by an acute treatment with leptin (Ricci *et al.* 2006). However, we have also observed that a chronic treatment with a low dose of this protein enhances

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the ovulatory process in comparison with control animals and partially prevents the negative effects produced by a severe malnutrition (Roman *et al.* 2005).

Although there is some evidence of leptin's ability to modulate HPG activity in the gonads and the pituitary, most of the information on this topic is concentrated on the mechanisms of leptin action within the central nervous system, especially on the hypothalamus.

Therefore, the objectives of the present study were to investigate the expression of leptin receptors in the rat HPO axis during the ovulatory process, and to assess whether different levels of leptin in this axis are able to modulate the expression of its receptors.

Materials and Methods

Animals

Adult female Sprague–Dawley rats with ten 15-day-old pups each were purchased from the School of Veterinarian Sciences (University of Buenos Aires, Argentina). Pups were weaned at 21 days of age and were maintained under controlled conditions of light (14 h light:10 h darkness), temperature (22 °C) and humidity, with free access to food and water. Animals were handled according to the Guiding Principles for the Care and Use of Research Animals approved by the Animal Care and Use Committee of the Centro de Estudios Farmacológicos y Botánicos (CEFYBO–CONICET) – School of Medicine (UBA).

In vivo *studies*

At 26–28 days of age, female rats were i.p. injected with 10 IU eCG (in 0·10 ml saline) to induce the growth of the first generation of pre-ovulatory follicles. After 48 h, the animals were i.p. injected with 10 IU hCG (in 0·10 ml saline) to induce ovulation, which usually occurs within 12 h after hCG administration in this rat colony. Simultaneously, some animals were injected with vehicle.

Experiment 1 To study the expression of Ob-R, eight to ten animals were killed by decapitation at different times during the gonadotropin or vehicle treatment. The medial basal hypothalamus (MBH), the anterior pituitary and both the ovaries were immediately dissected out, frozen on dry ice and stored at −72 °C. The MBH tissue fragment was bounded rostrally by the posterior border of the optic chiasma, laterally by the lateral sulcus and caudally by the mammillary bodies and cut to a depth of ∼2 mm.

Experiment 2 To study the effect of different levels of leptin on the expression of its receptors during the ovulatory process, rats received one of the two following treatments: 1) acute treatment (Ricci *et al.* 2006), in which the rats received 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 2) chronic treatment (Roman *et al.* 2005), at 22 days of age, in which the rats received an i.p. injection of either recombinant rat leptin (3 μg/0·15 ml PBS–BSA) or PBS–BSA alone (control) per day until the day the animals were killed. Both leptin and PBS–BSA were administered at the beginning of the light period to avoid the influence of this protein on daily feeding. Body weight and food intake were monitored daily. At 28 days of age, animals received the same gonadotropin treatment as before. The rats from these two leptin treatments (10–12 per group) were killed 10 h after the hCG injection by decapitation, since this is a pre-ovulatory moment when all factors involved in ovulation are at their highest levels (Faletti *et al.* 1999*a*). The MBH, the anterior pituitary and both the ovaries were immediately dissected out, frozen on dry ice and stored at −72 °C until the measurement of the expression of leptin receptors by western blot analysis.

In vitro *studies*

Tissue culture After 4 h of gonadotropin treatment, animals were killed by decapitation. The MBH, the anterior pituitary and both the ovaries were immediately removed and placed in different plates containing Dulbecco's modified Eagle's medium (DMEM)/F12 (1:1) medium (Bio-Rad Laboratories) with 25 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 0·5 μg/ml fungizone and 2 mM L-glutamine. Each hypothalamus or anterior pituitary was placed individually in a final volume of 300 μl/well culture medium at 37 °C under a humidified atmosphere (5% CO_2:95% O_2). After 30-min pre-incubation, the medium was replaced by fresh medium in either the presence or the absence of leptin (0·3–500 ng/ml) and incubated for 2 or 3 h for hypothalamus (Lebrethon *et al.* 2000) or pituitary (Fernandez-Fernandez *et al.* 2005) respectively. Ovaries were dissected free of fat and bursa and cut into pieces of approximately equal size (four slices per ovary). Ovarian slices (four slices/well) were placed in a final volume of 500 μl/well culture medium at 37 °C in a humidified atmosphere (5% CO_2:95% O_2). After 30-min pre-incubation, the medium was replaced by either fresh medium alone or medium containing different leptin concentrations (0·3–500 ng/ml) and incubated for 4 h (Ricci *et al.* 2006). The leptin doses used in these studies were obtained from previous reports (Spicer & Francisco 1997, Ricci *et al.* 2006).

After the respective incubation periods, all tissues were recovered and frozen on dry ice and stored at −72 °C to measure the expression of leptin receptors by western blot analysis. The medium samples from hypothalamic explants were boiled to inactivate endogenous protease activity and stored at −20 °C as the other culture media, until assayed for hormone content. At least three independent experiments were run for each culture condition using different tissue preparations.

Drugs and chemicals

hCG, recombinant rat leptin and protease inhibitors were purchased from Sigma–Aldrich. eCG was obtained from Syntex SA (Buenos Aires, Argentina). [1, 2, 6, 7-^{3}H]progesterone and [2, 4, 6, 7-^{3}H]17β-oestradiol were obtained from Amersham Pharmacia Biotech. The western blotting reagents were obtained from Sigma–Aldrich and Bio-Rad Laboratories.

Western blot analysis

Soluble tissue extracts were prepared as previously described (Ricci *et al.* 2006). Briefly, hypothalamus, anterior pituitary or ovarian tissues were homogenized in 20 mM ice-cold Tris–HCl buffer (pH 7·4) containing 0·25 mM sucrose, 1 mM EDTA, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 100 μg/ml phenylmethylsulphonyl fluoride and 10 μg/ml trypsin inhibitors. The homogenates were centrifuged at 7800 ***g*** at 4 °C for 15 min and the protein concentration in the supernatant was determined by the Bradford method with BSA as the standard. Homogenates were boiled for 5 min in buffer containing 0·3% (w/v) bromophenol blue and 1% (v/v) β-mercaptoethanol. Equal amounts of protein (100 μg) were loaded onto 4% (w/v) 0·125 M Tris–HCl (pH 6·8) stacking polyacrylamide gel, followed by a 7·5% (w/v) 0·375 M Tris–HCl (pH 8·8) separating polyacrylamide gel. Following electrophoresis, proteins were transferred to polyvinylidene difluoride membrane (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 mM Tris–HCl:150 mM NaCl, pH 7·5) containing 5% (w/v) of milk powder, and then incubated at 4 °C overnight with antibody raised in rabbit against Ob-R (H-300; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The final dilution of antibody was 1:200. The membranes were washed four times for 15 min each in Tris–HCl:saline containing 0·1% (v/v) Tween-20 (pH 7·5; TTBS). Then, the sections were incubated for 1 h at room temperature with goat anti-rabbit IgG (1:2500) as the secondary antibody (Santa Cruz Biotechnology). The antibody was then washed off in TTBS and the immunoreactive bands were visualized using chemiluminescence detection reagents (Sigma–Aldrich) and exposed to Kodak X-OMAT film. Negative controls were carried out by omitting the incubation with the primary antibody and no bands were detected. Positive controls were carried out using the extract of different rat tissues like brain and placenta. Before reuse, the membranes were stripped, blocked and reprobed according to the manufacturer's instructions. The membranes were reprobed with anti-actin antibody (A2066, Sigma–Aldrich of Argentina SA). Molecular weight standards (Kaleidoscope St, Bio-Rad Laboratories) were run under the same conditions to identify the protein bands. Blots were scanned using a scanning UMAX Astra 12205 and densitometry was analyzed using a Dekmate III Sigma Gel software package (Jandel Scientific software). The data were normalized to β-actin protein levels in each sample to avoid procedural variability.

Immunohistochemistry

Immunostaining of Ob-R in ovarian tissue from culture studies was performed as previously (Archanco *et al.* 2003). Ovarian tissues were fixed in neutral buffered formalin, embedded in paraffin and sliced in 6 μm thick sections. Then, the slices were deparaffinized with xylene and rehydrated with graded ethanol. Endogenous peroxidase activity was blocked by incubation in 0·5% (v/v) hydrogen peroxide (30%) in absolute methanol at room temperature for 30 min. Background blocking was performed incubating with 10% (v/v) normal goat serum (Sigma–Aldrich) for 1 h at room temperature, before incubation with the specific antiserum. The tissue sections were incubated overnight at 4 °C with Ob-R mouse monoclonal antibody (Santa Cruz Biotechnology) raised in mouse against Ob-R (B-3) diluted 1:50, as primary antibody. The detection system used was the peroxidase anti-peroxidase method (PAP). The sections were incubated with goat anti-mouse secondary antibody (Sigma–Aldrich; 1:50 dilution) at room temperature for 1 h and mouse PAP (Sigma–Aldrich; 1:100 dilution) at room temperature for 1 h. All antibodies were diluted in PBS containing 0·2% (v/v) Triton X-100. Colour development was performed with a solution containing 0·06% (w/v) 3,3′-diaminobenzidine (Sigma–Aldrich) plus 0·01% (v/v) hydrogen peroxide for 15–30 min. Control sections were performed by omitting the primary antibody. Finally, the sections were dehydrated, mounted with Entellan New (Merck) and observed with a Zeiss Axiophot light microscope.

Hormone assays

Progesterone and 17β-oestradiol were quantified by RIA in the ovarian culture medium as described previously (Roman *et al.* 2005, Ricci *et al.* 2006). Antisera were kindly provided by Dr G D Niswender (Colorado State University, Fort Collins, CO, USA). The sensitivity of both assays was 15 pg/ml. The cross-reactivities for oestradiol antiserum were 1% for progesterone and testosterone, 5% for oestriol and 10% for oestrone. The cross-reactivities for progesterone antiserum were $<2{\cdot}0\%$ for 20α-dihydro-progesterone and deoxycorticosterone and 1·0% for other steroids in the serum. Results were expressed as ng/mg protein.

Luteinizing hormone (LH)-releasing hormone (LHRH) was measured by RIA in the hypothalamic culture medium as described previously (Faletti *et al.* 1999*b*). The highly specific LHRH antiserum was kindly provided by Ayala Barnea (University of Texas Southwestern Medical Center, Dallas, TX, USA). The sensitivity of the assay was 0·2 pg per tube and the curve was linear up to 100 pg LHRH. The intra-assay coefficient of variation of the LHRH RIA ranged from 4 to 7·3%, and the inter-assay coefficient of variation was 8·9%. LH was measured by RIA in the pituitary culture medium using a double antibody and reagents kindly provided by the NIAMDD Rat Pituitary Programme. The sensitivity of the assay was 4 pg per tube and the curve was linear up to 1000 pg LH. The intra- and inter-assay coefficients of variation were 8 and 10% respectively.

LHRH and LH for iodination were purchased from Peninsula Laboratories. Iodine-125 for iodination was purchased from New England Nuclear. All samples were measured in duplicate. The results were expressed as pg per hypothalamus or pituitary.

Statistical analysis

All data are expressed as means ± S.E.M. Comparisons between groups were performed using a two-way ANOVA with Bonferroni post-tests for the *in vivo* experiments, and one-way ANOVA with Dunnett's multiple comparison test between each concentration and controls for the *in vitro* experiments. The difference between the leptin-treated group and the buffer group in the *in vivo* treatment with leptin was analysed using Student's *t*-test. Differences between groups were considered significant when $P<0{\cdot}05$.

Results

Effect of the gonadotropin treatment on the expression of leptin receptors

The expression of leptin receptors was evaluated at different times after the eCG/hCG or vehicle treatment by western blot analysis in the HPO axis. All these tissues revealed both the presence of long and short receptor isoforms by a polyclonal antibody raised against a recombinant protein corresponding to amino acids 541–840 mapping a region within an internal domain of Ob-R. Ob-R-immunoreactive proteins with apparent relative molecular masses of 210, 150 and 110 kDa were detected, as previously described with CHO cells (Matsuda *et al.* 1999), hypothalamus tissue (Pal & Sahu 2003), adipose tissue (Meli *et al.* 2004) and ovarian tissue (Ricci *et al.* 2006). Protein bands at 150 and 110 kDa were consistent with the predicted size of Ob-Rb and the short isoforms (Ob-Rs) respectively based on amino acid composition. Thus, these two bands were considered to be leptin receptors. Figures 1 and 2 show the results of leptin receptor expression in the different tissues assayed during the gonadotropin treatment. No differences were found in the expression of leptin receptor protein in the rats treated with vehicle. eCG induced an increase in the expression of both hypothalamic Ob-R proteins at 24-h post-treatment when compared with that obtained from animals without treatment (−48 h). These increases represented 73·7% ($P<0{\cdot}05$) and 73·5% ($P<0{\cdot}01$) for Ob-Rb and Ob-Rs respectively (Fig. 1B and C). Furthermore, these levels were significantly higher than those obtained from animals treated with vehicle ($P<0{\cdot}05$ and $P<0{\cdot}01$ for long and short proteins respectively). After 48 h of eCG treatment, the expression returned to values similar to those obtained without treatment and the hCG injection did not alter these levels.

Results obtained for the expression of the levels of pituitary and ovarian leptin receptor are shown in Fig. 2 (left and right panels respectively). eCG treatment produced an increase in the levels of the pituitary Ob-Rb expression at 24 h (79·7%; $P<0{\cdot}01$) and at 48 h (98·3%; $P<0{\cdot}01$) post-injection in comparison with that obtained from animals without treatment (−48 h; Fig. 2B). Furthermore, these levels were significantly higher than those obtained from animals treated with vehicle ($P<0{\cdot}05$ at 48-h post-eCG). These values remained high after hCG injection and decreased after ovulation (Fig. 2B). Similar results were obtained with the expression of the pituitary Ob-Rs protein, in which the increase was 184% at 24 h ($P<0{\cdot}05$) and 198% at 48 h ($P<0{\cdot}01$) post-eCG in comparison with that obtained from animals without treatment (−48 h; Fig. 2C).

The expression of both isoforms of leptin receptor in the ovarian tissue obtained from rats treated with eCG/hCG is shown in Fig. 2 (right panel). The administration of eCG induced an increase in the expression of Ob-Rb proteins at 24 h (49·9%; $P<0{\cdot}05$) and at 48 h (71·2%; $P<0{\cdot}01$) post-injection in comparison with that obtained from animals without treatment (−48 h). After hCG administration, the expression of this protein

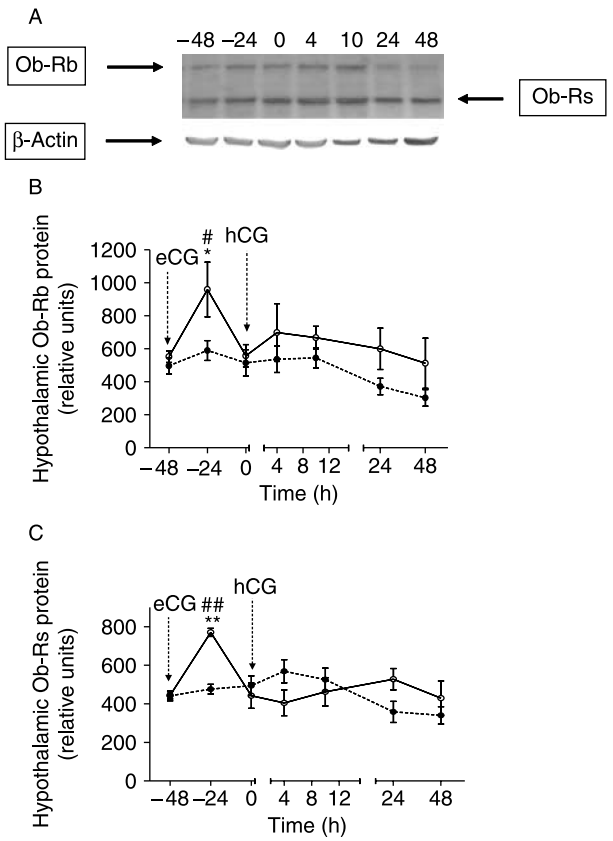

Figure 1 Pattern of changes in protein levels of the long (Ob-Rb) and short (Ob-Rs) isoforms of leptin receptor in the hypothalamic tissue obtained at different times from immature rats primed with eCG/hCG (—○—) or vehicle (- -●- -). (A) Expression of both Ob-R and β-actin, as protein control, by western blot analysis, from rats primed with eCG/hCG. (B and C) Quantitative analysis of immunoreactive bands for Ob-Rb (B) and Ob-Rs (C). Data points represent the mean ± S.E.M. for four to five samples per group. Each sample represents two hypothalami from different animals at the same time. $^{*}P<0{\cdot}05$, $^{**}P<0{\cdot}01$ vs −48 h. $^{\#}P<0{\cdot}05$, $^{\#\#}P<0{\cdot}01$ versus vehicle (two-way ANOVA and Bonferroni post-tests).

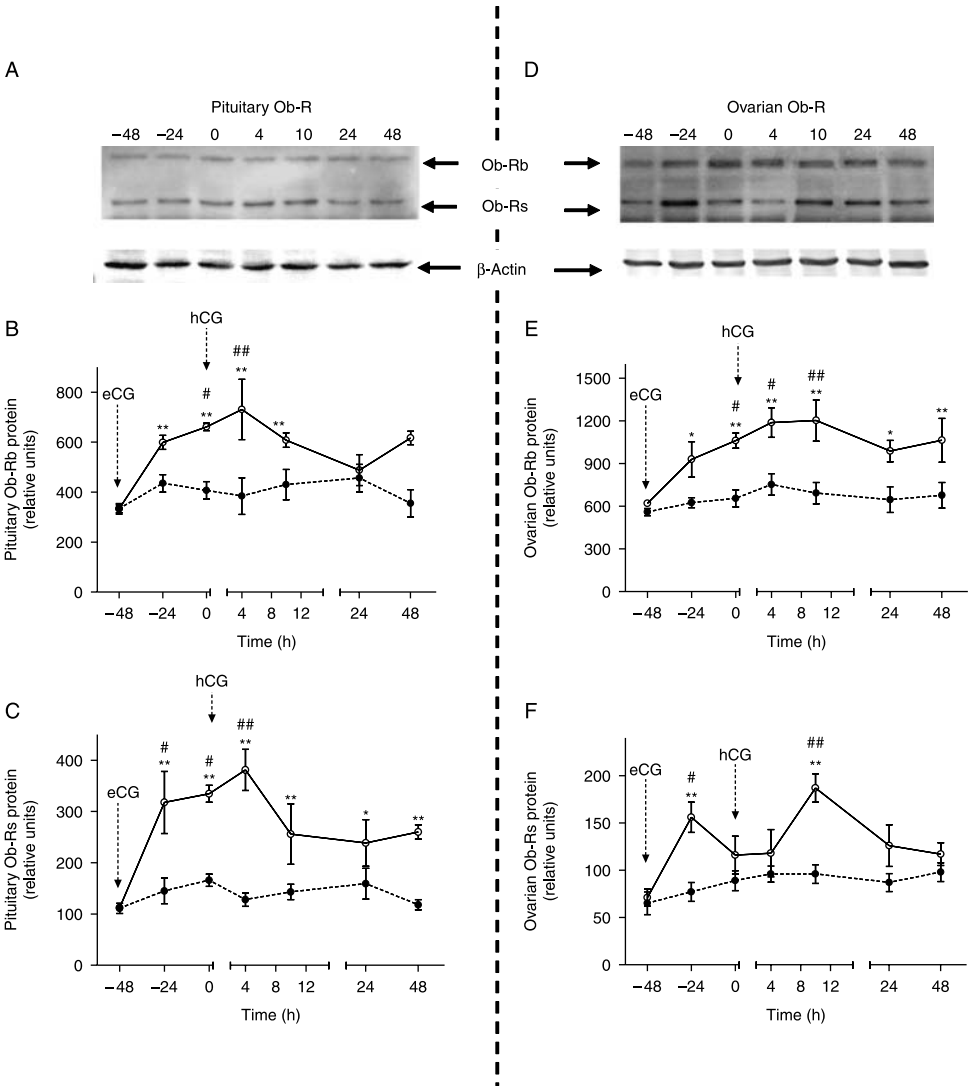

Figure 2 Pattern of changes in protein levels of the long (Ob-Rb) and short (Ob-Rs) isoforms of leptin receptor in the pituitary (left panel) and ovarian (right panel) tissue obtained at different times from immature rats primed with eCG/hCG (—○—) or vehicle (- -●- -). (A and D) Expression of both Ob-R isoforms and β-actin, as protein control, by western blot analysis, from rats primed with eCG/hCG. (B and C) Quantitative analysis of immunoreactive bands for Ob-Rb (B) and Ob-Rs (C) in pituitary tissue (left panel). (E and F) Quantitative analysis of immunoreactive bands for Ob-Rb (E) and Ob-Rs (F) in ovarian tissue (right panel). Data points represent the mean ± S.E.M. for four to seven samples per group. Each sample represents two pituitary or two to four ovaries from different animals at the same time. $^{*}P<0{\cdot}05$, $^{**}P<0{\cdot}01$ vs −48 h. $^{\#}P<0{\cdot}05$, $^{\#\#}P<0{\cdot}01$ versus vehicle (two-way ANOVA and Bonferroni post-tests).

remained high and reached a maximum at 10-h post-injection ($P<0{\cdot}01$). These levels were significantly higher than those obtained from animals treated with vehicle ($P<0{\cdot}05$ at 0 and 4 h, and $P<0{\cdot}01$ at 10-h post-hCG; Fig. 2E). In the case of the ovarian Ob-Rs, eCG induced an increase in their expression at 24-h post-treatment (119·7%; $P<0{\cdot}01$) when compared with that obtained from animals without treatment (−48 h). In addition, these levels were significantly higher than those obtained from animals treated with vehicle ($P<0{\cdot}05$). After 48 h of eCG treatment, the expression returned to values similar to those obtained from rats treated with vehicle. hCG induced an increase at 10-h post-administration ($P<0{\cdot}01$) and after ovulation, no differences were found in the expression of Ob-Rs between rats primed with eCG/hCG and those injected with vehicle.

Effect of different levels of leptin on the expression of leptin receptors

***In vivo* studies** Figure 3 shows the results obtained with the ovarian tissue from immature rats subjected to both the

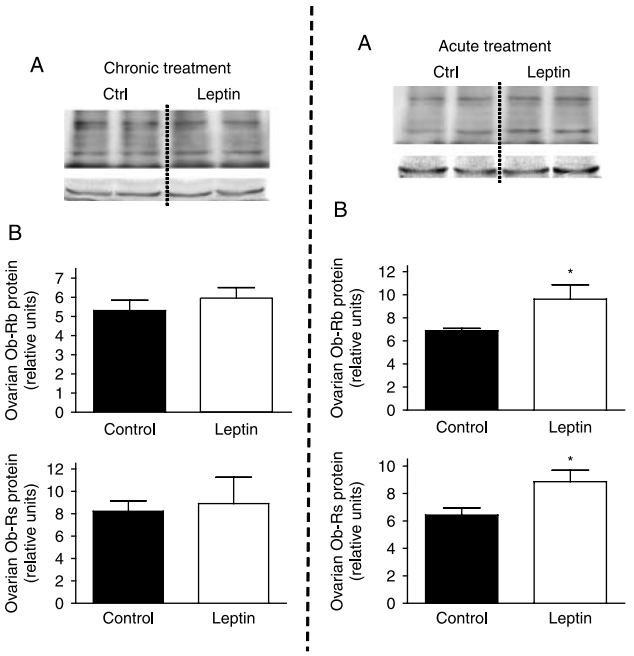

Figure 3 Effect of chronic (left panel) and acute (right panel) treatment of leptin on the expression of ovarian leptin receptors. In the chronic treatment, immature rats were daily injected with either PBS–BSA or 3 μg leptin. After a week, the animals were primed with eCG/hCG. In the acute treatment, immature rats were primed with either eCG/hCG and treated with PBS–BSA or 5 μg leptin at 1 h before hCG and at 150-min intervals. All the animals were killed 10 h after hCG administration. (A) Expression of both ovarian Ob-R isoforms and β-actin, by western blot analysis. (B) Quantitative analysis of immunoreactive bands for both Ob-R in ovarian tissue. Data points represent the mean±S.E.M. for four to five samples per group. Each sample represents two ovaries from different animals with the same treatment. *$P<0{\cdot}05$ versus control (Student's *t*-test).

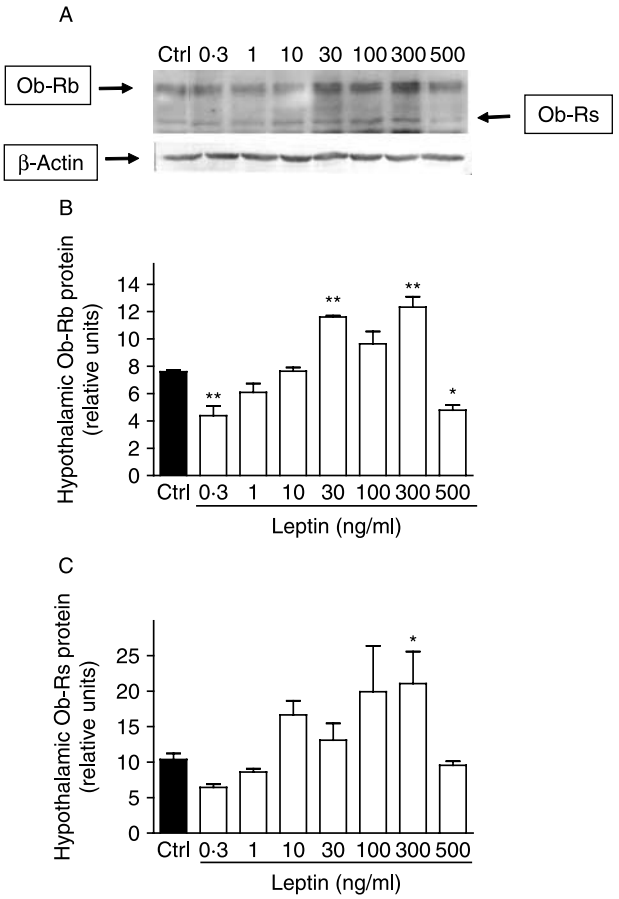

Figure 4 *In vitro* effect of leptin on the protein levels of hypothalamic long (Ob-Rb) and short (Ob-Rs) isoforms. Hypothalamic explants were obtained 4 h after hCG administration from immature rats primed with eCG/hCG and incubated for 2 h either in the presence or in the absence of different concentrations of leptin (30–500 ng/ml). (A) Expression of both Ob-R and β-actin, as protein control, by western blot analysis. (B and C) Quantitative analysis of immunoreactive bands for Ob-Rb (B) and Ob-Rs (C). Results are mean±S.E.M. of three independent experiments; each treatment was applied in quadruplicate culture well and each sample represents two hypothalami from different animals exposed at the same concentration ($n=6$). *$P<0{\cdot}05$, **$P<0{\cdot}01$ versus control (one-way ANOVA and Dunnett's multiple comparison test).

chronic and acute treatments. No differences were found in the expression of leptin receptors of either Ob-Rb or Ob-Rs in ovaries between animals treated with a daily dose of leptin and control animals (left panel). The same results were found with hypothalamic and pituitary tissue (data not shown). By contrast, the acute treatment with leptin significantly increased the expression of both leptin receptor isoforms ($P<0{\cdot}05$) in ovarian tissue (Fig. 3, right panel), but no differences were found in either hypothalamic or pituitary tissue (data not shown).

***In vitro* studies** Figures 4 and 5 show the expression of the leptin receptors in the hypothalamic, pituitary and ovarian tissues after incubation for 2, 3 or 4 h respectively, in the presence or absence of different levels of leptin (0·3–500 ng/ml). In the case of hypothalamus, the results show a biphasic effect of leptin on the expression of its receptors (Fig. 4). The addition of low concentrations of leptin (0·3 ng/ml) significantly reduced (−42%, $P<0{\cdot}01$) while high concentrations (30 and 300 ng/ml) increased the protein content of the Ob-Rb isoform (52 and 62% respectively; $P<0{\cdot}01$) when compared with controls, although the highest concentrations assayed produced a significant reduction ($P<0{\cdot}05$; Fig. 4B). The content of hypothalamic Ob-Rs protein showed the same pattern as that obtained with the long isoform, although the results were significant only at 300 ng/ml, when a densitometric analysis was performed (Fig. 4C).

In the case of pituitary tissue (Fig. 5, left panel), the expression of Ob-Rb protein was significantly increased at leptin concentrations of 10 ng/ml (74%, $P<0{\cdot}01$; Fig. 5B), while Ob-Rs isoforms reached a maximum at 30 ng/ml (130%, $P<0{\cdot}01$; Fig. 5C), both when compared with those obtained from controls. When ovarian tissues were incubated

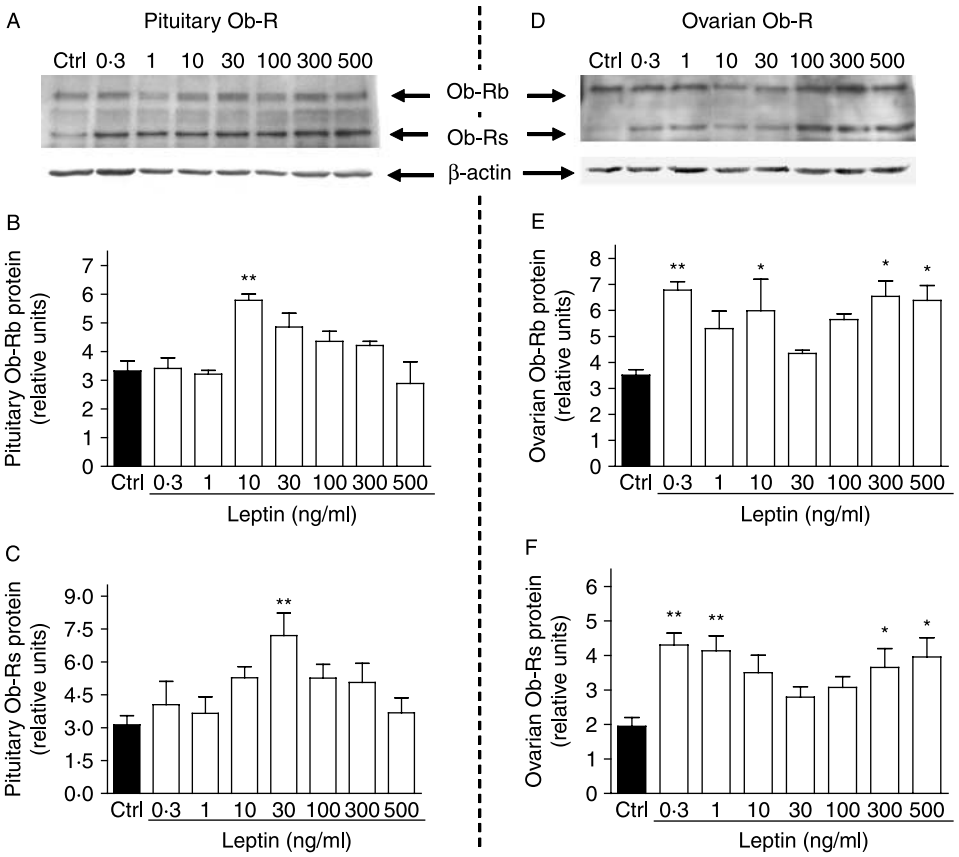

Figure 5 *In vitro* effect of leptin on the protein levels of pituitary (left panel) and ovarian (right panel) of both the long (Ob-Rb) and short (Ob-Rs) isoforms. Pituitary and ovarian explants were obtained 4 h after hCG administration from immature rats primed with eCG/hCG and incubated for 3 or 4 h respectively either in the presence or in the absence of different concentrations of leptin (0·3–500 ng/ml). (A and D) Expression of both Ob-R and β-actin, as protein control, by western blot analysis. (B and C) Quantitative analysis of immunoreactive bands for Ob-Rb (B) and Ob-Rs (C) in pituitary tissue (left panel). (E and F) Quantitative analysis of immunoreactive bands for Ob-Rb (E) and Ob-Rs (F) in the ovarian tissue (right panel). Results are mean±S.E.M. of three independent experiments; each treatment was applied in quadruplicate culture well and each sample represents two pituitary or two ovaries from different animals exposed at the same concentration ($n=6$). *$P<0·05$, **$P<0·01$ versus control (one-way ANOVA and Dunnett's multiple comparison test).

in the presence of different concentrations of leptin, the expression of both isoforms of leptin receptors significantly increased in different ranges of leptin levels: at 0·3, 10, 300 and 500 ng/ml for Ob-Rb and at 0·3, 1, 300 and 500 ng/ml for Ob-Rs (Fig. 5, right panel). These increases oscillated between 70 and 90% as compared with controls.

In order to study the effect of leptin on these tissues and relate it with the changes obtained in the expression of leptin receptors, the secretion of LHRH was measured in hypothalamic incubation medium, LH in pituitary incubation medium, and progesterone and 17β-oestradiol were measured in the ovarian incubation medium. These data are summarized in Table 1. The addition of leptin led to an increase in LHRH secretion to reach a maximum at 1 ng/ml compared with those obtained from controls, while higher concentrations did not alter this secretion. Leptin also induced an increase in LH release from pituitary cultures when compared with that obtained from controls, but it was significant at 30 ng/ml. In the ovarian culture, the presence of leptin produced a dose-dependent biphasic effect on the production of progesterone. Progesterone concentration was significantly increased at 1–10 ng/ml and significantly reduced at 300 ng/ml when compared with controls. No differences were found in 17β-oestradiol concentrations (Table 1).

In order to confirm the results obtained with the *in vitro* assays, and to localize Ob-R in ovarian tissue, immunolocalization was assessed. The whole ovarian tissue was incubated in the same conditions as those in the ovarian tissue culture in the presence or absence of some leptin concentrations that showed an increase in the content of Ob-R protein. Figure 6 shows the Ob-R immunostaining obtained. As expected, the Ob-R receptors were detected mainly in granulosa and

Table 1 *In vitro* effect of different leptin levels on the production of luteinizing hormone (LH)-releasing hormone (LHRH), LH, progesterone and 17β-oestradiol by cultures of tissue explants, from immature gonadotropins-primed rats. Hypothalamic, pituitary and ovarian explants were obtained 4 h after hCG administration from immature rats primed with eCG/hCG and incubated for 2, 3 or 4 h respectively either in the presence or in the absence of different concentrations of leptin (0·3–500 ng/ml). Values are expressed as means±S.E.M. of three independent experiments; each treatment was applied in quadruplicate culture well and each sample represents the concentrations of the hormone in the culture medium of one hypothalamus, one pituitary or two ovaries from different animals exposed at the same concentration (n=8–12)

	Ctrl	**Leptin** (ng/ml)						
		0·3	1	10	30	100	300	500
LHRH (pg/hypothalamus)	10±1	16±2	18±2*	15±2	12±3	9±3	8±1	10±3
LH (ng/pituitary)	54±5	87±15	85±16	94±24	174±35†	85±7	80±14	71±12
Progesterone (ng/mg protein)	18±2	22±1	27±2*	31±2†	21±2	15±1	8±2*	14±2
Oestradiol (ng/mg protein)	15±1	17±2	18±2	15±2	20±3	14±1	14±2	13±1

*P<0·05, †P<0·01 versus control (one-way ANOVA and Dunnett's multiple comparison test).

cumulus oophorus cells. Weaker immunostaining was observed in oocytes, theca and interstitial cells (Fig. 6A). A stronger immunostaining was observed in ovarian tissue incubated with low concentrations (0·3 ng/ml) of leptin (Fig. 6C) compared with control (Fig. 6B). Similar results were obtained when ovarian tissues were incubated with high concentrations (300 ng/ml) of leptin (Fig. 6F and G). Figure 6D, E, H and I show the negative controls from each sample.

Discussion

The reproductive function is regulated by a close relationship between the hypothalamus, the pituitary and the gonads, which form the reproductive axis. This axis is, in turn, modulated by multiple and complex metabolic and nutritional factors. Although there are a lot of experimental evidence about the mechanism involved in regulating the reproductive function, the complete system remains poorly understood. The discovery of leptin, a 16 kDa protein synthesized primarily by adipose tissue, opened up a new perspective in the complex endocrine system that controls the reproductive system. Since mRNA for leptin receptor has been detected in ovarian tissue, many studies have demonstrated that leptin may have direct effects on the ovarian function. However, most of these studies are contradictory, since both stimulatory and inhibitory actions on the ovarian function have been described (Ahima *et al.* 1997, Zachow & Magoffin 1997, Clément *et al.* 1998, Strobel *et al.* 1998, Agarwal *et al.* 1999, Barkan *et al.* 1999, 2005, Brannian *et al.* 1999, Zachow *et al.* 1999, Duggal *et al.* 2000, 2002*a*, Almog *et al.* 2001). Recently, we have demonstrated that leptin may be regulating the ovulatory process, as when the levels of this protein are low there is a positive relationship between leptin and the ovarian function (Roman *et al.* 2005), while when these levels are high, there is a negative relationship between them (Ricci *et al.* 2006). In this work, we studied the expression of leptin receptors in the rat HPO axis during the ovulatory process and found that gonadotropins regulate these receptors in a differential way. eCG induced an increase in the expression of both the long and short isoforms of leptin receptors in hypothalamus, although this result was only significant with the short forms. Ryan *et al.* (2003) found that plasmatic leptin decreases 70% after eCG administration in immature rats primed with eCG/hCG, and that the basal values were recovered 48-h post-eCG. These results appear to suggest that leptin receptors are sensitive to change in plasmatic leptin concentrations, in order to balance a ligand lack. In a recent study, Meli *et al.* (2004) observed that the plasmatic leptin concentration was increased in ovariectomized rats in a time-dependent manner. This increase was 50% in 1 week and 100% in 22 weeks after ovariectomy. Moreover, the expression of the long isoform decreased in hypothalamus in both 1 and 22 weeks after ovariectomy. These data confirm our findings, since the leptin receptors in the hypothalamus seem to balance the modification of plasmatic leptin levels.

In a previous study, we found that plasmatic leptin levels diminished 4 h after hCG administration in immature rats primed with eCG/hCG (Ricci *et al.* 2006). Similar results were found by Ryan *et al.* (2003) who reported a reduction of 80% leptin at 9-h post-hCG administration. In our studies, the expression of hypothalamic leptin receptors was not altered by hCG administration, while the content of these receptors was high in both the pituitary and the ovarian tissue. These results suggest that the hypothalamus is not responsive to plasmatic leptin changes during hCG treatment. Ryan *et al.* (2003) also reported that mRNA expression of both the short and long forms of the leptin receptor in the immature gonadotropin-primed rat ovary increases after hCG administration, followed by a dramatic reduction 24 h after this treatment. In the present study, we found that the ovarian content of both proteins (Ob-Rb and Ob-Rs) was increased before follicular rupture. In this study, we confirm the results obtained in a previous work with the long isoform (Ricci *et al.* 2006), where we found that the administration of eCG induced an increase in the expression of Ob-R protein, and that hCG administration did not modify this increase. All these data confirm that the production of leptin and its receptors is regulated by gonadotropins

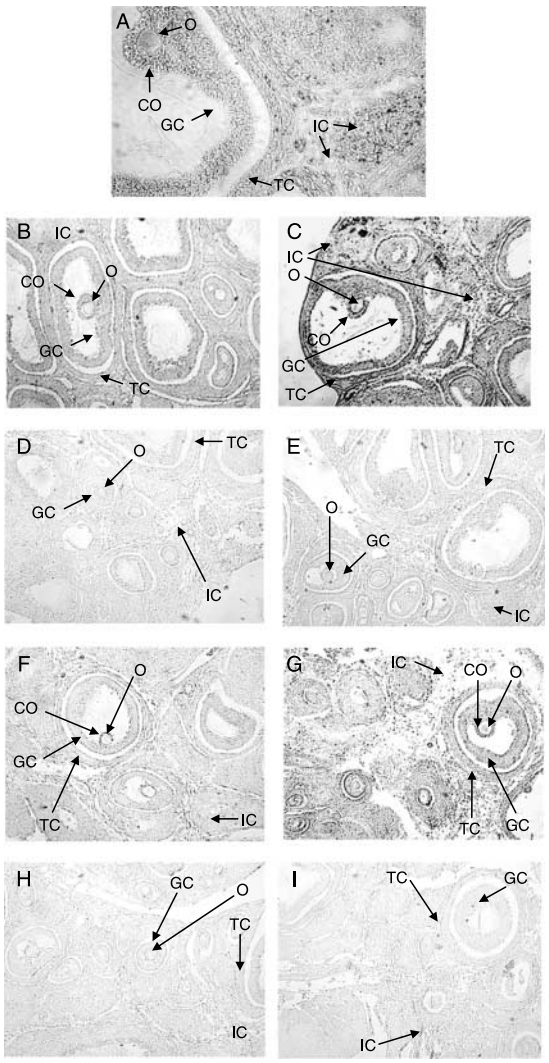

Figure 6 Representative photomicrographs of leptin effect on ovarian Ob-R. Ovarian tissues were obtained 4 h after hCG administration from immature rats primed with eCG/hCG and incubated for 4 h either in the presence or in the absence of different concentrations of leptin (0·3 or 300 ng/ml). (A) Immunostaining from ovarian control without incubation. (B and F) Immunostaining from ovarian control after incubation. (D and H) Negative control from B and F respectively. (C) Immunostaining from ovarian tissue exposed at 0·3 ng/ml leptin. (G) Immunostaining from ovarian tissue exposed at 300 ng/ml. (E and I) Negative control from C and G respectively. GC, granulosa cells; CO, cumulus oophorus cells; O, oocyte; IC, interstitial cells. Original magnification: 80×.

in a tissue-dependent manner, indicating their differential targets in different processes, like follicular growth and/or ovulation. The fact that leptin receptors from the pituitary and the ovarian tissues were responsive to hCG is not surprising, because these peripheral tissues are closely involved in the final phase of ovulation and are activated by the hypothalamus-produced gonadotropin-releasing hormones (GnRH), which are in turn, negatively regulated by the ovarian hormones. Since our studies were not able to distinguish the different short isoforms, further studies are necessary to clarify which isoforms are involved in the different events of the ovarian function. However, we firmly believe that these findings will be useful in future investigations, since we have shown for the first time that the expression of leptin receptors along the HPO axis is clearly modified by gonadotropins in a differential manner.

In previous studies using this biological model, it has been shown that eCG administration resulted in a gradual increase in plasmatic oestrogens, and that after hCG injection, these levels diminished to basal values a few hours before ovulation occurs (Espey *et al.* 1989, 1991, Ryan *et al.* 2003). These results, together with those obtained in this work, suggest that oestrogens could be exerting a negative feedback on the expression of leptin receptors in the hypothalamic tissue. These data support the results obtained by Bennett *et al.* (1998, 1999) who demonstrated that oestrogens modulate the expression of the hypothalamic leptin receptors. On the other hand, it is likely that the expression of leptin receptors in the peripheral tissues is also modulated by steroids, since a positive relationship between steroids and leptin receptors in both the pituitary and ovarian tissue seems to exist. All these data may suggest that leptin receptors in the HPO axis could be differentially regulated by steroids, depending on both the tissue and the gonadotropin treatment timing.

In previous studies, using immature rats primed with eCG/hCG, we found that an acute treatment with leptin inhibits the ovulatory process (Ricci *et al.* 2006), whereas a chronic administration with a low dose of this protein enhances it (Roman *et al.* 2005). Therefore, we investigated whether these treatments were able to modify the expression of leptin receptors. No differences were found in the expression of leptin receptors between animals subjected to the chronic treatment and controls. By contrast, the acute treatment with leptin significantly increased the expression of both the long and short isoforms of leptin receptors in the ovarian tissue, but no differences were found in either the hypothalamic or the pituitary tissue. We initially expected that the changes in the expression of leptin receptors produced by the gonadotropin treatment before ovulation would be altered by leptin administration. However, we found that neither the acute nor the chronic treatments showed significant alterations in their expressions, except for the response with high level of leptin on the ovarian tissue. In a previous study, the chronic administration of leptin for 4 weeks diminished the expression of the mRNA and protein of leptin receptor in the rat hypothalamus (Martin *et al.* 2000). However, other studies have shown no changes in the expression of leptin receptors after a chronic administration of low doses of leptin for 2 weeks (Pal & Sahu 2003). Although all these data suggest that both the dose and the timing of leptin administration are critical to obtain either a positive or a negative response, further studies are necessary to clarify this point.

In order to study the effect of a narrow range of leptin concentrations on its receptors, the expression of leptin

receptors was assessed in hypothalamic, pituitary and ovarian explant cultures either in the presence or in the absence of different leptin levels. We found that leptin differentially regulates its receptors in both dose- and tissue-dependent manner. Again, a similar pattern of responsiveness was obtained with both the long and short isoforms within the same tissue. In the hypothalamic culture, we found that leptin was able to produce a biphasic effect on leptin receptors after a 2-h incubation. The results obtained with low (0·3 ng/ml) leptin levels suggest again that leptin receptors in hypothalamus are sensitive to changes in the plasmatic leptin concentrations in order to balance modifications of its ligand. It has been reported that leptin levels in the cerebrospinal fluid of normally fed female rats (0·2–0·3 ng/ml) are about ten times lower than in the plasma (Grueso *et al.* 2001, Rocha *et al.* 2001). The other concentrations of leptin used in this study were, however, higher than those normally found in the cerebrospinal fluid. It was surprising that the hypothalamus was responsive to these levels of leptin. Although we cannot explain this response, we should point out that this is an *in vitro* system without the presence of numerous regulatory factors, and thus it is difficult to extrapolate. Low concentrations of leptin (1 ng/ml) were able to induce an increase in LHRH and an inhibition of the content of the leptin receptor, while high concentrations did not alter LHRH secretion and increased the content of leptin receptor when compared with controls. These results are consistent with other previous studies using different biological models. Yu *et al.* (1997), using hypothalamus explants from adult male rats, demonstrated a significant increase in LHRH only at the lowest concentrations tested (10^{-12}–10^{-10} M), whereas they did not find any changes at higher levels. Lebrethon *et al.* (2000), using hypothalamus explants from pre-pubertal male rats, also reported stimulatory effects on GnRH secretion by the presence of different concentrations of leptin. Watanobe (2002) examined the effects of direct intra-hypothalamic perfusions with leptin (1–10 ng/ml) on the *in vivo* release of GnRH in ovarian steroid-primed ovariectomized rats and found that in normally fed animals, the leptin infusion had no effect, while in 3-day-fasted rats, leptin was effective in stimulating GnRH secretion.

When we studied the effect of leptin on the peripheral tissues, we observed that the expression of leptin receptors increased in different ranges of leptin levels. The maximal increase in pituitary was at 10 and 30 ng/ml of leptin for Ob-Rb and Ob-Rs respectively. In turn, we found that 30 ng/ml of leptin was able to increase significantly the LH secretion in incubated anterior pituitary. Yu *et al.* (1997) found that leptin produced a dose-related increase in LH after 3 h of incubation of hemi-anterior pituitary of adult male rats. This increase reached peaks with 10^{-9} mol/l. De Biasi *et al.* (2001) demonstrated that the addition of increasing concentrations of leptin to the incubation medium of proestrus pituitaries produced a dose-related stimulation of LH release, where the maximal increase was obtained with 10 nmol/l (~16 ng/ml). Leptin also stimulates LH release by pituitaries from ovariectomized rats, and the treatment with steroid hormones leads to a marked increase in the response (De Biasi *et al.* 2001). Furthermore, leptin is able to stimulate LH release in pre-pubertal female rats (Dearth *et al.* 2000) and adult ovariectomized estrogen-primed rats (Yu *et al.* 1997) after a third ventricular injection of the peptide *in vivo*. All these results indicate that leptin plays an important role in controlling gonadotropin secretion by stimulatory hypothalamic and pituitary actions, at least in part, by modulating its receptors in a differential manner. It has been reported that plasmatic leptin levels are about 2–3 ng/ml in normally fed female rats (Watanobe & Suda 1999, Almog *et al.* 2001), 0·3 ng/ml in 3-day-fasted rats (Watanobe *et al.* 1999) and 10 ng/ml in mildly obese humans and rats (Watanobe *et al.* 2001). Women with mild obesity have been reported to have three to four times higher levels of circulating leptin than subjects of normal weight (Rissanen *et al.* 1999). All these data suggest that the anterior pituitary seems to be insensitive to leptin concentrations higher than those found in mildly obese humans and rats. Differently, the ovary responded to both lower (0·3 ng/ml) or higher (300–500 ng/ml) leptin concentrations than the pituitary. The results obtained with progesterone concentrations in the incubation medium are consistent with previous studies using different biological models. Roman *et al.* (2005) reported that chronic treatment with low doses of leptin produces a stimulatory effect on the plasmatic progesterone levels, while Ricci *et al.* (2006) observed an inhibitory effect on the plasmatic progesterone levels in rats treated with high levels of leptin. Furthermore, Ricci *et al.* (2006) confirmed this result with *in vitro* studies where the concentration of progesterone was reduced by the presence of high levels of leptin in ovarian explants and preovulatory follicle cultures. Although the mechanism involved for such inhibitory action has not been completely clarified, it has been suggested that leptin may modulate some transcriptional factors such as StAR and P450scc (Tena-Sempere *et al.* 2001) or c-Jun (Barkan *et al.* 1999). In this work, we found that leptin is able to induce a biphasic effect on progesterone production. A direct inhibitory action of leptin on steroid hormone secretion has been demonstrated independently by different authors in the ovary (Spicer & Francisco 1997, Zachow & Magoffin 1997, Agarwal *et al.* 1999, Barkan *et al.* 1999, Zachow *et al.* 1999, Ghizzoni *et al.* 2001, Kikuchi *et al.* 2001) and in other tissues (Tena-Sampere *et al.* 2001, Cameo *et al.* 2003). However, findings of both stimulation and inhibition effect with different levels of leptin in ovarian cells *in vitro* are in agreement with observations by Ruiz-Cortés *et al.* (2003) who found that the effects of leptin are biphasic with regard to stimulation and inhibition of progesterone synthesis. These authors have reported that leptin modulates steroidogenesis in a biphasic manner via STAT-3.

Finally, the reason for the apparently different results obtained between *in vivo* and *in vitro* treatments with leptin is not surprising considering that i) the effective amount of leptin that reaches its target in the *in vivo* experiments is

reduced due to the loss occurring in the peripheral route, thus making it difficult to be precise with the concentrations to which the tissue is exposed and ii) although the *in vitro* experiments are a good design to study the direct effect of many factors on their targets, we should point out that these systems lack numerous regulatory factors, and thus it is difficult to extrapolate the results. However, it is important to highlight the results obtained with the ovarian tissue, since the expression of leptin receptors was increased in rats subjected to the acute treatment with leptin and in the ovarian explants exposed to the highest concentrations.

In conclusion, all these results clearly suggest that leptin is able to modulate the expression of its own receptors in the hypothalamic–pituitary–ovarian axis in a differential way and that the positive or negative effect that leptin exerts on the ovulatory process may be dependent on this regulation.

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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