Differential effects of leptin on ovarian metalloproteinases and their tissue inhibitors between *in vivo* and *in vitro* studies

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Abstract

In this study, we investigated the effect of leptin on the ovarian metalloproteinase system in the rat during the ovulatory process. Ovulation was induced in immature rats primed with gonadotropins. In both *in vitro* and *in vivo* experiments, we measured i) the protein expression of the ovarian metalloproteinases (matrix metalloproteinases, MMPs) and their tissue inhibitors (TIMPs) by western blot; ii) the gelatinase activity of the ovarian MMPs by zymography; and iii) the inhibitory action of TIMPs by reverse zymography. Using cultures of ovarian explants, leptin increased the activity but not the protein expression of MMP-2 and MMP-9 in both culture medium and ovarian tissue, and the protein expression of TIMPs, without a higher inhibitory action of the gelatinase activity. These results suggest either that the increase in TIMP proteins was not sufficient or that the inhibitory actions of TIMPs were impaired to suppress the MMP activity when the ovaries were directly exposed to leptin. To study the *in vivo* effect, rats received an acute treatment with high doses of leptin to inhibit ovulation. This treatment increased the expression of both the latent and the active forms of MMP-2 but did not result in a greater activity of MMP-2. In addition, the inhibitory action of TIMP-2 was also increased by this treatment. These results suggest that the administration of high doses of leptin could be regulating the follicle wall degradation, at least in part, by increasing the action of the ovarian TIMP-2 as a result of an extraovarian mechanism or signaling pathway.

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Introduction

Leptin is produced mainly in adipose tissue and secreted into the blood stream (Zhang *et al.* 1994). 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 signaling and reproduction. The effects of leptin on ovulation are contradictory; both stimulatory (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) actions on the ovarian function have been described.

Ovulation is a dynamic and complex process that is initiated and synchronized by a series of biochemical events that culminate in the follicle rupture and extrusion of the oocyte. This process involves gonadotropins, steroid hormones, cytokines, prostaglandins, nitric oxide, plasmin–plasminogen system, and different proteolytic enzymes (Richards *et al.* 2002). Matrix metalloproteinases (MMPs) are a class of proteolytic enzymes involved in connective tissue remodeling throughout the body (Sellers & Murphy 1981). MMPs are a family of more than 20 structurally related enzymes that include four major classes: collagenases, gelatinases, stromelysins, and membrane-associated metalloproteinases (Nagase & Woessner 1999). These enzymes are synthesized as preproenzymes that are secreted to the extracellular space as inactive pro-MMPs, where they must be activated to cleave the extracellular matrix by the catalytic domain. The gelatinase class consists of two distinct MMPs, MMP-2 (72 kDa) and MMP-9 (92 kDa). These enzymes have a potent ability to bind to and cleave gelatin and therefore act to degrade major constituents of basement membranes, including type IV collagen, laminin, and fibronectin. The activity of MMPs in the extracellular space is regulated by both serum-borne and tissue-derived metalloproteinase inhibitors. MMPs and their endogenous inhibitors are referred to as the MMP system. The tissue inhibitors of MMPs (TIMPs) are abundant in reproductive tissues, hormonally regulated, and involved in numerous ovarian processes (McIntush & Smith 1998, Curry *et al.* 2001). There is much evidence to support a role for the MMP system in follicular development, ovulation, and early luteal development. During the ovulatory process, extensive remodeling is apparent in the dissolution of the granulosa cell basement membrane and fragmentation of the extracellular matrix of the follicular wall, allowing the oocyte

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release (Richards *et al.* 2002). There is substantial evidence that both MMPs and TIMPs play a critical role in these processes (Curry & Osteen 2003). Both MMPs and TIMPs are present in the ovaries of mice (Waterhouse *et al.* 1993, Inderdeo *et al.* 1996), rats (Curry *et al.* 1985, Reich *et al.* 1985, Hirsch *et al.* 1993, Butler & Woessner 1994, Okada *et al.* 1994, Nothnick *et al.* 1995, 1996, Balbín *et al.* 1996), monkeys (Duncan *et al.* 1996*a*), and humans (Curry *et al.* 1990, Duncan *et al.* 1996*b*). The localization of the MMP system and its responsiveness to hormonal stimulation therefore support a role for MMPs and TIMPs in follicular development and ovulation (Curry & Osteen 2003).

We have previously found that the ovulatory process is significantly inhibited in response to an acute treatment with leptin, and that this effect may be due, at least in part, to the direct or indirect impairment of some ovarian inflammatory factors, such as prostaglandins and nitric oxide (Ricci *et al.* 2006). Therefore, the objectives of this study were to investigate the effect of leptin on the MMP system during the ovulatory process and to assess whether this system is involved in the inhibitory action of an acute treatment of leptin on the ovulatory process in rats.

Materials and Methods

Animals

Immature female Sprague–Dawley rats aged 22 days were purchased from the School of Veterinarian Sciences (University of Buenos Aires, Argentina). All the animals were kept under controlled conditions of light (14 h light:10 h darkness), temperature (22 °C), and humidity, with free access to food and water. In all the experiments, at 26–27 days of age, rats were injected with 10 IU of equine chorionic gonadotropin (eCG) i.p. (in 0·10 ml saline) to induce the growth of 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 injected with 10 IU of human chorionic gonadotropin (hCG) i.p. (in 0·10 ml saline) to induce ovulation, which usually occurs within 12 h after hCG administration in this rat strain.

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 both the Centro de Estudios Farmacológicos y Botánicos (CEFYBO) and the Comité Institucional para el Cuidado y Uso de Animales de Laboratorio (CICUAL), Resolution 2079/07 from the School of Medicine of the University of Buenos Aires. The CICUAL adheres to the rules of the 'Guide for the Care and Use of Research Animal' (NIH), and the Institution has an Animal Welfare Assurance approved by the Public Health Service (PHS) with the assurance number A5801-01.

In vitro *studies*

Ovarian explant culture Animals were killed by decapitation 4 h after hCG administration, as described previously (Ricci *et al.* 2006). Briefly, the ovaries were immediately removed and dissected free of fat and bursa, and cut into pieces of approximately equal size (four slices per ovary). Ovarian slices were placed in 24-well plates containing 500 µl/well of 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 well contained eight slices obtained from the two ovaries of the same animal. After 30-min preincubation at 37 °C in a humidified atmosphere (5% CO_2:95% O_2), 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 in the same conditions as before. After the incubation periods, ovarian tissues were recovered and frozen on dry ice and stored at −72 °C to measure protein content by western blot analysis, MMP activity by zymography, and inhibitory action of TIMPs by reverse zymography. Culture media were stored at −20 °C until metalloproteinase activity, and the inhibitory action of TIMPs were assayed. The range of leptin concentrations was based both on previous studies (Spicer & Francisco 1998, Duggal *et al.* 2002, Ricci *et al.* 2006) and on the fact that plasma leptin levels are about 3–10 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 rats (Watanobe *et al.* 2001). In addition, the plasma leptin concentration can greatly vary in women with reproductive disorders such as anorexia nervosa, obesity, polycystic ovary syndrome, and preeclampsia (Mantzoros *et al.* 1997, Mise *et al.* 1998, Rissanen *et al.* 1999, Herpertz *et al.* 2000, Ludwig *et al.* 2007). For each culture condition, four to five independent experiments were run with two replicates using different tissue preparations.

In vivo *studies*

Since it has previously been demonstrated that ovulation is significantly inhibited by an acute treatment with leptin (Duggal *et al.* 2000, 2002, Ricci *et al.* 2006) and in order to study whether the MMP system is involved in this inhibitory action, rats received five i.p. injections of either recombinant rat leptin (5 µg/0·15 ml of PBS–BSA) or PBS–BSA alone (control). The first injection was given 1 h before hCG administration, and the other four at intervals of 150 min until killing. This treatment was based on previous studies (Duggal *et al.* 2000, 2002, Ricci *et al.* 2006). The original purpose of this treatment design was to maintain high levels of leptin after hCG administration to avoid the fall of this protein observed in circulation before ovulation as described previously (Ryan *et al.* 2003, Ricci *et al.* 2006). Rats were killed by decapitation 10 h after the hCG injection, since this is a preovulatory moment when some factors that induce

the follicle rupture are at their highest levels in control animals and are significantly inhibited with this leptin treatment (Ricci *et al.* 2006). Ovaries were immediately dissected out, frozen on dry ice, and stored at −72 °C.

Drugs and chemicals

Recombinant rat leptin, hCG, and protease inhibitors were purchased from Sigma–Aldrich, and eCG was obtained from Syntex SA (Buenos Aires, Argentina). Both the western blotting and the zymography reagents were obtained from Sigma–Aldrich and Bio-Rad Laboratories.

Western blot analysis

Soluble tissue extracts were prepared as described previously (Ricci *et al.* 2006). Equal amounts of protein (100 μg) were separated by SDS-PAGE (10% for MMPs and 15% for TIMPs). Following electrophoresis, proteins were transferred to polyvinylidene difluoride 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 mM Tris–HCl:150 mM NaCl, pH 7·5) containing 5% (w/v) milk powder and then incubated at 4 °C overnight with a specific primary antibody for the western blot assay. Mouse anti-human antibodies raised against MMP-2 (Ab-4) and MMP-9 (Ab-1; Calbiochem, San Diego, CA, USA), each diluted 1/500, rabbit anti-human antibody for TIMP-1 (H-150), TIMP-2 (H-140) and TIMP-3 (H-55; Santa Cruz Biotechnology, Santa Cruz, CA, USA), each diluted 1/200, and rabbit anti-rat actin antibody (Sigma–Aldrich Corp.), each diluted 1/500, were used. After washing, membranes were treated for 1 h at room temperature with goat anti-mouse IgG for MMPs and goat anti-rabbit IgG for TIMPs and actin diluted 1/2500 as secondary antibody (Santa Cruz Biotechnology). 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 conditioned media enriched in MMP-2 and MMP-9. Before re-use, membranes were stripped, blocked, and re-probed according to the manufacturer's instructions. Molecular weight standards (Kaleidoscope St, Bio-Rad Laboratories) were run under the same conditions to identify the protein bands. Blots were scanned using a UMAX Astra 12205 scanner, and densitometry was analyzed using a Dekmate III Sigma Gel software package (Sigma Chemicals Co., St. Louis, MO, USA). The data were normalized to β-actin protein levels in each sample to avoid procedural variability.

Zymography

Zymography was performed to evaluate the presence of gelatinase activity as described previously (Cooke *et al.* 1999). Ovarian tissues were homogenized in 0·05 M ice-cold Tris–HCl buffer, pH 7·6, containing 10 mM $CaCl_2$, 1 μM $ZnCl_2$, and 1% (v/v) Triton X-100. The homogenates were centrifuged at 10 000 ***g*** at 4 °C for 15 min. Protein concentration in these supernatants and culture media was determined by the Bradford method with BSA as the standard. Both homogenates and culture media were diluted

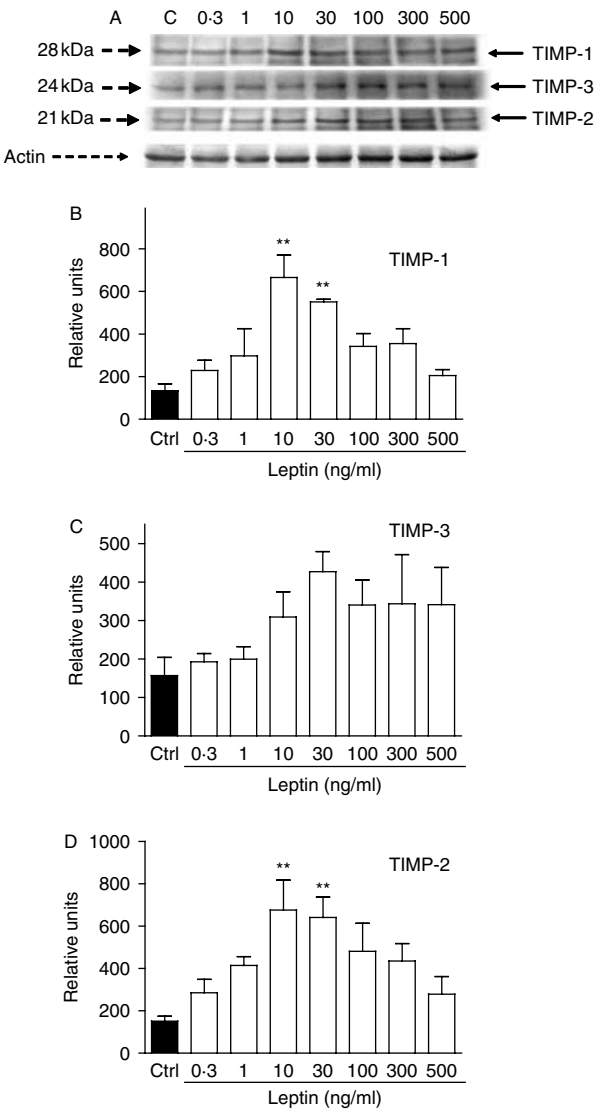

Figure 1 *In vitro* effect of leptin on the protein levels of ovarian TIMPs. Ovarian explants were obtained 4 h after hCG administration from immature rats primed with eCG/hCG and incubated for 4 h in the presence or absence of different concentrations of leptin (0·3–500 ng/ml). (A) Expression of TIMPs and β-actin, as protein control, by western blot analysis. (B–D) Quantitative analysis of immunoreactive bands for the different ovarian TIMPS detected. Results are expressed as the mean ± S.E.M. of four independent experiments, with two replicates per experiment. $^{**}P<0{\cdot}01$ versus control (one-way ANOVA and Dunnett's multiple comparison test).

in 0·05 M Tris–HCl buffer, pH 6·8, containing 10% (v/v) SDS, 18% (v/v) glycerol, and 0·3% (w/v) bromophenol blue. Pellets were kept for a subsequent re-homogenization as indicated under 'reverse zymography'.

Equal amounts of protein (20 μg) were subjected to 7·5% (w/v) SDS-PAGE (0·375 M Tris, 0·1% (w/v) SDS, pH 8·8) containing 0·1% (w/v) gelatin (type A from porcine skin). Following electrophoresis, the gels were washed in 2·5% (v/v) Triton X-100 for 60 min to remove SDS. The gels were washed three times for 10 min each in 50 mM Tris, pH 7·4, to remove the Triton solution. Then, the gels were incubated in 50 mM Tris buffer, pH 7·4, containing 0·15 mM NaCl, and 30 mM $CaCl_2$ at 37 °C for 60 h. The gels were stained with Coomassie blue, and then destained with 10% (v/v) acetic acid and 30% (v/v) methanol in water. Negative staining is indicative of the localization of active proteolytic bands. Enzymatic activities in the gel appear as bright bands against a dark background. These bands were quantified using a S2W 4300U scanner, and densitometry was analyzed using Image Processing and Analysis in Java (ImageJ). Zymography allows detection of both the latent and the active forms of MMPs, because exposure to SDS results in conformational changes associated with their activation. Molecular weight markers (Kaleidoscope St, Bio-Rad Laboratories) and positive controls (conditioned medium from HT-1080 enriched in MMP-9 and MMP-2) were run under the same conditions to identify the different MMPs and to allow the standardization of the results obtained in the different zymograms. Gelatinolytic activity was expressed as arbitrary densitometric units. These bands of enzymatic degradation are specific since they disappear in the presence of 0·01 M EDTA in the incubation buffer.

Reverse zymography

Reverse zymography was used to determine the inhibitory action of the ovarian TIMPs as described previously (Zhu & Woessner 1991). Ovarian tissues were treated as described under zymography, and the pellets obtained were re-homogenized in 0·05 M ice-cold Tris–HCl buffer, pH 7·5, containing 0·1 M $CaCl_2$ and 0·15 M NaCl, and incubated at 60 °C for 6 min and then centrifuged at 15 000 ***g*** at 4 °C for 15 min. Protein concentration in these supernatants (enriched in TIMPs) and culture media was determined by the Bradford method with BSA as the standard. Both homogenates and culture media were diluted in 0·5 M Tris–HCl buffer, pH 6·8, containing 10% (v/v) SDS, 18% (v/v) glycerol, and 0·3% (w/v) bromophenol blue. Equal amounts of protein (30 μg) were subjected to 15% (w/v) SDS-PAGE (0·375 M Tris–HCl, 0·1% (w/v) SDS, pH 8·8) containing 0·1% (w/v) gelatin (type A from porcine skin) and 10% (v/v) conditioned medium from MB49 (enriched in MMP-9) and 3T3 (enriched in MMP-2). Following electrophoresis, the gels were washed and treated as described under zymography. Molecular weight markers (Kaleidoscope St, Bio-Rad Laboratories) were run under the same conditions to identify the different TIMPs. MMPs in this conditioned medium digest the gelatin impregnated in the gel, whereas the presence of TIMPs in the samples inhibits their action. Thus, blue bands corresponding to the molecular weights reported for TIMPs appear on the gels on a clear background. These bands were quantified as described under 'zymography'.

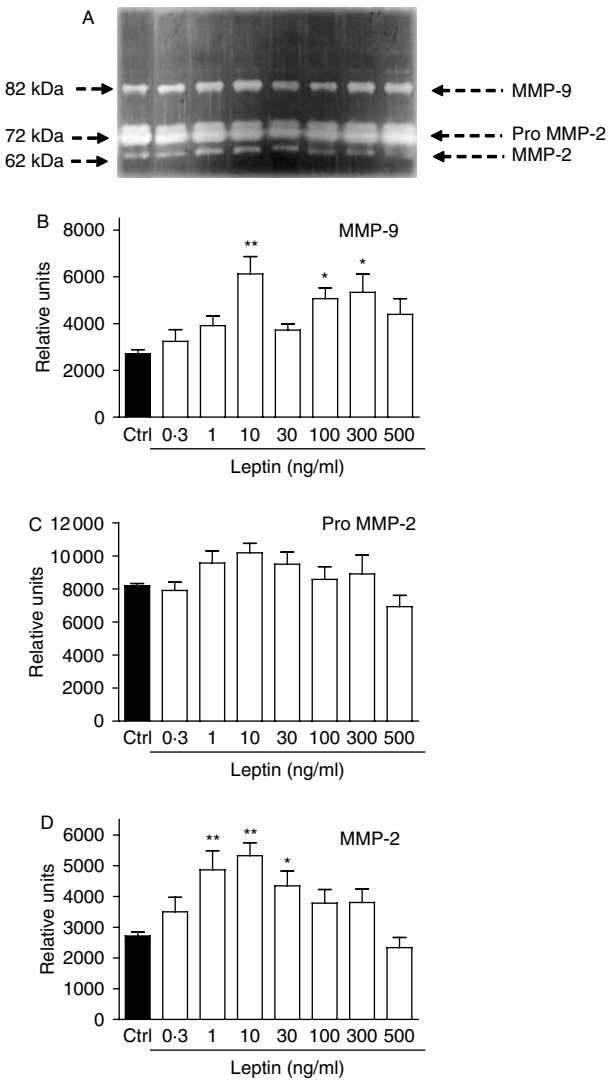

Figure 2 *In vitro* effect of leptin on the ovarian MMPs' activity in the culture medium. Ovarian explants were obtained 4 h after hCG administration from immature rats primed with eCG/hCG and incubated for 4 h in the presence or absence of different concentrations of leptin (0·3–500 ng/ml). (A) Representative zymogram depicting gelatinase activity. (B–D) Densitometric analysis of the activity for the different MMPs detected. The molecular size of each band is indicated. Results are expressed as the mean ± S.E.M. of five independent experiments, with two replicates per experiment. *$P<0·05$, **$P<0·01$ versus control (one-way ANOVA and Dunnett's multiple comparison test).

Statistical analysis

All data are expressed as mean±S.E.M. Comparisons were performed with 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 was analyzed using Student's *t*-test. Differences between groups were considered significant when $P<0{\cdot}05$.

Results

In vitro *studies*

Protein expression of both MMPs and TIMPs was determined in the ovarian tissue after incubation for 4 h in either the presence or absence of different concentrations of leptin (0·3–500 ng/ml) by western blot analysis. When the mouse anti-MMP-9 was used, a single band with relative molecular mass of 82 kDa was detected, whereas when the mouse anti-MMP-2 was used, two bands with relative molecular masses of 72 and 62 kDa were found. The size of these bands was consistent with the predicted size of MMP-9, the latent (proMMP-2) and the active forms of MMP-2, respectively, based on amino acid composition, as described previously (Salamonsen 1996). However, no differences were found in the expression of these MMPs after incubation with leptin compared with controls (data not shown). Using specific antibodies for TIMPs, we detected the expression of TIMP-1, TIMP-3, and TIMP-2 at 28, 24, and 21 kDa respectively (Duncan *et al.* 1998; Fig. 1A). The protein contents of both TIMP-1 and TIMP-2 showed a significant increase at leptin concentrations between 10 and 30 ng/ml ($P<0{\cdot}01$) when compared with those obtained from controls. These increases oscillated between 3·5- and 5·0-fold (Fig. 1B and D). No differences were found in the expression of TIMP-3 protein (Fig. 1C).

Since MMPs are synthesized as preproenzymes that are secreted to the extracellular space, we measured the activity of MMPs in both the culture medium (Fig. 2) and the ovarian tissue (Fig. 3) after incubation for 4 h in the presence or absence of different levels of leptin. As reflected in the gelatin gel zymogram of both extracts, gelatinase activity was detected at relative molecular masses of 82, 72, and 62 kDa (Figs 2A and 3A). These bands were consistent with the predicted size of the same MMPs detected in the western blot assay. In the case of the MMPs present in the culture medium (Fig. 2), the presence of leptin increased the MMP-2 activity at concentrations between 1 and 30 ng/ml, being maximum at 10 ng/ml ($P<0{\cdot}01$; Fig. 2D). The MMP-9 activity was increased at leptin concentrations of 10 and 100–300 ng/ml ($P<0{\cdot}05$; Fig. 2B). The results obtained with the ovarian tissue extract (Fig. 3) were similar to those obtained with the culture medium. However, in the ovarian tissue, the MMPs

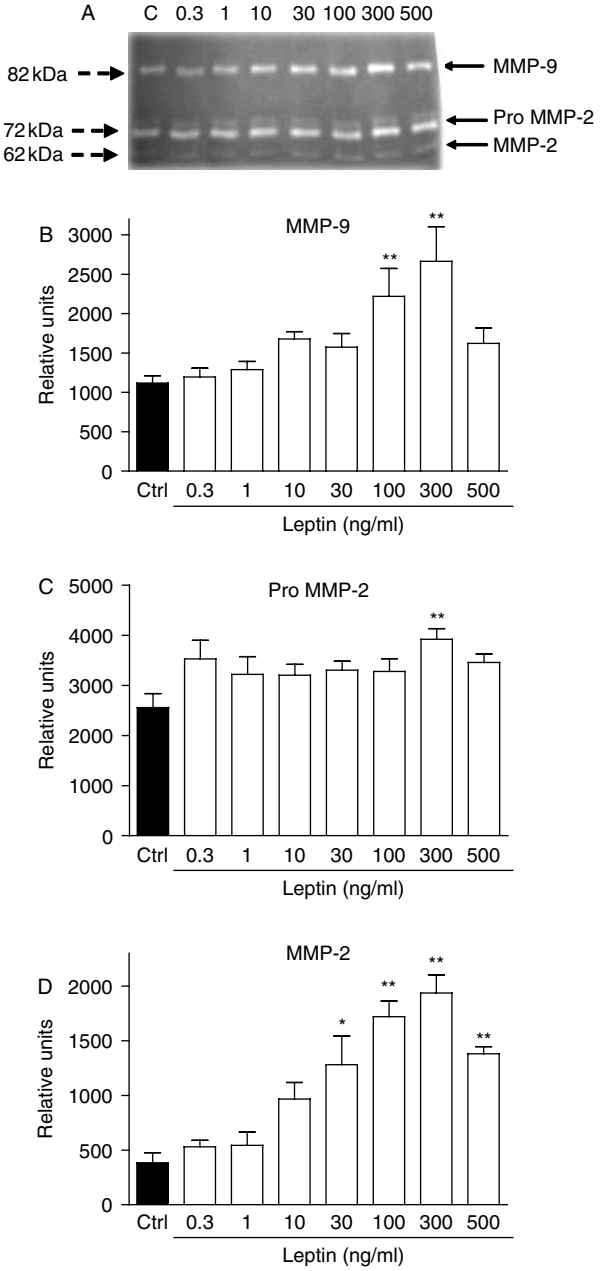

Figure 3 *In vitro* effect of leptin on the MMPs' activity in the ovarian tissue. Ovarian explants were obtained 4 h after hCG administration from immature rats primed with eCG/hCG and incubated for 4 h in the presence or absence of different concentrations of leptin (0·3–500 ng/ml). (A) Representative zymogram depicting gelatinase activity. (B–D) Densitometric analysis of the activity for the different MMPs detected. The molecular size of each band is indicated. Results are expressed as the mean±S.E.M. of five independent experiments, with two replicates per experiment. *$P<0{\cdot}05$, **$P<0{\cdot}01$ versus control (one-way ANOVA and Dunnett's multiple comparison test).

activity was increased at higher concentrations of leptin, which ranged between 30 and 500 ng/ml (Fig. 3B–D).

In order to study the effect of leptin on TIMPs, the action of these inhibitors was measured in both the culture medium and the ovarian tissue by reverse zymography. No expression was found with the culture medium extract, whereas three bands of inhibition of gelatinase activity were found at ~28, 24, and 21 kDa with the ovarian tissue extracts. These bands are consistent with the TIMPs detected by western blot assay. However, there were no substantial differences in the inhibition of the gelatinase activity in any of the TIMPs when leptin was included in the cultures compared with controls (data not shown).

In vivo *studies*

In order to study the *in vivo* effect of high levels of leptin on the ovarian MMP system and to assess whether this system is involved in the inhibitory action on the ovulatory process induced by an acute treatment with leptin, rats were injected with either recombinant rat leptin (5 μg) or PBS–BSA at 1 h before hCG injection and at 150-min intervals, as described in 'Materials and Methods', and the protein content of MMPs and their inhibitors was measured in the ovarian tissue. Figure 4 shows that the acute treatment with leptin was able to increase the ovarian expression of both proMMP-2 ($P<0{\cdot}01$) and MMP-2 ($P<0{\cdot}05$), but not that of MMP-9 (Fig. 4A–D) or TIMPs (data not shown).

When the activity of the different MMPs was assessed in these animals, no differences were found in the ovaries from rats treated with leptin compared with those obtained from the control animals (data not shown). In contrast, the *in vivo* leptin treatment significantly increased (46·9%, $P<0{\cdot}05$) the inhibitory action of TIMP-2, but not of TIMP-1 and TIMP-3 compared with controls (Fig. 5).

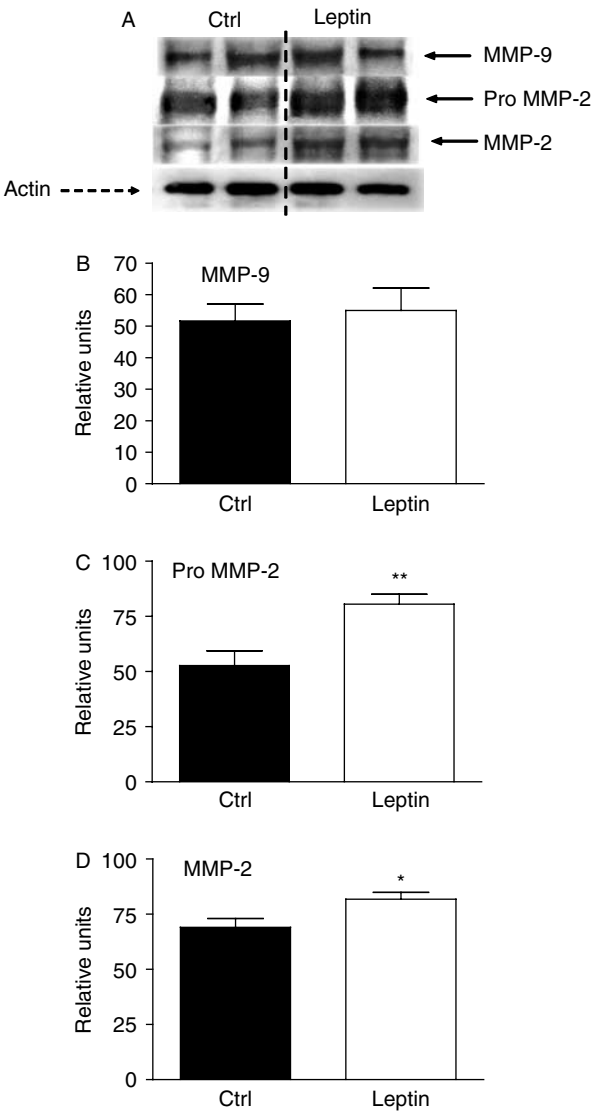

Figure 4 *In vivo* effect of acute treatment of leptin on ovarian MMP proteins. Immature rats were primed with eCG/hCG and treated with either PBS–BSA or 5 μg leptin at 1 h before hCG and at 150-min intervals. Ovarian tissue was obtained 10 h after hCG administration. (A) Expression of TIMPs and β-actin, as protein control, by western blot analysis. (B–D) Quantitative analysis of immunoreactive bands for the different ovarian MMPs detected. Data points represent the mean±S.E.M. for six to eight samples per group. Each sample represents both ovaries from each animal with the same treatment. *$P<0{\cdot}05$, **$P<0{\cdot}01$ versus control (Student's *t*-test).

Discussion

Many studies have demonstrated that leptin has direct effects on the ovarian tissue. Most of these studies were performed *in vitro* with ovarian cells and resulted in inhibitory effects on the stimulatory 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 authors have demonstrated that the ovulatory process in the rat is significantly inhibited by an acute treatment with leptin (Duggal *et al.* 2000, 2002, Ricci *et al.* 2006). In our previous studies, the serum progesterone level, the ovulation rate, and the ovarian prostaglandin E content were reduced in rats primed with eCG/hCG and treated with acute doses of leptin. These inhibitory effects were confirmed by *in vitro* studies, where the presence of leptin reduced the concentrations of progesterone, prostaglandin E, and nitrite in the media of both ovarian explants and preovulatory follicle cultures (Ricci *et al.* 2006). Thus, in this study, we wanted to investigate whether the MMP system is also involved in the inhibitory action of leptin on the ovulatory process. Using cultures of ovarian explants from rats primed with eCG/hCG, we found that the presence of leptin was able to increase the activity of MMPs in both the culture medium and the ovarian tissue, but not the protein content. These results are in agreement with those obtained by other authors with

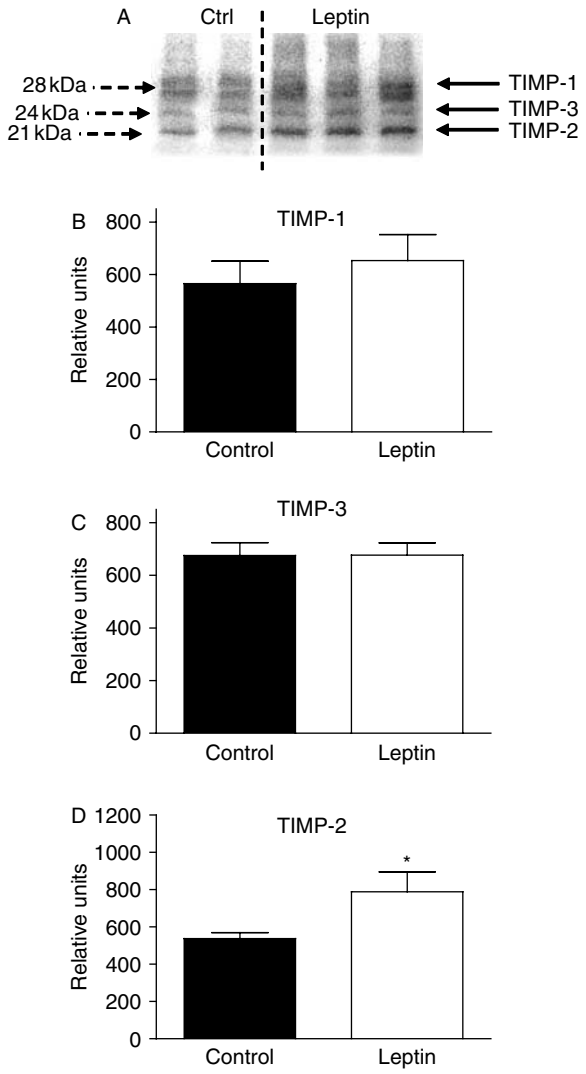

Figure 5 *In vivo* effect of acute treatment of leptin on the inhibitory action of the ovarian TIMPs on the MMP activity. Immature rats were primed with either eCG/hCG and treated with PBS–BSA or 5 μg leptin at 1 h before hCG and at 150-min intervals. Ovarian tissue was obtained 10 h after hCG administration. (A) Representative reverse zymogram of protein extracts from ovarian tissue. The bands are seen as dark against a lighter background, and the molecular size of each band is indicated on the left. (B–D) Densitometric analysis of the bands for the different ovarian TIMPs detected. Data points represent the mean ± S.E.M. for six to eight 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).

different *in vitro* biological models. Castellucci *et al.* (2000) demonstrated that leptin increases the secretion of immunoreactive MMP-2 and enhances the activity of MMP-9 in cultured cytotrophoblastic cells, in a dose-dependent manner. Schulz & Widmaier (2004) found that the ability of leptin to stimulate the trophoblast cell invasion is completely blocked in the presence of an inhibitor of MMP activity. Park *et al.* (2001) reported that leptin induces an increase in MMP-2 and MMP-9 expression in a dose-dependent manner in two different kinds of endothelial cells. The only report on the effect of leptin on ovarian metalloproteinases was that by Barkan *et al.* (2005), who demonstrated that leptin is able to induce the expression of ADAMTS-1 (a disintegrin and metalloproteinase with a thrombospondin-like motif) in isolated preovulatory follicles. We have previously found that the presence of leptin in ovarian culture is able to induce a dose-dependent biphasic effect on the progesterone production, reaching the highest and lowest levels at 10 and 300 ng/ml of leptin respectively (Di Yorio *et al.* 2008). Thus, these results together with the present data could suggest that leptin produces two different kinds of response in the ovarian function when the gonads are directly exposed to leptin: i) a clear stimulating action that occurs at concentrations around 10 ng/ml and ii) an unclear action at 300 ng/ml, since it induces an increase in MMP activity but inhibits progesterone and other mediators, as described previously (Ricci *et al.* 2006). However, both effects are simultaneous with an increase in the expression of the ovarian leptin receptors (Di Yorio *et al.* 2008). It is possible that these different responses are caused by different isoforms of the leptin receptor. Further studies are in progress to examine which isoform is activated by different levels of leptin.

The ability of leptin to increase the ovarian MMPs activity could induce the extracellular matrix breakdown that leads to the follicle wall rupture. This event would be a logical consequence if the *in vivo* leptin treatment enhanced the ovulatory process as observed in other studies (Barkan *et al.* 2005). However, the acute treatment with leptin used in our studies inhibited follicle rupture and impaired some proinflammatory factors as described previously (Ricci *et al.* 2006). Thus, we determined both the protein content and the inhibitory effect of the TIMPs in the same samples, where we evaluated the ovarian MMPs. Although we found that leptin was able to increase the protein content of both TIMP-1 and TIMP-2 at 10–30 ng/ml, these increases did not result in a higher inhibitory action of the gelatinase activity, but in a higher gelatinase activity in the ovaries treated with leptin. The LH surge, or the administration of exogenous hCG to mimic LH, induces the expression and activity of MMPs and TIMPs (Mann *et al.* 1991, Curry *et al.* 1992, Nothnick *et al.* 1995, 1996, Simpson *et al.* 2001). Therefore, MMPs degrade the follicle wall to facilitate the oocyte release, and TIMPs regulate the extent of the MMPs action. Although the inhibitory action of TIMPs was not increased in our *in vitro* studies, it is possible that the levels of TIMP proteins were increased to modulate the MMPs activity induced by leptin. However, either this increase was not sufficient or the inhibitory actions of these proteins were impaired to suppress the MMP activity when the ovaries are directly exposed to leptin.

Since these results do not seem to be involved in the *in vivo* inhibitory effect of leptin on the ovulatory process, the ovarian

MMP/TIMP system was assessed in rats treated with leptin, as described in 'Materials and Methods'. This treatment increased the expression of both the latent and the active forms of MMP-2 but did not result in a greater activity of MMP-2. No differences were found in the protein expression of TIMPs, but the inhibitory action of TIMP-2 was increased in the ovaries from rats exposed to leptin compared with control animals. It is well known that TIMP-2 has a high affinity for MMP-2 (Stetler-Stevenson *et al*. 1989, Leco *et al*. 1992, Curry & Osteen 2003). TIMP-2 binds tightly to the zymogen of MMP-2 forming a complex that is important in the cell surface activation of proMMP-2, while TIMP-1 forms a specific complex with proMMP-9 (Brew *et al*. 2000). Thus, the results obtained in our *in vivo* studies may suggest that the action of TIMP-2 is induced by the acute treatment with leptin to regulate the activity of MMP-2.

Our studies are the first to describe the *in vivo* action of leptin on the ovarian MMP/TIMP system and to compare it with its *in vitro* action. We cannot explain the apparent discrepancies obtained between the *in vivo* and the *in vitro* studies, but the fact that the *in vivo* treatment with leptin could not be reproduced by a direct exposure of the ovarian tissue to leptin suggests that the action of the *in vivo* treatment on the ovarian MMP/TIMP system is through an extraovarian mechanism or signaling pathway. However, it is important to highlight that leptin is able to regulate the ovarian MMP/TIMP system through either a direct (at gonadal level) or an indirect (at extragonadal level) effect.

Although further studies are necessary to clarify this point, it is possible that the resulting action depends on the circulating leptin levels and/or on the different activated isoforms of the leptin receptor, which, in turn, can trigger different cell signaling pathways.

In conclusion, in our *in vitro* studies, the presence of leptin was able to increase the activity of ovarian MMPs without altering their protein contents and the expression of TIMPs without changes in their inhibitory action. These results suggest either that the increase in the TIMP proteins was not sufficient or that their inhibitory actions were impaired to suppress the MMP activity when the ovaries are directly exposed to leptin. However, in our *in vivo* studies, treatment with high doses of leptin, which inhibits the ovulatory process, increased the protein expression of both the latent and the active forms of MMP-2, without altering its activity, and the inhibitory action of the ovarian TIMP-2. These results suggest that the administration of high doses of leptin could be regulating the follicle wall degradation, at least in part, by increasing the action of the ovarian TIMP-2 as a result of an extraovarian mechanism or signaling pathway.

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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