

Leptin receptor expression and *in vitro* leptin actions on prostaglandin release and nitric oxide synthase activity in the rabbit oviduct

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Abstract

In this study, we have examined the presence and the distribution of receptors for leptin (Ob-R) in the oviduct of rabbits, and the effects of leptin on the release of prostaglandin (PG) F2 α and PGE2 and on the activity of nitric oxide (NO) synthase (NOS) by oviducts cultured *in vitro*. Rabbits were killed during the follicular phase and the oviducts were incubated *in vitro* with leptin, PGF2 α , PGE2, NO donor and inhibitors of NOS and cyclooxygenase (COX). Using immunohistochemistry, Ob-R-like positive reaction was observed only in the cytoplasm of secretory cells, having stronger intensity in the in-

fundibulum and ampulla tracts than in the isthmus. Both leptin and NO donor inhibited PGE2 release, whereas they enhanced PGF2 α release; NOS inhibitor alone or with leptin increased PGE2 and decreased PGF2 α production; NOS activity was enhanced by leptin, while PGs did not affect this enzyme. This study suggests that the oviduct could be a potential target for endocrine regulation by leptin, whose circulating levels may act as a metabolic signal modulating oviductal PG release through mediation of the NOS/NO system.

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Introduction

The oviduct plays a key role in the reproductive process by enabling gamete transport, oocyte fertilization and early embryonic development through fine regulation of oviductal smooth muscle contractility, ciliar activity and synthesis of oviductal factors (Sayegh & Mastroianni 1991). The endocrine and paracrine control mechanism of oviductal functions involves several factors including ovarian steroids (Wollenhaupt *et al.* 2002), prostaglandin (PG) F2 α and PGE2 (Harper 1988), nitric oxide (NO) (Rosselli *et al.* 1996, Perez Martinez *et al.* 1997), growth factors (Makarevich & Sirotkin 1997) and cytokines (Wijayagunawardane *et al.* 2003).

Recent evidence suggests that leptin, a 16.4 kDa cytokine encoded by the *obese* gene (*ob*) and synthesized by adipocytes (Zhang *et al.* 1994) and many tissues of the female reproductive system (Masuzaki *et al.* 1997, Smith-Kirwin *et al.* 1998, Gonzalez *et al.* 2000, Ryan *et al.* 2002), is involved not only in the regulation of food intake, energy expenditure and metabolism (Barb 1999) but also in the control of reproductive functions (Zhang *et al.* 1994,

Chehab *et al.* 1996, Clark & Henry 1999, Cunningham *et al.* 1999, Brann *et al.* 2002, Williams *et al.* 2002).

Within the reproductive system, the action of leptin is exerted both centrally, in the hypothalamus and pituitary, and peripherally through binding with its specific receptor (Ob-R), which has been identified in various tissues of the gonadic axis of several species (Spicer & Francisco 1997, Finn *et al.* 1998, Jin *et al.* 2000, Kitawaki *et al.* 2000, Koshiba *et al.* 2001, Ryan *et al.* 2002) and recently also in the corpora lutea of rabbits (Zerani *et al.* 2004). These observations, together with the finding of the effects induced by leptin in different *in vitro* systems based on cells from the reproductive tract, suggest that leptin is involved in a large array of regulatory actions required for normal reproductive functions. In particular, given the wide distribution of Ob-R in different sites of the reproductive axis, 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).

All these reasons prompted us to verify, using immunohistochemistry, whether receptors for leptin also exist in

the oviduct of rabbits. We also examined, using *in vitro* studies, the possible effects of leptin on NO synthase (NOS) activity and on PGF2 α and PGE2 release by the oviduct.

Materials and Methods

Animals, hormonal regimen and tissue collection

Sexually mature unmated New Zealand White (HY/CR strain) rabbits (Charles River Italia, Lecco, Italy) of 5 months of age, weighing 3.5–3.8 kg, were caged individually in quarters at the University of Perugia Central Animal Facility. The animals were maintained under controlled conditions of light (14 h light:10 h darkness) and temperature (18 °C) and provided with commercial rabbit chow and tap water *ad libitum*.

Rabbits were killed during the follicular phase by cervical dislocation and reproductive tracts, promptly removed from each animal, were thoroughly washed with saline and transported to the nearby laboratory in glass containers filled with saline. Within a few minutes, the oviducts were separated from the utero-tubal junction and carefully trimmed of fat with fine forceps. The oviducts harvested from each rabbit were then immediately processed for immunohistochemistry and *in vitro* studies.

The protocols involving the care and the use of animals for these experiments were approved by the Bioethic Committee of the University of Perugia.

Immunohistochemistry of Ob-R

For the immunohistochemical studies, specimens were collected from the infundibulum, ampulla and isthmus regions of the uterine tubes of three rabbits.

The specimens were fixed by immersion in 4% formaldehyde in phosphate-buffered saline (pH 7.4) (PBS) for 24 h at room temperature, then dehydrated and routinely embedded in paraffin. On 6 μ m thick sections, the presence and the distribution of Ob-R were identified by an immunohistochemical technique, using the avidin–biotin–peroxidase complex and a diaminobenzidine–nickel solution (Vector Laboratories, Burlingame, CA, USA) to visualize the reaction site.

The primary antibody used in the immunohistochemical procedures was a mouse anti-Ob-R (1:50; sc-8391; Santa Cruz Biotechnology, Santa Cruz, CA, USA). This antibody is recommended for the detection of short and long forms of Ob-R, as reported in the manufacturer's datasheet.

Briefly, sections were dewaxed in xylene and hydrated through a series of graded ethanol and were microwaved for 5 min at 750 W in 10 mM citric acid (pH 6.0) for antigen retrieval. Afterwards, sections were incubated with normal goat serum at room temperature in a moist chamber for 30 min and then overnight with the primary antibody in the same conditions.

The primary antibody was washed off in three successive PBS washes, and then the sections were incubated with the secondary antibody (a biotinylated goat anti-mouse, 1:200 in PBS; Vector Laboratories) and then with the avidin–biotin–peroxidase complex (1:2500 in PBS; ABC Elite Kit; Vector Laboratories), both for 30 min at room temperature.

Sections in which the primary antibody was omitted or substituted by pre-immune mouse γ -globulin were used for the negative control of unspecific staining.

In vitro incubations

Tracts of the ampullary and infundibulum regions of the oviducts, approximately 1 cm in length, were randomly distributed (one segment/well) into incubation wells (Becton Dickinson Co., Clifton, NJ, USA) in 1 ml 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). Before treatment, the oviducts were 'opened' longitudinally inside each well using fine forceps. Each incubation set of wells was divided into 12 experimental groups of four wells as follows: (i) medium alone as control; (ii) leptin (100 nM; Calbiochem Corp., San Diego, CA, USA); (iii) PGF2 α (1 μ M; Sigma); (iv) PGE2 (1 μ M; Sigma); (v) NO donor (sodium nitroprus-side, 10 μ M; Sigma); (vi) cyclo-oxygenase (COX) inhibitor (acetylsalicylic acid, 10 μ M; Sigma); (vii) NOS inhibitor (N-nitro-L-arginine methyl ester, 10 μ M; Calbiochem Corp.); (viii) leptin plus COX inhibitor; (ix) leptin plus NOS inhibitor; (x) PGF2 α plus NOS inhibitor; (xi) PGE2 plus NOS inhibitor; (xii) NO donor plus COX 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 6 h of incubation and stored immediately at –20 °C for later determination of hormones. Each oviductal segment was weighed and stored immediately at –20 °C for later determination of NOS activity. Preliminary evidence led to our choosing the incubation conditions and the minimum effective doses of the substances used in the present *in vitro* study (Fig. 1). For *in vitro* studies, segments from the infundibulum and ampulla were purposely used due to their higher expression for Ob-R.

Determination of NOS activity

NOS activity was determined in the same oviductal tracts used for *in vitro* incubations by monitoring the conversion of [³H]L-arginine into [³H]L-citrulline with the NOS detect assay kit (Alexis Corp., Läufelfingen, Switzerland) as previously described (Boiti *et al.* 2000). The oviduct specimen of each well was homogenized in 1 ml cold fresh homogenization buffer (50 mM Tris, 1 mM EDTA and 1 mM EGTA, pH 7.4) and centrifuged at 20 000 g for

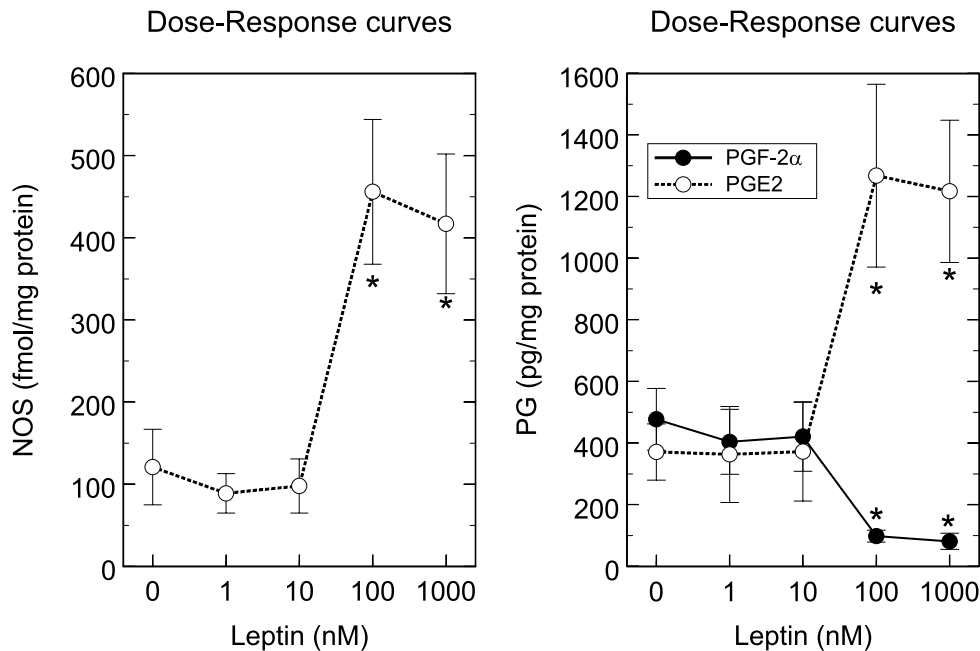


Figure 1 *In vitro* effects of increasing concentrations of leptin on total NOS activity (left), and release (right) of PGF2 α and PGE2 by oviducts of rabbits. Results are the means \pm s.d. of four replicate values. * $P \leq 0.01$ (Student's *t*-test).

60 min at 4 °C. Twenty-five microlitres of supernatant and 100 μ l incubation buffer (1.5 mM NADPH and 1 mM CaCl₂) containing 150 000 d.p.m. [³H]L-arginine (Sigma) were added to the incubation tube. After 30 min of incubation at room temperature, the enzymatic reaction was stopped by the addition of 2 ml blocking buffer (20 mM HEPES and 2 mM EDTA, pH 5.5). The mixture was applied to a pre-equilibrated column (20 mM sodium acetate, 2 mM EDTA and 0.2 mM EGTA, pH 5.5; 1 cm diameter) containing 1 ml Dowex AG50W-X8 (Sigma), and the material was eluted with 2 ml 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 NOS inhibitor (N-nitro-L-arginine methyl ester) excess to verify the specificity of the assay for production of [³H]L-citrulline by NOS (data not shown). Protein concentration was determined by the Bio-Rad (Hercules, CA, USA) protein assay kit.

Hormone RIA

PGF2 α and PGE2 were determined in each medium sample using the RIA protocols previously reported (Boiti *et al.* 2000). Intra- and interassay coefficients of variation and minimum detectable dose were respectively: 7%, 14% and 16 pg for PGF2 α and 9%, 16% and 19 pg for PGE2. PGF2 α and PGE2 antisera and non-radioactive hormones

were purchased from Sigma. Tritiated hormones were purchased from Amersham International plc (Amersham, Bucks, UK).

Statistics

All data relative to PG release and enzyme activity were submitted to ANOVA followed by Student's *t*-test (Sokal & Rohlf 1981).

Results

Oviduct expression of Ob-R

The cytoplasm of secretory cells was clearly stained and the Ob-R-like reactivity was more intense in the perinuclear and basal regions than in the apical cytoplasm (Fig. 2a, arrows). The intensity of staining for Ob-R was stronger in the infundibulum and ampulla tracts than in the isthmus (Fig. 2a-c). Positive control sections were correctly stained, while no reactivity was observed in the sections where primary antibody was replaced with normal serum (data not shown).

In vitro cultured oviducts

PGs Both leptin and NO donor decreased PGF2 α ($P \leq 0.01$) but increased PGE2 ($P \leq 0.01$) release (Fig. 3).

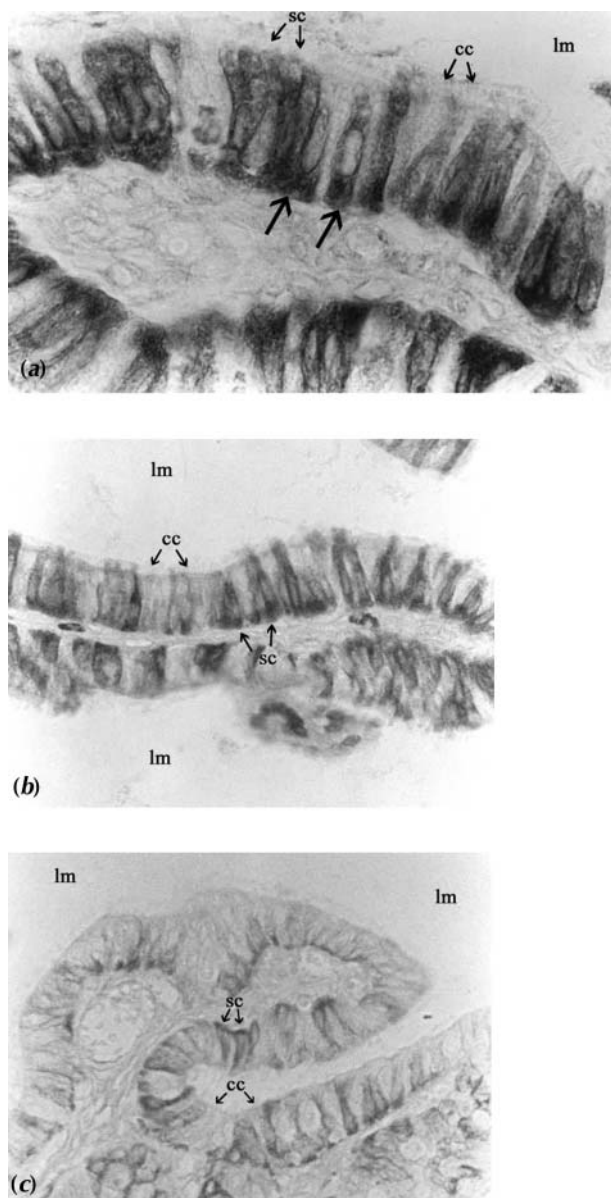


Figure 2 Immunohistochemical localization of Ob-R in different segments of the rabbit oviduct: (a) infundibulum, (b) ampulla and (c) isthmus. Strong positive reaction is shown especially in the cytoplasm of the epithelial secreting cells (sc), whereas it is less evident in ciliated cells (cc). The Ob-R-like immunoreaction was demonstrated all over the cell cytoplasm, but was particularly evident in the basal cell region ((a) larger arrows) opposite to the lumen (lm), where it is more likely localized in the rough endoplasmic reticulum. Magnifications: (a) 1000 \times ; (b) and c) 500 \times .

NOS inhibitor alone or with leptin increased PGF2 α ($P \leq 0.01$) and decreased PGE2 ($P \leq 0.01$) release (Fig. 3). The COX inhibitor decreased PG release ($P \leq 0.01$) in all experimental groups (Fig. 3). PGF2 α and PGE2

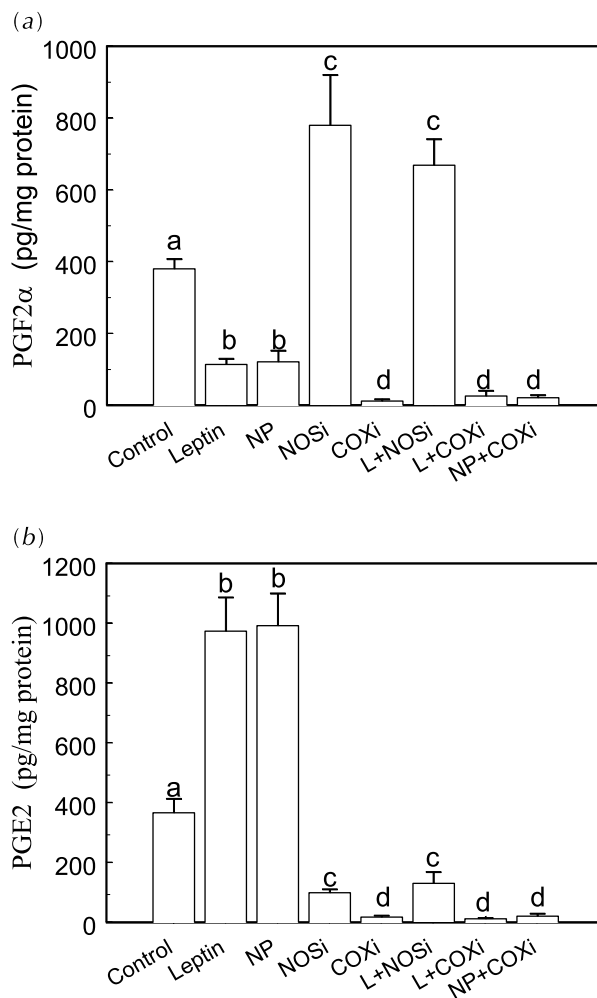


Figure 3 *In vitro* effects of leptin, NO donor (nitroprusside, NP), NOS inhibitor (NOSi), COX inhibitor (COXi), leptin plus NOSi (L+NOSi), leptin plus COXi (L+COXi) and NO donor plus COXi (NP+COXi) on (a) PGF2 α and (b) PGE2 release by rabbit oviducts. Results are the means \pm s.d. of five replicate values. Different letters above the bars indicate significantly different values ($P \leq 0.01$, Student's *t*-test).

values (the experimental groups with COX inhibitor were not considered) were negatively correlated ($n=20$, $r = -0.9821$, $P \leq 0.001$).

NOS Leptin alone or co-incubated with COX inhibitor increased NOS activity ($P \leq 0.01$) (Fig. 4). The NOS inhibitor decreased NOS activity ($P \leq 0.01$) in all experimental groups (Fig. 4). PGF2 α , PGE2 and the COX inhibitor did not affect NOS activity (Fig. 4). NOS and PGF2 α values (medium alone and leptin experimental groups only were considered) were negatively correlated ($n=8$, $r = -0.9195$, $P \leq 0.01$). By contrast, NOS and PGE2 values were positively correlated ($n=8$, $r = 0.9468$, $P \leq 0.01$).

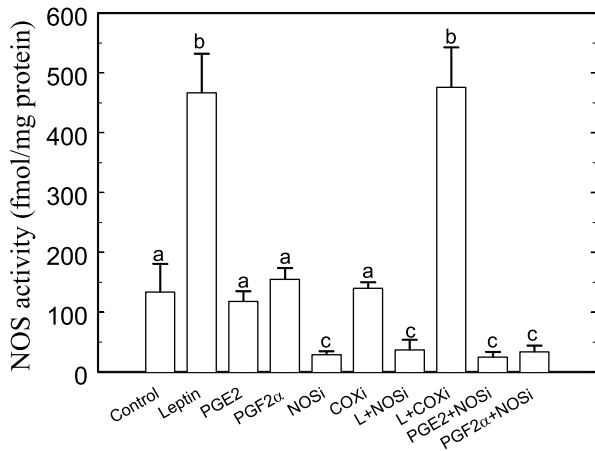


Figure 4 *In vitro* effects of leptin, PGE2, PGF2 α , NOS inhibitor (NOSi), COX inhibitor (COXi), leptin plus NOSi (L+NOSi), leptin plus COXi (L+COXi), PGE2 plus NOSi (PGE2+NOSi) and PGF2 α plus NOSi (PGF2 α +NOSi) on total NOS activity by rabbit oviducts. Results are the means \pm s.d. of five replicate values. Different letters above the bars indicate significantly different values ($P \leq 0.01$, Student's *t*-test).

Discussion

To the best of our knowledge, this is the first report to describe the presence and localization of the Ob-R in the mammalian oviduct. Immunohistochemistry revealed that Ob-R-like positive staining was present in the cytoplasm of the secretory cells of the rabbit oviducts, whereas it was completely absent from smooth muscle cells. The intensity of Ob-R staining was stronger in the epithelial cells of the infundibulum and ampulla than in the junctional tract of the oviduct. This finding, however, should be regarded with care because of the intrinsic limitation of the immunohistochemical technique used.

The Ob-R is a single membrane-spanning receptor and a member of the cytokine receptor superfamily (Sweeney 2002), which, upon binding with leptin, is internalized into specific cytoplasmic domains via clathrin-mediated endocytosis (Barr *et al.* 1999). Our immunohistochemical results have consistently shown a stronger Ob-R-like reaction in the basal cytoplasmic region of secretory cells where the rough endoplasmic reticulum is more likely localized. These findings are in agreement with recent reports which showed Ob-R-like immunoreactivity in the rough endoplasmic reticulum, Golgi apparatus and cytoplasmic matrix of hypothalamic neurones (Diano *et al.* 1998), glial cells (Funahashi *et al.* 2000), COS-7 cells (Barr *et al.* 1999) and different ovarian structures of rabbits (Zerani *et al.* 2004).

PGE2 and PGF2 α are the principal prostanoids produced in the endometrium, and regulate its function in an autocrine/paracrine manner (Sales & Jabbour 2003). In particular, these PGs interact with smooth muscle and

vascular receptors to control myometrial and vascular activity. PGE2 inhibits myometrial contractility and causes vasodilatation; by contrast, PGF2 α induces contractility and vasoconstriction (Sales & Jabbour 2003). The present work suggests that leptin is a modulator of oviductal PG synthesis, given that this hormone inhibited PGF2 α release, whilst it stimulated that of PGE2. This role in PG synthesis regulation was also confirmed, although indirectly, by our recent findings showing an increase of PGF2 α release induced by leptin in rabbit corpora lutea (Zerani *et al.* 2004). Even if no direct data exist on the interaction between blood leptin concentration and subsequent successful implantation, the idea that leptin might affect fertility and fecundity, via oviductal PG synthesis, is intriguing.

The role of NO in the regulation of COX enzymes and their arachidonic-derived products are well known in both physiology and physiopathology (Salvemini 1997). Increasing evidence supports the hypothesis that the NOS/NO system mediates the effects of leptin on various mammalian tissues: arterial relaxation (Kimura *et al.* 2000), preovulatory luteinizing hormone and prolactin surges (Watanabe & Sciöth 2001), lipolysis of white adipose tissue (Frühbeck & Gomez-Ambrosi 2001), fluidity and rigidity of erythrocyte membranes (Tsuda *et al.* 2002) and growth hormone production by blood mononuclear cells (Dixit *et al.* 2003). In our *in vitro* study, we found that leptin affects PG release by rabbit oviduct via a NOS-dependent mechanism. In fact, leptin increased NOS activity and NO donor mimicked the same effects of leptin on PG release, whereas the NOS inhibitor counteracted and reversed both leptin-dependent PGF2 α decrease and PGE2 increase. In agreement with these results, it has been found that the NOS inhibitor enhanced PGF2 α -induced contraction of the rat oviduct, proving that there is a basal release of NO which may reduce and/or modulate the contractile actions of PGF2 α (Perez Martinez *et al.* 1998). More recently, other studies have confirmed that NO inhibits prostanoid synthesis in the rat oviduct (Perez Martinez *et al.* 2000a). Differently from our study, these authors reported that NO decreases all prostanoids evaluated (6-keto-PGF2 α , PGF2 α , PGE2 and tromboxane B2). Perez Martinez *et al.* (2000b) also found that NO inhibition increases rat oviductal mobility and accelerates ovum transport, thus suggesting that NO could act as a paracrine signal between different layers of oviductal wall. In addition, Ribeiro *et al.* (2003) showed that the synthesis of COX metabolites is positively regulated by NO in the oestrogenized uterus of the rat.

Leptin acts through its binding to six isoform receptors (Ob-Ra-f), but only the long form Ob-Rb has full signalling capabilities and is able to activate the janus kinase (JAK)/signal transducer and activator of transcription (STAT), which is the major pathway used by leptin to promote its effects (Sweeney 2002, Hegyi *et al.* 2004).

However, some signalling events can be initiated by the short isoforms and/or involve other pathways, such as mitogen-activated protein kinase (MAPK) (Brann *et al.* 2002, Hegyi *et al.* 2004). Recently, we found Ob-R in the corpora lutea of rabbits, where leptin was shown to use different intracellular signalling pathways in affecting progesterone and PGF2 α , but not PGE2, release (Zerani *et al.* 2004). In fact, leptin inhibited progesterone production through the MAPK cascade, and enhanced PGF2 α release through the JAK/STAT pathway.

An increasing body of evidence unequivocally points to a close inter-link between nutrition and reproduction (Spicer & Francisco 1997). Besides long-term food deprivation and starvation, two conditions well known for their negative effects on fertility, interestingly even short-term fasting has been shown to impair reproduction in different species (Bonanno *et al.* 2002, Steiner *et al.* 2003). In rabbits fed a restricted diet, peripheral plasma leptin concentrations are lower compared with animals fed *ad libitum*, especially during the compensatory growth phase after week 12 (Rommers *et al.* 2004).

It was recently suggested that leptin has a dual effect on the reproductive mechanisms of mammals, determined by different thresholds and by its site of activity (Caprio *et al.* 2001). Blood levels of leptin above a minimal threshold are necessary to activate the hypothalamus–pituitary–gonadal axis, trigger puberty and maintain normal reproductive function. Leptin excess above a certain threshold, such as found in obesity, might impair testicular and ovarian steroidogenesis and have deleterious effects on reproduction.

Taken together, the *in vitro* effects of leptin on the rabbit oviduct suggest that this organ could be a potential target for fine-tuned regulation by leptin, whose circulating levels, reflecting whole body energy store, may act as a metabolic signal either centrally and/or peripherally to switch on or off reproduction activity respectively.

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