Expression of the long (OB-RB) and short (OB-RA) forms of the leptin receptor throughout the oestrous cycle in the mature rat ovary

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Leptin is secreted by adipocytes and exerts its effects by interacting with the long form of the leptin receptor, OB-RB. The leptin protein and leptin receptors have been localized in the ovary, and acute leptin treatment directly inhibits ovulation in the rat ovary. It was hypothesized that expression of the leptin receptor gene varies throughout the oestrous cycle to modulate the sensitivity of the ovary to leptin. In this study, expression of genes for the long and short isoforms of the leptin receptor in the adult ovary was investigated at different stages of the rat oestrous cycle. Vaginal cytology was used to determine the stage of the oestrous cycle. Ovaries were collected and RNA was extracted for real-time RT-PCR analysis of leptin receptor gene expression. OB-RB gene expression was low in pro-oestrus (3.13 ± 0.18 fg RNA per μ g total DNA) and dioestrus II (2.52 ± 0.19 fg RNA per μ g total DNA) of the oestrous cycle, whereas expression was high in oestrus (5.9 ± 0.27 fg RNA per μ g total DNA) and dioestrus I (4.6 ± 0.24 fg RNA per μ g total DNA) ($P < 0.001$). Expression of the gene for the short form of the leptin receptor (OB-RA) was at a maximum in dioestrus I (65.5 ± 0.8 fg RNA per ng total DNA), high in oestrus (39.0 ± 0.8 fg RNA per ng total DNA) and low at pro-

oestrus (5.0 ± 0.2 fg RNA per ng total DNA) and dioestrus II (1.1 ± 0.09 fg RNA per ng total DNA) ($P < 0.001$). Plasma oestradiol concentrations (pg ml^{-1}) were highest at pro-oestrus (19.38 ± 1.3), and similar at the remaining three stages studied (oestrus: 13.7 ± 1.9 ; dioestrus I: 12.4 ± 1.0 ; dioestrus II: 10.3 ± 0.9) ($P < 0.05$). Plasma progesterone concentrations (ng ml^{-1}) were higher in the luteal phases of the oestrous cycle (dioestrus I: 18.6 ± 2.3 ; dioestrus II: 14.7 ± 2.5) than during pro-oestrus (5.12 ± 0.6) and oestrus (5.9 ± 0.8) ($P < 0.05$). Plasma leptin concentrations were detectable only in pro-oestrus (0.35 ± 0.05 ng ml^{-1}) and were below the detection limit of the assay at other stages of the oestrous cycle. In summary, mRNA content for the long and short isoforms of the leptin receptor is lower in pro-oestrus and dioestrus II than in oestrus and dioestrus I of the rat oestrous cycle. The fluctuations in leptin receptor mRNA content may be a response to the concentrations of circulating steroid hormones and leptin. This research supports the initial hypothesis and shows that ovarian leptin receptor concentrations vary throughout the oestrous cycle in response to the changing environment of the ovary.

Introduction

Leptin is an adipocyte-derived hormone that plays an important role in the regulation of body fat mass and food intake. Two main sites of leptin action relating to the reproductive system are the brain and the ovary. The importance of the leptin protein in the brain has been well documented (Campfield *et al.*, 1996; McCowen *et al.*, 1998). Recent studies have demonstrated the significance of leptin in the regulation of reproductive processes in the human and rat ovary (Cioffi *et al.*, 1997; Agarwal *et al.*, 1999; Barkan *et al.*,

1999; Cunningham *et al.*, 1999; Kitawaki *et al.*, 1999; Zachow *et al.*, 1999) and the inhibitory effect of acute leptin treatment on ovulation in rats (Duggal *et al.*, 2000).

Leptin and the secretion of steroid hormones appear to be directly linked. Leptin concentrations in peripheral blood samples vary throughout the human menstrual cycle (Licinio *et al.*, 1998; Lukaszuk *et al.*, 1998; Messinis *et al.*, 1998): leptin concentrations peak in the luteal phase in a similar manner to oestradiol and progesterone concentrations (Sliker *et al.*, 1996; Lukaszuk *et al.*, 1998). Leptin gene expression in fat decreases with ovariectomy (Yoneda *et al.*, 1998), whereas leptin stimulates the production of oestrogen in periovarian fat and increases the production of progesterone and testosterone in the ovary (Cannady *et al.*,

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2000). Therefore, the variations in peripheral leptin concentrations throughout the menstrual cycle may alter the responsiveness of the ovary to leptin.

There is a short regulatory loop controlling leptin receptor mRNA synthesis in the brain. Six isoforms of the leptin receptor have been identified. The long isoform of the leptin receptor (OB-RB) is believed to be the main isoform responsible for leptin signal transduction. It has been suggested that the short form of the leptin receptor (OB-RA) is a transporter molecule for leptin (Stephens and Caro, 1998) that promotes leptin stability; however, its exact function is still unclear. OB-RB gene expression (Cioffi *et al.*, 1997; Spicer and Francisco, 1997; Zachow *et al.*, 1999) and the OB-RB receptor (Cioffi *et al.*, 1997; Karlsson *et al.*, 1997; Spicer and Francisco, 1998; Ruiz-Cortes *et al.*, 2000) have been localized in granulosa and theca cells of the ovary, whereas OB-RA expression and localization is ubiquitous. Several lines of evidence indicate that the leptin receptors are regulated by, and respond to, changes in circulating steroid hormones and leptin concentrations. Firstly, leptin receptors are differentially expressed in the hypothalamus at different stages of the oestrous cycle. The lowest concentration of total leptin receptor transcripts occurs in the choroid plexus at pro-oestrus, corresponding inversely to the highest concentration of oestradiol in the 4 day oestrous cycle of rats (Bennett *et al.*, 1999). Secondly, leptin concentrations vary with the duration of the photoperiod and with food deprivation (Saladin *et al.*, 1995; Caro *et al.*, 1996), and studies in Siberian hamsters have shown that leptin receptor mRNA expression also varies in response to these factors (Mercer *et al.*, 2000). Thirdly, leptin infusion into the brain results in an increase in leptin receptor transcripts (Bennett *et al.*, 1998, 1999). Finally, leptin-deficient mice (Mercer *et al.*, 1997) and fasting rats (Bennett *et al.*, 1998) have increased leptin receptor gene expression in the brain, and leptin treatment in these animals reduces the concentration of leptin receptor transcripts (Mercer *et al.*, 1997). This feedback loop also appears to work in the opposite direction in adipose tissue, as reduced copy number for the functional leptin receptor results in a diminished feedback signal to the leptin gene (Zhang *et al.*, 1997). Therefore, on the basis of studies in other organs, the concentration of leptin receptors may fluctuate in the ovary relative to circulating concentrations of steroids and leptin.

There is a species difference between rodents and humans in the circulating concentrations of leptin throughout the reproductive cycle: unlike the situation in humans, leptin concentrations remain constant throughout the oestrous cycle in mice (Chehab *et al.*, 1997) and rats (Bennett *et al.*, 1999). It was hypothesized that ovarian leptin receptor gene expression varies throughout the oestrous cycle in rats in response to the changing hormonal environment of the ovary, thus modulating ovarian sensitivity to the leptin protein. The purpose of the present study was to determine the relationship between plasma leptin concentrations, steroid hormone secretion and leptin receptor (OB-RB and OB-RA) gene expression at the various

stages of the oestrous cycle using the highly sensitive technique of real-time RT-PCR.

Materials and Methods

Animals

Pubertal female Sprague–Dawley rats (from the University of Adelaide colony) weighing approximately 80 g were maintained under controlled conditions of light (14 h light:10 h dark), temperature and humidity, with free access to pelleted food (21% protein, 6.9% fat, 5.4% fibre: equivalent to 34.4 kcal g⁻¹) and water. The rats were allowed 5 days to acclimatize to their new surroundings and then vaginal smears (Bronson *et al.*, 1966) were performed (between 09:00 and 11:00 h) for three consecutive oestrous cycles. Only mature, ovulating animals with three consecutive 4 day oestrous cycles were included in the study. Animals were handled in accordance with the Australian code of practice for the care and use of animals for scientific purposes, and the animal ethics committees of both The Queen Elizabeth Hospital and The University of Adelaide approved all experiments.

Tissue collection, RNA and DNA isolation

Given that leptin protein circulates in a diurnal pattern in rodents (Mistlberger *et al.*, 1998; Pickavance *et al.*, 1998) and rodents are nocturnal foragers, all animals were killed between 11:00 and 13:00 h, a time that is not associated with eating in rats. Rats were killed by s.c. administration of a mixture of the anaesthetic Ketamil (100 mg ketamine ml⁻¹; Troy Laboratories Pty Ltd, NSW) and the muscle relaxant Rompun (20 mg xylazine ml⁻¹; Bayer AG, Leverkusen). The ovaries and the attached oviducts were dissected out, placed into diethyl pyrocarbonate-treated water and the ampulla region of the oviducts (with or without ovulated oocytes), ovarian bursa and fat were removed. Left and right ovaries were weighed, frozen separately in liquid nitrogen and stored at -80°C. RNase-free conditions were maintained during the collection of tissues. RNA and DNA were isolated using Tri Reagent, the RNA, DNA and protein isolation reagent (Molecular Research Center Inc., Cincinnati, OH), in accordance with the manufacturer's protocol (1995). RNA and DNA were quantified using a fluorometer (Turner Designs, Sunnyvale, CA) as described in the manufacturer's protocol, using RiboGreen™ RNA quantitation reagent and PicoGreen® dsDNA quantitation reagent kits (Molecular Probes, Eugene, OR), respectively.

Reverse transcription of RNA

A master mix containing 1 × PCR buffer II (Perkin Elmer, Boston, MA), 50 nmol of each dNTP (Gibco BRL, Rockville, MD), 250 ng Oligo (dT)_{12–18} (Promega, Madison, WI), 50 U RNasin® ribonuclease inhibitor (Promega), 250 nmol MgCl₂ (Perkin Elmer), 500 U M-MLV reverse transcriptase (Gibco BRL) and 2 µl RNA was added to a final reaction

volume of 50 μ l. Reactions were added to a 96-well plate (Perkin Elmer), incubated for 30 min at 37°C and 5 min at 95°C in a thermal cycler (Perkin Elmer) and then stored immediately at –20°C until the PCR reaction. Both negative (no RNA in the RT reaction) and positive (RT-check™; Ambion Inc., Austin, TX) controls were included in the RT reaction. Total DNA from ovarian tissue was quantified to control for tissue-to-tissue variation.

Generation of standard OB-RB and OB-RA DNA in TOPO TA cloning vector

Primers used by Zachow *et al.* (1999), synthesized by Gibco BRL, were used to amplify both the long and short isoforms of the leptin receptor. A common forward primer (5'-ATGAAGTGGCTTAGAATCCCTTCG-3') for both OB-RB and OB-RA was used. The reverse primers for the long and short isoforms were 5'-ATATCACTGATTCTGCATGCT-3' and 5'-TACTTCAAAGAGTGTCCGCTC-3', respectively. A PCR master mix containing 1 $\times$ PCR buffer, 1.5 mmol MgCl_2 l^{-1} , 0.2 mmol l^{-1} of each dNTP, 400 nmol l^{-1} of each OB-RB primer or 200 nmol l^{-1} of each OB-RA primer, 2.5 U Taq Polymerase (Perkin Elmer) and 2 μ l of the cDNA (to a final volume of 50 μ l) was placed in a thermal cycler and a two-step PCR protocol (denaturing at 94°C and annealing at 55°C for 30 s each) of 40 cycles was applied. A single band of the expected size (346 bp for OB-RA and 375 bp for OB-RB) was visualized using gel electrophoresis and confirmed by enzymatic digestion and direct sequence. The gel bands were excised and the DNA purified using a QIAprep gel extraction kit (QIAGEN, Valencia, CA). Both DNA fragments were inserted into TOPO TA cloning vectors (Invitrogen, Carlsbad, CA), transformed into TOP10F' cells and plated on Luria Bertoni agar plates with 50 μ g ampicillin ml^{-1} (Fisher Scientific, Fair Lawn, NJ), according to manufacturer's instructions (1999). Positive clones (white colonies) were considered to have an insert in the TOPO TA cloning vector and these colonies were cultured overnight at 37°C in LB media with 50 μ g ampicillin ml^{-1} . Vector insert DNA was extracted using QIAprep spin miniprep kits (QIAGEN) and the presence of the insert was identified by EcoRV restriction enzyme digestion (Gibco BRL) and visualized using gel electrophoresis. Vector inserts were linearized using KpnI (Promega) for OB-RB and HindIII (Gibco BRL) for OB-RA. The concentration of DNA was quantified and dilutions prepared for the synthesis of standards for real-time PCR.

Real-time PCR

A common hybridization probe (Operon, Alameda, CA) was constructed, 5'-CCTTGTGCCAGGAACAATTCAAGG-3', with the reporter fluorochrome (6-carboxyfluorescein; 6-FAM) at the 5' end and a quencher fluorochrome (6-carboxy-tetramethyl-rhodamine; TAMRA) at the 3' end. The probe had a melting temperature (T_m) of approximately 15°C above that of the PCR primers and had four additional base pairs at the 3' end to promote a hairpin structure of the hybridization oligonucleotide to minimize background

fluorescence. The PCR master mix consisted of: 1 $\times$ PCR buffer, 1.5 mmol MgCl_2 l^{-1} , 0.2 μ mol l^{-1} of each dNTP, 400 nmol l^{-1} of primers for OB-RB detection, 200 nmol l^{-1} of primers for OB-RA detection and 2.5 U platinum Taq DNA polymerase (all from Gibco BRL). Ten microlitres of the RT reaction mixture was added to the appropriate wells, in duplicate. Hybridization oligonucleotide (500 nmol l^{-1}) was added to each well of the 96-well I-cycler plate (Bio-Rad, Hercules, CA) under low intensity light conditions. Real-time RT-PCR was carried out using the two-step procedure described above and data were recorded by the Bio-Rad I-cycler program (Bio-Rad).

Progesterone, oestradiol and leptin assays

Immediately before the animals were killed, a blood sample was collected from each animal by cardiac puncture, for plasma progesterone and oestradiol and leptin analyses. Blood was transferred to a lithium heparin separator tube (Vacuette Greiner Labortechnik, Kremsmünster, Bad Hollestr) and centrifuged at 13 000 *g* for 10 min at 4°C. The upper clear layer was stored at –80°C before assaying. All assay kits were purchased from DSL Inc. (Webster, TX) and analyses performed according to the manufacturer's instructions. Progesterone (DSL-3400) and oestradiol (DSL-4400) concentrations in rat plasma samples were analysed using radioimmunoassay kits. Leptin (DSL-10-24100) concentrations were measured by using an Active™ murine leptin ELISA kit, which is sensitive for both rat and mouse plasma leptin. The minimum detection limits of the progesterone, oestradiol and leptin assays were 0.09 ng ml^{-1} , 4.6 pg ml^{-1} and 0.04 ng ml^{-1} , respectively. The intra-assay coefficient of variation for each assay was < 7%. Samples for progesterone and oestradiol analysis were assayed in duplicate. The option of using 100 μ l in the assay was included to quantify the low concentrations of serum leptin.

Statistical analyses

Samples for analysis were assayed on a total of four I-cycler plates, with one plate dedicated for one ovary, to quantify both receptors. The inter-plate variation was < 6% and < 4% for OB-RB and OB-RA, respectively. RNA samples with threshold cycle numbers higher than the negative control values in the real-time RT-PCR assays were omitted from the study. The statistical analyses ($n = 6$ or 7 rats) were performed using the Bonferroni multiple comparisons test after significance was established using an ANOVA. In all studies, statistical significance was accepted when the *P* value was < 0.05.

Results

Generation of standard OB-RB and OB-RA DNA in TOPO TA cloning vector

Fragments of the long and short isoforms of the leptin receptor were generated by PCR using the appropriate

Fig. 1. Standard concentration of RNA (fg or 10^{-15} g) for the (a) short (OB-RA) and (b) long (OB-RB) form of the leptin receptor in the rat ovary throughout the oestrous cycle. The oestrous cycle of mature Sprague–Dawley rats was determined by vaginal cytology (P: pro-oestrus; E: oestrus; DI-I: dioestrus I; DI-II: dioestrus II). Ovaries were collected for RNA extraction and processed for real-time RT–PCR analyses. Data are expressed as the mean $\pm$ SEM standard concentration of RNA for each receptor, from both ovaries. Different letters indicate significant differences between groups ($P < 0.05$).

primers and cloned into the TOPO TA cloning vectors. The identity of the inserts was confirmed by direct sequencing and restriction mapping. The vector inserts were linearized for the preparation of standards for OB-RB and OB-RA. Standards were diluted to provide a range of concentrations to prepare a standard curve, and real-time PCR was performed. The standard curve data are expressed as the amount (fg) of insert DNA alone (from the insert vector), therefore, the results reflected the amount of full-length cDNA generated from mRNA samples. The threshold cycle, the cycle at which point the reporter dye emission intensity is above background noise, was used to determine the cycle number at which PCR amplification was in an exponential phase.

Real-time RT–PCR of tissues at different stages of the oestrous cycle, OB-RA and OB-RB detection

Real-time RT–PCR for OB-RA and OB-RB isoforms was performed to establish the expression pattern of the leptin receptor genes throughout the oestrous cycle (Fig. 1). OB-RA real-time RT–PCR analysis revealed that leptin receptor expression was high in oestrus and dioestrus I and the maximum expression was noted in dioestrus I (Fig. 1a). Real-time RT–PCR of OB-RB revealed a similar expression pattern: expression of OB-RB was highest at oestrus and dioestrus I, whereas lower expression was recorded at pro-oestrus and dioestrus II (Fig. 1b). PCR products were also visualized using gel electrophoresis to confirm the production of a single, correctly sized product. After the 40

cycles, gel electrophoresis revealed that the end quantity of the PCR products of each tube, regardless of starting concentrations, were identical. Therefore, by using real-time RT–PCR it was possible to quantify accurately the small differences among samples by analysing data in the exponential phase.

Negative and positive controls

Negative controls included omitting RNA in the RT step or omitting cDNA in the real-time RT–PCR step. Negative controls resulted in background level noise on the I-cycler PCR machine and no PCR product was visualized by gel electrophoresis. The RT-check™ kit was used as an endogenous control to test the integrity of the RT step and the amplification efficiency of the PCR. The PCR products produced in the RT-check™ step were labelled with [32 P]dCTP, visualized by gel electrophoresis and the bands were excised and counted in a scintillation counter. The radioactivity counts from the various PCR products validated that the RT step and amplification efficiency was similar from tissue to tissue ($< 8\%$ variation across all tissues). Owing to the low tube-to-tube variation, data generated from the real-time experiments required no correction. Genomic DNA was quantified in ovaries to control for variation among tissues. Data are expressed as fg RNA per µg total DNA.

Body weight, ovarian mass, and plasma steroid hormone and leptin concentrations

Body weights of animals at the time of tissue removal were similar among groups (Table 1). Left and right ovaries of animals, regardless of the stage of the oestrous cycle, were of similar mass (Table 1). Plasma oestradiol concentrations (pg ml^{-1}) were highest at the time of pro-oestrus (19.38 ± 1.3), and similar in the remaining three stages of the oestrous cycle studied (oestrus: 13.7 ± 1.9 ; dioestrus I: 12.4 ± 1.0 ; dioestrus II: 10.3 ± 0.9) ($P < 0.05$ for pro-oestrus versus the remaining stages studied). Plasma progesterone concentrations (ng ml^{-1}) were higher in the luteal phases of the cycle (dioestrus I: 18.6 ± 2.3 ; dioestrus II: 14.7 ± 2.5) than at pro-oestrus (5.12 ± 0.6) and oestrus (5.9 ± 0.8) ($P < 0.05$). Plasma leptin concentrations were detectable only in pro-oestrus ($0.35 \pm 0.05 \text{ ng ml}^{-1}$) and were below the detection limit of the assay at other stages of the oestrous cycle.

Discussion

Gene expression of the long form (OB-RB) of the leptin receptor has been detected in granulosa, theca, interstitial and cumulus oophorus cells of the human ovary (Cioffi *et al.*, 1997; Karlsson *et al.*, 1997; Agarwal *et al.*, 1999). The short form (OB-RA) of the leptin receptor has been detected in granulosa, theca and cumulus oophorus cells of the human ovary (Cioffi *et al.*, 1997; Karlsson *et al.*, 1997; Agarwal *et al.*, 1999). In rats, OB-RA and OB-RB are

Table 1. Body weights, ovarian masses and concentrations of serum leptin, oestradiol and progesterone in rats at different stages of the oestrous cycle

	Pro-oestrus	Oestrus	Dioestrus I	Dioestrus II
Body weight (g)	184 ± 13	163 ± 9	152 ± 5	168 ± 6
Ovarian mass (mg)	310 ± 11	262 ± 27	295 ± 3	292 ± 2
Serum leptin (ng ml ⁻¹)	0.35 ± 0.05	ND	ND	ND
Oestradiol (pg ml ⁻¹)	19.38 ± 1.3 ^a	13.7 ± 1.9 ^b	12.4 ± 1.0 ^b	10.3 ± 0.9 ^b
Progesterone (ng ml ⁻¹)	5.12 ± 0.6 ^a	5.9 ± 0.8 ^a	18.6 ± 2.3 ^b	14.7 ± 2.5 ^b

All measurements reported are at the time of tissue collection.

Values are mean ± SEM per animal (*n* = 6–7).

Different letters indicate significant differences between groups (*P* < 0.05).

ND: not detected.

expressed in granulosa cells, but expression of only OB-RA is detectable in theca cells of hypophysectomized rats (Zachow *et al.*, 1999). However, the expression of OB-RA and OB-RB in the ovary at different times of the oestrous cycle has not been quantified in any species. The current study was designed to investigate the direct effect of the stage of the oestrous cycle on leptin receptor (OB-R) expression in the rat ovary using the specific and quantitative technique of real-time RT–PCR.

This study demonstrates that OB-R expression varies in response to the changing environment of the ovary. The onset of oestrus and dioestrus I was correlated with an increase in the expression of OB-RA and OB-RB genes, and lower expression was observed at pro-oestrus and dioestrus II. The experimental design allowed specific identification of the natural expression patterns of the ovarian OB-R genes in response to normal fluctuations of steroid hormones and the leptin protein, throughout the oestrous cycle. With this design it was possible to focus on the ovary while it remained an intact unit with the hypothalamic–pituitary–ovarian axes, and the findings indicate that OB-R gene expression throughout the oestrous cycle responds to cyclical changes.

There is controversy with regards to the interaction between leptin and steroid hormones. Some studies have shown that oestradiol does not affect leptin secretion (Haffner *et al.*, 1997; Castracane *et al.*, 1998). However, several other studies have reported an interaction between leptin and steroid hormones in the human menstrual cycle (Hardie *et al.*, 1997; Licinio *et al.*, 1998; Lukaszuk *et al.*, 1998; Mannucci *et al.*, 1998; Messinis *et al.*, 1998, 1999; Teirmaa *et al.*, 1998; Gower *et al.*, 2000). This research prompted measurement of leptin and steroid hormone concentrations in rats throughout the oestrous cycle in the present study. The results revealed that steroid hormone concentrations vary throughout the oestrous cycle and that maximum oestradiol concentrations occur at pro-oestrus (Butcher *et al.*, 1974; Kalra and Kalra, 1974; Bennett *et al.*, 1999). It has been proposed that leptin can regulate oestradiol oscillations in humans (Licinio *et al.*, 1998) and that leptin gene expression is regulated by ovarian steroid hormones: ovariectomized rats have lower *ob* gene ex-

pression in fat (Yoneda *et al.*, 1998; Machinal *et al.*, 1999). In support of previous research, the present study showed that leptin concentrations are at a maximum at pro-oestrus, a time when oestradiol is at its peak. Research indicates that, after an increase in oestradiol at pro-oestrus, the amount of OB-R transcript decreases in the rat choroid plexus (Bennett *et al.*, 1999). Results from the present study show that this pattern of expression is also present in the ovary. OB-R mRNA concentrations increased in oestrus and dioestrus I after the oestradiol surge, and decreased again in dioestrus II, immediately before the oestradiol surge at pro-oestrus. The results indicate that oestradiol may be an important factor in fluctuations of leptin and OB-R at pro-oestrus. Studies on pig ovaries have shown that OB-R expression increases with maximum progesterone concentrations (Ruiz-Cortes *et al.*, 2000). Leptin has a stimulatory effect on luteal function in pigs: leptin mRNA and protein expression increases with progesterone accumulation *in vitro* (Murphy and Dobias, 1999; Pescador *et al.*, 1999). Therefore, it was hypothesized that leptin concentrations vary with progesterone concentrations during the oestrous cycle. In the present study, OB-R content was at a maximum in stages of the oestrous cycle anticipatory of maximum progesterone concentrations (oestrus and dioestrus I) and decreased markedly after high progesterone concentrations.

This study analysed OB-R expression in whole ovarian tissue; therefore, the findings relate to the change in OB-R expression in the ovary as a whole. It is possible that luteal cells, endothelial cells and leucocytes contribute to the differences in OB-R expression observed in oestrus and dioestrus I. The results presented for oestrus and dioestrus I are from a time after ovulation and development of corpora lutea. Owing to the changing physiology of the ovary, with granulosa cells becoming luteal cells, the increase in the content of OB-R transcripts probably leads to increases in intact OB-R protein. OB-R expression and OB-R gene expression increase in pig granulosa cells as they luteinize (Ruiz-Cortes *et al.*, 2000); therefore, the findings of the present study are in agreement with previous research and support the hypothesis that luteinization in the ovary promotes an increase in OB-R. Our laboratory has located dense staining for OB-R in the theca-luteal layer in mouse

corpora lutea (Ryan *et al.*, 2002). The role of leptin in angiogenesis (Bouloumie *et al.*, 1998) may also provide an explanation for the increase in OB-R at oestrus and dioestrus I, as luteinization results in neovascularization in the ovary due to ovulation at oestrus. Leucocytes and cytokines are important components of the ovulatory cascade and infiltrate the ovary at ovulation (Brannstrom and Norman, 1993). OB-R has been identified on leucocytes (Gainsford *et al.*, 1996; Lord *et al.*, 1998; Santos-Alvarez *et al.*, 1999), and the contribution of these cells to the total expression of OB-R in the ovary expression cannot be dismissed. Whether the increase in OB-R arises as a result of the influx of leucocytes at ovulation remains to be defined.

In the present study, leptin concentrations were detectable only in pro-oestrus and not at the other stages of the oestrous cycle. Other researchers have reported unchanged circulating leptin concentrations throughout the oestrous cycle in mice (Chehab *et al.*, 1997) and rats (Bennett *et al.*, 1999). The reason for the discrepancy between the findings may be attributed to the strain of the animal or the assay technique used (radioimmunoassay versus ELISA). In the present analysis, a larger volume of serum was used in the leptin assay, in an attempt to detect low concentrations of leptin. Future work in this laboratory may clarify leptin concentrations throughout the oestrous cycle in this strain of rat.

Although previous research (Duggal *et al.*, 2000) demonstrated that excessive leptin is inhibitory to ovulation, it has also been suggested that insufficient leptin–OB-R interaction, due to leptin resistance, results in anovulation (Schwartz *et al.*, 1996; Cunningham *et al.*, 1999). It is possible that, in the study Duggal *et al.* (2000), acute leptin administration ‘programmed’ the ovary to be in a post-ovulatory stage of the oestrous cycle. Physiologically, post-ovulation is a time of increased leptin action due to the increases in OB-R. Thus, in the earlier study, the response of the ovary to acute leptin administration was inhibition of ovulation. The changes in the content of OB-R throughout the oestrous cycle provides a mechanism for the regulation of normal physiological processes to allow different responses to leptin throughout the oestrous cycle, during which leptin concentrations may otherwise vary only slightly. Such a mechanism would not be unexpected given the variability in the number of receptors of other key regulators, such as the LH receptor, in granulosa cells at different stages of the oestrous cycle.

In summary, the present study investigated the effect of the stage of oestrous cycle on OB-R expression in the mature rat ovary. Significant decreases were found in OB-R expression at pro-oestrus and dioestrus II, stages that are preparatory for ovulation. The stages after ovulation appeared to show an increase in OB-R gene expression. The increase in OB-R gene expression at these stages may be a mechanism to decrease the action of leptin before ovulation while increasing the action of leptin for the corpora lutea. A simultaneous increase in OB-R may promote the stability of leptin at times of high OB-R action, as OB-R is

thought to be a transporter molecule for leptin. The unique relationship between leptin and the ovarian axis, at a physiological level, requires clarification. Several studies have demonstrated the inhibitory action of leptin on steroidogenesis (Zachow and Magoffin, 1997; Spicer and Francisco, 1998) and ovulation (Duggal *et al.*, 2000). Further investigations will concentrate on the effects of high concentrations of exogenous leptin and steroid hormone treatment on OB-R expression in the ovary.

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