

Ob receptor in rabbit ovary and leptin *in vitro* regulation of corpora lutea

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Abstract

We studied leptin involvement in rabbit corpora lutea (CL) activity, and its post-transcriptional signalling pathway. The expression of leptin receptor (Ob-R) in rabbit ovary at day 9 of pseudopregnancy was evaluated by immunohistochemistry and Western blot analysis. The specificity of the Ob-R receptor antibodies was characterised by immunoprecipitation and competition with blocking peptide. Day 9 CL were incubated *in vitro* with leptin alone or with inhibitors of PLC (phospholipase C), PLD (phospholipase D), AC (adenylate cyclase), JAK (janus kinase), MAPK (mitogen-activated protein kinase) and both cAMP- and cGMP-specific PDE (phosphodiesterase). Prostaglandin F₂ α (PGF₂ α), PGE₂ and progesterone levels were measured in the culture medium, while NOS (nitric oxide synthase) and cAMP- and

cGMP-specific PDE activities were measured in CL tissue. Positive staining for Ob-R was found within the cytoplasm of large luteal cells of CL as well as in granulosa cells of follicles and oocytes. Immunoblots detected a band of about 99 kDa size in Ob-R immunoprecipitates from CL homogenates. This band was not detectable after pre-incubation of the primary antibody with the immunising leptin peptide. Leptin increased PGF₂ α and cAMP-specific PDE, decreased basal progesterone and did not affect PGE₂ and NOS levels. Leptin used the JAK pathway in increasing PGF₂ α , and MAPK and cAMP-specific PDE in decreasing progesterone. This study supports a permissive luteolytic role for leptin in rabbit CL.

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Introduction

Corpus luteum (CL) is a transient endocrine gland whose primary secretory product, progesterone, is required for the establishment and maintenance of pregnancy. The principal luteotrophic and luteolytic signals in mammals are luteinizing hormone (LH), prostaglandin F₂ α (PGF₂ α) and (PGE₂), however, it is becoming increasingly evident that new factors modulate the lifespan of the CL (Webb *et al.* 2002).

Leptin is a 16.4 kDa cytokine encoded by the *obese* gene and primarily secreted by adipocytes (Zhang *et al.* 1994). Leptin, upon binding to specific receptors in different areas of the hypothalamus (Morash *et al.* 1999), is involved in the control of satiety and energy metabolism through the regulation of several neurotransmitters (Harris 2000).

An increasing body of evidence suggests, however, that leptin is also implicated in several key points of the mammalian reproductive functions (Mantzoros 2000) such as steroidogenesis (Spicer & Francisco 1997, Agarwal *et al.* 1999, Brannian *et al.* 1999), ovulation (Cunningham *et al.*

1999, Ryan *et al.* 2002), pregnancy (Mounzih *et al.* 1998, Mukherjea *et al.* 1999) and menstrual cycles (Quinton *et al.* 1999, Ludwig *et al.* 2000). In particular, leptin may act as the critical link between adipose tissue and the reproductive system, indicating whether adequate energy reserves are present for normal reproductive function (Moschos *et al.* 2002).

Recently, leptin receptor (Ob-R) has been detected in various tissues of the reproductive axis such as pituitary gonadotrope cells (Jin *et al.* 2000); granulosa, theca and interstitial ovary cells (Karlsson *et al.* 1997); endometrium (Kitawaki *et al.* 2000) and Leydig cells (Caprio *et al.* 1999). The leptin receptor shows six isoforms (Ob-Ra-f) arising from mRNA splice variants (Houseknecht *et al.* 1998, Sweeney 2002). The extracellular and transmembrane domains are identical in Ob-Ra-d and Ob-Rf (Sweeney 2002, Zabeau *et al.* 2003). Ob-Rb, the receptor single long form, includes a long intracellular domain (302 amino acids) that activates the janus kinase (JAK)/signal transducer and activator of transcription (STAT) (Baumann *et al.* 1996, Brann *et al.* 2002). In particular, ligand binding

to the Ob-Rb, which lacks intrinsic tyrosine kinase activity, results in homodimerisation of the receptors, activation of JAK2 preassociated with the receptor and the subsequent tyrosine phosphorylation of Ob-Rb cytoplasmic tail, and, therefore tyrosine-phosphorylating STAT1, STAT3, STAT5 and STAT6 through interaction with the SH2 domain in STATs (Baumann *et al.* 1996). Ob-Ra, Ob-Rc-d and Ob-Rf have a short intracellular domain (32–42 amino acids) that can activate the MAPK pathway (Bjørnbæk *et al.* 1997, Houseknecht *et al.* 1998). Ob-Re is a soluble receptor isoform consisting of the extracellular domain only, lacking both cytoplasmic and transmembrane domains (Lahlou *et al.* 2000).

To date, nothing is known about the role of leptin in rabbit CL. The present work was undertaken to localise the cell distribution of Ob-R in different ovarian structures, and to assess *in vitro* the specific downstream post-transcriptional pathway used by leptin for the regulation of progesterone and PG releases, used as markers of luteal function.

Materials and Methods

Animal and hormonal regimen

Sexually mature New Zealand White female rabbits, weighing 3.5–4 kg, were housed individually in an indoor facility under controlled conditions of light (14 h light/10 h darkness) and temperature (18 °C). Each animal had free access to food and water. Pseudopregnancy was induced with 20 IU pregnant mare serum gonadotrophin (PMSG) (Folligon, Intervet, Milan, Italy) given *i.m.*, followed 3 days later by an *i.m.* injection of 0.8 µg gonadotrophin-releasing hormone (GnRH) analogue (Receptal, Hoechst-Roussel Vet, Milan, Italy). Previous experiments in our laboratory showed that this hormonal protocol was effective in inducing ovulation in does (Stradaoli *et al.* 1997). The day of GnRH injection was designated day 0. All rabbits were euthanised by cervical dislocation on day 9 of pseudopregnancy. Reproductive tracts were promptly removed after sacrifice, and both ovaries and harvested CL were immediately processed for immunohistochemistry, protein expression of Ob-R, and *in vitro* studies. The protocols involving the care and use of animals for these experiments were approved by the Bioethics Committee of the University of Perugia.

Immunohistochemistry

For the immunohistochemical detection of Ob-R, the ovaries of three animals were fixed by immersion in 4% paraformaldehyde and 0.1 M phosphate-buffered saline (PBS, pH 7.4) for 24 h and then processed for embedding in paraffin following routine tissue preparation procedures.

Serial 7 µm thick sections, mounted on poly-L-lysine coated glass slides, were brought into water through

graded ethanol and microwaved for 15 min in 10 mM citric acid (pH 6.0) for antigen retrieval. All subsequent steps were carried out in moist chambers at room temperature. To prevent non-specific binding of primary antibody, the sections were pre-incubated for 30 min with normal goat serum (1:10, Santa Cruz Biotechnology, Santa Cruz, CA, USA).

The sections were left overnight with primary monoclonal antibody, mouse anti-Ob-R (1:50, sc-8391, Santa Cruz Biotechnology). This antibody is recommended for detection of short and long forms of Ob-R, as reported in the manufacturers datasheet. The next day, sections were washed in PBS and incubated with biotinylated secondary antibody to anti-mouse IgG, (1:200, Vector Laboratories, Burlingame, CA, USA) for 30 min. After PBS washes, sections were exposed to avidin-biotin complex (ABC, Vector Elite Kit, Vector Laboratories) for 30 min, followed by the chromogen 3,3'-diaminobenzidine tetrachloride (DAB, Vector Laboratories) for 5 min. After washing in tap water, the luteal sections were counter-stained with haematoxylin, dehydrated and mounted in Canada Balsam Natural (BDH, Poole, Dorset, England). Positive reactions were recognised as reddish brown precipitates. Sections in which the primary antibodies were omitted or substituted by pre-immune mouse gamma globulin were used for the negative control of unspecific staining. As a further control of the specificity of the staining, sections were incubated with the primary antibody that had been pre-incubated with the corresponding antigen (50 µg of the blocking peptide (sc-8391 P, Santa Cruz Biotechnology) in 1 ml of the working solution).

Western blot analysis

For Western blot analysis of the Ob-R proteins, CL were collected from the ovaries of two rabbits. Non-luteal tissue was carefully dissected away with fine forceps under stereoscopic magnification. For each rabbit, total luteal proteins were extracted from a pool of 10 CL that was homogenised in 1 ml of ice-cold RIPA buffer (PBS containing 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS) with added EDTA-free protease inhibitor cocktail (Roche, Mannheim, Germany) using an Omni-µU mixer (Analytical Control, Dasit, Milan, Italy). After incubation at 4 °C for 20 min, the homogenates were centrifuged at 25 000 × g for 60 min at 4 °C. The supernatants were collected and their protein concentrations measured using the Protein Assay Kit (Bio-Rad Laboratories Inc., Hercules, CA, USA) with BSA as standard. Total luteal protein extracts were appropriately diluted with PBS at a final concentration of 500 µg/ml.

Two µg of goat anti-Ob-R antibody (sc-1834, antibody for detection of short and long forms of Ob-R, Santa Cruz Biotechnology) was then added to an aliquot of 500 µg luteal proteins and gently stirred overnight at 4 °C. After incubation, 50 µl of agarose-protein G slurry

(sc-2002, Santa Cruz Biotechnology) was added to each sample and stirred in an ice-cold bath for 40 min. Following centrifugation (2500 *g* for 2 min), the pellets containing the immunoprecipitated proteins were washed two times with cold PBS and re-suspended in 50 μ l PBS. The supernatants, containing soluble proteins, were also collected. Thereafter, 50 μ l Laemmli sample buffer was added to 50 μ l suspended pellet and 50 μ l supernatant-derived solution and boiled for 4 min.

For each luteal protein extract processed, two equivalent volumes (20 μ l) from pellet- and supernatant-derived solutions were electrophoretically separated in duplicate by discontinuous 10% SDS-PAGE with 4% stacking gel for 40 min at 200 V/500 mA, after which proteins were transferred onto a nitrocellulose membrane (Protran BA 85, Schleicher & Schuell, Dassel, Germany) for 1 h at 100 V/350 mA. After transfer, membranes were blocked with PBS containing 0.5% casein (PBSC). The first membrane was incubated overnight at 4 °C with anti-Ob-R monoclonal antibodies (1:100, sc-8391, Santa Cruz Biotechnology). The second membrane was similarly incubated with the same mAb anti-Ob-R that was pre-adsorbed for 3 h at 4 °C with its blocking peptide (10 μ g of antigen in 1 ml of the working solution).

Membranes were then probed with a chicken anti-mouse IgG-peroxidase antibody (Santa Cruz Biotechnology) at 1:2000 dilution for 60 min at room temperature under gentle agitation. All antibody incubations were performed in PBSC and washings in PBS with 0.05% Tween-20. Immunoreactive bands were developed using 4CN substrate for peroxidase (Bio-Rad Laboratories) at room temperature for 20 min. Pre-stained SDS-PAGE MW Standards (Bio-Rad Laboratories, Dual Color Standard) were used as molecular weight references. Blot images were acquired with HP scanner.

In vitro incubations

CL were randomly distributed (one CL/well) into incubation wells (Becton Dickinson Co., Clifton, NJ, USA) in 1 ml of culture medium 199 with Earles Balanced Salt Solution (GIBCO, Grand Island, NY, USA) containing 2.2 mg/ml sodium bicarbonate, 2.3 mg HEPES (Sigma, St Louis, MO, USA), and 3% BSA (Sigma), referred to here as M199. Before treatment, the CL were quartered inside each well using fine forceps. Each incubation set of wells was divided into 12 experimental groups of 5 wells as follows: (I) medium alone as control; (II) leptin (100 nM, Calbiochem Corporation, San Diego, CA, USA); (III) leptin plus PGF2 α (3 μ M, Sigma); (IV) leptin plus PLC inhibitor (compound 48/80, 2 μ M, Sigma); (V) leptin plus PLD inhibitor (propranolol, 10 μ M, Sigma); (VI) leptin plus AC inhibitor (2-O-methyladenosine, 2 μ M, Sigma); (VII) leptin plus JAK inhibitor (AG490, 2 μ M, Calbiochem Corporation); (VIII) leptin plus MAPK/extracellular signal-regulated kinase kinase 1 and

2 (MEK1/2) inhibitor (English *et al.* 2002) (PD98059, 10 μ M, Tocris); (IX) leptin plus cAMP specific PDE inhibitor (zardaverine, 2 μ M, Tocris); (X) leptin plus cGMP specific PDE inhibitor (zaprinast, 2 μ M, Tocris); (XI) PGF2 α plus cAMP-specific PDE inhibitor; (XII) PGF2 α plus cGMP-specific PDE inhibitor. The culture plates were incubated at 37 °C in air with 5% CO₂ as reported elsewhere (Gobbetti *et al.* 1999, Boiti *et al.* 2000). The media of each well were collected after 4 h of incubation and stored immediately at -20 °C for later determination of hormones. CL were weighed and stored immediately at -20 °C for later determination of NOS and PDE activities. Preliminary evidence led to our choosing the incubation conditions and the minimum effective doses of the substances used in the present *in vitro* study (data not shown).

Phosphodiesterase assay

Cyclic AMP- and cGMP-specific PDE activities were assessed by using the two-step assay as previously reported (Hermsdorf & Dettmer 1998). Half the CL of each well were homogenised in 1 ml of cold fresh homogenating buffer (50 mM Tris, 1 mM EDTA and 1 mM EGTA, pH 7.4), and centrifuged at 20 000 \times *g* for 60 min at 4 °C. Incubation was performed in a total volume of 300 μ l 100 mM Tris-HCl (pH 7.4), 5 mM MgCl₂, 3.75 mM mercaptoethanol with 100 000 d.p.m. [³H]cAMP or [³H]cGMP (Sigma) for 20 min at 30 °C. The reaction was stopped by boiling the mixtures for 3 min. After cooling in an ice bath, samples were incubated for 15 min at 30 °C in 0.3 alkaline phosphate followed by inactivation by 3 min in boiling water. To separate [³H]adenosine from [³H]cAMP or [³H]guanosine from [³H]cGMP, 1 ml of a slurry (DOWEX 1 \times 2200-400 mesh, Cl form, resin:water 1:4 v/v, Sigma) was added and mixed for 1 min. After centrifugation for 3 min at 12 000 \times *g*, 250 μ l of supernatant was transferred into vial for liquid scintillation counting. Recovery of the ³H-labelled products was estimated by using [³H]adenosine or [³H]guanosine (data not shown). Protein concentration was determined by Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA, USA).

NO synthase activity determination

NOS activity was determined, in the CL used for *in vitro* incubations, by monitoring the conversion of [³H]L-arginine into [³H]L-citrulline with the NOS detect Assay Kit (Alexis Corporation, Läufelfingen, Switzerland) (Boiti *et al.* 2000). Half the CL of each well was homogenised in 1 ml of cold fresh homogenating buffer (50 mM Tris, 1 mM EDTA and 1 mM EGTA, pH 7.4), and centrifuged at 20 000 \times *g* for 60 min at 4 °C. Twenty-five microlitres of supernatant and 100 μ l of incubation buffer (1.5 mM NADPH, 1 mM CaCl₂) containing 150 000 d.p.m. [³H]L-arginine (Sigma) were added to the incubation tube.

After 30 min incubation at room temperature, the enzymatic reaction was stopped by the addition of 2 ml of blocking buffer (20 mM HEPES, 2 mM EDTA, pH 5.5). The mixture was applied to a pre-equilibrated column (20 mM sodium acetate, 2 mM EDTA, 0.2 mM EGTA, pH 5.5; 1 cm diameter) containing 1 ml of Dowex AG50W-X8 (Sigma) and the material was eluted with 2 ml of water. [³H]L-Citrulline was quantified in a liquid scintillation system LS 1801 (Beckman Instruments, Fullerton, CA, USA). Additional determinations were performed in the presence of excess of NOS inhibitor (L-NAME, Sigma) to verify the specificity of the assay for production of [³H]L-citrulline by NOS (data not shown). Protein concentration was determined by Bio-Rad Protein Assay Kit.

Hormone radioimmunoassay

PGF2 α , PGE2 and progesterone were determined in the medium samples following the RIA previously reported (Boiti *et al.* 2000). Intra- and inter-assay coefficients of variation and minimum detectable dose were PGF2 α : 8%, 12%, 19 pg; PGE2: 7%, 13%, 18 pg; progesterone: 5%, 9%, 10 pg. PGF2 α , PGE2, progesterone antisera and non-radioactive hormones were purchased from Sigma (St Louis, MO, USA). Tritiated hormones were purchased from Amersham International (Amersham, Buckinghamshire, UK).

Statistics

Data relative to hormone releases and enzymatic activities were analysed by an analysis of variance (ANOVA) (Sokal & Rohlf 1981) followed by Duncan's multiple range test (Duncan 1955).

Results

Immunolocalization of Ob-R

Using a monoclonal antibody, positive staining for Ob-R was detected in the ovary of pseudopregnant rabbits. Within the ovary, strong Ob-R-like immunoreactivity was found in granulosa cells of primary, secondary and tertiary follicles and in oocytes (Fig. 1, panel A). Within the CL, a strong positive reaction for Ob-R was localised primarily in the large luteal cells as indicated by the staining in their cytoplasm (Fig. 1, panel B) and also in the thecal layer cells, although less evident. Staining was completely abolished when the primary antibody was substituted with non-immune serum or pre-adsorbed with the blocking peptide (data not shown).

Western blot analysis

Western blot analysis of Ob-R protein expression revealed a strong band of approximately 99 kDa size only in the

immunoprecipitated-derived fraction from whole luteal protein extracts of the two rabbits examined (Fig. 2, left). As expected, in the supernatant-derived samples (Ob-R-stripped fraction), the corresponding 99 kDa band was not visible. Pre-incubation of mAb anti Ob-R of mouse origin with the immunising peptide completely neutralised the primary antibody as a band of comparable size was not detectable (Fig. 2, right).

In vitro cultured corpora lutea

Hormones Leptin increased PGF2 α ($P \leq 0.01$) (Fig. 3) and decreased progesterone ($P \leq 0.01$) (Fig. 4) release in *in vitro* cultured CL. PGF2 α decreased progesterone release ($P \leq 0.01$) (Fig. 4). The progesterone decreasing induced by leptin was lower ($P \leq 0.01$) than those induced by PGF2 α (Fig. 4). JAK inhibitor counteracted ($P \leq 0.01$) the leptin effects on PGF2 α release (Fig. 3), while MEK1/2 and cAMP-specific PDE inhibitors counteracted ($P \leq 0.01$) those on progesterone (Fig. 4). PLC, PLD, AC and cGMP-specific PDE inhibitors did not affect the leptin effects on hormone release (Figs. 3, 4). In all experimental groups PGE2 release did not change (data not shown).

Enzymes Leptin increased cAMP-specific PDE activity ($P \leq 0.01$), while PGF2 α did not affect the activities of either PDE (Fig. 5). Leptin did not affect NOS activity, whereas PGF2 α increased enzyme activity ($P \leq 0.01$) (data not shown).

Discussion

The present study, for the first time, indicates the presence of Ob-R in the ovaries of pseudopregnant rabbits, within the cytoplasm of large luteal cells of the CL, in granulosa cells of follicles at different stages of development and in oocytes. Our findings, which are similar to those obtained recently in other species, support a direct role of leptin in the regulation of several ovarian functions. In fact, Ob-R has been identified in the adult human ovaries (Cioffi *et al.* 1996, 1997) and rat ovaries (Zamorano *et al.* 1997), human granulosa and theca cells (Karlsson *et al.* 1997, Agarwal *et al.* 1999), mouse oocytes (Matsuoka *et al.* 1999) and porcine CL (Lin *et al.* 2000, Ruiz-Cortes *et al.* 2000). Our HIC data confirm that Ob-R are mainly internalised after binding to its agonist as reported by Barr *et al.* (1999).

Western blotting of the immunoprecipitated protein fractions derived from luteal extracts yielded a strong band at 99 kDa when probed with a mAb raised against a peptide mapping at the carboxy terminus of Ob-R of mouse origin. The immunoprecipitation of CL extracts with an affinity-purified goat polyclonal antiserum raised against peptides corresponding to amino acids 32–51 and 877–894, mapping respectively at the amino- and carboxy-termini of mouse Ob-R, greatly improved the efficiency of the Western blotting. The addition of the blocking peptide

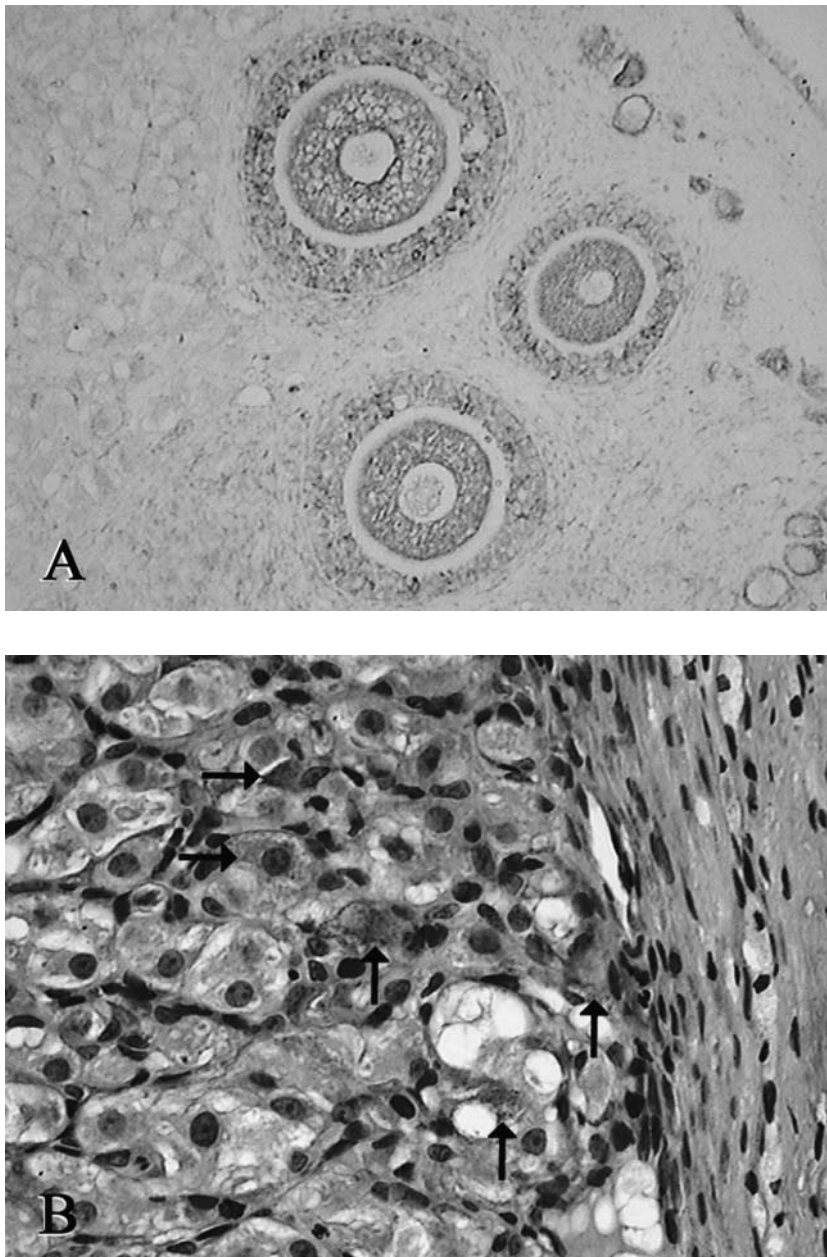


Figure 1 Immunohistochemical localisation of leptin receptor (Ob-R) in the ovary (panel A) and in the corpus luteum (panel B) of pseudopregnant rabbits. In the ovary ($\times 250$) Ob-R is observed in the granulosa cells and in the oocyte cytoplasm of follicles at different stages of development. In the CL ($\times 1000$) the positive reaction (arrows) is evident in the cytoplasm of both large luteal and thecal layer cells.

to the primary antibody completely masked the target 99 kDa band, thus indirectly confirming the specificity of the antibodies here employed to evaluate Ob-R of rabbit origin. As the sequence of rabbit Ob-R gene is still unknown, its similarity with mouse Ob-R transcript, used to raise both the polyclonal and monoclonal antibodies here

employed, remains to be disclosed. However, it should be noted that for the region of the Ob-R that the primary monoclonal IgG was directed against, there is a 100% identity, exact amino acid sequence, for mouse, rat, human, pig and rhesus monkey (personal communication from Santa Cruz Biotechnology). To the best of our

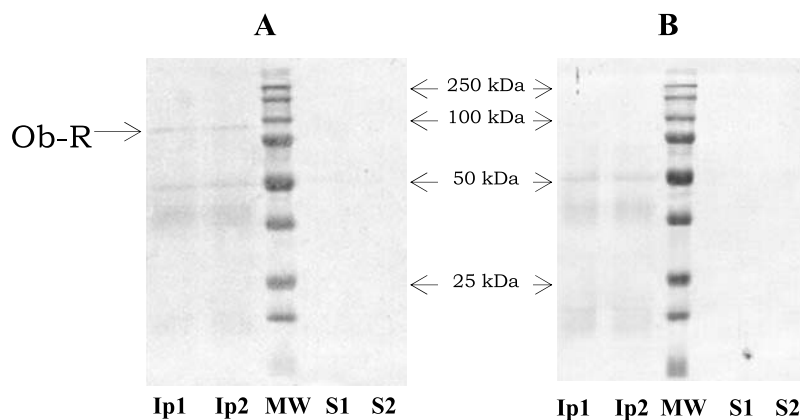


Figure 2 Representative Western blots of Ob-R protein in the Ob-R immunoprecipitated fractions from whole homogenates of CL collected at day 9 of pseudopregnancy from two rabbits (Ip1 and Ip2) and in the corresponding Ob-R-stripped fractions (S1 and S2). MW identifies the molecular weight standards. Blots of membrane A were incubated with primary mAb anti Ob-R of mouse origin, whereas those of membrane B were tested with the same mAb pre-incubated with its immunising peptide. The left immunoblot (membrane A, Ip1 and Ip2) shows Ob-R specific bands at an apparent MW of 99 kDa. Experiments were performed at least three times.

PGF2 α release by CL cultured in vitro

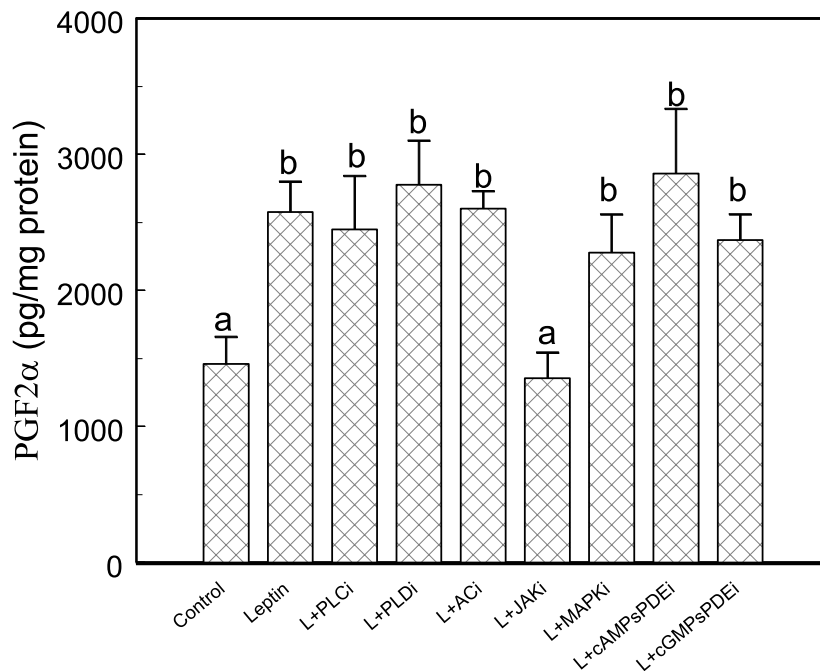


Figure 3 *In vitro* effects of leptin (L) and L co-incubated with inhibitors of PLC, PLD, AC, JAK, MEK1/2, cAMP-specific PDE, and cGMP-specific PDE on PGF2 α release by pseudopregnant rabbit CL. Results are the means \pm S.D. of five replicate values. Different letters above the bars indicate significantly different values ($P \leq 0.01$, Duncan's multiple range test).

Progesterone release by CL cultured in vitro

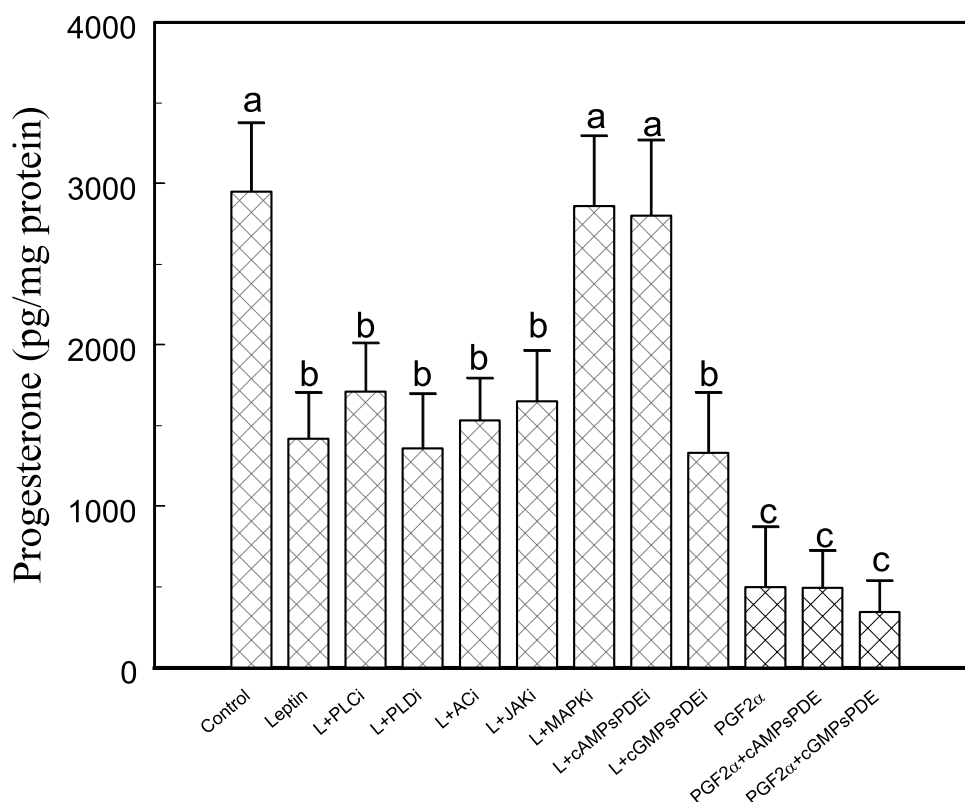


Figure 4 *In vitro* effects of leptin (L), PGF2 α and inhibitors of PLC, PLD, AC, JAK, MEK1/2, cAMP-specific PDE and cGMP-specific PDE on progesterone release by pseudopregnant rabbit CL. Results are the means \pm s.d. of five replicate values. Different letters above the bars indicate significantly different values ($P \leq 0.01$, Duncan's multiple range test).

knowledge this is the first report on Ob-R expression in rabbits and, therefore, comparative analysis with results obtained in other species is of limited relevance. Since both Ob-R antibodies used for Western blot recognise all transmembrane Ob-R splice variants, it remains to be established which type of leptin receptors, either long or short, is indeed expressed in the ovary of rabbits.

Recent studies carried out on theca, granulosa and luteinised granulosa cells show that leptin *in vitro* exerts a direct inhibition on rodent, bovine and primate steroidogenesis intracellular signalling pathway (Karlsson *et al.* 1997, Spicer & Francisco 1997, 1998, Zachow & Magoffin 1997, Agarwal *et al.* 1999, Brannian *et al.* 1999). Similarly, our study suggests that leptin *in vitro* affects steroidogenesis in rabbit too, and in fact this hormone decreases progesterone basal release by CL. In untreated human luteinised granulosa cells, leptin has no effect on progesterone basal synthesis, but it has been shown to decrease progesterone production only when stimulated with human chorionic gonadotrophin (Brannian *et al.* 1999).

Nothing is known about leptin effects on PG release, except that this hormone stimulates PGE2 and PGF2 α in rat hypothalamus (Brunetti *et al.* 1999). The present work shows a direct effect of leptin on PG release by rabbit CL, given that PGF2 α release is increased by leptin *in vitro*. Differently from Brunetti *et al.* (1999) however, leptin had no effect on PGE2.

From our data, it emerges that leptin uses different intracellular signalling pathways in affecting progesterone and PGF2 α . In fact, leptin exhibits an inhibitory effect on progesterone through MAPK cascade and a stimulatory one on PGF2 α , through JAK in rabbit CL. These results suggest that leptin could use different receptors (long Ob-R and short Ob-R?) and/or affect different (small and large?) CL cells in the modulation of progesterone and PGF2 α release by rabbit CL. However, because the co-expression of both long and short types of leptin receptor is not supported by immunoblot analysis of luteal homogenates, these intriguing results need further study, given that the long isoform has well-known signalling

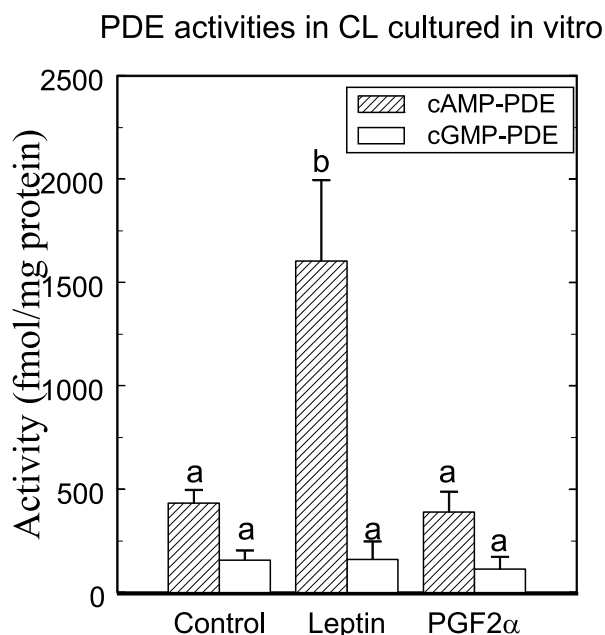


Figure 5 *In vitro* effects of leptin and PGF2 α on cAMP-specific and cGMP-specific PDE activities in pseudopregnant rabbit CL. Results are the means \pm S.D. of five replicate values. Different letters above the bars indicate significantly different values ($P \leq 0.01$, Duncan's multiple range test).

capability, whereas the short form is largely expressed in different tissues (Tartaglia 1997).

Recently, Cauzac *et al.* (2003) reported that leptin stimulated the transcription of the *c-fos* gene and cell proliferation in human placental cells through the rapid phosphorylation of p42–44 MAPK, while the JAK/STAT pathway is not required for leptin to transduce proliferative signals in these cells.

There are two main mechanisms regulating the signal transduction in the MAPK cascades: the docking interaction and the scaffolding (Tanoue & Nishida 2003). The docking interaction is achieved through specific conserved regions on MAPKs and MAPK-interacting molecules. Scaffolding generally requires a third molecule to join enzyme and substrate (Tanoue & Nishida 2003). PDE is a substrate of MAPKs and shows a specific conserved motif for the docking interaction (Mackenzie *et al.* 2000, Fantz *et al.* 2001, Tanoue & Nishida 2003).

The effects of leptin on progesterone release by rabbit CL suggest that the MAPK could regulate its signal through interaction with a cAMP-specific PDE, in fact, the inhibitor of this PDE counteracted the progesterone decrease induced by leptin. In a recent study it was proposed that PDE4 (cAMP-specific PDE) isoenzymes integrate cAMP and p42 MAPK intracellular signal transducing and that these isoenzymes may be activated, inhibited or insensitive to p42 MAPK (Baillie *et al.* 2000). In particular, Baillie *et al.* (2001) suggest that PDE4D

isoenzyme is activated by protein kinase A through a process ablated by the MEK1/2 inhibitor PD98059 in human aortic smooth muscle cells.

Due to the existing cross-talk and interference between different MAPK modules in transducing intracellular signals, the antisteroidogenic action evoked *in vitro* by leptin might also have been elicited by the stress-activated JNK/p38/MAPK pathways leading to apoptosis of luteal cells (Cano & Mahadevan 1995).

Nitric oxide is deeply involved in the regulation of rabbit CL function, as PGE2 exerts its luteotrophic effect through NOS down-regulation while the PGF2 α luteolytic effect is mediated by NOS up-regulation (Boiti *et al.* 2000). Present results show that PGF2 α increased the enzymatic activity of NOS in agreement with what we found in previous studies (Boiti *et al.* 2000). Leptin, however, does not exert any influence on luteal NOS activity, at least under present experimental conditions. Leptin increased PGF2 α CL release, but probably this prostaglandin increase did not reach the threshold to affect NOS activity.

Finally, although considerable progress has been achieved in understanding the reproductive actions of leptin over the past few years, much work is needed before we can arrive at a complete understanding of its physiological role, if any, in controlling the life span of rabbit CL. At the moment our data, based on the observation that this hormone increases PGF2 α and decreases progesterone release by rabbit CL *in vitro*, suggest a permissive luteolytic role for leptin, whose physiological relevance remains to be better elucidated. This hypothesis might be indirectly suggested by the findings that luteolysis is impaired in feed-restricted gilts when circulating leptin levels are low. In fact, plasma progesterone was significantly higher in fasting than in full-fed gilts from 5 to 53 h after the induction of luteolysis by the prostaglandin analogue, alfaprostol (Prunier & Quesnel 2000). Caprio *et al.* (2001) have proposed that leptin has a dual action on mammal reproductive function, determined by different thresholds and by its site of activity. Leptin concentrations above a minimal threshold are necessary in the hypothalamus to activate the hypothalamus–pituitary–gonadal axis, for triggering puberty and maintaining normal reproductive function. Leptin excess above a certain threshold, such as is found in obesity, might impair testicular and ovarian steroidogenesis and have deleterious effects on reproduction (Caprio *et al.* 2001). The *in vitro* effects of leptin on rabbit CL seem in agreement with the current view on the role of leptin in considering that this hormone has showed a permissive luteolytic role.

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