The ovarian expression of mRNAs for aromatase, IGF-I receptor, IGF-binding protein-2, -4 and -5, leptin and leptin receptor in cycling ewes after three days of leptin infusion

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Abstract

An experiment was carried out to determine the pattern of follicular expression of mRNAs for aromatase, IGF-I receptor (IGF-IR), IGF-binding protein (IGFBP)-2, -4 and -5, leptin and the long form of the leptin receptor (Ob-Rb) in ten ewes infused with human recombinant leptin ($n = 5$; 1 µg/h) or saline ($n = 5$) for 72 h in the luteal phase of the oestrous cycle. At the end of infusion a follicular phase was induced with a luteolytic dose of a prostaglandin F2α analogue and the ovaries were collected 32 h later. One ovary from each ewe was serially sectioned at 10 µm using a cryostat at -20 °C. All follicles >1 mm in diameter were counted and probed with specific oligoprobes for aromatase, IGF-IR and IGFBP-2, -4 and -5 and specific riboprobes for leptin and Ob-Rb. Leptin mRNA was detected in theca and granulosa cells and Ob-Rb mRNA was detected only in granulosa cells, of some, but not all antral follicles. Leptin doubled the number of follicles with a diameter ≥ 3.5 mm (1.0 ± 0.36 (S.E.M.) vs 2.4 ± 0.24 ; control vs leptin; $P < 0.02$) but had no effect on the number of $\geq 1 < 3.5$ mm follicles. Leptin had no effect on the number of follicles expressing aromatase mRNA but it decreased significantly the number of follicles expressing mRNA for IGF-IR (10.7 ± 0.79 vs 7.4 ± 0.81 ; control vs leptin; $P < 0.05$), IGFBP-2 (10.0 ± 0.82 vs 5.2 ± 0.87 ; control vs leptin; $P < 0.05$) and IGFBP-5 (5.2 ± 1.60 vs 1.2 ± 0.30 ; control vs leptin; $P < 0.05$). Leptin increased the diameter of IGFBP-2 mRNA-positive follicles (1.5 ± 0.15 vs 2.2 ± 0.31 mm; control vs leptin; $P < 0.05$) and increased follicular mRNA expression for IGFBP-2 (0.30 ± 0.021 vs 0.39 ± 0.027 arbitrary units; control vs leptin; $P < 0.05$) and IGFBP-5 (0.46 ± 0.019 vs 0.25 ± 0.053 arbitrary units; control vs leptin; $P < 0.05$). The mRNA for IGFBP-4 was detected in the theca of only two follicles from the control group. Leptin increased the number of follicles expressing Ob-Rb mRNA (0.25 ± 0.25 vs 1.40 ± 1.17 ; control vs leptin; $P < 0.05$) but had no effect on the number expressing leptin mRNA. Leptin decreased plasma concentrations of oestradiol ($P < 0.05$) and increased concentrations of FSH ($P < 0.001$) and insulin ($P < 0.001$), with no effect on glucose concentrations. These data show that: (i) ovine granulosa cells express mRNA for Ob-Rb and leptin and (ii) leptin increased the number of follicles ≥ 3.5 mm. Furthermore, the data suggest that suppression of oestradiol production by leptin is not mediated by inhibition of aromatase gene expression. Finally, the data indicate that the action of leptin in ovarian follicles is mediated by the IGF system, because leptin increased mRNA expression of IGFBP-2 and -5. Leptin also decreased the number of follicles expressing IGF-IR and IGFBP-2 and -5. We suggest that these actions of leptin on the IGF system decrease the bioavailability of IGF-I, resulting in decreased oestradiol production.

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Introduction

The nutritional regulation of folliculogenesis involves, among other factors, the direct action of nutrients and

nutritional signals on the follicle (Spicer 2001). In recent years there has been a concerted effort to identify these nutrients and signals and to elucidate their mechanisms of action on the follicle (Clarke & Henry 1999, Spicer 2001).

One such putative signal is the adipocyte hormone, leptin, and since its discovery in 1994 (Zhang *et al.* 1994) there has been a continued interest in establishing the precise role of the leptin system as a mediator of nutritional influences on the follicle (Brannian *et al.* 1999, Spicer 2001). The intrafollicular insulin-like growth factor (IGF) system is a second candidate system that probably mediates nutritional effects on the follicle and there has been similar interest in establishing its role (Monget *et al.* 1993, 2002).

The presence of either leptin or its receptor (Ob-Rb) in the ovary has proven surprisingly difficult to demonstrate and no reports have been published showing their presence in sheep ovarian tissues. However, the presence of mRNA for Ob-Rb, detected using RT-PCR, has been reported for human granulosa lutein cells (Cioffi *et al.* 1997, Karlsson *et al.* 1997, Löffler *et al.* 2001) and for porcine (Ruiz-Cortés *et al.* 2000) and rodent (Zamorano *et al.* 1997) granulosa cells. Leptin has been detected in human granulosa cells of pre-antral follicles and in theca from healthy and atretic antral follicles (Löffler *et al.* 2001).

The intrafollicular effects of leptin may be direct but there are probably interactions with both the insulin-glucose system and the IGF system (Zachow & Magoffin 1997, Zachow *et al.* 1999, Muñoz-Gutiérrez *et al.* 2002, 2004). Leptin inhibits IGF-I-stimulated oestradiol secretion from cultured granulosa cells (Spicer & Francisco 1997, Zachow & Magoffin 1997, Zachow *et al.* 1999) and human granulosa lutein cells (Agarwal *et al.* 1999, Greisen *et al.* 2000, Ghizzoni *et al.* 2001, Tsai *et al.* 2002). These findings suggest that leptin has a negative interaction with the intrafollicular IGF system. We have *in vivo* confirmation of these observations in sheep with an ovarian autotransplant (Kendall *et al.* 2004). These data clearly suggest a direct *in vivo* role for leptin in ovarian steroidogenesis.

In this context, changes in peripheral concentrations of insulin, IGF-I and leptin have been associated with the initiation of follicle waves in cattle and it has been suggested that follicular oestradiol regulates the secretion of these metabolic hormones (Armstrong *et al.* 2003). Furthermore, leptin expression in adipose tissue is correlated with hepatic IGF-I expression (Houseknecht *et al.* 2000) and hepatic IGF-I is a major factor controlling both the acute secretion of leptin (Marie *et al.* 2001, Kadokawa *et al.* 2003) and follicular function (Monget *et al.* 1993, 2002, Wang & Chard 1999).

In this paper we report the results of an experiment to determine the effect of a physiological dose of exogenous leptin, infused at that stage of the oestrous cycle when other nutritional treatments are known to stimulate folliculogenesis and ovulation rate (Smith & Stewart 1990, Scaramuzzi *et al.* 1993, Viñoles 2003), on the circulating concentration of oestradiol and on the localization and pattern of mRNA expression for aromatase and components of the IGF and leptin systems in sheep ovarian follicles.

Materials and Methods

Experimental animals and leptin infusion

All animal procedures were conducted in accordance with the requirements of the UK Home Office and in compliance with the Animal (Scientific Procedures) Act, 1986.

Ten mature adult Welsh Mountain ewes were used during the natural breeding season. Their oestrous cycles were synchronized using progestagen sponges (Chronogest; Intervet Ltd, Milton Keynes, Northamptonshire, UK) for 12 days. On day 11 of the synchronized cycle the ewes were randomly allocated to two treatment groups: (i) with a freely available maintenance diet of oaten straw and infused i.v. with saline (controls; $n = 5$), and (ii) with a freely available maintenance diet of oaten straw and infused i.v. with human recombinant leptin (leptin-infused; $1 \mu\text{g/h}$; $n = 5$). The infusions were started on day 12 and lasted 72 h, ending on day 15 of the oestrous cycle, at which time a single i.m. injection of $125 \mu\text{g}$ of the prostaglandin $\text{F}_{2\alpha}$ analogue (PG), cloprostenol (Estrumate; Intervet) was given to induce luteolysis.

Blood sampling and hormone analyses

On day 11 of the synchronized oestrous cycle, bilateral venous jugular cannulae were inserted under xylocaine-induced local anaesthesia (Williams *et al.* 2001). One catheter was used exclusively for infusions and the other exclusively for blood sampling. A 5 ml blood sample was drawn from the sampling catheter every 8 h starting 8 h (1600 h) before the commencement of the infusions (midnight) and continuing until the ewes were killed 104 h after the start of treatment. The syringe of blood was emptied into a heparinized tube (20 IU/ml). The blood was then centrifuged for 15 min at $1000g$ at 4°C and the plasma removed and stored at -20°C .

The plasma concentrations of oestradiol $17\text{-}\beta$ were determined in every third sample (i.e. daily intervals) until PG injection and then every 8 h to the end of the experiment. Oestradiol was assayed using a commercial human RIA kit (KE2D; Diagnostic Product Co., Los Angeles, CA, USA) adapted for sheep (Meikle *et al.* 1997, Viñoles 2003). The detection limit of the assay was 4 pmol/l ; the intra-assay and interassay coefficients of variation were 8 and 14% respectively.

The plasma concentration of glucose was measured every 8 h using the 'infinity' hexokinase reagent (Sigma UK Ltd, Poole, Dorset, UK) in 96-well plates (Nunc clone plates; Nunc (Europe) Ltd, Hereford, UK) and at read 340 nm on a Benchmark plate reader. The lowest standard used was 25 mg/dl and all samples in the experiment had plasma concentrations that were above this lower limit. The intra-plate coefficient of variation was 4.5% and the interassay coefficient of variation was 6.6%.

The plasma concentrations of insulin were measured by RIA (Williams *et al.* 2001). The detection limit of the assay was 50 pg/ml ; intra-assay and interassay coefficients of

variation were 10.0 and 12.5% respectively. The plasma concentrations of follicle-stimulating hormone (FSH) were measured by RIA (Campbell *et al.* 1994, Souza *et al.* 1997). The detection limit of the assay was 0.1 ng/ml; the intra-assay and interassay coefficients of variation were 7 and 12% respectively.

Collection of ovaries

Thirty-two hours after the end of the infusions and PG injections (i.e. during the mid-follicular phase of the oestrous cycle) the ewes were killed with a captive bolt pistol. The ovaries were removed within 5 min of death and examined visually. The ovary with the presence of at least one large, presumably dominant follicle was then frozen rapidly in liquid nitrogen vapour and stored at -80°C . The remaining ovary was placed in sterile medium and all follicles >1 mm in diameter were dissected free, counted and used to provide granulosa cells for culture experiments that are not reported in this paper.

Follicle counts

The whole frozen ovaries were serially sectioned at $10\text{ }\mu\text{m}$ using a cryostat at -20°C . The sections were mounted onto electrostatically charged glass slides (Superfrost plus $75 \times 25 \times 1.0\text{ mm}$; BDH Benchmark Plate Reader, Biorad Laboratories (UK) Ltd, Hemel Hempstead, Hertfordshire, UK), fixed with neutral buffered formalin (BDH Gurr), washed six times (four times with $1 \times \text{PBS}$, once with 70% ethanol and once with 95% ethanol) and then stored in 95% ethanol at 4°C . Each ovary produced between 500 and 680 sections and the sections were mounted, four per slide. Every 20th slide was stained with Harris's haematoxylin and eosin and all antral follicles were counted and their maximum diameter estimated from the mean of two measurements of the follicle taken at right angles to each other. The number of follicles from the sectioned ovary was then added to the number of follicles from the dissected ovary to provide an estimate of total follicle population for each ewe. The follicles were classified into two arbitrary classes based on their size; $\geq 1 < 3.5\text{ mm}$ and $\geq 3.5\text{ mm}$. Follicles $<1\text{ mm}$ in diameter were not included.

Oligoprobes for cytochrome P_{450} aromatase, IGF-I receptor (IGF-IR) and IGF-binding protein (IGFBP) -2, -4 and -5

Established *in situ* hybridization procedures were used to measure the mRNA expression for cytochrome P_{450} aromatase (Muñoz-Gutiérrez *et al.* 2002, 2004), IGF-IR, IGFBP-2, -4 and -5 (Perks *et al.* 1995, Perks & Wathes 1996, Muñoz-Gutiérrez *et al.* 2004) on dehydrated and fixed sections. Sequences of every 20th slide were probed with labelled ($[\alpha\text{-}^{35}\text{S}]\text{dATP}$, SJ 1334; Amersham Pharmacia Biotech, Amersham, Bucks, UK) specific oligoprobes (sense; 45 mer synthetic oligonucleotides; Table 1). There were four sections on each slide; two were used for the sense probe and two for the antisense probe. The sections were impregnated with labelled probe diluted to a concentration of 100 000 c.p.m./ml in hybridization buffer by spreading $100\text{ }\mu\text{l}$ over the sections using a small piece of Parafilm. First, $100\text{ }\mu\text{l}$ of 'labelled' sense probe was spread over two sections and then $100\text{ }\mu\text{l}$ of 'labelled' antisense probe was spread over the other two sections. The slides were then incubated overnight in a humidified box at 42°C for aromatase and 50°C for the other oligoprobes. After incubation, the sections were washed at room temperature with citrate buffer in a shaking bath for 30 min followed by 1 h at 60°C . The sections were then dehydrated in a gradient of ethanol, air dried and exposed to Biomax MR-1 film for 21 days (Kodak Biomax MR-1; Kodak, Hemel Hempstead, Hertfordshire, UK).

Photographic development and image analysis were carried out as reported (Muñoz-Gutiérrez *et al.* 2002, 2004). Briefly, the autoradiographic images of the ovarian sections were quantified for labelling using an image analysis system (Seescan plc, Cambridge, UK) to measure the absorbance in the theca and granulosa cell layers. The slides were then emulsion-coated, developed and counterstained with haematoxylin and eosin to confirm the cellular localization of the radiographic signal. The results from the autoradiographs were expressed as arbitrary units of absorbance with linear range of 0.01–2.10. The non-specific counts (estimated from the sense probe) were subtracted from the total counts (estimated from the

Table 1 Nucleotide sequences for the sense oligoprobes for aromatase, IGF-IR and IGFBP-2, -4 and -5.

Probe	Sense sequence	Reference	Homology with sheep [%]
P_{450} aromatase	376–420 of bovine aromatase gene 5'-TGCGCAAAGCCTTAGAGGATGATGTCATCGATGGCTACCCGGTGA-3'	Genbank Z69249, Furbass <i>et al.</i> (1997)	97
IGF-IR	352–396 of human IGF-IR gene 5'-CTCACGGTCATCCGCGGCTGGAAACTCTTCTACAACTACGCCCTG-3'	Genbank NM000875, Stoeltzing <i>et al.</i> (2003)	—
IGFBP-2	452–496 of ovine IGFBP-2 gene 5'-GCGCCAGCCCCGAGCAGGTTGCAGACAATGGCGAGGAGCACTCTG-3'	Genbank S44612, Delhanty & Han (1992)	100
IGFBP-4	118–162 of ovine IGFBP-4 gene 5'-CTGGGCAGGGGAATGCGCTGTGGGGTGTACACCCCGACTGCGGC-3'	Genbank S77394, Carr <i>et al.</i> (1994)	93
IGFBP-5	291–335 of bovine IGFBP-5 gene 5'-CTACTCGCCCAAGATCTTCCGGCCCAAGCACACCCGCATCTCCGA-3'	Genbank S52657, Moser <i>et al.</i> (1992)	100

anti-sense probe) to produce a mean value for the specific hybridization in each follicle.

Riboprobes for leptin and Ob-Rb

A 308 bp cDNA was generated by RT-PCR from placental RNA using primers to the partial ovine sequence (Dyer *et al.* 1997, Genbank accession number U62124) 5'-GATGAGATGGTGCCAACTA-3' (120-141) and 5'-TGGGT-TTCTATTTCCCATGATC-3' (427-406) (Mercer *et al.* 1998, Williams *et al.* 1999). The PCR product was cloned directly into pGEM-T (Promega, Southampton, UK). A 501 bp cDNA, was isolated by RT-PCR from ovine adipose tissue RNA using the following primers to the ovine leptin sequence (Simmons *et al.* 1997, Genbank accession number U84247) 5'-ATGCGCTGTGGACCCCTGT-3' (1-19) and 5'-GCACCCGGGACTGAGGTCC-3' (501-483). The RT-PCR product was cloned directly into pGEM-T (Promega). Plasmids were linearized using NcoI or SacI and transcribed with T7 or SP6 RNA polymerase respectively, to generate sense and antisense riboprobes for *in situ* hybridization.

Established *in situ* hybridization procedures (Mercer *et al.* 1998, Williams *et al.* 1999, Archer *et al.* 2002) were used with minor modifications. Briefly, slides were fixed in 4% paraformaldehyde, acetylated and hybridized overnight at 58 °C using ³⁵S-labelled riboprobes at a concentration of 1–1.5 × 10⁷ c.p.m./ml. Slides were then treated with RNase A, desalted with a final high stringency wash (30 min), in 0.5 × saline–sodium citrate at 60 °C, dried and exposed on Hyperfilm β-max film (Amersham Pharmacia) for 21 days. Frozen hypothalamic (Ob-Rb) and placental (leptin) tissues sectioned at 10 μm were used as positive controls and sequences of every 20th slide from the serially sectioned ovaries were probed for Ob-Rb and leptin. Photographic development and image analysis were carried out as described above for the oligoprobes.

Statistical analyses

The effect of treatment on the plasma concentrations of glucose, insulin and oestradiol were analysed using a split-plot ANOVA and the effect of time was then compared by further post-hoc testing using Tukey's

method (SAS 1995). The effect of treatment on the (i) number of follicles, (ii) relative concentration of mRNA, and (iii) follicle diameter was analysed using one-way ANOVA. Fisher's exact test was used to test effects of treatment on the proportion of follicles expressing mRNA for each probe. The effects of treatment on patterns of mRNA co-expression were analysed as proportions, also using Fisher's exact test.

Results

Daily monitoring of plasma progesterone concentrations was used to confirm the physiological status of the ewes. These data show that one control ewe had a persistent corpus luteum. This animal has been excluded.

Number and distribution of follicles

There was no treatment effect on the number of follicles in the ≥ 1 < 3.5 mm size class (Table 2). However, there was a significant effect of treatment on the number of follicles in the ≥ 3.5 mm diameter size class; leptin infusion increased the number of follicles ≥ 3.5 mm approximately 2-fold (*P* < 0.02; Table 2).

Ovarian P₄₅₀ aromatase

Aromatase mRNA expression was assessed in 93 follicles > 1 mm in diameter and expression was detected in the membrana granulosa of ten (10.8%) follicles from eight ovaries. In seven of these, the aromatase-positive follicle was also the largest follicle present. The average number of aromatase-positive follicles was not affected by leptin infusion (Table 3), neither was the maximum diameter (Table 3) nor the concentration of aromatase mRNA (Table 3).

The ovarian IGF system

The known presence (Reynolds *et al.* 1997, Gadd *et al.* 2000) of mRNAs for: IGF-IR, in endometrial glands and caruncular stroma; IGFBP-2, in placentome capsule; IGFBP-4, in endometrial glands, placentome capsule and caruncular stroma; and IGFBP-5, in luminal epithelium, placentome capsule, caruncular stroma and

Table 2 Mean ± S.E.M. number of follicles in two size classes and the proportion of follicles with mRNA expression for aromatase, IGF-IR, IGFBP-2 IGFBP-5, the signalling form of the leptin receptor (Ob-Rb) leptin in follicular phase ovaries from Welsh Mountain ewes infused with saline (control) or 1 μg/h human recombinant leptin (leptin) for 72 h in the late luteal phase of the oestrous cycle.

Treatment	Follicle diameter (mm)	Mean number of follicles	Probe						
			Aromatase	IGF-IR	IGFBP-2	IGFBP-4	IGFBP-5	Ob-Rb ¹	Leptin ¹
Control (n = 4)	≥ 3.5	1.0 ± 0.36 ^a	3/4 ^a	4/4 ^a	1/4 ^a	0/4 ^a	0/4 ^a	0/4 ^a	2/3 ^a
Leptin (n=5)		2.4 ± 0.24 ^b	5/8 ^a	7/8 ^a	7/8 ^b	0/8 ^a	1/8 ^a	2/7 ^a	2/3 ^a
Control (n=4)	≥ 1 < 3.5	23.2 ± 2.70 ^a	2/43 ^a	30/43 ^a	25/43 ^a	2/43 ^a	13/43 ^a	1/38 ^a	2/7 ^a
Leptin (n=5)		24.2 ± 2.88 ^a	0/38 ^a	22/38 ^b	14/38 ^b	0/38 ^b	4/38 ^b	5/36 ^a	5/13 ^a

Values with different superscripts between treatments within columns differ significantly (*P* < 0.05).

¹ Not all follicles were assessed with both these probes.

Table 3 Mean $\pm$ S.E.M number, maximum diameter and concentration of mRNA for aromatase, IGF-IR and IGFBP-2, -4 and -5, long signalling form of the leptin receptor (Ob-Rb) and leptin in follicular phase ovaries from Welsh Mountain ewes infused with saline (control) or 1 μ g/h human recombinant leptin (leptin) for 72 h in the late luteal phase of the oestrous cycle.

Probe	Treatment	Number of positive follicles per ovary	Diameter of positive follicles (mm)	mRNA concentration (arbitrary units of absorbance)
Aromatase	Control	1.2 $\pm$ 0.5	4.1 $\pm$ 1.36	0.07 $\pm$ 0.02
	Leptin	1.0 $\pm$ 0.0	5.0 $\pm$ 0.26	0.10 $\pm$ 0.00
IGF-IR	Control	10.7 $\pm$ 0.8 ^a	1.8 $\pm$ 0.20	0.15 $\pm$ 0.01
	Leptin	7.4 $\pm$ 0.8 ^b	2.0 $\pm$ 0.24	0.14 $\pm$ 0.02
IGFBP-2	Control	10.0 $\pm$ 0.8 ^a	1.5 $\pm$ 0.15 ^a	0.30 $\pm$ 0.01 ^a
	Leptin	5.2 $\pm$ 0.9 ^b	2.2 $\pm$ 0.31 ^b	0.39 $\pm$ 0.02 ^b
IGFBP-4	Control	2	2.3, 2.1*	0.13, 0.13*
	Leptin	—	—	—
IGFBP-5	Control	5.2 $\pm$ 1.60 ^a	1.9 $\pm$ 0.14	0.46 $\pm$ 0.01 ^a
	Leptin	1.2 $\pm$ 0.300 ^b	2.0 $\pm$ 0.70	0.25 $\pm$ 0.05 ^b
Ob-Rb ¹	Control	0.25 $\pm$ 0.25 ^a	1.57*	0.28*
	Leptin	1.40 $\pm$ 1.17 ^b	2.77 $\pm$ 0.59	0.17 $\pm$ 0.08
Leptin ¹	Control	1.00 $\pm$ 1.00	3.41 $\pm$ 0.83	0.10 $\pm$ 0.01
	Leptin	1.40 $\pm$ 0.91	2.70 $\pm$ 0.63	0.07 $\pm$ 0.01

For each probe, within columns, values with different superscripts differ significantly ($P < 0.05$).

* Individual values.

¹ Not all follicles were assessed with these two probes.

endometrial glands of the placentome was confirmed (data not shown). Sections of placentome were used as positive controls for the analysis of ovarian tissues.

The expression of mRNAs for IGF-IR, IGFBP-2, -4 and -5 was detected (Fig. 1) in a variable proportion of the ovarian follicles examined. Of the follicles that were examined, IGF-IR mRNA was detected in 63 out of 93 (6.7%) follicles from all nine ewes (Table 2). For the three binding proteins the corresponding figures are as follows: IGFBP-2 detected in 47 out of 93 follicles (50.3%) from eight of nine ewes; IGFBP-4 detected in only two follicles from the control group; and IGFBP-5 detected in 18 out of 93 follicles (19.4%) from seven of nine ewes (Table 2).

Leptin infusion reduced the number of IGF-IR-positive follicles ($P < 0.05$) without affecting their diameter or mRNA concentration (Table 3). The expression of IGF-IR mRNA was detected as previously reported in both the membrana granulosa and the thecal layers of the follicle (Perks *et al.* 1995, Muñoz-Gutiérrez *et al.* 2004).

The number of follicles with mRNA for IGFBP-2 was reduced by leptin infusion ($P < 0.05$; Table 3) but the maximum diameter of IGFBP-2-positive follicles ($P < 0.05$) and the concentration of mRNA ($P < 0.05$) were both increased significantly by leptin infusion (Table 3). As previously reported (Muñoz-Gutiérrez *et al.* 2004) the expression of IGFBP-2 mRNA was confined to the membrana granulosa of the follicle.

The mRNA for IGFBP-4 was detected in the theca cell layer in only 2 out of 93 follicles that were examined (Table 3); both of these were in the control group.

Leptin infusion reduced the number of IGFBP-5-positive follicles without affecting their mean follicular diameter but it increased the concentration of mRNA ($P < 0.05$; Table 3). The expression of IGFBP-5 mRNA was detected

in both the membrana granulosa and the thecal layers of the follicle. There were 18 follicles (19.4%) > 1 mm in diameter that were positive for IGFBP-5, and in all of these mRNA expression was confined to the membrana granulosa. Many follicles < 1 mm in diameter were also positive for IGFBP-5 and although these were not counted it was noted that when any of these small follicles expressed mRNA for IGFBP-5, expression was confined to the thecal cell layer.

Ob-Rb and leptin expression

The presence of mRNA for Ob-Rb was confirmed in the ventromedial and arcuate nuclei of the ovine hypothalamus (Williams *et al.* 1999, Archer *et al.* 2002) and sections of ovine hypothalamus were used as a positive control for the analysis of ovarian tissues. Similarly, leptin mRNA expression previously detected by RT-PCR in ovine placenta (Buchbinder *et al.* 2001, Hoggard *et al.* 2001, Thomas *et al.* 2001) was confirmed here by *in situ* hybridization in sections of ovine placentome that were used as positive controls for the analysis of ovarian tissues.

Low but uniform expression of mRNA for Ob-Rb was detected in the granulosa cells of eight (9.4%) follicles of a total of 85 that were examined (Fig. 2). The pattern of expression for leptin and Ob-Rb were indistinguishable in individual follicles from control and treated animals. However, leptin infusion did increase the number of Ob-Rb mRNA-positive follicles ($P = 0.05$; Table 3) without affecting their mean diameter or the mRNA concentration (Table 3).

Leptin mRNA expression was detected in the theca and granulosa cells (Fig. 3) of 11 (42.3%) follicles from a total of 26 follicles that were examined. Leptin infusion did not affect the number of follicles with mRNA for leptin, their

Figure 1 Autoradiographic localization of mRNA for IGF-IR (a and b), IGFBP-2 (c, d), IGFBP-4 (e and f) and IGFBP-5 (g and h) in follicular phase ovaries from Welsh Mountain ewes infused with saline (control) or 1 µg/h human recombinant leptin for 72 h in the late luteal phase of the oestrous cycle. Antisense (AS; a, c, e and g) and sense (S; b, d, f and h) probes are illustrated. Scale bars represent 2.5 mm.

mean diameter or the level of expression of leptin mRNA (Table 3).

Co-expression in aromatase-positive follicles

Co-expression was determined by analysis of adjacent sections of the same follicle. Co-expression of mRNA for IGF-IR, IGFBP-2 or -5 with aromatase was detected in ten out of ten, six out of ten and one out of ten follicles respectively. There was no effect of treatment on these proportions for IGF-IR and IGFBP-5 but for IGFBP-2 the effect was significant (one out of five vs five out of five for control and leptin-infused ewes; $P=0.024$). Aromatase expression was not related to the presence of mRNA for IGF-IR or IGFBP-2 or -5.

Co-expression of mRNA for Ob-Rb or leptin with aromatase was detected in three out of ten follicles for Ob-Rb and in four out of six follicles for leptin. There was no effect of treatment on this pattern for leptin but for Ob-Rb the effect approached significance (none out of five vs three out of five for control and leptin-infused; $P=0.083$). Aromatase expression was not related to the presence of mRNA for leptin or Ob-Rb.

Oestradiol, FSH, glucose and insulin

During the leptin infusion, plasma oestradiol concentrations declined steadily in leptin-infused ewes (Fig. 4) and the concentration of oestradiol in leptin-infused ewes was significantly lower than controls at 48 h ($P<0.01$).

Figure 2 Autoradiographic localization of mRNA for the long signalling of leptin receptor (Ob-Rb) in follicular phase ovaries from Welsh Mountain ewes infused with saline (control) or 1 µg/h human recombinant leptin for 72 h in the late luteal phase of the oestrous cycle. Note the localization of Ob-Rb in the inner layer of the membrana granulosa bordering the antrum and its absence in theca cells. Antisense (AS; a, c and e) and sense (S; b, d and f) probes are illustrated. Code: fa: follicular antrum; g: granulosa cells; t: theca cells. Each scale bars represent (a and b) 2.5 mm and (c–f) 10 µm.

and 72 h ($P < 0.05$). Following the end of the infusion and the induction of luteolysis with a PG injection, oestradiol concentrations increased significantly in both groups (Fig. 4) by 8 h and remained significantly ($P < 0.05$) elevated for the remainder of the experiment in both control (19.9 ± 2.30 (S.E.M.) pmol/l) and leptin-infused ewes (16.1 ± 1.36 pmol/l). However, oestradiol concentrations post-infusion were significantly lower in the leptin-infused ewes than control ewes 8 h ($P < 0.01$), 16 h ($P < 0.05$) and 32 h ($P < 0.05$) after the end of the infusion (Fig. 4).

The plasma concentration of FSH had increased significantly ($P < 0.01$) by 24 h after the start of infusion and it remained elevated for the duration of the infusion, decreasing to control levels by 24 h after the end of the leptin infusion (Fig. 4). The mean plasma concentration of

FSH was significantly increased both during (1.36 ± 0.05 vs 1.95 ± 0.09 ng/ml; $P < 0.001$) and after (1.26 ± 0.16 vs 1.70 ± 0.17 ng/ml; $P < 0.003$) the leptin infusion.

The mean plasma concentration of glucose was not affected by leptin infusion (51.8 ± 1.75 and 52.3 ± 4.15 mg/dl for control and leptin-infused groups respectively) and there was no significant effect of time, nor were there significant time by treatment interactions.

The mean plasma concentration of insulin was significantly increased by leptin infusion (0.24 ± 0.01 vs 0.75 ± 0.12 ng/ml; $P < 0.001$). The plasma concentration of insulin had increased significantly ($P < 0.01$) by 8 h after the start of infusion and it remained elevated for the duration of the infusion decreasing to control levels by 8 h after the end of the leptin infusion (Fig. 5).

Figure 3 Antisense (AS; a, c and e) and sense (S; b, d and f) autoradiographic localization of mRNA for leptin in follicular phase ovaries from Welsh Mountain ewes infused with saline (control) or 1 µg/h human recombinant leptin for 72 h in the late luteal phase of the oestrous cycle. Code: fa: follicular antrum; g: granulosa cells; t: theca cells. Each scale bars represent (a and b) 2.5 mm and (c–f) 10 µm.

Discussion

An important finding from this experiment is the detection of mRNA for Ob-Rb in ovine granulosa cells (Fig. 2) from a proportion of the antral follicle population. The expression was weak but unequivocal, confirming that the granulosa cell is a physiological target for leptin. The presence of mRNA for leptin in granulosa and theca cells of antral follicles (Fig. 3) suggests that leptin is produced locally and that it may exert autocrine and/or paracrine effects on granulosa cell function.

The results of the present study and those of Kendall *et al.* (2004) show that human recombinant leptin is physiologically active in sheep at an infusion rate of

1 µg/h. This is a considerably lower dose than that used in other published studies (Spicer & Francisco 1998, Greisen *et al.* 2000, Spicer *et al.* 2000) and suggests that in some of these earlier studies the dose of leptin used may not have been physiological. The infusion rate used in this experiment produced blood concentrations of leptin (Kendall *et al.* 2004) that fell within the 0.5–2 ng/ml reported physiological blood concentrations for leptin in sheep (Blache *et al.* 2000, Delavaud *et al.* 2000, Kauter *et al.* 2000, Kadokawa *et al.* 2003). A short-term infusion of leptin at a dose of 1 µg/h stimulated folliculogenesis by increasing the number of follicles ≥ 3.5 mm in diameter but with no effect on the number of follicles in the $\geq 1 < 3.5$ mm class (Tables 2 and 3). Since most of the

Figure 4 Mean $\pm$ S.E.M. plasma concentration of oestradiol-17 β and FSH in Welsh Mountain ewes infused with saline (control, $n = 4$) or 1 μ g/h human recombinant leptin (leptin, $n = 5$) for 72 h in the late luteal phase of the oestrous cycle (* $P < 0.05$ between treatment within time). Prostaglandin F2 α (PG, 125 μ g) was injected at the end of the infusion period (72 h) and the ewes were killed 32 h later (104 h after the start of infusion).

Figure 5 Mean $\pm$ S.E.M. plasma concentration of insulin in Welsh Mountain ewes infused with saline (control, $n = 4$) or 1 μ g/h human recombinant leptin (leptin, $n = 5$) for 72 h in the late luteal phase of the oestrous cycle (* $P < 0.05$ between treatment within time). Prostaglandin F2 α (PG, 125 μ g) was injected at the end of the infusion period (72 h) and the ewes were killed 32 h later (104 h after the start of infusion).

follicles positive for leptin and Ob-Rb were <3.5 mm (Tables 2 and 3) these results suggest that leptin is acting on these smaller recruited follicles, stimulating their growth and thus increasing the number of follicles ≥ 3.5 mm. In ewes, the follicles that ovulate are those that avoid atresia and reach the final stage of development (Oldham *et al.* 1990, Scaramuzzi *et al.* 1993) and follicles >3.5 mm are generally considered capable of ovulating (Scaramuzzi *et al.* 1993, Rhind & McNeilly 1998). In the rat, physiological doses of leptin increased the number of ovulations 2-fold, and accelerated follicular maturation by attenuating atresia and increasing the ratio of the anti-apoptotic Bcl₂–Bax dimer (Almog *et al.* 2001). These findings lead us to suggest that in the ewe improved nutrition and higher body weight, which both elevate plasma leptin concentrations (Dyer *et al.* 1997, Blache *et al.* 2000, Delavaud *et al.* 2000, Kauter *et al.* 2000, Marie *et al.* 2001, Muñoz-Gutiérrez *et al.* 2002, Kosior-Korzecka & Bobowiec 2003, Narro *et al.* 2003) may increase ovulation rate by a leptin-mediated mechanism.

In our work, the number of large potentially ovulatory follicles was doubled in leptin-infused ewes. However, the number of large follicles is not necessarily a reliable indicator of ovulation rate, because they must also be oestrogenic if they are to ovulate (Webb *et al.* 1989) and the number of follicles that were aromatase-positive was not increased by leptin infusion.

In the present study, the plasma concentration of oestradiol was decreased by leptin and further low oestradiol production was not associated with reduced aromatase mRNA expression. Early reports demonstrated that leptin could inhibit *in vitro* oestradiol production in cultured granulosa cells from the rat (Zachow & Magoffin 1997, Zachow *et al.* 1999), cow (Spicer & Francisco 1997) and in human granulosa lutein cells (Agarwal *et al.* 1999, Greisen *et al.* 2000, Ghizzoni *et al.* 2001) under a variety of conditions. Our findings provide further *in vivo* confirmation for these *in vitro* studies and suggest that leptin has a direct physiological action on the follicle. The finding also indicates that the suppressive effect of leptin on follicular oestradiol production is not caused by reduced aromatase mRNA expression and suggests that the negative modulation of oestradiol production is caused by some other aspect of aromatase function such as its translation, activation or activity *per se* (Zachow *et al.* 1999).

Much of the published evidence suggests that the ovarian actions of leptin involve an interaction with the ovarian IGF system. In human granulosa lutein cells, leptin directly attenuates IGF-I-stimulated oestradiol production (Huang *et al.* 2002). In bovine theca and granulosa cells cultured *in vitro*, insulin-induced progesterone, androstenedione and oestradiol production are all inhibited by leptin (Spicer & Francisco 1997, 1998). The results of our experiment (Table 3) show that some components of the IGF system are directly altered by leptin and provide *in vivo* evidence that supports a role for leptin as a modulator of the ovarian IGF system.

The numbers of follicles that were positive for IGF-IR or IGFBP-2 or -5 were all reduced by leptin (Table 3), and in particular those follicles between 1.3 and 2.5 mm in diameter. These are recruited follicles (McNatty *et al.* 1982, Scaramuzzi *et al.* 1993, Souza *et al.* 1998, Bartlewski *et al.* 1999) in a rapid growth phase (Scaramuzzi *et al.* 1993, Monget & Martin 1997, Driancourt 2001). These findings suggest that leptin is most effective at modifying the IGF system of rapidly growing follicles around 2 mm in diameter.

In follicles that were ≥ 3.5 mm in diameter, the effects of leptin on the IGF system were predominantly on mRNA expression for IGFBP-2. Virtually all the follicles ≥ 3.5 mm in diameter were positive for IGF-IR mRNA, and leptin had no effect on the pattern of expression. However, leptin infusion significantly decreased the proportion of follicles ≥ 3.5 mm in diameter that were positive for mRNA for IGFBP-2 (Table 2). Only one follicle that was ≥ 3.5 mm in diameter had any detected IGFBP-5 mRNA expression.

No mRNA expression for IGFBP-4 was detected in any follicle from the leptin-infused group of ewes and only two IGFBP-4-positive follicles were detected in the control ewes. Nevertheless, IGFBP-4 is an essential component of follicular growth and development (Mazerbourg *et al.* 2001, Monget *et al.* 2002, Richards *et al.* 2002) and its presence in sheep follicles, especially atretic follicles, as both protein and message has been widely reported (Besnard *et al.* 1996, Perks & Wathes 1996, Mazerbourg *et al.* 2001). The inhibition of IGFBP-4's biological activity by proteolytic cleavage (Mazerbourg *et al.* 2001, Wright *et al.* 2002) is a common feature of preovulatory follicles in human, ovine and bovine ovaries. The low incidence of IGFBP-4-positive follicles in this study suggests that mRNA expression for IGFBP-4 is also inhibited during the late follicular phase of the oestrous cycle.

The results of the present experiment show that leptin can modify IGFBP-2 and -5 mRNA expression and imply that leptin modulation of follicular function involves these two binding proteins as intermediaries. The binding proteins could have direct stimulatory effects on the follicle because IGFBP-2 and -5 can bind to components of the extracellular matrix (Robinson *et al.* 2000), which is involved in follicular remodelling. Our results are consistent with those of Monniaux *et al.* (2000), who reported decreased IGFBP-2 and -5 protein expression during terminal follicular growth. Our data suggest that IGFBP-2 is acting to attenuate follicular development (Table 3) because leptin induced both increased expression of IGFBP-2 and suppression of oestradiol production.

The infusion of leptin increased the number of follicles that expressed mRNA for Ob-Rb (Table 2). Ob-Rb mRNA expression was seen predominantly in follicles around 2.0 mm in diameter, follicles that had already been recruited and were rapidly growing. We suggest that endogenous leptin acts on recruited follicles to inhibit

oestradiol secretion (Fig. 4) and enhance their survival by blocking IGFBP-2 and -5 expression (Table 3) to increase the number of large follicles (Table 2).

Leptin has also been implicated in other metabolic pathways including insulin (McClain 1998). Leptin increased insulin plasma concentrations without altering glucose concentrations, suggesting that in the ewe leptin acts as a trigger for insulin secretion independently of blood glucose concentrations. Leptin stimulates insulin secretion by a direct action on pancreatic insulin-producing β -cells (Ukropec *et al.* 2001). Our results agree with Zieba *et al.* (2003), who demonstrated that leptin has a dose-dependent bimodal influence on pancreatic β -cells, and low doses of leptin stimulated pancreatic β -cell responses while the high doses attenuated them. These findings and our own both suggest that care is required when interpreting the physiological consequences of leptin in experiments that use non-physiological doses of leptin (Henry *et al.* 1999, Morrison *et al.* 2001).

The lack of effect of leptin on blood glucose concentrations in the presence of elevated insulin concentrations suggests that leptin induced a mild degree of insulin resistance in our animals. This suggestion is consistent with leptin-attenuated insulin-stimulated functions such as lipogenesis, glycogen synthesis and amino acid uptake. However, in sheep, leptin signals body fat and nutrient stores rather than short-term changes in glucose utilization (Kauter *et al.* 2000). The effects of leptin on glucose utilization are indirect, at a neuronal level, and reduce whole body insulin sensitivity (Houseknecht & Portocarrero 1998) leading to increased insulin concentrations without affecting blood glucose concentrations.

We conclude that in the ewe, a short-term (72 h) physiological infusion of leptin at 1 μ g/h has a stimulatory effect on ovine follicular development and increases the number of large follicles (≥ 3.5 mm in diameter). However, leptin also inhibited follicular oestradiol production leading to an increase in blood concentrations of FSH. The action of leptin in follicles appears to be mediated by the IGF system because leptin reduced the number of follicles with mRNA for IGF-IR and IGFBP-2 and -5 without affecting the number with mRNA for aromatase. We suggest that these actions of leptin on the IGF system decrease bioavailability of IGF-I, resulting in decreased oestradiol production.

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