Effects of leptin administration and feed restriction on thecal leucocytes in the preovulatory rat ovary and the effects of leptin on meiotic maturation, granulosa cell proliferation, steroid hormone and PGE₂ release in cultured rat ovarian follicles

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Leptin is expressed by adipocytes and is thought to play a role in regulating food intake and in reproduction. It has been demonstrated that acute leptin administration to immature gonadotrophin-primed rats *in vivo* inhibits ovulation and causes a decline in food intake. However, feed restriction alone does not inhibit ovulation. Two experiments were designed to investigate the mechanism of leptin-induced inhibition of ovulation. In the first experiment, which was prompted by the importance of ovarian leucocytes in ovulation, the role of leucocytes in leptin-induced inhibition of ovulation was investigated. The second experiment investigated whether high leptin concentrations could inhibit other factors important to ovulation, such as meiotic competence of oocytes, granulosa cell proliferation, steroid or PGE₂ release, and interleukin 1 β production, *in vitro*. In the first experiment, the populations of neutrophils and monocytes–macrophages in the preovulatory follicles of gonadotrophin-primed, leptin-treated and -untreated rats were examined. A decrease in food intake, as a result of either leptin treatment or feed restriction, specifically reduced the numbers of neutrophils and monocytes–macrophages infiltrating the theca interna of preovulatory follicles without

affecting the numbers found in the stroma. The findings show that reduced infiltration of thecal neutrophils and macrophages into preovulatory follicles is a response to reduced food intake. Furthermore, this reduction is not the direct cause of the leptin-induced inhibition of ovulation. In the second experiment, ovarian follicles were cultured for 4 or 12 h in the presence or absence of the following hormones: FSH (500 mIU), insulin-like growth factor I (IGF-I) (50 ng ml⁻¹), LH (100 ng ml⁻¹) and leptin (300 ng ml⁻¹). The results demonstrated that high concentrations of leptin in follicle culture do not affect meiotic maturation or steroid release, but tend to inhibit release of PGE₂ (although this result was not significant). DNA synthesis in granulosa cells was not inhibited by leptin in FSH- and IGF-I-supplemented culture media. These results are in agreement with previous studies that have shown that leptin inhibits the stimulatory effects of IGF-I on FSH-stimulated oestradiol production in rat granulosa cells without affecting progesterone production. In summary, leptin does not appear to have an adverse effect on the components of ovulation tested in this study, and therefore must impact on the ovulatory cascade in a way that remains to be defined.

Introduction

Leptin is produced and secreted by adipocytes (Zhang *et al.*, 1994) and is known to be a key regulator of food intake and energy expenditure (Houseknecht *et al.*, 1998). This hormone can have significant negative effects on cellular components of the ovary (Spicer and Francisco, 1997, 1998; Zachow and Magoffin, 1997; Agarwal *et al.*, 1999). Furthermore, acute administration of leptin to immature

gonadotrophin-primed rats inhibits ovulation independent of caloric restriction (Duggal *et al.*, 2000).

The leptin protein and the functional leptin receptor have been localized in the ovary, indicating that leptin may have a direct effect on the ovary. Several lines of evidence indicate that high leptin concentrations can inhibit the stimulatory actions of many hormones important in the selection of dominant follicles, including insulin-like growth factor I (IGF-I), transforming growth factor β (TGF- β), FSH and insulin: (i) high leptin concentrations block the stimulatory effects of IGF-I on FSH-stimulated oestradiol production, but not progesterone production, in rat

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granulosa cells (Zachow and Magoffin, 1997; Agarwal *et al.*, 1999); (ii) leptin suppresses the positive effect of TGF- β on FSH-dependent oestrone and oestradiol concentrations in cultured rat granulosa cells, aromatase mRNA expression and aromatase activity (Zachow *et al.*, 1999); (iii) leptin inhibits insulin-induced oestradiol production in granulosa cells and decreases the insulin-induced increases in progesterone production in bovine granulosa cells (Spicer and Francisco, 1997); (iv) leptin suppresses FSH- and dexamethasone-induced pregnenolone, progesterone and 20 α -hydroxy-4-pregnen-3-one production in rat granulosa cells, an effect caused in part by reduction of an enzyme in the P450_{scc} electron transport system (adrenodoxin) (Barkan *et al.*, 1999); and (v) leptin inhibits insulin-stimulated progesterone production from human luteinized granulosa cells (Brannian *et al.*, 1999). Hypophysectomized rat thecal cells lack a functional leptin receptor (Zachow *et al.*, 1999); therefore leptin research in the rat ovary has focused mainly on the effects of leptin on granulosa cells. However, several studies have demonstrated that leptin has inhibitory effects on human and bovine thecal cells (Spicer and Francisco, 1998; Agarwal *et al.*, 1999).

Leucocytes are thought to be important cells in the ovulatory process (Norman *et al.*, 1997; Van der Hoek *et al.*, 2000). Evidence from studies in rodents indicates that neutrophils and macrophages are actively recruited into the preovulatory follicle after the LH surge, via chemotaxis. This recruitment results in an eightfold increase in the density of thecal neutrophils and a fivefold increase in the density of macrophages (Herriot *et al.*, 1986; Brannstrom *et al.*, 1993). Leucocyte supplementation of *in vitro* perfused rat ovaries results in an increase in ovulation rate (Hellberg *et al.*, 1991). Furthermore, administration of a neutrophil-depleting antibody *in vivo* causes a reduction in ovulation rate in rats (Brannstrom *et al.*, 1995) and macrophage depletion results in a decrease in ovulation rate in mice (Van der Hoek *et al.*, 2000). The mechanism by which neutrophils and macrophages contribute to the ovulatory process has not been defined, but may be linked to the local cytokine network of macrophage secretory products in the pre-ovulatory follicle that leads to follicle wall breakdown and subsequent oocyte release (Brannstrom and Norman, 1993; Norman *et al.*, 1997). It is now established that leptin is capable of interactions with the immune system. Studies *in vitro* have shown that leptin can stimulate human monocyte proliferation by inducing production of tumour necrosis factor α and interleukin 6 (IL-6) (Santos-Alvarez *et al.*, 1999) and that leptin increases Th1 and suppresses Th2 cytokine production in CD4⁺ T cells from mice (Lord *et al.*, 1998). Furthermore, the effects of leptin on food intake and body temperature are believed to be mediated by the leucocyte-secreted cytokine IL-1 (Luheshi *et al.*, 1999).

Two experiments were designed to investigate the cause of inhibition of ovulation in leptin-treated, gonadotrophin-primed immature rats. The aim of the first experiment was to assess the effect of leptin treatment on ovarian leucocyte infiltration into the preovulatory follicles of gonadotrophin-

primed rats. The aim of the second experiment was to investigate whether other key factors in the ovulatory process, namely meiotic maturation, granulosa cell DNA synthesis, steroid and prostaglandin release and IL-1 secretion, were disturbed by high concentrations of leptin *in vitro*.

Materials and Methods

Experiment 1: effect of acute leptin treatment on infiltration of neutrophilic granulocytes and monocytes-macrophages into the ovary

Immature female Sprague-Dawley rats aged 21–28 days (from the University of Adelaide colony) and weighing 60–80 g were maintained under controlled conditions of light (14 h light: 10 h dark), temperature (22°C) and humidity, with free access to pelleted food and water. 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. The rats were primed with gonadotrophins as described by Duggal *et al.* (2000). In brief, an Alzet® mini-osmotic pump (Alza Corporation, Palo Alto, CA) containing 8 iu hFSH (Organon, Oss) was inserted s.c. on day 1. Two days later the rats were injected i.p. with either leptin (30 μ g in 200 μ l saline) or saline only (200 μ l) at 08:00 h and at intervals of 3 h thereafter for 12 h. hCG (10 iu) was administered i.p. at 12:00 h to induce ovulation. There were three treatment groups: (1) FSH + saline, in which animals received an 8 iu FSH minipump and saline injections on day 3; (2) FSH + leptin, in which animals received an 8 iu FSH minipump and leptin injections on day 3; and (3) pair fed: FSH + saline, in which animals were treated as in group 1 but were fed only the amount of food consumed by animals in group 2. The rats were killed at 23:00 h and both ovaries frozen in Jung tissue freezing media (Leica Instruments, Nussloch) using isopentane (BDH, Poole) and liquid nitrogen. Tissue was then stored at –80°C. Each pair of ovaries was then cut into sections every 130 μ m (6 μ m thick sections). The sections were examined for neutrophils and monocytes-macrophages using the mouse anti-rat MCA149A and ED1 antibodies (Serotec, Oxford), respectively, as described by Brannstrom *et al.* (1993). The signal was amplified using a secondary sheep anti-mouse antibody linked to horseradish peroxidase (Amersham Pharmacia Biotech, Piscataway, NJ), and the complex was visualized using Sigma Fast™ diaminobenzidine (DAB) (Sigma Chemical Co., St Louis, MO) and counterstained with haematoxylin (Surgipath Medical Industries, Richmond, IL). Preovulatory follicles were defined as healthy follicles > 576 μ m in the largest cross-section. A cell showing the brown DAB signal was defined as a positive cell. The percentage positivity was determined by dividing the number of positively stained cells (brown stain: neutrophils or monocytes-macrophages in the theca

interna) by the total stain (brown stain and blue haematoxylin counterstain) in the preovulatory follicle using video image analysis software (Leading Edge Pty Ltd, Adelaide). The video image analysis software uses contrast for analysis and requires limits to be set manually. The mean positivity (three random areas) for each preovulatory follicle in the ovarian sections was tallied and this value was divided by the total number of follicles in all ovarian sections. The mean positivity of the stromal regions for each ovarian section was evaluated by analysing six random stromal regions per section and calculating the average for the ovary. Positive control tissue (spleen, liver) and negative controls (in which primary antibody incubation was omitted) were included. Video image analysis was always carried out by two observers using a blind protocol, and data were compared. Data were averaged from each observer and never varied by > 15%. Both ovaries of four rats from each treatment group were analysed. The statistical analyses were performed using a parametric ANOVA with Tukey–Kramer multiple comparisons test and significance was accepted when the *P* value was < 0.05, where the two ovaries from each rat were averaged to represent the data from one rat.

Experiment 2: effect of leptin treatment on important factors in ovulation

Hormones, chemicals and animals. Equine chorionic gonadotrophin (eCG) was purchased from Intervet (Boxmeer); recombinant human FSH was donated by Organon (Oss); IGF-I was purchased from GroPep (Adelaide); culture medium M199 and sodium bicarbonate were purchased from Gibco (Grand Island, NY); heat shock treated BSA (fraction V) was obtained from Boehringer Mannheim (Mannheim); penicillin and streptomycin sulphate were purchased from CSL Biosciences (Adelaide); the anaesthetic Ketamil (100 mg ketamine ml⁻¹) and the muscle relaxant Rompun (20 mg xylazine ml⁻¹) were purchased from Troy Laboratories Pty Ltd (NSW) and Bayer AG (Leverkusen), respectively; ovine LH (oLH-26no. AFP-5551B, specific activity of 2.3 U mg⁻¹) was kindly provided by NIADDK (Bethesda, MD); human recombinant leptin was purchased from Diagnostic Systems Laboratories (Webster, TX); and androstenedione and 3-isobutyl-1-methyl xanthine (IBMX) were purchased from Sigma-Aldrich (St Louis, MO).

Immature female Sprague–Dawley rats aged 25–28 days (from the University of Adelaide colony) and weighing 60–80 g were maintained under controlled conditions of light (14 h light: 10 h dark), temperature (22°C) and humidity, with free access to pelleted food and water. 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.

Follicle culture for 4 h and 12 h. Follicle growth was

induced in immature rats by a s.c. injection of 10 iu eCG at 12:00 h for the 4 h of follicle culture on day –2 and at 00:00 h for the 12 h of follicle culture on the night of day –2. Rats were killed 46–48 h later using ketamine–xylazine anaesthesia and cervical dislocation. Both ovaries were dissected free of the ovarian fat and bursa and placed in Hepes–M199–0.1% BSA medium on a 37°C warming tray. Rat follicles were cultured as described by Tornell *et al.* (1995). Follicles were dissected with the aid of a stereo-microscope and fine forceps. Approximately 45 preovulatory follicles were obtained from six ovaries and these were collected and pooled. Aliquots of bicarbonate–M199–0.1% BSA medium (five follicles per 1 ml) were added to clean rubber-capped Vacutainer® (no additive) tubes from Becton Dickinson (Franklin Lakes, NJ). Follicles were cultured in the presence or absence of leptin (300 ng ml⁻¹) in combination with FSH (500 iu), IGF-I (50 ng ml⁻¹) or LH (100 ng ml⁻¹). The tubes were gassed with a gas mixture of 5% CO₂:95% O₂ from BOC gases (Sydney) and incubated in a slowly gyrating waterbath set at 37°C. Preliminary follicle culture (4 h) experiments, in which follicles were incubated with various concentrations of leptin in the presence of LH or FSH and IGF-I, indicated a similar response to meiotic maturation and steroid synthesis at all concentrations of leptin tested. Therefore, the final dose of leptin chosen was in the high physiological range to evaluate the response of follicles to high leptin concentrations. After incubation, the follicles were recovered, opened with a 20-gauge needle to release the oocyte into Hepes–M199 medium with 0.1% BSA and 100 µmol IBMX l⁻¹ and the stage of meiosis determined by an independent observer using contrast microscopy. Oocytes with intact germinal vesicles were considered immature and those with germinal vesicle breakdown were considered to have resumed meiosis. Polar bodies were seen in some oocytes, which was considered to be equivalent to an observation of germinal vesicle breakdown.

DNA synthesis in granulosa cells. Immature rats were killed using ketamine–xylazine and their ovaries were placed in cold Hepes–M199 medium. Granulosa cells were aspirated using a fine mouth pipette to isolate sheets of granulosa cells into single cell populations and the cells were kept on ice in Hepes–M199 medium. Cells were centrifuged for 5 min at 1400 *g*, the media discarded and granulosa cells were resuspended in sufficient bicarbonate–M199 medium to result in a granulosa cell count of 1 × 10⁵ cells per well. Cell viability was determined by using Trypan blue and a haemocytometer. Culture was initiated when cell viability was > 50%. Granulosa cell cultures were plated in flat-bottomed 96-well dishes (Falcon®; Franklin Lakes, NJ) in 125 µl bicarbonate–M199 medium for 18 h, followed by a 6 h pulse of 0.8 µCi [³H]thymidine (20 Ci mmol⁻¹) (ICN, Costa Mesa, CA) as an indicator of granulosa cell DNA synthesis. DNA synthesis was assessed as described by Gilchrist *et al.* (2000). Data are expressed as proliferation relative to the control (c.p.m. per 1000 viable cells).

Granulosa cell culture. The procedure described by Zachow and Magoffin (1997) was followed exactly, except cells were cultured in M199 with 0.1% BSA. Granulosa cells were obtained from immature rats and approximately 60 000 cells plated per well. Designated wells were challenged with androstenedione ($0.1 \mu\text{mol l}^{-1}$) in the presence of leptin (0, 0.1, 10, 100, 600 ng ml^{-1}), FSH (100 mIU) with or without IGF-I (50 ng ml^{-1}) and cultured for 48 h.

Progesterone, oestradiol, prostaglandin and IL-1 β assays. Progesterone and oestradiol were analysed using a Johnson & Johnson Vitros ECI chemiluminescent immunoassay system (Orthoclinical Diagnostics; Amersham) with sensitivities of 0.3 nmol l^{-1} and 10 pmol l^{-1} , respectively. The progesterone and oestradiol assays had interassay coefficients of variation (CV) of $< 8\%$ and $< 6\%$, respectively. PGE₂ radioimmunoassay (RPA 530) and IL-1 β ELISA (RPN 2743) kits were obtained from Amersham and used according to the manufacturer's protocol. The sensitivities of the PGE₂ radioimmunoassay and IL-1 β ELISA were 1 and 5 pg ml^{-1} , respectively. The PGE₂ radioimmunoassay and IL-1 β ELISA assays had intra-assay CVs of $< 6\%$ and $< 9\%$, respectively.

Statistical analyses

The statistical analyses for all components of this study ($n \geq 6$ –7, unless stated otherwise) were performed using a parametric ANOVA. In all studies, statistical significance was accepted when the P value was < 0.05 .

Results

Experiment 1: effect of acute leptin treatment on infiltration of neutrophilic granulocytes and monocytes–macrophages into the ovary

As in previous studies (Brannstrom *et al.*, 1993, 1995), both neutrophils and monocytes–macrophages were distributed throughout the thecal interna layer of preovulatory follicles, but were virtually absent in the granulosa layer of these follicles. The numbers of thecal neutrophils (Fig. 1a) and monocytes–macrophages (Fig. 1b) were significantly lower ($P < 0.05$) in ovaries of rats treated with leptin and those subjected to feed restriction compared with the numbers in rats treated with saline. There was no significant difference among treatments in the percentage of positive cells in the stroma.

Experiment 2: effect of leptin treatment on factors important in ovulation (follicle culture for 4 h and 12 h)

Meiotic maturation. Meiotic maturation of cultured follicles was assessed after 4 and 12 h of incubation. The rate of meiotic maturation was not affected by leptin treatment, regardless of the duration of incubation (Fig. 2). Analysis of follicles revealed that meiotic maturation was

Fig. 1. The effects of exogenous leptin treatment on the infiltration of (a) neutrophilic granulocytes and (b) monocytes–macrophages into the theca interna of rat preovulatory follicles. Ovaries of rats treated with FSH + saline, FSH + leptin or FSH + feed restriction (Pair fed) were sectioned and stained immunohistochemically for (a) neutrophilic granulocytes and (b) monocytes–macrophages. Bars represent the mean $\pm$ SEM positivity (percentage brown stain of the total stain) in three theca interna regions of preovulatory follicles in both ovaries of each animal ($n = 3$ –4 rats per group). Asterisks indicate values that are significantly different from the FSH + saline group ($P < 0.05$).

not induced in control cultures or cultures with IGF-I or leptin, whereas culture with the gonadotrophins FSH and LH induced meiotic maturation of follicle-enclosed oocytes. Leptin did not inhibit meiotic maturation in any instance.

Steroid hormone, prostaglandin and IL-1 β release. Oestradiol and progesterone production was assessed after follicle culture for 4 and 12 h. Control, leptin or IGF-I treatments stimulated only low oestradiol production after 4 or 12 h of incubation (Table 1). The addition of the gonadotrophins FSH or LH stimulated oestradiol production at 4 and 12 h, regardless of whether IGF-I or leptin was added. Progesterone production was stimulated above control values when FSH was added; however, the stimulation was far greater when LH was added. Addition of IGF-I in combination with FSH enhanced progesterone production above that observed with FSH alone. Addition of leptin to the cultures did not influence progesterone production at either time point. PGE₂ concentrations were measured after 12 h of culture. The addition of LH, FSH, or FSH and IGF-I to the cultures enhanced PGE₂ production above basal values. The addition of leptin to cultures incubated with LH alone, FSH and IGF-I (FSH + IGF-I), or FSH, IGF-I and LH (FSH + IGF-I + LH) tended to decrease PGE₂ production,

Fig. 2. Percentage of germinal vesicle breakdown (GVBD) after culture of rat follicles for (□) 4 h or (■) 12 h with FSH (500 mIU), LH (100 ng ml⁻¹), insulin-like growth factor I (IGF-I) (50 ng ml⁻¹) or leptin (LEP) (300 ng ml⁻¹). Data are expressed as percentage GVBD $\pm$ SEM ($n = 30$ follicles per group).

Table 1. Analysis of culture media for oestradiol, progesterone and prostaglandin synthesis after culture of rat follicles for 4 and 12 h with insulin-like growth factor I (IGF-I), FSH, LH and leptin alone or in combination

	Control	IGF-I	FSH	LH	FSH + IGF-I	FSH + LH + IGF-I	Leptin	LH + leptin	FSH + IGF-I + leptin	FSH + LH + IGF-I + leptin
Oestradiol per follicle at 4 h (nmol l ⁻¹)	1.5 $\pm$ 0.2	1.48 $\pm$ 0.5	5.5 $\pm$ 2.1 ^a	6.5 $\pm$ 2.0 ^a	4.7 $\pm$ 0.4 ^a	6.8 $\pm$ 1.2 ^a	2.0 $\pm$ 0.7	6.1 $\pm$ 1.5 ^a	3.7 $\pm$ 0.8 ^a	6.4 $\pm$ 1.1 ^a
Progesterone per follicle at 4 h (nmol l ⁻¹)	0.2	0.3	3.2 $\pm$ 1.0 ^a	27.3 $\pm$ 4.3 ^b	5.4 $\pm$ 1.9 ^c	31.5 $\pm$ 5.4 ^b	0.9 $\pm$ 0.7	38.2 $\pm$ 2.1 ^b	4.4 $\pm$ 1.6 ^a	36.0 $\pm$ 2.0 ^b
Oestradiol per follicle at 12 h (nmol l ⁻¹)	3.0 $\pm$ 0.6	NT	5.8 $\pm$ 1.4 ^a	6.7 $\pm$ 2.2 ^a	6.7 $\pm$ 0.7 ^a	6.8 $\pm$ 0.6 ^a	4.4 $\pm$ 0.7	6.6 $\pm$ 2.0 ^a	6.1 $\pm$ 1.4 ^a	7.1 $\pm$ 1.3 ^a
Progesterone per follicle at 12 h (nmol l ⁻¹)	3.7 $\pm$ 1.9	NT	6.9 $\pm$ 1.5 ^a	21.6 $\pm$ 5 ^b	11.4 $\pm$ 2.4 ^c	23.0 $\pm$ 4.0 ^b	2.5 $\pm$ 0.7	21.4 $\pm$ 4.4 ^b	8.2 $\pm$ 3.0 ^a	27.3 $\pm$ 4.3 ^b
PGE ₂ per follicle at 12 h (ng ml ⁻¹)	0.09 $\pm$ 0.02	NT	1.2 $\pm$ 0.3 ^a	3.4 $\pm$ 0.5 ^a	1.6 $\pm$ 0.2 ^a	3.9 $\pm$ 0.6 ^a	0.1 $\pm$ 0.02	2.3 $\pm$ 0.6 ^a	1.0 $\pm$ 0.3 ^a	3.6 $\pm$ 0.5 ^a

Data are expressed as steroid synthesis $\pm$ SEM ($n = 6-7$). Letters indicate values that are significantly different from the remaining groups, as analysed by the Dunnett's multiple comparisons test ($P < 0.05$).

NT: not tested.

although this result did not reach significance. IL-1 β concentrations in the culture media of treated follicles were similar (66.8 ± 1.26 pg ml⁻¹) regardless of the culture treatment (data not shown).

DNA synthesis in granulosa cells. Incorporation of ³H into the DNA of cells challenged with FSH and IGF-I in the presence or absence of leptin was used to assess the effect of leptin on granulosa cell proliferation (Fig. 3). Addition of FSH enhanced proliferation above the basal value. IGF-I treatment or addition of leptin to cultures incubated with FSH and IGF-I did not alter cell proliferation (Fig. 3).

Granulosa cell culture. Granulosa cells were challenged with androstenedione (0.1 μ mol l⁻¹) in the presence of FSH, IGF-I and leptin. FSH induced both oestradiol and progesterone production above control values (Fig. 4). The addi-

tion of IGF-I with FSH induced oestradiol and progesterone production above values seen with FSH alone. There was no significant difference between treatment with FSH + IGF-I and treatment with FSH + IGF-I in the presence of 0.1 ng leptin ml⁻¹. Leptin concentrations above 10 ng ml⁻¹, in the presence of FSH and IGF-I, resulted in an inhibitory effect on oestradiol production, whereas progesterone production was unaffected.

Discussion

The inhibitory effects of high leptin concentrations on the ovary have been described in many studies. It has been demonstrated that chronic leptin treatment *in vivo* inhibits ovulation (Duggal *et al.*, 2000). The current study was designed to investigate the cause of the inhibition of ovulation reported previously.

Fig. 3. DNA synthesis in rat granulosa cells (relative to control) after treatment with FSH, insulin-like growth factor I (IGF-I), or FSH + IGF-I in the presence of various concentrations of leptin ($n = 4$). C: control. Asterisks indicate values that are significantly different from the control ($P < 0.05$).

Experiment 1 demonstrated that the number of neutrophils and monocytes–macrophages recruited at ovulation decreases significantly in response to a decrease in food intake, initiated by feed restriction or leptin treatment. In the study of Duggal *et al.* (2000), ovulation rates in pair fed rats were not significantly different from those in FSH/saline-treated controls and hence were unaffected by reduced food intake. Therefore, the reduction in the numbers of thecal neutrophils or monocytes–macrophages observed here is not likely to be the cause of the inhibition of ovulation observed in leptin-treated rats, as was hypothesized. The numbers of neutrophils and monocytes–macrophages found in the stroma were not affected by any of the treatments. Therefore, the decrease in infiltrating neutrophils and monocytes–macrophages in leptin-treated and feed-restricted animals was a specific event in the theca interna of preovulatory follicles in response to decreased food intake. The current data extend previous observations (Hudson *et al.*, 1999) that a small (5%) reduction in body weight is able to alter markedly the numbers of macrophages in the mouse uterus.

Other studies have shown that leucocytes are present in the ovarian follicle at ovulation (Brannstrom *et al.*, 1993, 1995; Norman *et al.*, 1997). The present study is the first to indicate that even a small decrease in food intake (approximately 3.1 g; Duggal *et al.*, 2000), in otherwise normal animals, results in a decrease in leucocyte infiltration of preovulatory follicles. The cause of the specific leptin-induced inhibition of ovulation in the study of Duggal *et al.* (2000), independent of calorific restriction, is not clear. Future studies will aim to ascertain the threshold value at which ovulation rate begins to decrease as a response to a decrease in infiltrating leucocytes.

Studies *in vitro* in dispersed ovaries treated with IGF-I indicate that leptin can impair LH action (Agarwal *et al.*, 1999). The results of Duggal *et al.* (2000) also indicated that

leptin may inhibit LH action in the ovary. Therefore, the aim of the second experiment was to investigate the effects of leptin on LH action in isolated rat follicles. Gonadotrophin-stimulated follicle culture produced similar concentrations of oestradiol and progesterone, as reported by Tornell *et al.* (1995). Leptin did not influence steroid synthesis when follicles were cultured in the presence of FSH, LH or IGF-I. These results contradict previous findings with isolated components of the ovary: granulosa cell cultures have clearly demonstrated that leptin has an adverse effect on the action of sensitizing agents, such as IGF-I. However, the culture system used in the present study provides a new dimension to current leptin research in the ovary. The culture system minimizes follicle–follicle interaction. Previous research has shown that co-culture of more than one follicle with direct contact between follicles results in the growth and development of one follicle while the growth of the others is depressed (Nayudu and Osborn, 1992; Spears *et al.*, 1996). In the present study, granulosa cells were cultured in the presence of androstenedione and leptin, and oestradiol and progesterone production were measured, to confirm previous findings and validate the activity of the hormones used in the study. The data confirm previous findings (Zachow and Magoffin, 1997) and show that culture of isolated granulosa cells with leptin inhibits oestradiol production, which is a different result from that found with whole cultured follicles.

In agreement with other studies (Gutierrez *et al.*, 1997), in the present study, physiological concentrations of FSH led to proliferation of granulosa cells, as indicated by DNA synthesis, and the inclusion of IGF-I also enhanced proliferation. As reported in other studies, granulosa cell proliferation was unaffected by leptin treatment (Spicer *et al.*, 1997, 1998). The results indicate that high leptin concentrations inhibit ovulation via a pathway other than that of granulosa cell DNA synthesis. Systemic changes in response to food

Fig. 4. Concentration of the steroid hormones (a) oestradiol and (b) progesterone produced by rat granulosa cells challenged with androstenedione after treatment with FSH, insulin-like growth factor (IGF-I), or FSH + IGF-I in the presence of various concentrations of leptin ($n = 4$). C: control. Asterisks and letters indicate values that are significantly different from the remaining groups ($P < 0.05$).

availability and hence the concentration of leptin are reflected in parallel to changes that occur in follicular fluid, essentially as the result of leptin diffusion from the blood. This follows as leptin concentrations in blood and follicular fluid correlate in both humans (Agarwal *et al.*, 1999) and pigs (Galeati *et al.*, 2000). Leptin treatment (10 ng ml⁻¹) of oocytes retrieved from prepubertal gilts led to a reduction in the number of oocytes with germinal vesicles from 60 to 33% (Galeati *et al.*, 2000). Preliminary data on the culture of mouse oocytes *in vitro* in this laboratory also indicate that leptin inhibits germinal vesicle breakdown. However, the data show that the meiotic competence of oocytes is not compromised by high concentrations of leptin in rats, indicating that follicle-enclosed oocytes will respond to gonadotrophin stimulus regardless of the concentration of leptin. The results indicate that there is an important species difference in the way leptin interacts with oocytes.

The importance of prostaglandins in ovulation has been established (Espey and Lipner, 1994). PGE and PGF begin to increase in ovarian follicles during the first few hours of the ovulatory process. These prostaglandins reach a peak at the time of follicular rupture, and concentrations decline and return to preovulatory values after ovulation. Prostaglandins have roles in pro- and anti-inflammation. Therefore it was hypothesized that chronic leptin treatment may diminish the prostaglandin concentrations at the time of ovulation. Leptin tended to decrease prostaglandin production; however, future studies will investigate how ovulation is inhibited,

as the mechanism of action remains uncertain. The data indicate that high leptin concentrations are able to exert a direct inhibitory effect on ovarian function by inhibiting PGE₂. However, treatment of neonatal rat hypothalami with leptin appears to stimulate the production of PGE₂ (Brunetti *et al.*, 1999).

The processes within the ovary that follow the LH surge and lead to ovulation have been well characterized (Norman *et al.*, 1996, 1997, 1999). LH initiates an increase in IL-1 β production as well as the induction of a variety of other cytokines, collagenases, plasminogen activators and enzymes (Norman *et al.*, 1999). Follicular fluid contains detectable concentrations of immunoactive cytokines, including interleukins, and granulosa luteal cells are capable of producing detectable concentrations of interleukins (Norman *et al.*, 1996). In the present study, the concentration of IL-1 β in the follicle culture media was similar among treatments, indicating that IL-1 β was not secreted from the follicles into the media. Future experiments are required to investigate the effects of leptin on IL-1 β production within ovarian follicles.

In summary, this study demonstrated that the number of neutrophils and monocytes-macrophages recruited at ovulation decreases significantly in response to a decrease in food intake, initiated by feed restriction or leptin treatment. The effect of high leptin concentrations *in vitro* was also investigated. High concentrations of leptin were unable to inhibit germinal vesicle breakdown in oocytes of

gonadotrophin-stimulated enclosed follicles. Steroid synthesis was unaffected by leptin, whereas IL-1 β concentrations were similar regardless of the treatment. Prostaglandin release was marginally inhibited by leptin treatment, whereas DNA synthesis in granulosa cells was unaffected. Leptin treatment of isolated granulosa cells cultured in the presence of androstenedione, FSH and IGF-I had an inhibitory effect on oestradiol production. Further investigations (Duggal *et al.*, 2002) will concentrate on the effects of pharmacodynamic and physiological concentrations of leptin throughout the oestrous cycle on ovarian leptin receptors.

The authors are grateful to Rob Gilchrist and Lou Warnes for their insight during the course of this work. They also thank Jennifer Hayes, Carole Woodhouse and Clyde Milner for technical expertise throughout these investigations. This research was funded by the National Health & Medical Research Council (Australia). A portion of this research was presented at the 14th Annual Meeting of the Australian Society for Reproductive Biology. P. Duggal is supported by a Reproductive Medicine Laboratories Postgraduate Scholarship, The University of Adelaide.

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Received 17 September 2001.

First decision 18 December 2001.

Revised manuscript received 8 February 2002.

Accepted 14 February 2002.