REPRODUCTION
RESEARCH

Direct *in vivo* effects of leptin on ovarian steroidogenesis in sheep

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Abstract

Leptin, the metabolic fat hormone, has been shown to have effects on reproduction in mice and to modulate steroid production by cultured ovarian somatic cells in a number of species. However, a direct role of leptin on normal ovarian function *in vivo* has not been shown. In this paper the effect of passive immunisation against leptin (experiment 1; 20 ml antiserum or non-immune plasma i.v.; $n = 6$/treatment) and direct ovarian infusion of leptin (experiment 2; 0, 2 or 20 μg recombinant ovine leptin; $n = 4$/treatment) during the early follicular phase was investigated in sheep with ovarian autotransplants, which allow recovery of ovarian venous blood and regular non-invasive scanning of the ovary.

Passive immunisation against leptin resulted in an acute increase ($P < 0.05$) in ovarian oestradiol secretion but had no effect on gonadotrophin concentrations, ovulation or subsequent luteal function. Conversely, direct ovarian arterial infusion of the low dose of leptin resulted in an acute decline ($P < 0.05$) in ovarian oestradiol secretion whereas the high dose, which resulted in supra-physiological leptin concentrations, had no effect on oestradiol production compared with the controls. Neither dose of leptin had any effect on gonadotrophin concentrations or ovulation but both doses resulted in an increase ($P < 0.05$) in progesterone concentrations over the subsequent luteal phase.

In conclusion, together these data provide strong *in vivo* evidence that leptin can modulate ovarian steroidogenesis directly and acutely in ruminants and suggest that leptin is an alternate regulatory system whereby nutritional status can regulate reproductive activity.

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Introduction

Level of nutrition is one of the most important environmental factors leading to subfertility and even infertility in both humans and farm animals. Severe under-nutrition is associated with anovulatory infertility (e.g. anorexia nervosa; Katz & Vollenhoven 2000) whereas over-nutrition and obesity are generally associated with impaired folliculogenesis, reduced oocyte quality and embryonic failure (Crosignani *et al.* 1994, Norman & Clark 1998, Bellver *et al.* 2003). Further, the duration and severity of post-partum anovulation is directly associated with the nutritional status of the mother and the amount of nutritional supplementation (milk) provided to the suckling young (McNeilly *et al.* 1983, Butler & Smith 1989). Finally, nutrition profoundly affects the presentation and natural history of polycystic ovary syndrome, which is known to affect 5–10% of women of reproductive age and is the most common cause of anovulatory infertility (Franks 1995). While the effects of nutrition on reproduction are generally well described, the physiological mechanisms that underlie these observations are poorly understood.

Over recent years, research in domestic ruminants has examined the endocrine and cellular mechanisms underlying the effect of nutrition on ovarian follicle and oocyte development, early embryo development and uterine receptivity. These studies confirm that nutrition modulates reproductive activity at multiple levels through changes in both circulating metabolic hormones (e.g. growth hormone, insulin, insulin-like growth factor (IGF-I)), gene expression and the synthesis of local paracrine and

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autocrine factors (e.g. IGF-II, IGF-binding protein) that modulate the action of the gonadotrophins. Although the IGF/insulin system has been well characterised in ruminants (Webb *et al.* 2003), an alternate, not so well-characterised hormone that links reproduction with nutritional status is leptin.

Leptin, a 16 kDa polypeptide with 146 amino acids and a disulphide linkage between Cys 96 and Cys 146, is secreted primarily by adipocytes and the blood concentration of leptin is positively correlated with the total amount of body fat. Studies have led to the cloning of the gene for the leptin receptor from the mutant db/db mouse (Tartaglia *et al.* 1995, Chen *et al.* 1996) and the Zucker fa/fa rat (Chen *et al.* 1996). Both the fa/fa rat and the db/db mouse are obese and infertile. Subsequent research has shown that there are six splice variants of the leptin receptor, designated Ob-Ra, Ob-Rb, Ob-Rc, Ob-Rd, Ob-Re and Ob-Rf. The Rb receptor is the long form and appears to be the main mediator of the cellular actions of leptin; the others are short forms and may act as leptin carriers and transporters. The leptin receptor gene is expressed in several regions of the brain including the hypothalamus, choroid plexus and brain stem; it is also expressed in the lungs, kidneys (Tartaglia *et al.* 1995) and ovary (Cioffi *et al.* 1997, Karlsson *et al.* 1997), including granulosa cells (Cioffi *et al.* 1997, Karlsson *et al.* 1997), thereby implicating the follicle as a normal target for the action of leptin. Leptin binds to cell surface receptors and signals by the STAT/JAK signal transduction pathway (Ghilardi *et al.* 1996, Vaisse *et al.* 1996).

Leptin has long been known to have a role in reproduction, because the sterility of the ob/ob mouse can be reversed by the administration of exogenous leptin (Berisha *et al.* 1996, Malik *et al.* 2001). Leptin receptors have been observed in both the granulosa and theca cells of the human (Cioffi *et al.* 1997, Karlsson *et al.* 1997) and cattle (Spicer & Francisco 1997, 1998). Leptin mRNA itself was not found in human follicles Karlsson *et al.* (1997), suggesting the ovarian follicle to be a signal receiver. Subsequently, both leptin and leptin receptor mRNA have been found in the granulosa cells of human follicles (Loffler *et al.* 2001), although Karamouti *et al.* (2003) found no expression of leptin mRNA in cultured human luteinized granulosa cells. Recent data from sheep have shown that mRNA for both leptin and leptin receptor can be detected in the membrana granulosa cell layer of antral follicles (M Munoz-Gutierrez & R J Scaramuzzi, unpublished observations), suggesting that leptin is a local factor in the control of ovarian function in this species. Studies in a number of species using *in vitro* cell culture have shown that leptin is generally inhibitory to steroidogenesis in both granulosa (bovine: Spicer & Francisco 1997, Spicer *et al.* 2000, rodent: Zachow & Magoffin 1997, Zachow *et al.* 1999, human: Brannian *et al.* 1999) and theca (bovine: Spicer & Francisco 1998, human: Agarwal *et al.* 1999) cells. These data provide further evidence that leptin has a direct role in controlling ovarian function. However, to date, the only direct evidence of an effect of leptin on ovarian function *in vivo* is the demonstration by Duggal *et al.* (2000) that leptin depressed ovulation rate in immature gonadotrophin-primed rats.

The existing evidence therefore supports the hypothesis that leptin can control follicular function by directly modulating the sensitivity of ovarian somatic cells to gonadotrophic stimuli (follicle-stimulating hormone (FSH) and luteinising hormone (LH)). Because leptin can act centrally on the hypothalamo–pituitary axis to modulate gonadotrophin secretion it is possible that leptin may be exerting effects both centrally and locally. In the present experiment, we have utilised ewes with ovarian autotransplants to examine the *in vivo* effects of leptin on ovarian steroidogenesis and follicle growth. This experimental model allows the infusion of test substances, such as locally active growth factors (Campbell *et al.* 1994), metabolic factors (Campbell *et al.* 1995*a*) or hormones (Campbell *et al.* 1995*b*, Campbell & Scaramuzzi 1995), directly into the ovary to expose follicular cells to high local concentrations without greatly increasing peripheral concentrations. Further, the preparation allows repeated collection of ovarian venous blood from conscious animals and transdermal ultrasound scanning of the ovary, therefore greatly facilitating the assessment of the effects of treatment on ovarian steroidogenesis and follicle development.

Materials and Methods

Experimental animals

Mature, 4- to 8-year-old Merino cross Scottish Blackface (experiment 2) and Merino cross Finnish Landrace (experiment 1) ewes with ovarian autotransplants were used (Goding *et al.* 1967). Experiment 1 was carried out during anoestrus (March) and ewes were treated with intravaginal progestagen impregnated sponges (medroxy-progesterone-acetate (MAP); Dunlop, Dumfries, UK) inserted for 10 days prior to the start of blood sampling. Experiment 2 was carried out during the breeding season (January) and oestrous cycles were synchronised using MAP-impregnated sponges inserted for 10 days with injection of cloprostenol (Estrumate; Intervet, Cambridge, Cambs, UK) at the time of sponge withdrawal (0 h). In both experiments, the absence or presence of oestrous behaviour prior to sponge insertion was confirmed by inclusion of a keeled Scottish Blackface Merino cross ram within the flock.

On the day prior to the start of blood sampling all animals had cannulae inserted into both jugular veins and the carotid artery (experiment 2 only) as described previously (Campbell *et al.* 1994). Following cannulation all sheep were placed in metabolism crates in ventilated rooms and treated prophylactically with antibiotics (3 ml/3 days i.m.; Clamoxil; SmithKline Beecham, Weybridge, Surrey, UK) and heparin (experiment 2, 5000 IU/12 h i.v.; Leo Laboratories, Aylesbury, Bucks, UK). The sheep were

well habituated to these housing conditions and frequent handling.

Experiment 1: passive leptin immunisation

Leptin antibody

Nine ewes were immunised against 100 μg recombinant human leptin (R&D systems Inc., Minneapolis, MN, USA) in Freund's complete adjuvant and subsequently boosted monthly with leptin (100 μg) in Freund's incomplete adjuvant on four occasions. Antibody titres were determined monthly, using ^{125}I-labelled human leptin as a tracer incubated with serial dilutions of plasma as previously described (Webb *et al.* 1985), with the animals slaughtered after the last immunisation. The serum was collected and titrated and the serum that gave the highest antibody titre (sheep 6E-546: 54% binding at 1:10 000 dilution) was used for the passive immunisation study. The specificity of this antibody was confirmed by Western blot analysis which showed that the leptin antibody was able to bind ovine leptin, which migrated in the gel identically to human leptin (C G Gutierrez, unpublished observations).

Experimental design

Twelve Merino cross ewes, with ovarian autotransplants, were stimulated with a pulsatile regime of gonadotrophin-releasing hormone (GnRH) injections, designed to mimic the normal follicular phase and induce ovulation (Campbell & Scarmuzzi 1996). Briefly, following sponge withdrawal all ewes received native GnRH injections (150 ng i.v.; Sigma Chemical Co., Poole, Dorset, UK), at 3-hourly intervals for the first 12 h, 2-hourly intervals for the following 12 h and hourly intervals for the following 36 h. These injections were delivered in a volume of 1 ml over 1 min via a jugular venous cannula which was connected to a syringe-driven pump controlled by a timer.

Ewes were treated with a single 20 ml i.v. bolus injection of either non-immune serum ($n = 6$) or leptin antiserum ($n = 6$) 12 h after sponge withdrawal. Samples of ovarian venous blood (5 ml) were taken every 4 h from 12 h prior until 80 h after sponge withdrawal. In addition, in order to examine the pulsatile pattern of pituitary LH release and ovarian steroid secretion, more frequent blood samples were taken at 10-min intervals between 23.5 and 26 h after sponge withdrawal. Finally, jugular venous blood samples (5 ml) were taken every day for the 7 days after the end of the intensive experimental period in order to assess post-ovulatory luteal function.

Experiment 2: ovarian arterial infusion

Infusion

Recombinant ovine leptin (Gertler *et al.* 1998) was prepared as a stock solution at a concentration of 1 mg/ml in 0.9% (w/v) saline with 0.1% (v/v) normal sheep plasma and sterilised by filtration through a 0.22 μm filter. This stock solution was used to prepare the infusate at two dose levels, 6.7 and 0.67 μg/ml, in normal saline with 1% normal sheep plasma. Control (0 μg/ml) sheep were infused with normal saline with 1% normal sheep plasma. Solutions were infused into the carotid artery at a flow rate of 3 ml/h to give nominal leptin infusion rates of 20, 2 and 0 μg/h, using syringe-driven pumps fitted with plastic 50 ml syringes. Blood flow was directed to the ovary by occlusion of the carotid artery cranial to ovarian carotid arterial anastomosis using a paediatric sphygmomanometer (Downs Surgical, Mitcham, Surrey, UK) inflated to a pressure of 200 mmHg as previously described (Campbell *et al.* 1994).

Experimental design

Arterial infusion was performed over a 12-h period during the early follicular phase, 9–21 h after prostaglandin-induced luteal regression. This time-period was chosen as previous studies have shown that this is when follicular selection normally occurs (Souza *et al.* 1997*a*). The three treatment groups were: (i) vehicle only controls ($n = 4$), (ii) low dose of leptin at 2 μg/h ($n = 4$) and (iii) high dose of leptin at 20 μg/h ($n = 4$). Samples of ovarian venous blood (5 ml) were taken every 6 h from 6 h prior until 84 h after prostaglandin. In addition, there were three periods of more frequent blood sampling (10-min intervals), designed to examine the effect of infusion on the pulsatile pattern of pituitary LH release and ovarian steroid secretion. These were carried out immediately prior to infusion (6–8.5 h post prostaglandin), during the infusion (15–17.5 h post prostaglandin) and immediately after the infusion (21–23.5 h post prostaglandin). Samples of jugular venous blood (5 ml) were taken at the start, middle and end of the infusion period in order to estimate the effect of infusion on peripheral concentrations. Finally, jugular venous samples (5 ml) were taken every day for the 7 days after the end of the intensive experimental period in order to assess post-ovulatory luteal function.

Hormone assays

Blood samples were centrifuged at 4 °C and 3000 ***g*** for 20 min to obtain plasma which was stored at −20 °C. In experiment 1, oestradiol, LH, FSH and progesterone concentrations were determined by RIA (Souza *et al.* 1997*b*). In experiment 2, plasma samples were assayed for oestradiol by double-antibody RIA (Webb *et al.* 1985) after extraction with diethyl ether (Fisher Chemicals, Loughborough, Leics, UK). Intra- and interassay coefficients of variation were less than 6.4 and 14.1% respectively. LH concentrations in samples from experiment 2 were determined by RIA using reagents supplied by the NIH. Ovine LH (NIDDK-NIH AFP9598B) was used for iodinations and preparation of the standard curve. NIDDK-anti-ovine LH-1 (AFP-192279) was used at a final dilution of 1:250 000 and under these conditions the sensitivity of this assay was 0.16 ng/ml with intra- and interassay coefficients of variation of 14.8 and 20.0% respectively. Progesterone

was assayed directly using the method described by Souza *et al.* (1997*b*) with intra- and interassay coefficients of variation of 13 and 10% respectively. Leptin was assayed within one RIA (Blache *et al.* 2000), which had an intra-assay coefficient of variation of 18.7%.

Ovarian scanning procedure

In order to estimate ovulation rate animals were scanned 7 days after the end of intensive blood sampling in both experiments (Souza *et al.* 1997*a*,*b*, Campbell *et al.* 1998) using a real-time Aloka 500 ultrasound scanner with a linear 7.5 MHz transducer probe (Dynamic Imaging, Livingston, West Lothian, UK).

Statistical analyses

All hormone data was log transformed prior to statistical analysis. The oestradiol profiles were normalised to the peak of the preovulatory LH surge in both experiments. Hormone profile data was analysed using repeated measures ANOVA. The characteristics of pulsatile LH and oestradiol secretion were determined using the Munro pulse analysis program (Zaristow software, Haddington, East Lothian, UK). Analysis of pulse characteristics in experiment 1 was performed by unpaired *t*-test. In experiment 2, analysis during and post-infusion was carried out on SPSS 10 (SPSS UK Ltd, Woking, Surrey) using univariate ANOVA with preinfusion pulse characteristics as covariates with individual group differences quantified using the Bonferroni test.

Results

Experiment 1

LH pulsatility, ovulation and subsequent luteal function

GnRH injections induced normal LH pulses with amplitudes that did not differ between treatment groups (2.5 ± 0.5 vs 1.7 ± 0.2 ng/ml in controls and immunised respectively). All animals responded to this regime and exhibited an LH surge of normal magnitude and duration which was not significantly different between treatments that occurred at 37.3 ± 0.8 and 38.0 ± 1.6 h after sponge withdrawal in controls and immunised ewes respectively. All ewes ovulated (assessed by progesterone profiles) and had normal patterns of progesterone over the 7 day post-ovulatory period. There were no treatment effects or treatment by time interactions in progesterone concentrations (data not shown) and ultrasound scanning confirmed that there was no difference between groups in the number of corpora lutea (1.9 ± 0.3 vs 1.8 ± 0.2 in controls and immunised respectively).

Ovarian oestradiol secretion

In response to the stimulatory GnRH regime, oestradiol concentrations in ovarian venous plasma increased with time in both treatment groups ($P < 0.001$) to a peak that occurred 4 h prior to the peak of the LH surge (Fig. 1). Oestradiol concentrations did not differ between treatment groups over the period before passive immunisation, but thereafter were significantly higher ($P < 0.05$) in immunised animals until the occurrence of the LH surge (Fig. 1). No treatment effect was observed in the period following the LH surge. Examination of the pulsatile pattern of oestradiol secretion revealed that immunisation resulted in an increase ($P < 0.05$) in both pulse amplitude (1.4 ± 0.3 vs 3.2 ± 0.2 nmol/l) and mean concentration (1.9 ± 0.6 vs 3.5 ± 0.5 nmol/l) in controls and immunised ewes respectively.

Experiment 2

Leptin concentrations in venous blood

Leptin concentrations in jugular venous blood at the start of the experimental period ranged between 1.2 and

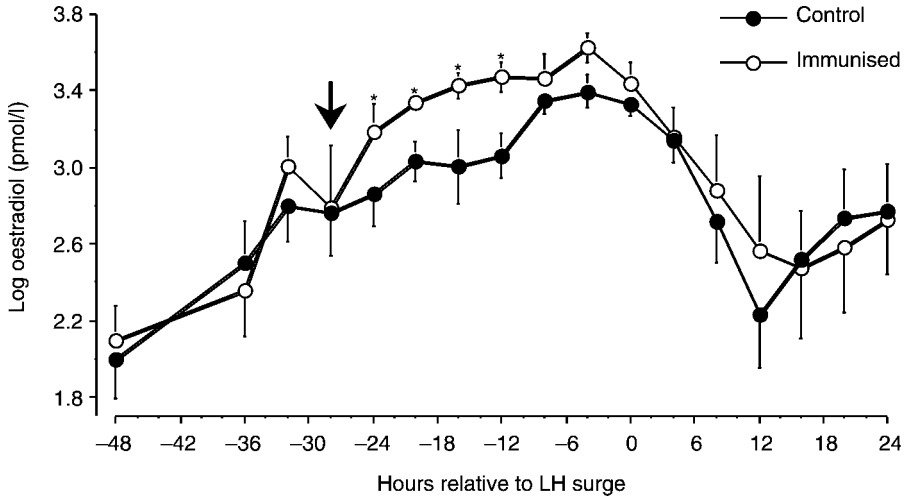

Figure 1 Experiment 1: mean ± S.E.M. concentrations of oestradiol in the ovarian venous plasma of ewes which received an intravenous 20 ml bolus injection (indicated by the arrow) of either non-immune (●; $n = 6$) or leptin antisera (○; $n = 6$) during the early follicular phase. Data have been normalised to the peak of the preovulatory LH surge. $*P < 0.05$ compared with controls at the same time-point.

2.2 ng/ml and there were no differences between treatment groups (Fig. 2). Preinfusion leptin concentrations in ovarian venous blood ranged between 2 and 2.9 ng/ml and did not differ from jugular venous concentrations, indicating negligible leptin secretion by the ovary. There were no preinfusion differences in leptin concentrations in ovarian venous blood between treatment groups. Infusion of the high dose of leptin resulted in a 30-fold increase ($P < 0.05$) in leptin concentrations in ovarian venous blood over controls during the infusion period and, although concentrations were still elevated 12 h after the end of infusion, this effect was not significant. By 42 h after the end of the infusion, leptin concentrations had returned to normal. In contrast, concentrations of leptin in ovarian venous blood from ewes receiving the low dose of leptin did not differ from the controls at any time over the infusion period (Fig. 2).

Ovarian oestradiol secretion

In control animals, oestradiol concentrations in ovarian venous plasma exhibited the expected increase with time after prostaglandin ($P < 0.001$), to a peak that occurred just prior to the LH surge (Fig. 3). Oestradiol concentrations in ewes which received an arterial infusion of the low dose of leptin did not differ from controls over the preinfusion period but fell over the infusion period, deviating significantly ($P < 0.05$) from controls during the infusion period and tending ($P < 0.1$) to remain lower for the remainder of the follicular phase (Fig. 3a). In contrast, oestradiol secretion did not differ from controls in the ewes that received the high dose of leptin at any time-point (Fig. 3b).

Examination of the pulsatile pattern of oestradiol secretion over the infusion period confirmed the responses detected by 6-hourly blood samples with decreased oestradiol during the infusion period for the low dose sheep. These results are presented in Table 1 and show that oestradiol pulse amplitude, pulse area and mean level were all significantly lower ($P < 0.05$) during the infusion period in ewes that received the low dose of leptin, when compared with controls. However, infusion of the high dose of leptin had no effect on the parameters of pulsatile ovarian oestradiol secretion (Table 1). There were no effects of any treatment on oestradiol pulse interval (Table 1) and there was no effect of treatment on the pulsatile pattern of LH secretion (data not shown).

Ovulation and subsequent luteal function

There were no treatment effects on LH or FSH concentrations (data not shown) and all ewes exhibited a normal preovulatory gonadotrophin surge that occurred within 58–67 h of prostaglandin (58–67, 58–61 and 58–61 for controls, low and high dose respectively). All ewes exhibited an increase in peripheral progesterone concentrations in the period following the LH surge ($P < 0.001$), indicating ovulation, but progesterone concentrations were significantly elevated over controls ($P < 0.05$) in both treatment groups between days 6 and 9 of the subsequent luteal phase (Fig. 4). There were no differences in progesterone concentrations between the low and high leptin infusion group. Ultrasound scanning confirmed the presence of corpora lutea and there were no differences between groups in mean ovulation rate (1.50 ± 0.58,

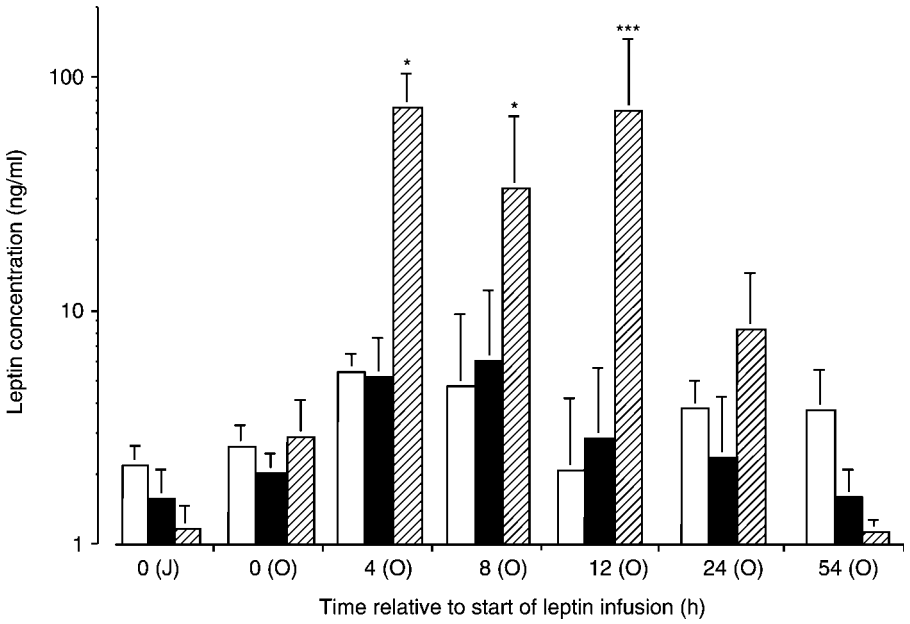

Figure 2 Experiment 2: mean ± S.E.M. concentration of leptin in either jugular (J) or ovarian (O) venous plasma in ewes infused with carrier (□; $n=4$), 2 μg/h leptin (■; $n=4$) or 20 μg/h leptin (▨; $n=4$) via the ovarian artery over a 12-h period during the early follicular phase, 9–21 h after induction of luteal regression. $^*P < 0.05$ and $^{***}P < 0.001$ when compared with both control and low dose groups at the same time-point.

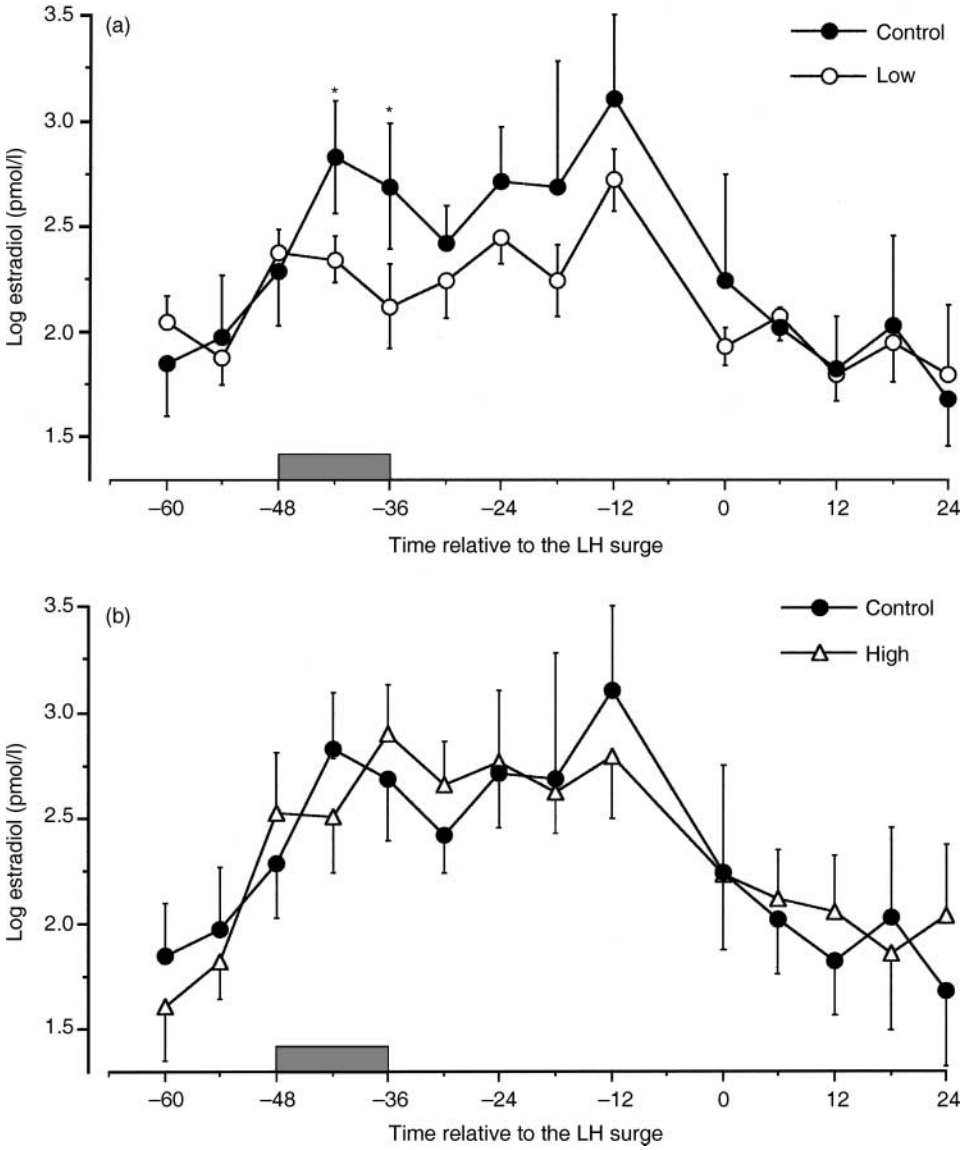

Figure 3 Experiment 2: mean ± S.E.M. concentrations of oestradiol in the ovarian venous plasma of ewes which received an ovarian arterial infusion of (a) low (2 μg/h; ○) or (b) high (20 μg/h; △) dose of leptin compared with control animals (carrier; ●). Data have been normalised to the peak of the preovulatory LH surge and the horizontal bars indicate the period of leptin infusion. $*P < 0.05$ compared with controls at the same time-point.

1.50 ± 0.58, 1.75 ± 0.50 for control, low and high infused groups respectively).

Discussion

The results of this experiment showed that passive immunisation against leptin during the follicular phase of the sheep oestrous cycle increased ovarian oestradiol secretion, whereas direct ovarian exposure to physiological doses of leptin at the same stage of the cycle depressed ovarian oestradiol secretion although a supraphysiological dose did not. These changes in ovarian steroidogenesis occurred without any detectable change in the level of gonadotrophic stimulation (plasma FSH and LH concentrations). Together, these data provide strong *in vivo* evidence that leptin can modulate ovarian function directly.

These experiments showed that physiological doses of leptin have inhibitory actions on ovarian somatic cell steroidogenesis *in vivo*. This result therefore supports the results of a number of studies that have reported an inhibitory effect of leptin on steroidogenesis by cultured granulosa cells (Spicer & Francisco 1997, Zachow & Magoffin 1997, Agarwal *et al.* 1999, Zachow *et al.* 1999). The dose effects of leptin on somatic cell steroidogenesis, however, are complex and seem to differ between the *in vivo* and *in vitro* situation. Plasma concentrations of leptin are generally below 10 ng/ml (Delavaud *et al.* 2000, León *et al.*

Table 1 Parameters of pulsatile ovarian oestradiol secretion during and after infusion of low leptin (2 μg/h), high leptin (20 μg/h) and control carrier infusion. Values are means± S.D.

	Control	High	Low	*P*
Mean pulse interval (min)				
Infusion	36.3 ± 11.8	31.7 ± 9.3	28.5 ± 6.3	0.556
Post infusion	42.1 ± 18.7	36.4 ± 10.7	28.3 ± 4.6	0.318
Mean pulse amplitude (ng/ml)				
Infusion	2383 ± 2448	2213 ± 1767	155 ± 99	0.025
Post infusion	1830 ± 1533	2213 ± 3601	197 ± 93	0.252
Mean pulse area (arbitary units)				
Infusion	42566 ± 46932	34373 ± 28099	2388 ± 1714	0.027
Post infusion	35602 ± 26641	52578 ± 92422	3072 ± 1296	0.220
Mean measured level (ng/ml)				
Infusion	1143 ± 929	1609 ± 1731	209 ± 100	0.029
Post infusion	1828 ± 1957	1546 ± 2469	252 ± 170	0.187

2004) and concentrations of leptin above this physiological level have been generally shown to inhibit steroid production *in vitro* when IGF-I is either absent or present at low concentrations (Spicer & Francisco 1997, Zachow & Magoffin 1997, Agarwal *et al.* 1999, Zachow *et al.* 1999, Campbell *et al.* 2000, Ruiz-Cortes *et al.* 2003). In contrast, doses of leptin within the physiological range have also been reported to have a stimulatory effect on steroidogenesis by both granulosa (Campbell *et al.* 2000) and theca cells (Rodriguez A & C G Gutierrez, unpublished observations) when IGF-I is either absent or present at low concentrations. In contrast, in the current *in vivo* study we found that the dose of leptin that resulted in physiological concentrations of leptin (2 μg/h) had an inhibitory effect on ovarian steroidogenesis, whereas the high dose of leptin (20 μg/h) which produced plasma concentrations of leptin well above the normal physiological range (supraphysiological) had no effect on ovarian oestradiol secretion. The reason for this difference between dose effects *in vivo* and *in vitro* is unclear, but these results do illustrate the importance of working within the normal physiological range and the value of confirming the validity of cell culture studies by direct animal experiments utilising models such as the ovarian autotransplant. The most likely explanation for the lack of effect of infusion of supraphysiological concentrations of leptin on ovarian steroidogenesis is that greatly elevated plasma concentrations of leptin activate alternate pathways which stimulate ovarian function. Examples of such pathways would include the hypothalamic–pituitary axis (see below), the insulin/IGF axis (Williams *et al.* 2002) and the renin angiotensin system (Fortuno *et al.* 2002). The supraphysiological dose of leptin may also down-regulate the leptin receptor as has been seen with down-regulation of leptin receptor in adrenal glands when leptin was administered to rats (Tena-Sempere *et al.* 2000).

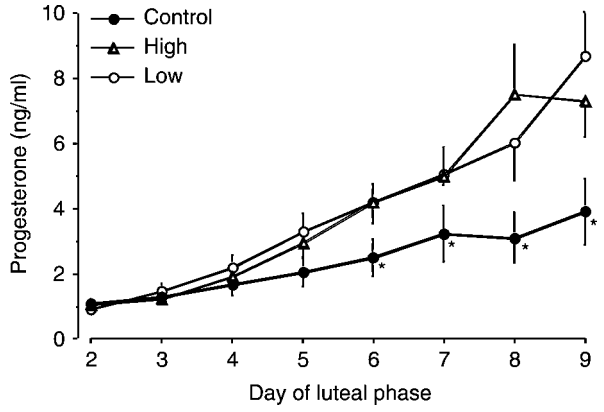

Figure 4 Experiment 2: mean± S.E.M. concentrations of progesterone in the jugular venous plasma over the subsequent luteal phase of ewes which received an infusion of carrier (●), low dose leptin (2 μg/h; ○) or high dose leptin (20 μg/h; △) via the ovarian artery during the early follicular phase. $*P < 0.05$ for both treatment groups compared with controls at the same time-point.

In addition to direct effects on the ovary, leptin may also effect pituitary gonadotrophin release through hypothalamic receptors (Williams *et al.* 2002). As LH is the primary drive for ovarian steroidogenesis (Baird & Scaramuzzi 1976), these central mechanisms offer an alternate means by which leptin could modulate ovarian steroid secretion. In the present experiments, however, there is little evidence to support this possibility because experiment 1 utilised exogenous GnRH to control the pattern of LH stimulation to the ovary and in experiment 2 there was no effect of intra-ovarian leptin on the pulsatile pattern of LH secretion, even in animals exposed to the high dose where marked elevations in leptin concentrations in blood were observed.

A striking feature of the effects of immunisation against leptin (experiment 1) and infusion of leptin (experiment 2) on ovarian oestradiol secretion was that these effects were extremely acute and did not persist beyond the period of immediate exposure, so that induction of the LH surge and ovulation occurred at the normal time in treated

animals. This suggests that leptin is modulating ovarian steroid secretion directly, rather than by inducing changes in the developmental potential of the ovulatory follicle, as supported by *in vitro* data.

An unexpected and surprising result was the effect of leptin infusion on subsequent luteal function. As ovulation rate did not differ between groups, it appears that follicular phase leptin infusion resulted in an increase in the steroidogenic capacity of the corpus luteum. It has been reported that the corpus luteum in pigs (Ruiz-Cortes *et al.* 2000) and cattle (Nicklin *et al.* 2004) express leptin receptor but, as exposure occurred in the follicular phase in the present experiment, these data suggest that exposure of the somatic cells of the ovulatory follicle to leptin affects the steroidogenic potential of these cells following transformation into luteal cells. Leptin could aid the development of the corpus luteum in a number of ways: leptin enhances the expression of StAR by luteinising granulosa cells (Ruiz-Cortes *et al.* 2003); it has been observed that expression of leptin receptor (Ob-Rb) is highest during dioestrus (Duggal *et al.* 2002); leptin binding increases parallel to progesterone production (Ruíz-Cortes *et al.* 2000); leptin antagonising the vasoconstrictor effect of angiotensin II (Fortuno *et al.* 2002) could directly increase the blood flow to the corpus luteum (Berisha *et al.* 2002, Schams *et al.* 2003). To our knowledge, this is the first report of an increase of progesterone production after priming of a preovulatory follicle with leptin. However, the numbers of animals used within this study were low and could be prone to type II errors. Further experiments are therefore required to confirm this observation and to subsequently examine possible mechanisms.

In conclusion, this work provides strong *in vivo* evidence that leptin can modulate ovarian steroidogenesis directly and acutely in ruminants and suggests that leptin is an alternate regulatory system whereby nutritional status can regulate reproductive activity.

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