REPRODUCTION
RESEARCH

Inhibitory effect of leptin on the rat ovary during the ovulatory process

A G Ricci[1], M P Di Yorio[1] and A G Faletti[1,2]

[1]*Dpto de Química Biológica, Facultad de Ciencias Exactas y Naturales (FCEyN), Universidad de Buenos Aires (UBA), Buenos Aires, Argentina and* [2]*Centro de Estudios Farmacológicos y Botánicos (CEFYBO), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Facultad de Medicina, UBA, Paraguay 2155, 16 Piso, Buenos Aires C1121ABG, Argentina*

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

Abstract

The aims of this study were to investigate the negative action of leptin on some intraovarian ovulatory mediators during the ovulatory process and to assess whether leptin is able to alter the expression of its ovarian receptors. Immature rats primed with gonadotrophins were used to induce ovulation. Serum leptin concentration was diminished 4 h after human chorionic gonadotrophin (hCG) administration, whereas the ovarian expression of leptin receptors, measured by western blot, was increased by the gonadotrophin treatment. Serum progesterone level, ovulation rate and ovarian prostaglandin E (PGE) content were reduced in rats primed with equine chorionic gonadotrophin (eCG)/hCG and treated with acute doses of leptin (five doses of 5 μg each). These inhibitory effects were confirmed by *in vitro* studies, where the presence of leptin reduced the concentrations of progesterone, PGE and nitrites in the media of both ovarian explants and preovulatory follicle cultures. We also investigated whether these negative effects were mediated by changes in the expression of the ovarian leptin receptors. Since leptin treatment did not alter the expression of ovarian leptin receptor, the inhibitory effect of leptin on the ovulatory process may not be mediated by changes in the expression of its receptors at ovarian level, at least at the concentrations assayed. In summary, the ovulatory process was significantly inhibited in response to an acute treatment with leptin, and this effect may be due, at least in part, to the direct or indirect impairment of some ovarian factors, such as prostaglandins and nitric oxide.

Reproduction (2006) **132** 771–780

Introduction

Leptin, a recently discovered 16 kDa protein, is synthesised primarily by adipose tissue and secreted into the blood stream. The protein was first identified as the gene product found deficient in the obese *ob*/*ob* mouse (Zhang *et al.* 1994). The administration of leptin to *ob*/*ob* mice, which lack circulating leptin, resulted not only in the restoration of normal body weight and food intake, but also in re-establishment of fertility (Chehab *et al.* 1996, Cioffi *et al.* 1997, Mounzih *et al.* 1997). These observations have suggested that leptin may participate in the control of reproduction, in conjunction with that of food intake and energy expenditure. The hypothalamus seems to be the main target of leptin action, since leptin receptors have been localised in various neuronal populations (Mercer *et al.* 1996, Schwartz *et al.* 1996). However, the occurrence of leptin receptors has also been identified on different peripheral structures of the reproductive system. This suggests that, in addition to the hypothalamus, leptin might also exert actions at these sites (Tartaglia 1997).

The effects of leptin on ovulation are contradictory and both stimulatory and inhibitory actions on ovarian function have been described. In addition to the effects on the hypothalamic–pituitary axis, we can mention some negative actions: (i) leptin can directly suppress insulin, insulin-like growth factor-I (IGF-I), transforming growth factor-β and glucocorticoid-induced steroidogenesis of ovarian granulosa cells of rat (Zachow & Magoffin 1997, Barkan *et al.* 1999, Brannian *et al.* 1999, Zachow *et al.* 1999) or human (Agarwal *et al.* 1999) and (ii) acute administration of leptin to immature gonadotrophin-primed rats inhibits ovulation (Duggal *et al.* 2000, 2002). Likewise, leptin is able to produce some positive effects: (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 GnRH-deficient mice

ISSN 1470–1626 (paper) 1741–7899 (online)
DOI: 10.1530/rep.1.01164
Online version via www.reproduction-online.org

(Barkan *et al.* 2005) and eCG/hCG-primed rats (Roman *et al.* 2005).

Nevertheless, the precise mechanism by which leptin affects the ovulatory process is completely unknown. Ovulation is a complex process involving gonadotrophins, steroid hormones and many mediators common to inflammatory reactions, such as cytokines, prostaglandins, leukotrienes, plasminogen, nitric oxide and histamine. Prostaglandins (PGs) and nitric oxide (NO) are of particular interest, as they have been shown to play a role in follicle rupture (Brännström & Janson 1991, Ellman *et al.* 1993, Shukovski & Tsafriri 1994). In a previous study, we have demonstrated that during the ovulatory process, the increase in ovarian nitric oxide synthase (NOS) activity results in an increase in NO, which stimulates PGs production and enhances the inflammatory process, facilitating follicle rupture (Faletti *et al.* 1999).

Therefore, the aim of the present study was to investigate the negative action of leptin on some of these ovarian preovulatory factors during the ovulatory process by performing both *in vivo* studies using prepuberal rats stimulated with gonadotrophins and *in vitro* studies, using ovarian explants and follicle cultures as biological models. The expression of leptin receptors in the ovarian tissues was also evaluated.

Materials and Methods

Animals

Immature female Sprague–Dawley rats aged 26–28-days old and weighing 60–70 g obtained from the School of Veterinarian Sciences of the University of Buenos Aires (UBA), Argentina, were used to induce the first ovulation and to avoid the confounding effects of the presence of different types of follicles and corpora lutea from previous cycles. Rats 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 guide for the care and use of laboratory animals approved by the Animal Care and Use Committee of Centro de Estudios Farmacológicos y Botánicos (CEFYBO-CONICET) – School of Medicine (UBA).

Drugs and chemicals

hCG, PGE_2, recombinant rat leptin, progesterone and proteases inhibitors were purchased from Sigma-Aldrich. eCG was obtained from Syntex SA (Buenos Aires, Argentina). [^{3}H]-PGE_2 (181 Ci/mmol) and [1,2,6,7-^{3}H]-progesterone were obtained from Amersham Pharmacia Biotech. The western blotting reagents were obtained from Sigma-Aldrich and Bio-Rad Laboratories.

In vivo *studies*

Animals were injected i.p. with 10 iu eCG (in 0.10 ml saline) to induce the growth of the first generation of preovulatory follicles. Forty-eight hours later, the animals were injected i.p. with 10 iu hCG (in 0.10 ml saline) to induce ovulation, which usually occurs within 12–14 h after hCG administration in this rat colony.

To study the serum concentration of leptin and the expression of leptin receptors (Ob-R), animals were killed by decapitation at different times during the gonadotrophin treatment. Trunk blood was collected, serum was harvested and stored at −20 °C until RIA studies. Ovaries were dissected out quickly, frozen on dry ice and stored at −70 °C. Eight or ten animals were killed at each time, resulting in 16–20 ovaries/group.

To study the effect of high level of leptin during the ovulatory process, rats received five i.p. injections of either recombinant rat leptin (5 μg/0.15 ml PBS-BSA) or PBS-BSA (control) 1 h before hCG administration and at intervals of 150 min. To study the ovulation rate, some animals (four to five per group) were killed 20 h after the hCG injection by cervical dislocation. The remaining rats (four to five per group) were killed 10 h after the hCG injection by decapitation and trunk blood was collected. Sera were harvested and stored at −20 °C until assayed for progesterone by specific RIA. The ovaries were dissected out quickly, weighed and frozen on dry ice and stored at −70 °C. One ovary from each animal was used to determine prostaglandin E (PGE) content by RIA and the other one to measure the expression of leptin receptors by western blot analysis. Previously, we have shown that gonadotrophin administration increases ovarian NOS activity and PGs content, and that these increases peak at 10 h after hCG administration (Faletti *et al.* 1999). Therefore, we chose this time as a preovulatory moment when NOS activity and PGs content are at their highest levels before ovulation occurs. The experiments were repeated at least two times.

Ovulation rate

Twenty hours after hCG injection, the rats were killed by cervical dislocation, ovaries were immediately removed and oviducts were dissected and examined by means of a stereoscopic microscope to assess the number of oocytes present within, as described previously (Faletti *et al.* 1995).

Ovarian PGE content

PGE was extracted from the ovaries as described previously (Faletti *et al.* 1997). Briefly, one ovary from each animal was homogenised in absolute ethanol, centrifuged at 1000 ***g*** and the supernatants evaporated to dryness. The residues were stored at −70 °C and reconstituted before being assayed. PGE was quantified

by RIA as in previous studies (Faletti *et al.* 1997) using rabbit antiserum (P5164) from Sigma-Aldrich. The sensitivity of this assay was 15 pg/ml and the cross-reactivity of PGE_2 was 100% with PGE_1 and lower than 0.1% with other prostaglandins. The intra- and interassay coefficients of variation were 8.2 and 12% respectively.

Western blot analysis

Soluble tissue extracts were prepared as described previously (Faletti *et al.* 2003). Briefly, ovaries were homogenised 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 phenylmethylsulfonyl fluoride and 10 μg/ml trypsin inhibitors. The homogenates were sonicated and centrifuged at 7800 ***g*** at 4 °C for 15 min and protein concentration in the supernatant was determined by the Bradford method with BSA as the standard. Homog enates 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 PVDF 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). Negative controls were carried out by omitting the incubation with the primary antibody. No bands were detected. 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 visualised using chemiluminescence detection reagents (Sigma-Aldrich) and exposed to Kodak X-OMAT film. Before reuse, membranes were stripped, blocked and reprobed according to the manufacturer's instructions. Membranes were reprobed with anti-actin antibody (A2066, Sigma-Aldrich). 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 analysed using a Dekmate III Sigma Gel software package (Jandel Scientific software). The data were normalised to β-actin protein levels in each sample to account for procedural variability.

In vitro *studies*

Ovarian explant culture

At days 26–28, animals were injected i.p. with 10 iu eCG (in 0.10 ml saline) to induce the growth of a first generation of preovulatory follicles. Forty-eight hours later, the animals were injected i.p. with 10 iu hCG (in 0.10 ml saline) to induce ovulation and were killed by cervical dislocation 4 h later. Both the ovaries were immediately removed and dissected free of fat and bursa, and were cut into pieces of approximately equal size (four slices/ovary). Ovarian slices (four slices/well) were placed in 24-well plates containing Dulbecco's modified Eagle medium (DMEM)/F12 (1:1; Bio-Rad Laboratories) medium with 25 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.5 μg/ml fungizone and 2 mM L-glutamine. Ovarian slices were incubated in a final volume of 500 μl/well in either the presence or the absence of leptin (30–500 ng/ml) at 37 °C in a humidified atmosphere (5% CO_2:95% O_2) for 4 h. The dose of leptin used in these studies was obtained from previous reports (Spicer & Francisco 1997). After the incubation period, ovarian tissues were recovered, weighed and the protein content was determined by the Bradford method with BSA as the standard. Culture media were stored at −20 °C until assay for progesterone, PGE and nitrite (as a stable metabolite of NO production) concentrations. At least three independent experiments were run for each culture condition using different ovarian tissue preparations.

Culture of ovarian follicles

At days 26–28, animals were primed with 10 iu eCG i.p. (in 0.10 ml saline) to induce the growth of a first generation of preovulatory follicles and 48 h later, the animals were killed by cervical dislocation. Preovulatory follicles (>550 μm in diameter) were dissected from the ovaries with the aid of a stereomicroscope and fine forceps. Approximately 126 preovulatory follicles were obtained from 12 ovaries and these were collected and pooled. Follicles were placed in 24-well containing 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. Five follicles per well were incubated in a final volume of 500 μl fresh medium in either the presence or the absence of leptin (30–500 ng/ml) in combination with follicle-stimulating hormone (FSH) (100 ng/ml) or luteinizing hormone (LH) (100 ng/ml) at 37 °C in a humidified atmosphere (5% CO_2:95% O_2) for 4 h. Both the doses of leptin (Spicer & Francisco 1997) and gonadotrophins (Carnegie & Tsang 1984, Duggal *et al.* 2002) used in these studies were obtained from previous studies. After incubation, culture media (without follicles) were stored at −20 °C until assayed for progesterone, PGE and nitrite concentrations. At least three independent experiments were run for each culture using different preparations.

Hormone assays

Serum concentration of leptin was quantified by RIA as described previously (Roman *et al.* 2005) using a highly specific anti-mouse leptin provided by Dr A F Parlow (NHPP, Terrance, CA, USA). Recombinant rat leptin was used for standards and serial dilutions of the samples showed parallelism with the standard curve. The limit of sensitivity was 50 pg/ml and the intra- and interassay coefficients of variation were 7.5 and 9.5% respectively. Results were expressed as pg/ml serum.

Progesterone was quantified by RIA in both serum samples extracted with diethyl ether and culture medium. The progesterone antiserum was kindly provided by Dr G D Niswender (Colorado State University, Fort Collins, CO, USA). The sensitivity of these assays was 15 pg/ml. The cross-reactivities were $<2.0\%$ for 20α-dihydro-progesterone and deoxy-corticosterone and 1.0% for other steroids normally in the serum. The intra- and interassay coefficients of variation were 7.5 and 10.5% respectively.

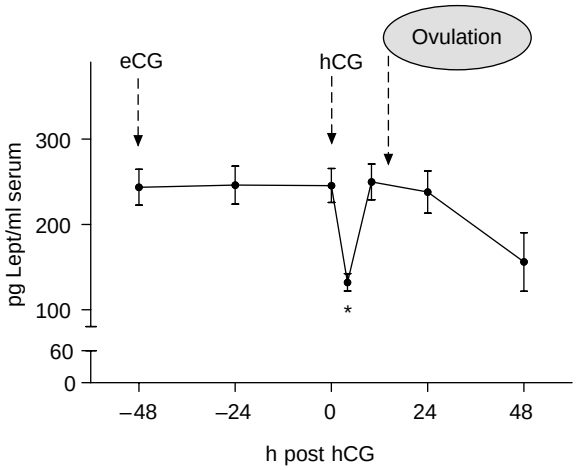

Figure 1 Pattern of changes in serum leptin level obtained at different times from immature rats primed with eCG/hCG. Leptin was quantified by RIA and expressed as pg leptin per ml serum. Each bar represents the mean ± S.E.M. for 8–10 animals per group. *$P<0.001$ versus −48 h (ANOVA and Student–Newman–Keuls multiple comparison test).

Nitrites assay

Levels of nitric oxide metabolites were measured in the media of both ovarian explants and follicle cultures, using the Griess reagents as described previously (Polisseni *et al.* 2005).

Statistical analysis

All data are expressed as mean ± S.E.M. The difference between two groups was analysed using Student's *t*-test. Comparisons between more than two groups were performed using a one-way ANOVA and Student–Newman–Keuls multiple comparison test. Differences between groups were considered significant when $P<0.05$.

Results

In vivo *studies*

Serum leptin concentration was measured at different times, after the eCG injection, by specific RIA as described previously (Roman *et al.* 2005) and expressed as pg leptin per ml serum. The administration of eCG did not alter the serum level of leptin at the times of study. However, the treatment with hCG induced a significant decrease in leptin levels at 4 h (132 ± 10), when compared with those observed at 0 h (246 ± 20; Fig. 1).

The expression of leptin receptors was evaluated at different times after the eCG injection, by western blot analysis to study the effect of the gonadotrophin treatment. This revealed all leptin receptor isoforms by a polyclonal antibody raised against a recombinant protein corresponding to amino acids 541–840 mapping within an internal domain of Ob-R. Three Ob-R-immunoreactive proteins with apparent relative molecular masses of 110, 150 and 210 kDa were detected, as described previously with CHO cells (Matsuda *et al.* 1999) and adipose tissue lysates (Meli *et al.* 2004). Protein bands at 150 kDa, corresponding to the predicted size of Ob-Rb based on amino acid composition, had higher expression than the others in all ovaries assayed and samples obtained from hypothalamus used as positive control (data not shown). Thus, only the approximately 150 kDa bands were assessed as leptin receptors expression (Fig. 2A). Densitometric analysis revealed that the administration of eCG induced increase of the expression of Ob-R protein in comparison with that obtained from animals without treatment, being significant only at 48 h postinjection (Fig. 2B). After the hCG administration, the expression of leptin receptors increased again to reach a maximum. Twenty-four hours after hCG administration and after ovulation occurred, the expression decreased returning to values similar to those obtained at 0 h after hCG administration.

In order to study the effect of high levels of leptin on the ovulatory process, rats were injected with either recombinant rat leptin (5 μg) or PBS-BSA at 1 h before hCG injection and at 150-min intervals. Data on body and ovarian weight, ovulatory rate, serum progesterone and ovarian PGE content, obtained at 10 h after hCG administration, are summarised in Table 1. No differences were found in either food intake (data not shown) or body weight between animals treated with leptin and control animals along this period, but ovarian weight was significantly decreased (Table 1). We examined the ovulation rate 20 h after the hCG

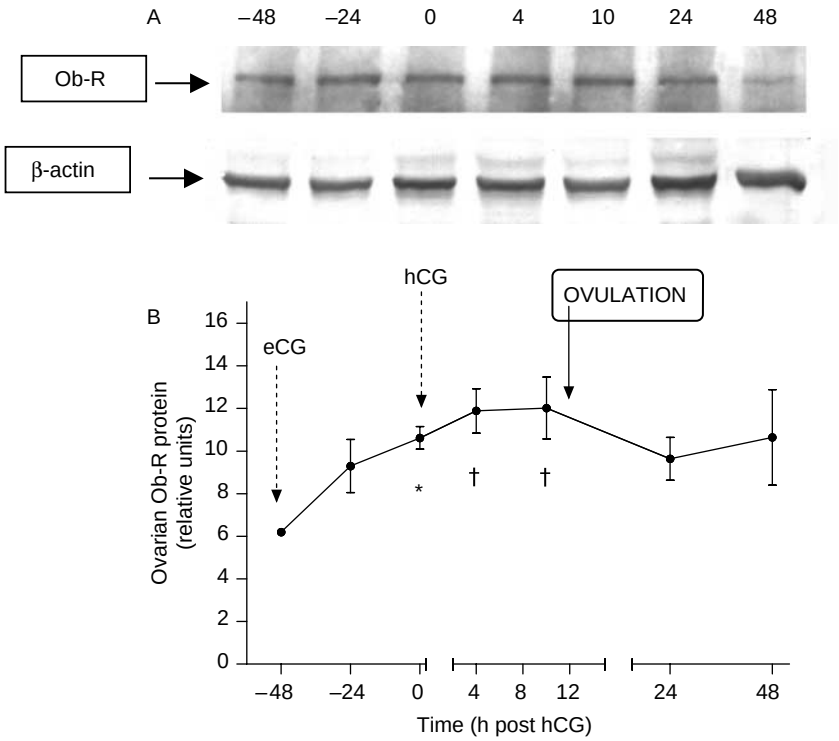

Figure 2 Pattern of changes in ovarian expression of leptin receptors obtained at different times from immature rats primed with eCG/hCG. (A) Expression of ovarian leptin receptor and ß-actin, as protein control, by western blot analysis. (B) Quantitative analysis of ovarian expression of leptin receptor during gonadotrophin treatment. Data points represent the mean±S.E.M. for four samples per group. Each sample represents two to four ovaries from different animals at the same time. *$P<0.05$, †$P<0.01$ versus −48 h (ANOVA and Student–Newman–Keuls multiple comparison test).

administration. The number of oocytes within oviducts was significantly lower in rats treated with leptin than that in control animals (20±3 vs 11±2; $P<0.05$; Table 1).

To study whether the inhibitory effect of high doses of leptin on the ovulatory process was related to ovarian prostaglandins production, PGE was measured 10 h after the hCG injection in ovaries from immature rats primed with eCG/hCG and expressed as pg PGE per mg wet mass. The administration of five doses of leptin (5 µg/injection) significantly reduced the preovulatory ovarian content of this prostanoid in comparison with that of the buffer control (256±42 vs 519±28; $P<0.001$). Likewise, the serum levels of progesterone, expressed as ng/ml serum, were significantly reduced in the animals treated with leptin (53±5 vs 32±2, $P<0.01$; Table 1).

In vitro *studies*

To investigate the source of the inhibition of ovulation by leptin, the concentration of PGE and progesterone were assessed in ovarian explants cultured for 4 h in either the presence or the absence of leptin and expressed as pg per mg wet weight. Since nitric oxide is involved in ovulation in rats as it stimulates the production of PGs (Faletti *et al.* 1999), NO production in these cultures was also quantified by measuring the concentration of nitrites as stable metabolites of NO and expressed as ng per mg wet weight. Ovarian explants were obtained from immature rats primed with eCG/hCG to closely imitate the conditions of *in vivo* experiments. The direct effect of recombinant rat leptin on ovarian production of PGE, nitrites and progesterone is shown in Fig. 3. Addition of 300 ng/ml or more of leptin led to a significant inhibition of PGE and nitrites production. Neither PGE nor nitrites concentrations were significantly affected by the presence of lower concentrations of leptin when compared with those of controls ($P>0.05$; Fig. 3A and B). All doses of leptin assayed also reduced progesterone production in comparison with that of control (Fig. 3C). When the results were expressed per mg protein, the responses obtained with these factors were the same (data not shown).

Table 1 *In vivo* effect of acute treatment of leptin on the rat ovulatory process.

Treatment	**Body weight** (g)	**Ovarian weight** (mg)	**Ovulation rate** (oocytes/ovary)	**Serum progesterone** (ng/ml)	**Ovarian PGE** (pg/mg wet weight)
Control	69±3	43±4	20±3	53±5	519±28
Leptin	68±2	31±3*	11±2*	32±2†	256±42‡

Immature rats primed with eCG/hCG were treated with 5 µg leptin 1 h before hCG and 150-min intervals. Ovulation rate was examined 20 h after hCG administration. The remaining rats were killed 10 h after hCG administration. $n=8$–10 animals. To ovarian weight and ovulation rate, each value represents the mean of both ovaries from each animal. *$P<0.05$, †$P<0.01$, ‡$P<0.001$. Significance compared with control (Student's *t*-test).

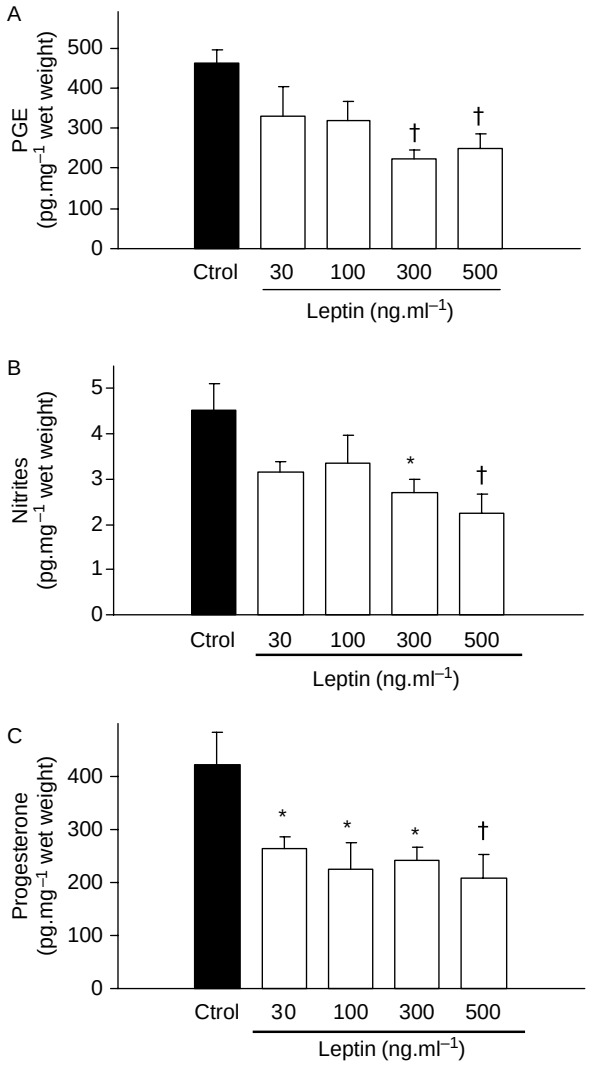

Figure 3 *In vitro* effect of acute treatment of leptin on the production of (A) PGE, (B) nitrites and (C) progesterone by cultures of ovarian explants. Ovarian explants were obtained 4 h after hCG administration from immature rats primed with eCG/hCG and incubated for 4 h in either the presence or the absence of different concentrations of leptin (30–500 ng/ml). Results are mean ± S.E.M. of three independent experiments, within each replicate experiment; each treatment was applied in quadruplicate culture well. $^{*}P<0.05$, $^{\dagger}P<0.01$ versus control (ANOVA and Student–Newman–Keuls multiple comparison test).

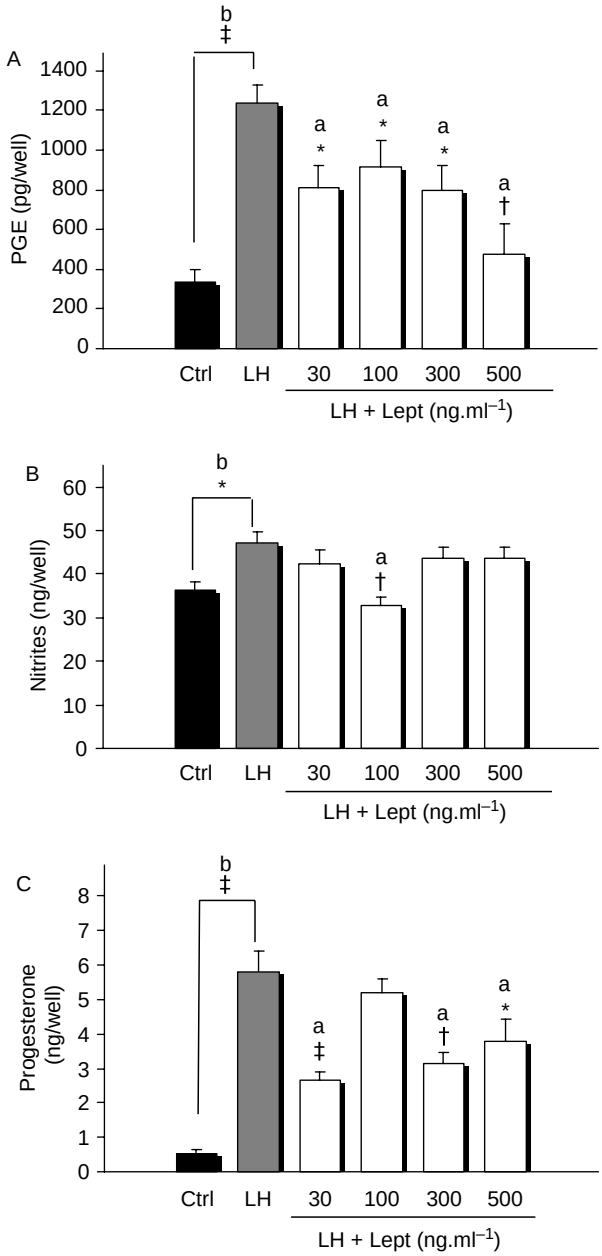

Figure 4 *In vitro* effect of acute treatment of leptin on the production of (A) PGE, (B) nitrites and (C) progesterone by cultures of preovulatory follicles. Preovulatory follicles (>550 μm in diameter; five per well) were obtained from ovaries of immature rats 48 h after eCG injection and incubation for 4 h in either the presence or the absence of leptin (30–500 ng/ml) in combination with LH (100 ng/ml). Results are mean ± S.E.M. of three independent experiments; each treatment was applied in quadruplicate culture well. $^{*}P<0.05$, $^{\dagger}P<0.01$, $^{\ddagger}P<0.001$; a. versus LH, b. versus control (ANOVA and Student–Newman–Keuls multiple comparison test).

In order to confirm the inhibitory effects of leptin on these mediators at cellular level, PGE, NO and progesterone were quantified in cultures of ovarian follicles in the presence of the same concentrations of leptin as in the ovarian explants cultures. Preovulatory follicles were obtained from ovaries of immature rats 48 h after the eCG injection. The effect of leptin was studied in either the presence or the absence of LH or FSH and is shown in Figs 4 and 5 respectively. As expected, both FSH and LH (100 ng/ml) stimulated PGE, NO and progesterone production ($P<0.05$). All doses of leptin assayed significantly reduced the production of both PGE and progesterone induced by FSH or LH, but not that of progesterone production when the concentration of leptin was 100 ng/ml in presence of LH (Fig. 4C). These inhibitory effects were not total in most of the concentrations assayed, because neither PGE nor progesterone production returned to basal values. Leptin was able to abolish completely the FSH-stimulated NO production in

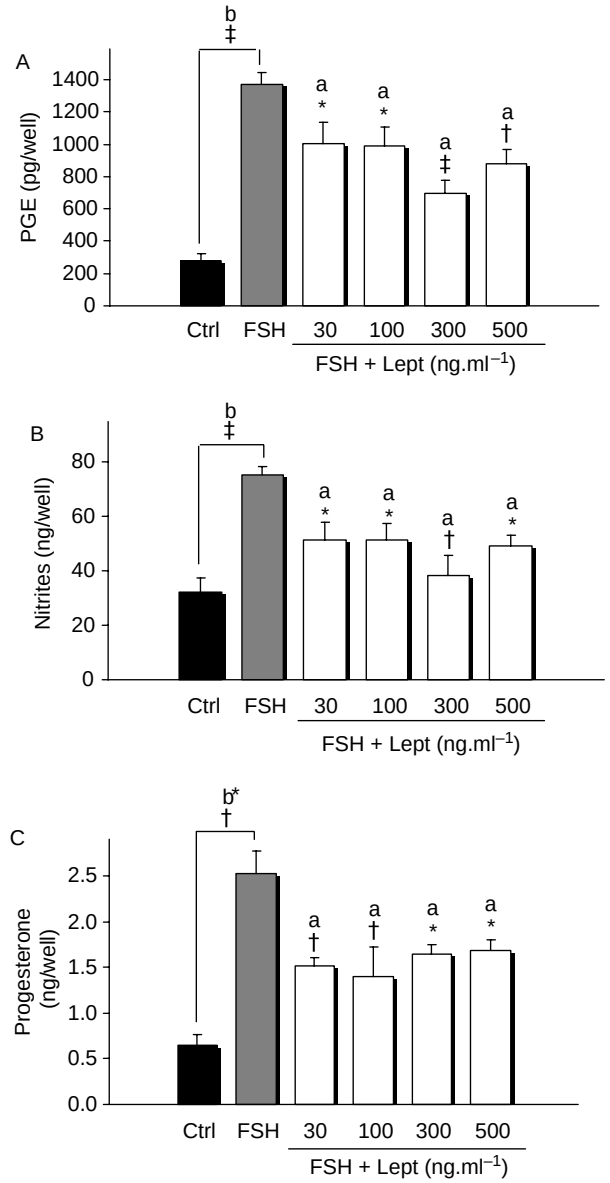

Figure 5 *In vitro* effect of acute treatment of leptin on the production of (A) PGE, (B) nitrites and (C) progesterone by culture of preovulatory follicles. Preovulatory follicles (>550 µm in diameter; five per well) were obtained from ovaries of immature rats 48 h after eCG injection and incubation for 4 h in either the presence or the absence of leptin (30–500 ng/ml) in combination with FSH (100 ng/ml). Results are mean±S.E.M. of three independent experiments; each treatment was applied in quadruplicate culture well. $^{*}P<0.05$, $^{\dagger}P<0.01$, $^{\ddagger}P<0.001$; a. versus FSH, b. versus control (ANOVA and Student–Newman–Keuls multiple comparison test).

all doses probed (Fig. 5B), but it only reduced the LH-stimulated NO production at 100 ng/ml (Fig. 4B).

Effect of leptin treatment on expression of its receptors

Western blot analysis was used to determine whether the inhibitory effect of leptin was correlated with changes in the expression of its ovarian receptors. Figure 6 shows

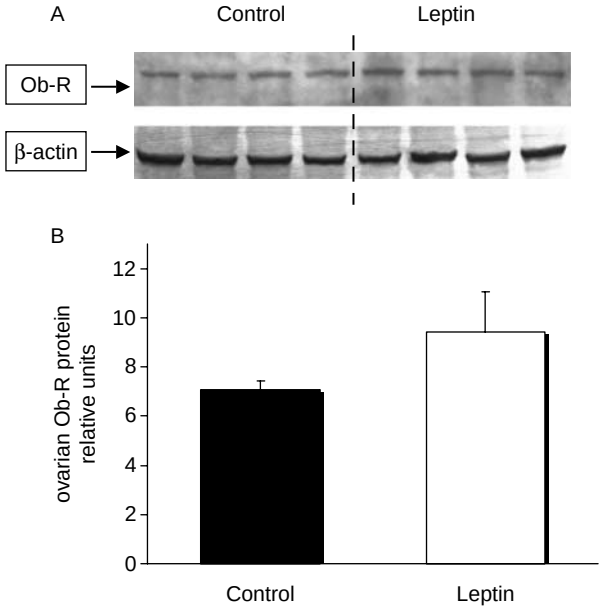

Figure 6 Effect of acute treatment of leptin on the expression of ovarian leptin receptor. Immature rats were primed with eCG/hCG and treated with PBS-BSA or 5 µg leptin at 1 h before hCG and 150-min intervals. Animals were killed 10 h after hCG administration. (A) Expression of ovarian Ob-R protein and β-actin by western blot analysis. (B) Quantitative analysis of ovarian Ob-R expression. Data points represent the mean±S.E.M. for four to five samples/group. Each sample represents two to four ovaries from different animals with the same treatment.

that acute leptin treatment did not significantly alter the ovarian leptin receptor expression in comparison with that of the buffer control, when a densitometric analysis was performed (Fig. 6B).

Discussion

Many studies have demonstrated that leptin may have direct effects on ovarian tissue. Most of these studies have performed *in vitro* experiments with ovarian cells and have resulted in inhibitory effects on the stimulating actions of many hormones that are important in the ovarian function (Zachow & Magoffin 1997, Spicer & Francisco 1998, Agarwal *et al.* 1999, Zachow *et al.* 1999). Other *in vivo* studies have reported that leptin also has inhibitory effects on ovarian factors, but these studies were performed with acute high doses (Duggal *et al.* 2000, 2002). Elevated leptin in the circulation as a result of exogenous injections can affect the ovarian function directly via its specific receptors or indirectly via central receptors. Therefore, the present study was designed to evaluate the source of this effect and some factors involved in the inhibition produced on the ovulatory process. At first, we determined the serum concentration of leptin and the expression of leptin receptors in immature animals primed with gonadotrophins to induce ovulation. We showed that serum concentration of leptin decreases dramatically 4 h after hCG administration and then returns to the levels

obtained before hCG administration. Ryan *et al.* (2003) have found that plasma leptin increases 47 h after eCG administration and 16 h after hCG administration, and that it decreases 2 and 9 h after hCG administration. Although these findings seem to contrast with our results, this may not be so since the times at which these authors measured leptin concentration were not the same as in our studies. We did not observe any increase of leptin levels in serum after the eCG injection at the times assayed, but the administration of hCG produced an important decrease of these levels before ovulation occurred, in agreement with the results obtained by Ryan *et al.* (2003). Other studies have shown no changes in leptin levels (Amico *et al.* 1998) or just a decrease before ovulation (Luukkaa *et al.* 2001). It might be possible that the individual level and timing of endogenous leptin production presents a high variability within the same species.

Duggal *et al.* (2000) have demonstrated that acute leptin treatment inhibits ovulation. In other study, these authors investigated the cause of this inhibition, but they could not demonstrate what ovulatory factors were involved in this negative action (Duggal *et al.* 2002). Therefore, the action of leptin on some ovarian preovulatory factors were evaluated during the ovulatory process by performing both *in vivo* studies with prepuberal rats stimulated with gonadotrophins and *in vitro* studies, using ovarian explants and follicle cultures as biological models. The purpose of the treatment design was to maintain high levels of leptin after hCG administration to avoid the fall of this protein observed in circulation before ovulation. The administration of five doses of 5 μg leptin, at 150-min intervals, to immature eCG/hCG-primed rats during the ovulatory process, was sufficient to inhibit the number of ovulated oocytes. This result confirmed those previously obtained by Duggal *et al.* (2000), although they administered higher doses at different intervals. Since the administration of leptin was during the light period (between 0700 and 1800 h), it was not necessary to use a group of rats with pair-fed to the leptin-treated animals, because no differences were found in either food intake or body weight between this group and control animals along this period.

It was of interest to study the action of acute administration on some ovarian mediators involved in the ovulatory process. Prostaglandins and nitric oxide are of particular interest, as they have been shown to play a role in follicle rupture (Brännström & Janson 1991, Ellman *et al.* 1993, Shukovski & Tsafriri 1994, Faletti *et al.* 1999). The ovarian concentration of PGs increases after LH surge and gonadotrophin stimulation (Brown & Poyser 1984, Faletti *et al.* 1995). The present study investigated the effect of high levels of leptin on ovarian PGE production by performing *in vivo* and *in vitro* assays. Preovulatory PGE content was significantly inhibited in ovaries from rats primed with eCG/hCG and treated with acute administration of leptin. This result was confirmed with *in vitro* studies, because the concentration of this prostanoid was reduced by the presence of leptin in the culture media of ovarian explants and FSH- or LH-stimulated preovulatory follicles from rats primed with gonadotrophin. In a previous study, it has been shown that gonadotrophin administration resulted in an increase in nitric oxide synthase activity and this increase results in an increase in NO, which stimulates prostaglandins production and enhances the inflammatory process, facilitating follicle rupture (Faletti *et al.* 1999). The present study investigated whether high levels of leptin were able to alter directly the ovarian production of nitric oxide, by measuring the concentration of nitrites, as stable metabolites, in the same culture media of ovarian explants and preovulatory follicles where PGE was determined. The production of nitrites was reduced by the presence of leptin in the cultures assayed, although this result was not significant at all the concentrations assayed. These findings appear to contrast with those reported by Huang *et al.* (2005), who have found that the inhibitory effect of leptin on human granulose cells is mediated by NO, because the exposure of human GCs to leptin at concentrations of 10 ng/ml decrease the IGF-I-stimulated or IGF-I plus FSH-stimulated 17β-oestradiol production, and the presence of L-NAME (NG-nitro-L-arginine methyl ester), an inhibitor of NOS, in the culture medium significantly attenuates this negative effect. Furthermore, the concentrations used by these authors (3–30 ng/ml) induce a dose- and time-dependent NO production, but they were lower than those used in our studies. Without considering the biological differences between both cellular systems used, it would not be the first time that leptin is able to produce a dual effect on the biological function. Recently, we have found that a chronic treatment with low doses of leptin induces a swift increase in the ovarian endothelium NOS expression in comparison with that of control animals and this treatment completely reverses the inhibitory effect on this protein expression produced by a severe food restriction (Roman *et al.* 2005).

A direct inhibitory action of leptin on steroid hormone secretion has been demonstrated independently by different groups in the ovary (Spicer & Francisco 1997, 1998, Zachow & Magoffin 1997, Agarwal *et al.* 1999, Barkan *et al.* 1999, Zachow *et al.* 1999, Ghizzoni *et al.* 2001, Kikuchi *et al.* 2001) and other tissues (Tena-Sempere *et al.* 2001, Cameo *et al.* 2003). The mechanism involved for such inhibitory action has not been completely clarified, but it has been suggested that this effect is mediated by a modulation on transcriptional factors, such as StAR and P450scc (Tena-Sempere *et al.* 2001) or overexpression on c-Jun (Barkan *et al.* 1999). In this paper, leptin treatment also induced significant inhibition in serum level of progesterone and again, this result was confirmed by *in vitro* studies where the concentration of progesterone was reduced by the

presence of leptin in cultures of ovarian explants and preovulatory follicles. Again, the findings of both stimulations by low doses and inhibition by high doses of leptin in ovarian cells *in vitro* concur with observations by Ruiz-Cortés *et al.* (2003), where the effects of leptin are shown to be 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. Some *in vivo* studies performed with acute high doses have reported that leptin may have inhibitory effects on ovarian factors (Duggal *et al.* 2000). But other studies have also demonstrated positive action with chronic treatment with leptin, since it has been able to accelerate the onset of puberty in rodents (Ahima *et al.* 1997, Almog *et al.* 2001) and human (Garcia-Mayor *et al.* 1997, Strobel *et al.* 1998). We have found in a previous study that a chronic administration of low leptin level is able to enhance the ovulatory process. These results are consistent with those obtained by Barkan *et al.* (2005), who have found that leptin is able to mimic FSH and LH actions or to induce an LH-independent ovulation. All these data suggest that both the dose and timing of leptin administration are critical to obtain either a positive or a negative response.

Functional leptin receptors are expressed in the ovary of numerous species, including humans (Cioffi *et al.* 1996, Karlsson *et al.* 1997), mice (Kikuchi *et al.* 2001) and rats (Zamorano *et al.* 1997, Zachow *et al.* 1999). The expression of ovarian leptin receptor was evaluated at different times, after eCG/hCG injection, by western blot analysis to study the effect of the gonadotrophin treatment. This expression varied across the gonadotrophin treatment, with significant increases 48 h after eCG administration and 10 h after hCG administration that tend to reduce after ovulation. These results agree with those obtained by Ryan *et al.* (2003), who have used quantitative RT-PCR to measure leptin receptor expression. All these data confirm that the production of leptin and its receptors is regulated within the ovary by gonadotrophins, indicating a possible involvement in several ovarian functions such as the ovulatory process. Further studies are necessary to determine which form of this receptor protein is involved and how this expression is regulated.

We initially expected that the increase in the expression of leptin receptor before ovulation would be blocked or partly attenuated by the acute leptin treatment. However, we found that this treatment did not show significant alteration in its expression. This suggests that the inhibitory effect of leptin on the ovulatory process in our *in vivo* studies was not mediated by changes in the content of receptors protein at ovarian level, at least at the concentrations assayed. Further studies are necessary to clarify this point.

Finally, we firmly believe that leptin may be regulating the ovulatory process by at least in part, modulating prostaglandins, nitric oxide and steroids, since when the levels of leptin are low, there is a positive correlation between leptin and ovarian functions (Roman *et al.* 2005), but when these levels are high, as in the present study, there is a negative correlation among them.

Acknowledgements

The authors thank Daniel Eduardo González and Eduardo José Nieves for their technical assistance. This work was supported by Grant PIP 2533 from Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), SigmaXi 503078 and UBACYT X006 from Universidad de Buenos Aires. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

References

Agarwal SK, Vogel K, Weitsman SR & Magoffin DA 1999 Leptin antagonizes the insulin-like growth factor-1 augmentation of steroidogenesis in granulosa and theca cells of the human ovary. *Journal of Clinical Endocrinology and Metabolism* **84** 1072–1076.

Ahima RS, Dushay J, Flier SN, Prabakaran D & Flier JS 1997 Leptin accelerates the onset of puberty in normal female mice. *Journal of Clinical Investigation* **99** 391–395.

Almog B, Gold R, Tajima K, Dantes A, Salim K, Rubinstein M, Barkan D, Homburg R, Lessing JB, Nevo N, Gertler A & Amsterdam A 2001 Leptin attenuates follicular apoptosis and accelerates the onset of puberty in immature rats. *Molecular and Cellular Endocrinology* **183** 179–191.

Amico JA, Thomas A, Crowley RS & Burmeister LA 1998 Concentrations of leptin in the serum of pregnant, lactating, and cycling rats and of leptin messenger ribonucleic acid in rat placental tissue. *Life Science* **63** 1387–1395.

Barkan D, Jia H, Dantes A, Vardimon L, Amsterdam A & Rubinstein M 1999 Leptin modulates the glucocorticoid-induced ovarian steroidogenesis. *Endocrinology* **140** 1731–1738.

Barkan D, Hurgin V, Dekel N, Armsterdam A & Rubinstein M 2005 Leptin induces ovulation in GnRH-deficient mice. *FASEB Journal* **19** 133–135.

Brannian JD, Zhao Y & McElroy M 1999 Leptin inhibits gonadotrophin-stimulated granulosa cell progesterone production by antagonizing insulin action. *Human Reproduction* **14** 1445–1448.

Brännström M & Janson PO 1991 Biochemistry of ovulation. In *Ovarian Endocrinology*, pp 132–166. Ed. S Hiller. Oxford: Blackwell Sci.

Brown CG & Poyser NL 1984 Studies on ovarian prostaglandin production in relation to ovulation in rat. *Journal of Reproduction and Fertility* **72** 407–412.

Cameo P, Bischof P & Calvo JC 2003 Effect of leptin on progesterone, human chorionic gonadotropin, and interleukin-6 secretion by human term trophoblast cells in culture. *Biology of Reproduction* **68** 472–477.

Carnegie JA & Tsang BK 1984 The calcium-calmodulin system: participation in the regulation of steroidogenesis at different stages of granulosa cell differentiation. *Biology of Reproduction* **30** 515–522.

Chehab FF, Lim ME & Lu R 1996 Correction of the sterility defect in homozygous obese female mice by treatment with the human recombinant leptin. *Nature Genetics* **12** 318–320.

Cioffi JA, Shafer AW, Zupancic TJ, Smith-Gbur J, Mikhah A, Platika D & Snodgrass HR 1996 Novel B219/OB receptor isoforms: possible role of leptin in hematopoiesis and reproduction. *Nature Medicine* **2** 585–589.

Cioffi JA, Van Blerkom J, Antczak M, Shafer A, Wittmer S & Snodgrass HR 1997 The expression of leptin and its receptors in pre-ovulatory human follicles. *Molecular Human of Reproduction* **3** 467–472.

Clément K, Vaisse C, Lahlou N, Cabrol S, Pelloux V, Cassuto D, Gourmelen M, Dina C, Chambaz J, Lacorte JM, Basdevant A, Bougneres P, Lebouc Y, Froguel P & Guy-Grand B 1998 A mutation in the human leptin receptor gene causes obesity and pituitary dysfunction. *Nature* **392** 398–401.

Duggal PS, Van der Hoek KH, Milner CR, Ryan NK, Armstrong DT, Magoffin DA & Norman RJ 2000 The *in vivo* and *in vitro* effects of exogenous leptin on ovulation in the rat. *Endocrinology* **141** 1971–1976.

Duggal PS, Ryan NK, Van der Hoek KH, Ritter LJ, Armstrong DT, Magoffin DA & Norman RJ 2002 Effects of leptin administration and feed restriction on thecal leukocytes in the preovulatory rat ovary and the effects of leptin on meiotic maturation, granulosa cell proliferation, steroid hormone and PGE2 release in cultured rat ovarian follicles. *Reproduction* **123** 891–898.

Ellman C, Corbett JA, Misko TP, McDaniel M & Beckerman KP 1993 Nitric oxide mediates interleukin-1-induced cellular cytotoxicity in the rat ovary. A potential role for nitric oxide in the ovulatory process. *Journal of Clinical Investigation* **92** 3053–3056.

Faletti A, Viggiano JM & Gimeno MAF 1995 Beta-endorphin inhibits prostaglandin synthesis in rat ovaries and blocks induced ovulation. *Prostaglandins* **9** 93–103.

Faletti A, Jawerbaum A, Viggiano J & Gimeno MAF 1997 Naltrexone enhances ovulation and prostaglandin synthesis in the rat ovary. *Prostaglandins* **54** 665–675.

Faletti A, Pérez-Martínez S, Perotti C & Gimeno MAF 1999 Activity of ovarian nitric oxide synthase (NOS) during ovulatory process in the rat: relationship with prostaglandins (PGs) production. *Nitric Oxide: Biology and Chemistry* **3** 330–347.

Faletti AG, Mohn C, Farina M, Lomniczi A & Rettori V 2003 Interaction among beta-endorphin, nitric oxide and prostaglandins during ovulation in rats. *Reproduction* **125** 469–477.

Garcia-Mayor RV, Andrade MA, Rios M, Lage M, Dieguez C & Casanueva FF 1997 Serum leptin levels in normal children: relationship to age, gender, body mass index, pituitary–gonadal hormones, and pubertal stage. *Journal of Clinical Endocrinology and Metabolism* **82** 2849–2855.

Ghizzoni L, Barreca A, Mastorakos G, Furlini M, Vottero A, Ferrari B, Chrousos GP & Bernasconi S 2001 Leptin inhibits steroid biosynthesis by human granulosa-lutein cells. *Hormone and Metabolic Research* **33** 323–328.

Huang HF, Wang B, Yang XF, Luo Q & Sheng JZ 2005 Nitric oxide mediates inhibitory effect of leptin on insulin-like growth factor I augmentation of 17beta-estradiol production in human granulosa cells. *Biology of Reproduction* **72** 102–106.

Karlsson C, Lindell K, Svensson E, Bergh C, Lind P, Billig H, Carlsson LM & Carlsson B 1997 Expression of functional leptin receptors in the human ovary. *Journal of Clinical Endocrinology and Metabolism* **82** 4144–4148.

Kikuchi N, Andoh K, Abe Y, Yamada K, Mizunuma H & Ibuki Y 2001 Inhibitory action of leptin on early follicular growth differs in immature and adult female mice. *Biology of Reproduction* **65** 66–71.

Luukkaa V, Savontaus E, Rouru J, Virtanen KA, Boss O, Huhtaniemi I, Koulu M, Pesonen U & Huupponen R 2001 Effects of estrous cycle and steroid replacement on the expression of leptin and uncoupling proteins in adipose tissue in the rat. *Gynecological Endocrinology* **15** 103–112.

Matsuda J, Yokota I, Tsuruo Y, Murakami T, Ishimura K, Shima K & Kuroda Y 1999 Development changes in long-form leptin receptor expression and localization in rat brain. *Endocrinology* **140** 5233–5238.

Meli R, Pacilio M, Raso GM, Esposito E, Coppola A, Nasti A, Di Carlo C, Nappi C & Di Carlo R 2004 Estrogen and raloxifene modulate leptin and its receptor in hypothalamus and adipose tissue from ovariectomized rats. *Endocrinology* **145** 3115–3121.

Mercer JG, Hoggard N, Williams LM, Lawrence CB, Hannah LT & Trayhurn P 1996 Localization of leptin receptor mRNA and the long form splice variant (Ob-Rb) in mouse hypothalamus and adjacent brain regions by *in situ* hybridization. *FEBS Letters* **387** 113–116.

Mounzih K, Lu R & Chehab FF 1997 Leptin treatment rescues the sterility of genetically obese ob/ob males. *Endocrinology* **138** 1190–1193.

Polisseni F, Faletti AG, Pereira VM, Reis AM, Camargos AF & Reis FM 2005 Prolactin inhibits oocyte release after gonadotropin stimulation in the rat: putative mechanism involving the ovarian production of beta-endorphin and prostaglandin. *Fertility & Sterility* **83** 1119–1124.

Roman EA, Ricci AG & Faletti AG 2005 Leptin enhances ovulation and attenuates the effects produced by food restriction. *Molecular and Cellular Endocrinology* **242** 33–41.

Ruiz-Cortés ZT, Martel-Kennes Y, Gévry NY, Downey BR, Palin MF & Murphy BD 2003 Biphasic effects of leptin in porcine granulosa cells. *Biology of Reproduction* **68** 789–796.

Ryan NK, Van der Hoek KH, Robertson SA & Norman RJ 2003 Leptin and leptin receptor expression in the rat ovary. *Endocrinology* **144** 5006–5013.

Schwartz MW, Seeley RJ, Campfield LA, Burn P & Baskin DG 1996 Identification of targets of leptin action in rat hypothalamus. *Journal of Clinical Investigation* **98** 1101–1106.

Shukovski L & Tsafriri A 1994 The involvement of nitric oxide in the ovulatory process in the rat. *Endocrinology* **135** 2287–2290.

Spicer LJ & Francisco CC 1997 The adipose obese gene product, leptin: evidence of a direct inhibitory role in ovarian function. *Endocrinology* **138** 3374–3379.

Spicer LJ & Francisco CC 1998 Adipose obese gene product, leptin, inhibits bovine ovarian theca cell steroidogenesis. *Biology of Reproduction* **58** 207–212.

Strobel A, Issad T, Camoin L, Ozata M & Strosberg AD 1998 A leptin missense mutation associated with hypogonadism and morbid obesity. *Nature Genetics* **18** 213–215.

Tartaglia LA 1997 The leptin receptor. *Journal of Biological Chemistry* **272** 6093–6096.

Tena-Sempere M, Manna PR, Zhang F-P, Pinilla L, González LC, Diéguez C, Huhtaniemi I & Aguilar E 2001 Molecular mechanisms of leptin action in adult rat testis: potential targets for leptin-induced inhibition of steroidogenesis and pattern of leptin receptor messenger ribonucleic acid expression. *Journal of Endocrinology* **170** 413–423.

Zachow RJ & Magoffin DA 1997 Direct intraovarian effects of leptin: impairment of the synergistic action of insulin-like growth factor-I on follicle-stimulating hormone-dependent estradiol-17β production by rat ovarian granulosa cells. *Endocrinology* **138** 847–850.

Zachow RJ, Weitsman SR & Magoffin DA 1999 Leptin impairs the synergistic stimulation by transforming growth factor-ß of follicle-stimulating hormone-dependent aromatase activity and messenger ribonucleic acid expression in rat ovarian granulosa cells. *Biology of Reproduction* **61** 1104–1109.

Zamorano PL, Mahesh VB, De Sevilla LM, Chorich LP, Bhat GK & Rrann DW 1997 Expression and localization of the leptin receptor in endocrine and neuroendocrine tissues of the rat. *Neuroendocrinology* **65** 223–228.

Zhang Y, Proenca R, Maffei M, Barone M, Leopold L & Friedman JM 1994 Positional cloning of the mouse obese gene and its human homologue. *Nature* **372** 425–432.

Received 20 March 2006
First decision 17 May 2006
Revised manuscript received 31 May 2006
Accepted 24 July 2006