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Regulation of the ovarian oxidative status by leptin during the ovulatory process in rats

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Abstract

Leptin exerts both stimulatory and inhibitory effects on the ovulatory process. In this study, we investigated whether these opposite effects involve changes in the oxidative status in response to different levels of leptin. To this end, we performed both *in vivo* and *in vitro* assays using ovaries of immature rats primed with gonadotropins to induce ovulation. Superoxide dismutase (SOD) and catalase (CAT) activity, lipid peroxidation, glutathione (GSH) content, and reactive oxygen species (ROS) were studied as oxidative damage-related parameters. The expression of BCL2, BAX, and caspase 3 were measured by western blot as apoptosis-related biomarkers. The acute treatment with leptin, which inhibits ovulation, decreased SOD activity and increased active caspase 3 expression. No differences were found in CAT activity, lipid peroxidation, or total GSH. In contrast, the daily administration of leptin, which induces ovulation, decreased GSH content, ROS levels, and Bax and active caspase 3 expression, but caused no changes in other parameters. In addition, the daily administration of leptin induced follicular growth, measured by the number of antral follicles in ovarian sections. Using ovarian explant cultures, we found increased BCL2 expression and decreased SOD activity at low and high concentrations of leptin respectively. Thus, leptin can modulate the oxidative status of the ovarian tissue, during the ovulatory process, by acting on different targets according to its circulating levels. At low concentration, leptin seems to play a protective role against the oxidative stress, whereas at high concentrations, this protein seems to be involved in cell death.
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Introduction

Leptin, a circulating protein mainly synthesized by adipocytes, acts as an important regulatory hormone in multiple functions, including ovarian function (Zhang *et al*. 1994). Leptin acts through its receptors, which are widely distributed in many tissues, including the ovary (Karlsson *et al*. 1997, Zamorano *et al*. 1997, Agarwal *et al*. 1999, Duggal *et al*. 2002*a*). Both leptin and its receptors are expressed in granulosa, theca, interstitial, and cumulus cells of humans (Karlsson *et al*. 1997, Agarwal *et al*. 1999) and rats (Zachow *et al*. 1999, Archanco *et al*. 2003). Leptin is able to cause both stimulatory (Ahima *et al*. 1997, Almog *et al*. 2001, Barkan *et al*. 2005) and inhibitory (Duggal *et al*. 2000, 2002*b*, Lin *et al*. 2009) effects on the ovarian function. Similarly, some studies have demonstrated that leptin can exert a biphasic effect on the ovarian function (Ruiz-Cortés *et al*. 2003, Karamouti *et al*. 2008, 2009).

Ovulation is a complex process, considered as an inflammatory process (Richards *et al*. 2002), which is mediated by different molecules, including prostaglandins, proteolytic enzymes, cytokines, leukotrienes, nitric oxide, steroid hormones, and reactive oxygen species (ROS; Agarwal *et al*. 2012, Rizzo *et al*. 2012). Although oxidative stress may be a physiological state in certain conditions, it may increase when the production of ROS exceeds or saturates the antioxidant levels. ROS are of particular interest, as they have been shown to play a role in follicle rupture induced by gonadotropins (Agarwal *et al*. 2005, Shkolnik *et al*. 2011). Mitochondria and the metabolic processes are the main sources of ROS production in steroidogenic tissues (Fujii *et al*. 2005, Hanukoglu 2006), but excessive ROS can affect a tissue by producing excessive lipid peroxidation, oxidation, and different lesions in the DNA, which may, in turn, result in apoptosis (Valko *et al*. 2007). Tissues, including the ovary, have a delicate balance between ROS and antioxidant species. Ovarian cells have both enzymatic and nonenzymatic antioxidant defenses to counteract oxidant species (Agarwal *et al*. 2005, Angelucci *et al*. 2006). The ratio between the

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levels of oxidant and antioxidant defenses changes depending on the hormonal conditions. After the gonadotropin surge (human chorionic gonadotropin (hCG) or luteinizing hormone (LH)), ROS levels increase while the expression of antioxidants decrease to enhance ovulation (Laloraya *et al*. 1988, Sato *et al*. 1992, Shkolnik *et al*. 2011). Using *in vivo* and *in vitro* assays, it has been shown that antioxidants significantly reduce the LH-induced cumulus mucification/expansion, progesterone production, and activation of two signaling pathways (PKA and MAPK), all events indispensable for the ovulatory process (Shkolnik *et al*. 2011). Indeed, preovulatory follicles isolated from immature rats stimulated with gonadotropin to induce ovulation produce high levels of superoxide anion after LH administration (Kodaman & Behrman 2001). ROS, such as superoxide anion radical and hydrogen peroxide, are considered essential to enhance steroidogenesis and ovulation (Sawada & Carlson 1996, Shkolnik *et al*. 2011). Thus, and considering that i) LH-induced ROS production seems to be an essential preovulatory signaling event by being involved in the activation of different signaling pathways (MAPK and probably PKA) (Shkolnik *et al*. 2011); ii) leptin regulates the ovulatory process by modifying steroidogenesis (Bilbao *et al*. 2013); and iii) some evidences indicate that leptin decreases ROS levels (Balasubramaniyan *et al*. 2007) in parallel with an increase in the antioxidant defenses (Sailaja *et al*. 2004), we hypothesize that the leptin action may affect the oxidative status, such as ROS production or antioxidant defense bioavailability, depending on its circulating levels.

We have previously found that an acute treatment with leptin inhibits (Ricci *et al*. 2006), whereas a daily administration of low doses of leptin enhances (Roman *et al*. 2005) the ovulatory process in comparison with control animals. Moreover, we found that the dual action of leptin seems to occur by regulating steroidogenesis (Bilbao *et al*. 2013), which, in turn, is regulated, at least in part, by both ERK and STAT signaling pathways (Di Yorio *et al*. 2013). Thus, and considering the relationship between ovulation and ROS, we were interested in studying whether the dual and opposite effects of leptin on the ovulatory process involve changes in the balance of oxidative stress at ovarian level in response to this protein in rats.

Materials and methods

Animals

Immature female Sprague–Dawley rats aged 21 days were purchased from the School of Veterinarian Sciences of Buenos Aires University, Argentina. Animals were kept under controlled conditions of light (12 h light:12 h darkness cycle), 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, and all the protocols were approved by the Institutional Committee of the School of Medicine of Buenos Aires University (CICUAL) by Resolution 2950/10.

Experimental design

In vivo studies

As shown in Fig. 1, prepubertal rats were i.p. injected with 15 IU of equine chorionic gonadotropin (eCG) to induce the first generation of preovulatory follicles and to avoid the confounding effects of the presence of different types of follicles and corpora lutea from previous cycles. After 48 h, the animals were i.p. injected with 15 IU of hCG to induce ovulation.

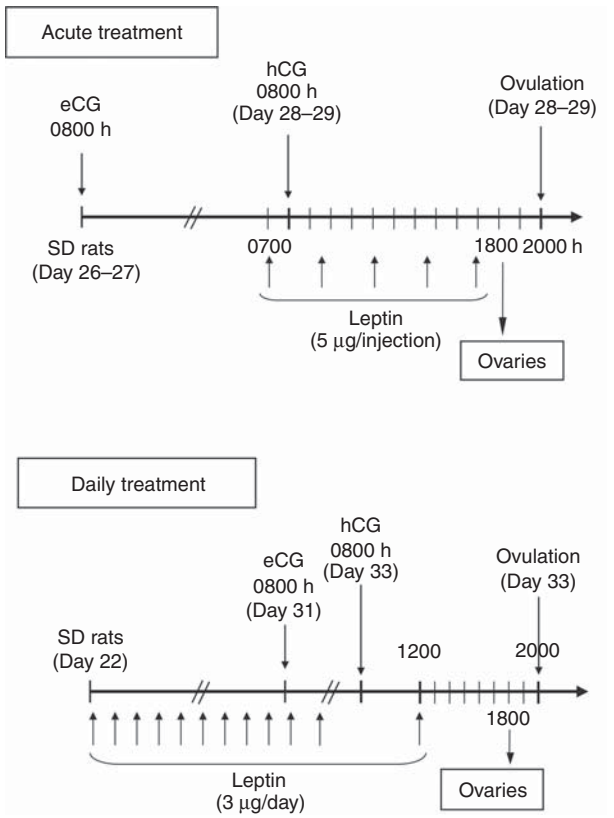

Figure 1 Schematic of the *in vivo* leptin treatments performed in our experiments. Acute treatment (upper panel): rats were injected with 15 IU eCG at 26–27 days of age and 15 IU hCG 48 h later. Both injections were administered at 0800 h. Leptin (5 µg/injection) was administered during the time period between 1 h before hCG administration and killing through five injections of recombinant rat leptin or vehicle at 150-min intervals (0700, 0930, 1200, 1430, and 1700 h; up arrow). Rats were killed by decapitation at 10 h (1800 h) after hCG administration. Daily treatment (bottom panel): at 22 days of age, rats were daily injected with 3 µg leptin or vehicle at 1200 h until killing. In total, the animals received 12 injections. In addition, all rats received 15 IU eCG at 31 days of age and 15 IU hCG 48 h later. Both injections were administered at 0800 h. Rats were killed by decapitation at 10 h (1800 h) after hCG administration. In this rat strain, ovulation occurs within 12 h after hCG administration. SD, Sprague–Dawley.

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

Both treatments attempt to simulate rats exposed to: i) high levels of leptin (acute treatment), as it occurs in obese rats, which inhibit ovulation (Duggal *et al*. 2000, Ricci *et al*. 2006) and ii) leptin levels that induce ovulation (daily treatment; Almog *et al*. 2001, Roman *et al*. 2005). All the rats were killed by decapitation 10 h after hCG administration and both ovaries were immediately dissected out, homogenized as indicated below or frozen on dry ice, and stored at −78 °C until use. We used only ovaries with an adequate size and weight that represent gonadotropin-induced stimulation (Espey *et al*. 2000).

In vitro studies

Animals were killed by decapitation 4 h after hCG administration and the ovarian tissue was incubated as described previously (Ricci *et al*. 2006). Briefly, ovarian slices (four slices per ovary per well) were randomly distributed in 24-well plates containing 500 μl/well of DMEM/F12 (1:1) medium (Bio-Rad Laboratories) with 25 mmol/l HEPES, 100 μg/ml streptomycin, 0.5 μg/ml fungizone, and 2 mmol/l L-glutamine, and different leptin concentrations (0.3–300 ng/ml) (Duggal *et al*. 2000, 2002*b*, Ricci *et al*. 2006) at 37 °C in a humidified atmosphere (5% CO_2:95% O_2). After 4 h of incubation, ovarian tissues were recovered, homogenized as indicated below or frozen on dry ice, and stored at −78 °C until use. Each experiment was repeated at least six times.

Quantification of the activity of antioxidant enzymes: superoxide dismutase and catalase

Each ovary, immediately dissected out after killing the rats, was homogenized in 20 mmol/l ice-cold Tris–HCl buffer (pH 7.6) containing 1 mmol/l EDTA, 150 mmol/l KCl, 1 mmol/l 2-mercaptoethanol, and 500 mmol/l sucrose, and centrifuged at 800 ***g*** for 10 min at 4 °C. Supernatants were used for superoxide dismutase (SOD) and catalase (CAT) assays and to measure the protein concentration by the Bradford method with BSA as the standard.

SOD activity was assessed as described previously (Misra & Fridovich 1972). This assay is based on the ability of SOD to inhibit the autoxidation of superoxide-induced epinephrine to adrenochrome, which is quantified by a spectrophotometer at an absorbance of 480 nm. The reaction mixture contained 30 mmol/l epinephrine in 0.05% (v/v) acetic acid, 50 mmol/l glycine buffer (pH 10.2), and different volumes of supernatants. The change in SOD activity was measured for 4 min at 10-s intervals. Results are expressed as units of SOD per milligram of protein.

CAT activity was assayed as described previously (Aebi 1984), based on the ability of CAT to catalyze the conversion of hydrogen peroxide (H_2O_2) to molecular oxygen and water. To this end, supernatants were incubated with 1 mmol/l H_2O_2 in 50 mmol/l PBS, pH 7.2. The rate of decrease in H_2O_2 absorbance was measured for 1 min at 10-s intervals by a spectrophotometer at an absorbance of 240 nm. Results are expressed as micromoles of CAT per milligram of protein.

Oxidative damage-related parameters

Each ovary, immediately dissected out after killing the rats, was homogenized in 0.5% (w/v) trichloroacetic acid at 4 °C and centrifuged at 10 000 ***g*** for 10 min at 4 °C. Supernatants were used to measure the presence of both malondialdehyde (MDA, as an index of lipid peroxidation) and total glutathione (GSH, as an indicator of antioxidant defense consumption). Protein concentration in these supernatants was determined by the Bradford method, with BSA as the standard.

Lipid peroxidation

As an index of lipid peroxidation, we used the formation of thiobarbituric acid-reactive species (TBARS) by measuring the amount of MDA, formed from the breakdown of polyunsaturated fatty acids during an acid heating reaction, as described previously (Ohkawa *et al*. 1979). Briefly, the supernatants were mixed with 0.67% (w/v) thiobarbituric acid (Merck) and heated in a boiling water bath for 20 min. After cooling, the absorbance of each sample was determined at 535 nm using a Beckman Coulter u.v./Visible (DU800). TBARS content was expressed in micromoles of MDA formed per milligram of protein.

Total GSH content

GSH content was determined by an enzymatic assay as described previously (Tietze 1969). Briefly, the supernatants were incubated with 0.24 mmol/l NADPH, 0.75 mmol/l 5,5′-dithiobis-2-nitrobenzoic acid in PBS 0.05 M, pH 7.4, and 2.5 IU GSH reductase, and the development of the chromophoric product was recorded every 30 s for 6 min at an absorbance of 412 nm using a Beckman Coulter u.v./Visible (DU800). Total GSH content was expressed in micromoles GSH per milligram of protein.

In situ ROS detection

Whole ovaries, immediately dissected out after killing the rats, were used to determine the presence of ROS, as described previously (Tsai-Turton & Luderer 2006). Each ovary was incubated with 100 μmol/l 2′,7′-dichlorofluorescein diacetate (H_2DCFDA) (Sigma–Aldrich) in DMEM/F12 (1:1) containing 0.5 μg/ml fungizone and 2 μg/ml gentamicin at 37 °C in a humidified atmosphere (5% CO_2:95% O_2) for 30 min. After incubation, ovaries were washed with PBS, fixed in buffered formalin, fixed in 70% ethanol, dehydrated, and finally included in paraffin. Then, the tissues were serially sectioned at 5 μm thickness and mounted onto glass slides, and fluorescence intensity was measured every 20 sections. H_2DCFDA diffuses easily into the cells, where it is converted by cytosolic esterases

to the nonfluorescent compound dichlorofluorescein (H_2DCF), which is, in turn, oxidized to the fluorescent dichlorofluorescein (DCF) in the presence of ROS. As DCF fluorescence is considered a direct indicator of the presence of ROS, the fluorescence intensity was quantified under a Zeiss LSM 511 META laser scanning confocal microscope using a Plan-Neofluor 10/0.3 objective with Cy2/alexa/fluorescein isothiocyanate excitation and a BP-500-530 filter for emission. Four sections from the same ovary and four to five ovaries from different animals with the same treatment were analyzed in each group and the ratio between the fluorescent area and the total area was used for statistical analyses using the freely available Image Pro Plus and ImageJ Software.

Protein isolation by western blot

Soluble tissue extracts, obtained from one ovary of each rat, were prepared as described previously (Bilbao *et al.* 2013). Briefly, proteins were isolated from the organic phase of the frozen tissues using TRI Reagent (Molecular Research Center) according to the manufacturer's instructions. Equal amounts of protein (100 μg) were separated by SDS–PAGE (12%). Proteins were transferred onto PVDF membranes (Bio-Rad Laboratories) for 60 min in a cold chamber using a Bio-Rad transblot apparatus. Membranes were first blocked at 4 °C overnight in Tris–HCl:saline (50 mmol/l Tris–HCl:150 mmol/l NaCl, pH 7.5) containing 5% (w/v) of non-fat milk powder, and then incubated at 4 °C for 48 h with specific antibodies. Rabbit anti-caspase 3 (H-277), anti-Bax (N-20), and anti-Bcl-2 (N-19) polyclonal antibodies (Santa Cruz Biotechnology), and rabbit anti-actin polyclonal antibody (A2066, Sigma–Aldrich) each diluted 1/200 were used. After washing, membranes were treated for 1 h at room temperature with a goat anti-rabbit IgG (diluted 1/2500) as the secondary antibody (Santa Cruz Biotechnology). Immunoreactive bands were visualized using chemiluminescence detection reagents (Sigma–Aldrich) and Image Quant RT ECL (General Electric, Amersham Bioscience Argentina SA), and quantified using the ImageJ Software. Before reuse, membranes were stripped, blocked, and reprobed according to the manufacturer's instructions. Negative controls were carried out by omitting the incubation with the primary antibody and no bands were detected. Molecular weight standards (Kaleidoscope, Bio-Rad Laboratories) were run under the same conditions to identify the protein bands. The data were normalized to β-actin protein levels in each sample to avoid procedural variability.

Ovarian histology and follicle counting

To assess the effect of the daily treatment with leptin on follicular growth, antral follicles were counted in histological sections prepared from ovaries as described previously (Barreiro *et al.* 2011). Briefly, each ovary was fixed in formalin, dehydrated and serially sectioned at 5 μm thickness and stained with hematoxylin and eosin according to standard protocols and analyzed using an Olympus CX21 microscope. Ovarian antral follicles were analyzed every ten sections and only follicles containing an oocyte were counted to avoid double counting the follicles. Ten sections from the same ovary and five or six ovaries from different animals with the same treatment were analyzed in each group. Follicle abundance was normalized by the total ovarian area in the section, as reported previously (Cheng *et al.* 2002, Barreiro *et al.* 2011). Ovarian area was measured using Image J (version 1.42q) and expressed per 10 mm^2.

Statistical analysis

All data are expressed as means±S.E.M. Differences between two groups were analyzed using Student's *t*-test. Comparisons between more than two groups were performed using one-way ANOVA and Dunnett's multiple comparison test. Levene's test and a modified Shapiro–Wilk test were used to assess homogeneity of variances and normal distribution respectively. If these assumptions were not met, a logarithmic transformation was applied to the data before ANOVA. If the transformation did not result in homogeneous variances, two nonparametric tests, the Mann–Whitney *U* and Kruskal–Wallis tests, were used to assess the differences between two groups and more than two groups respectively. Differences between groups were considered significant when $P<0.05$.

Results

In vivo *studies*

Table 1 shows the results obtained regarding SOD and CAT activities and MDA and total GSH contents in the

Table 1 Effect of both the acute and the daily treatments with leptin on the activity of two antioxidant enzymes, superoxide dismutase (SOD), and catalase (CAT), and two oxidative damage-related parameters, the concentration of malondialdehyde (MDA), as an indicator of lipid peroxidation, and the concentration of total glutathione (GSH) in rat ovaries. Values are expressed as means±S.E.M. (n=6–8) and were obtained by separate experiments.

	Acute treatment		Daily treatment	
	Control	Leptin	Control	Leptin
SOD (IU/mg protein)	148±13	111±8*	65±9	66±7
CAT (μmol/mg protein)	85±22	93±17	222±31	153±24
GSH (μmol/mg protein)	21±2	23±2	45±9	25±2†
MDA (μmol/mg protein)	131±12	140±9	106±16	106±5

Acute treatment: *$P<0.05$ vs control. Student's *t*-test was used for SOD, CAT, and GSH, and a nonparametric test, the Mann–Whitney *U* test, was used for MDA. Daily treatment: †$P<0.05$ vs the respective control. Student's *t*-test was used for SOD and CAT, and a nonparametric test, the Mann–Whitney *U* test, was used for GSH and MDA.

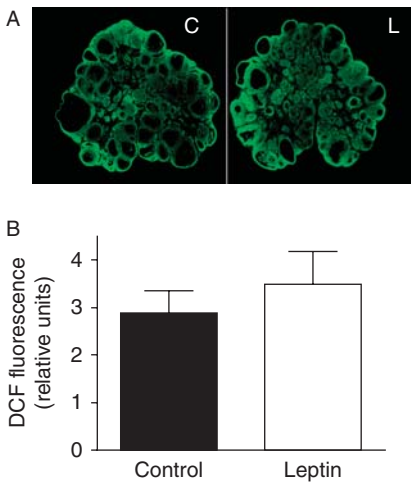

Figure 2 *In vivo* effect of the acute treatment with leptin on reactive oxygen species (ROS) levels in the ovarian tissue by dichlorofluorescein (DCF) fluorescence. Immature rats were primed with eCG/hCG and treated either with vehicle (control) or with 5 μg leptin 1 h before hCG administration and at 150-min intervals. Animals were killed 10 h after hCG administration. ROS production was assessed by measuring DCF fluorescence by laser scanning confocal microscopy as detailed in the Materials and methods section. (A) Representative images show DCF fluorescence in both the control (C) and leptin-treated tissue (L). (B) Mean±S.E.M. of DCF fluorescence intensity expressed as relative units (n=4–5 ovaries/group).

ovarian tissue from rats that had received both the acute and daily treatments with leptin. After the acute treatment, SOD activity was significantly lower (25%, $P<0.05$) than that of control animals, without changes in the other parameters evaluated. However, after the daily treatment, the ovaries had a significant decrease in total GSH content (44%, $P<0.05$) compared with controls, without differences in the other parameters.

When we studied the presence of ROS in the ovarian tissue, we found no differences between rats acutely treated with leptin and controls, as the fluorescence intensity of DCF was similar in both groups (Fig. 2). In contrast, the ovaries from animals treated daily with leptin exhibited an abrupt decrease in the fluorescence (67%, $P<0.001$) compared with controls (Fig. 3).

By western blot, all the ovarian tissues obtained from the *in vivo* experiments revealed the presence of BCL2 (26 kDa), BAX (23 kDa), procaspase 3 (42 kDa), and caspase 3 p17 (17 kDa), the latter of which represents active caspase 3. The acute treatment with leptin did not alter the ovarian expression of BCl2 or BAX (Fig. 4A) when compared with controls. However, this treatment was able to increase the expression of caspase 3 p17 (56%, $P<0.01$) but not that of procaspase 3 (Fig. 4B). The daily treatment with leptin decreased the expressions of both BAX (20%, $P<0.05$) (Fig. 5A) and caspase 3 p17 (38%, $P<0.01$) (Fig. 5B) compared with controls but not those of Bcl2 or procaspase 3 (Fig. 5).

To complete previous studies where a daily stimulus with low doses of leptin accelerated the follicular maturation (Almog *et al.* 2001) and increased both steroidogenesis (Roman *et al.* 2005) and the ovulation rate (Almog *et al.* 2001, Roman *et al.* 2005), antral follicles were counted to study whether this treatment was able to increase follicular growth. Figure 6 shows the results of the histological evaluation of the ovarian sections. The number of antral follicles was 5.6-fold higher in the leptin-treated animals than in control animals ($P<0.01$).

In vitro *studies*

To study the direct effect of a narrow range of leptin concentrations, some parameters studied in the *in vivo* experiments were measured in ovarian explant cultures after 4 h of incubation (results are shown in Table 2). Leptin showed no effects on CAT activity, GSH content, or MDA levels. However, the ovarian tissue exposed to high concentrations of leptin clearly exhibited lower activity of SOD than the control tissues, although this reduction was only significant at 100 ng/ml ($P<0.05$; Table 2). Unlike that observed in the *in vivo* experiments, we were not able to detect the bands corresponding to caspase 3 p17 and the presence of leptin did not alter the expression of procaspase 3 (data not shown). Nevertheless, BCL2 expression increased at 1 ng/ml (201%, $P<0.01$) of leptin when compared with controls, whereas Bax expression showed no changes (Fig. 7).

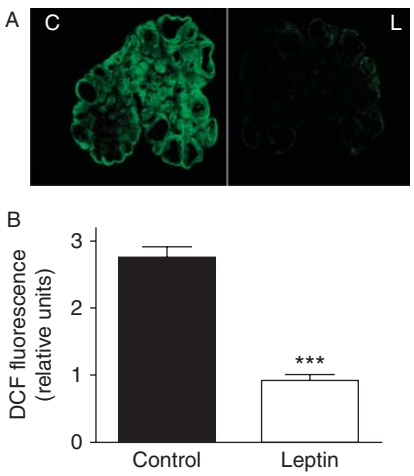

Figure 3 *In vivo* effect of the daily treatment with leptin on reactive oxygen species (ROS) levels in the ovarian tissue by DCF fluorescence. On day 22 of age, rats received injections of either vehicle (control) or 3 μg leptin/day. After 10 days of treatment, animals received eCG/hCG stimulation as indicated in the Materials and methods section. Animals were killed 10 h after hCG administration. (A) Representative images show DCF fluorescence in both the control (C) and leptin-treated tissue (L). (B) Mean±S.E.M. of DCF fluorescence intensity expressed as relative units (n=4–5 ovaries/group). ***$P<0.001$ vs controls (Student's t-test).

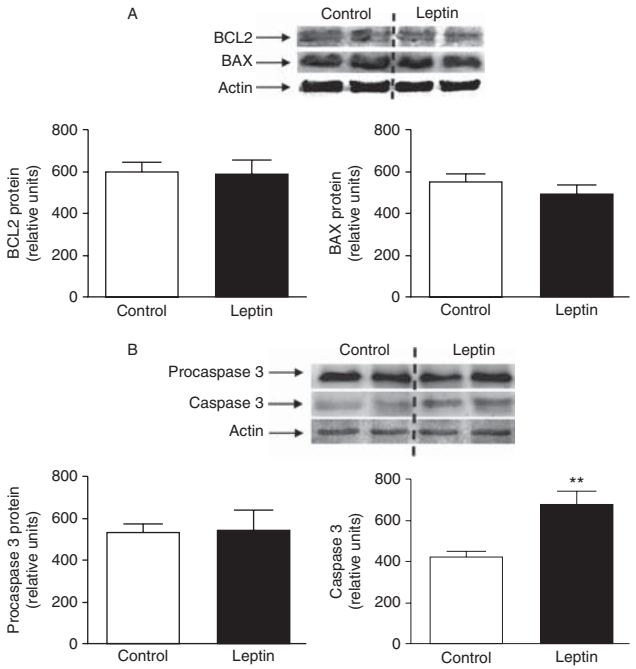

Figure 4 *In vivo* effect of the acute treatment with leptin on the ovarian expression of BCL2, BAX (A), procaspase 3, and caspase 3 (B). Immature rats were primed with eCG/hCG and treated either with vehicle (control) or with 5 µg leptin 1 h before hCG administration and at 150-min intervals. Animals were killed 10 h after hCG administration. (A and B) Representative blots (upper panel) showing protein expression of BCL2, BAX (A), procaspase 3, caspase 3 (B), and β-actin (as protein control) by western blot, and quantitative analysis (bottom panel) of immunoreactive bands. Results are expressed as the mean ± S.E.M. for six to eight samples per group. Each sample represents one ovary from each animal with the same treatment ($n=6$–8). $^{**}P<0.01$ vs control (Student's *t*-test).

Discussion

In the present work, we studied whether the dual action of leptin in the ovulatory process involves changes in the oxidative status by evaluating some antioxidant defenses, ROS levels, and some oxidative damage-related parameters. SOD is the first enzymatic defense against ROS generated by the tissue as this enzyme catalyzes the reaction of superoxide anion radicals and dismutation to hydrogen peroxide, which is then converted into water by other enzymes, such as CAT or GSH peroxidase (GPx; Fridovich 1995, Hanukoglu 2006). As the acute administration of leptin caused a decrease in SOD activity, it is expected that this inhibitory effect results in a decrease in the levels of hydrogen peroxide. While it is difficult to compare an *in vivo* effect caused by a tissue directly exposed to effective and known doses, this result is consistent with that obtained in our *in vitro* studies as the presence of a high concentration of leptin inhibited ovarian SOD activity. These results also support those obtained in other *in vitro* studies, where leptin caused an increase in ROS levels (Yamagishi *et al.* 2001, Xu *et al.* 2004, Chetboun *et al.* 2012). Steroidogenesis is an important source of free radical production as the enzymes involved in the different steps in the biosynthesis of steroids (cytochrome P450 family and others) transfer electrons to different molecules producing ROS (Rapoport *et al.* 1995, Hanukoglu 2006). SOD expression in developing follicles correlates positively with steroidogenesis (Suzuki *et al.* 1999) and seems to play a role generating hydrogen peroxide, which drives the peroxidase–ascorbate system, responsible for production of progesterone (Agrawal & Laloraya 1977, Laloraya *et al.* 1988, Suzuki *et al.* 1999). Therefore, a decrease in SOD activity, caused by the acute treatment with leptin, which implies lower production of hydrogen peroxide, could be altering the peroxidase–ascorbate system involved in the conversion of pregnenolone to progesterone. This hypothesis is consistent with the results obtained from previous studies where we found that the acute treatment with leptin inhibited the plasma progesterone levels (Ricci *et al.* 2006) and the expression of cytochrome P450 side chain cleavage (Bilbao *et al.* 2013), an enzyme involved in the conversion of pregnenolone to progesterone.

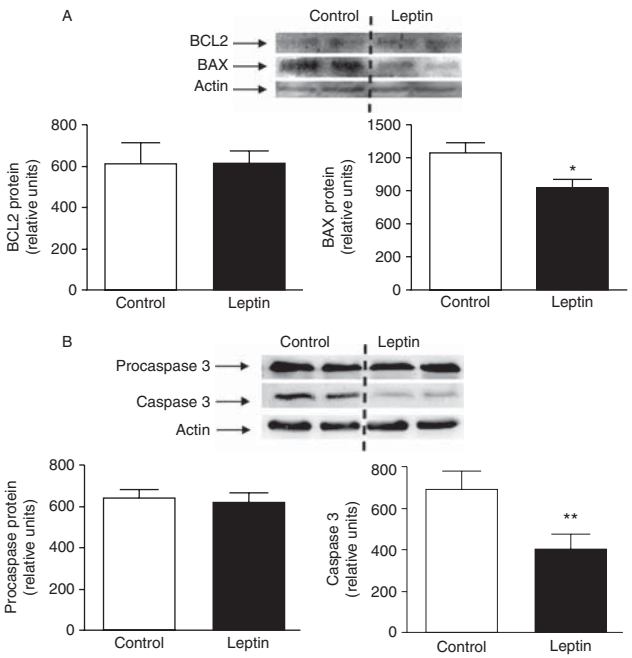

Figure 5 *In vivo* effect of the daily treatment with leptin on the ovarian expression of BCL2, BAX (A), procaspase 3, and caspase 3 (B). On day 22 of age, rats received injections of either vehicle (control) or 3 µg leptin/day. After 10 days of treatment, animals received eCG/hCG stimulation as indicated in the Materials and methods section. Animals were killed 10 h after hCG administration. (A and B) Representative blots (upper panel) showing protein expression of BCL2, BAX (A), procaspase 3, caspase 3 (B), and β-actin (as protein control) by western blot, and quantitative analysis (bottom panel) of immunoreactive bands. Results are expressed as the mean ± S.E.M. for six to eight samples per group. Each sample represents one ovary from each animal with the same treatment ($n=6$–8). $^{*}P<0.05$ and $^{**}P<0.01$ vs control (Student's *t*-test).

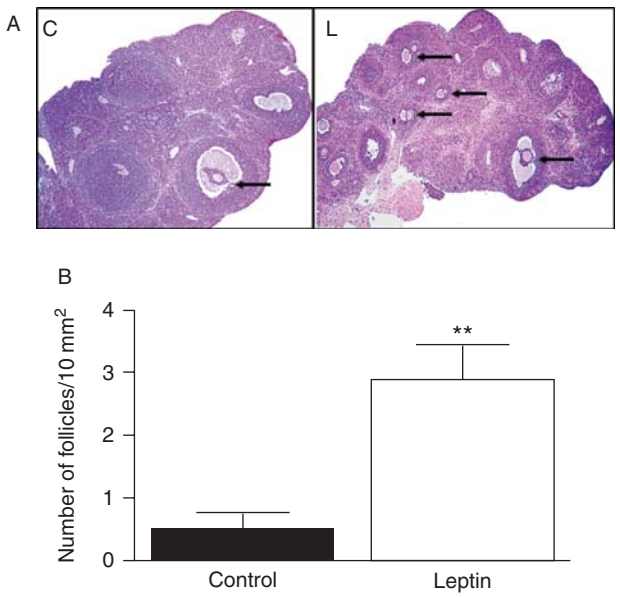

Figure 6 *In vivo* effect of the daily treatment with leptin on follicular growth by measuring the number of antral follicles in histological sections. On day 22 of age, rats received injections of either vehicle (control) or 3 µg leptin/day. After 10 days of treatment, animals received eCG/hCG stimulation as indicated in the Materials and methods section. Animals were killed 10 h after hCG administration. (A) Histological appearance of ovarian sections from animals treated with vehicle (C) or leptin (L) stained with hematoxylin and eosin. (B) Values are expressed as the number of follicles per 10 mm^2. Data represent the mean±S.E.M. for six ovaries from different animals with the same treatment, and whose values represent the mean of ten sections from the same ovary. Arrow: antral follicles. $^{**}P<0.01$ vs vehicle (Control) (Student's *t*-test). Magnification: ×100.

Unlike that observed in rats treated with the acute treatment, rats treated daily with leptin exhibited lower levels of total GSH in the ovarian tissue when compared with control animals. GSH regulates the cellular redox status as a scavenger of different free radicals such as hydroxyl radicals, superoxide anions, and peroxynitrite, or by acting as a substrate for both GPx and GSH S-transferase enzymes to detoxify hydrogen peroxide and lipid peroxides (Griffith 1999, Espey & Richards 2002, Devine *et al.* 2012). These reactions induce the formation of GSH disulfide (GSSG), its oxidized form, which, in turn, is reduced to GSH by GSH reductase. Thus, under physiological conditions, the concentration of GSH is higher than that of GSSG to protect the cell from further oxidative damage. In addition, GSH is synthesized by two sequential enzymatic reactions that are catalyzed by glutamate cysteine ligase (GCL) and GSH synthase, whose activities are induced by oxidative stress (Lu 2009). Tsai-Turton & Luderer (2005) demonstrated that gonadotropins regulate ovarian synthesis of GSH by regulating the expression of the rate-limiting enzyme, GCL, in a follicle stage-dependent manner. All these data suggest that daily stimulation with low doses of leptin causes a lower generation of GSH probably by regulating the activity of GCL and/or GSH synthase. On the other hand, and although no changes were found in SOD and CAT activities or MDA concentrations, the ovarian tissue from animals treated daily with leptin exhibited an important decrease in the fluorescence intensity of DCF, which indicates an important decrease in ROS levels. It has been reported that cytochrome *c* is a potent catalyst of H_2DCF oxidation and that the increased DCF production can be attributed to an increased cytosolic concentration of the cytochrome rather than an actual change in the ROS status (Kooy *et al.* 1997, Lawrence *et al.* 2003). In addition, Lawrence *et al.* (2003) showed that the rate of H_2DCF oxidation is highly sensitive to GSH concentrations, and that GSH and H_2DCF compete for oxidation by the same oxidizing species. The mechanism by which a daily leptin stimulus downregulates GSH content or ROS levels remains yet to be determined. However, and considering all these data and our results, it is reasonable to suggest that: i) GSH formation is not induced in the absence of oxidative stress and ii) the low concentrations of GSH caused by the daily treatment with leptin may be the result of its oxidation by other oxidant agents or by regulation on the activity of the enzymes involved in its synthesis.

Cell death by apoptosis is the mechanism involved in both follicular atresia and luteolysis. Activation of caspase 3 and increased expression of Bax, among others, are biomarkers of cell apoptosis. Both proteins are

Table 2 *In vitro* effect of different concentrations of leptin on the activity of two antioxidant enzymes, the superoxide dismutase (SOD) and catalase (CAT), and two oxidative damage-related parameters, the concentration of malondialdehyde (MDA), as an indicator of lipid peroxidation, and the concentration of total glutathione (GSH) by cultures of ovarian explants, from immature gonadotropin-primed rats. Values are expressed as means±S.E.M. ($n=8$).

	Ctrl	Leptin (ng/ml)						
		0.3	1	3	10	30	100	300
SOD (IU/mg protein)	21±3	21±2	21±2	15±2	15±2	15±1	11±4*	12±2
CAT (µmol/mg protein)	274±23	307±26	268±29	273±39	265±14	288±35	239±10	259±44
GSH (µmol/mg protein)	81±18	79±8	82±9	85±11	115±19	86±6	122±23	92±18
MDA (µmol/mg protein)	121±11	114±2	108±2	106±3	109±4	131±15	123±8	122±12

*$P<0.05$ vs control (Ctrl). One-way ANOVA and Dunnett's multiple comparison test were used for SOD and CAT, and a nonparametric test, the Kruskal–Wallis test, was used for GSH and MDA.

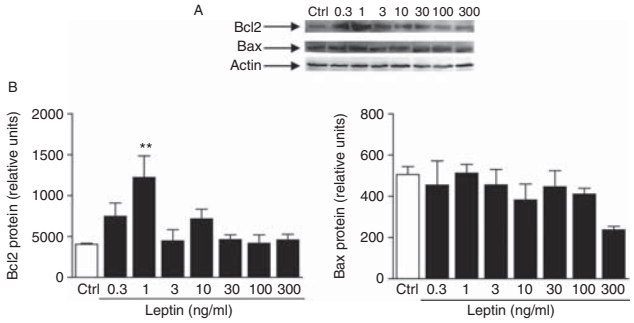

Figure 7 *In vitro* effect of leptin on the ovarian expression of Bcl2 and Bax. Ovarian explants were obtained 4 h after hCG administration from immature rats primed with eCG/hCG and incubated for 4 h either in the presence or in the absence (control) of different concentrations of leptin (0.3–300 ng/ml). (A) Representative blots showing the protein expression of Bcl2, Bax, and β-actin (as protein control) by western blot. (B) Quantitative analysis of immunoreactive bands. Results are expressed as the mean ± S.E.M. for five to six samples per group. Each sample represents one ovary from each animal with the same treatment. $^{**}P<0.01$ vs control (Ctrl) (one-way ANOVA and Dunnett's multiple comparison test).

mainly expressed in atretic follicles while anti-apoptotic BCL2 is abundant in healthy preantral follicles (Slot *et al.* 2006). Caspase 3 activation is an irreversible event during cell apoptosis. The fact that the acute treatment with leptin increased the expression of active caspase 3 but showed no changes in BAX and BCL2 or in ROS levels may indicate an involvement of the death receptor pathway rather than the mitochondrial pathway in caspase 3 activation. However, further studies are necessary to investigate the mechanism by which leptin activates caspase 3.

Unlike the acute treatment, the daily administration of leptin decreased the ovarian expression of both Bax and active caspase 3, which is consistent with the fact that: i) these animals exhibited a higher follicular development and ii) the ovarian tissue directly exposed to physiological concentrations of leptin caused an increase in BCL2 expression. These results are supported by previous studies that demonstrated that similar daily administrations with leptin reduce the incidence of follicular apoptosis after 7 days of leptin injection and that leptin may be involved in accelerating follicular maturation by attenuating follicular atresia through an increase in the BCL2/BAX ratio (Almog *et al.* 2001). In addition, using different *in vitro* systems, other authors have found that leptin reduces apoptosis and induces cell proliferation, at least in part, by upregulating BCL2 expression (Brown & Dunmore 2007, Sirotkin & Grossmann 2007, Lam *et al.* 2010), and that leptin may act as an anti-apoptotic factor in luteinized porcine granulosa cells by increasing BCL2 expression and decreasing caspase 3 activity (Dineva *et al.* 2007). It is noteworthy that neither the acute nor the daily treatment altered procaspase 3 expression. This result indicates that leptin acts specifically on the cleavage process of the protein and consequently regulates the active protein without significantly altering procaspase 3 expression.

In conclusion, leptin can modulate the oxidative status according to its circulating levels. The fact that a daily stimulus with low doses of leptin, which induces follicular growth, decreases ROS levels and BAX and active caspase 3 expression indicates that leptin seems to exert a protective role in the ovarian tissue, as an antioxidant molecule, by attenuating follicular atresia. In contrast, at high concentrations, leptin may be involved in the loss of cell viability by activating caspase 3 through the death receptor pathway rather than through the mitochondrial pathway. All these data suggest that leptin seems to have a mediating role in reproduction by acting on different targets, at both intra- and extra-ovarian levels, as it can either upregulate or downregulate the ovarian function according to its circulating concentration. Thus, specific leptin levels are necessary to ensure a normal ovarian function.

Declaration of interest

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

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