Level of nutrition affects leptin concentrations in plasma and cerebrospinal fluid in sheep

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Abstract

In mature male sheep, the level of nutrition acutely influences the secretion of reproductive hormones. The mechanism involved is not fully understood but findings in humans and laboratory rodents would suggest a major role for leptin that is secreted from adipose tissue and then travels via the circulation to the central nervous system. Before we can begin to test this hypothesis, we need to be able to measure leptin concentrations in blood plasma and cerebrospinal fluid. We have therefore developed a radioimmunoassay using antibodies raised against biologically active recombinant bovine–ovine leptin. Using this assay, we found that plasma concentrations of leptin were highly correlated to back-fat thickness and to the ratio of back-fat thickness to liveweight, in female and castrated male sheep. Plasma concentrations of leptin were higher in female sheep than in castrated or intact male sheep. Serial samples (every 5 min) suggested that the secretion of leptin in male sheep is episodic but it does not appear to show clear pulsatility, increases post-prandially, or a diurnal rhythm. Leptin concentrations in both plasma and cerebrospinal fluid increased within 5 days in male sheep fed a diet with a high content of energy and protein that also stimulates the secretion of LH pulses. These data suggest that in sheep, as in other species, leptin production is correlated with the mass of adipose tissue and that the hormone passes from the circulation to the cerebrospinal fluid and then to hypothalamic sites. There, it may affect appetite and perhaps GnRH secretion. The role of leptin in the link between nutrition and reproduction needs further investigation.

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Introduction

In mature male Merino sheep, nutrition is arguably the most powerful exteroceptive factor that affects fertility. Within a few days after the level of nutrition is increased, there is an increase in the frequency of luteinising hormone (LH) pulses and in circulating follicle-stimulating hormone (FSH) concentrations, indicating activation of the gonadotrophin-releasing hormone (GnRH) neuroendocrine system (Martin *et al.* 1994*a*). The mechanism underlying this response seems to involve both nutrients (i.e. fatty acids but not glucose) and metabolic hormones, such as insulin (Hötzel *et al.* 1995, Miller *et al.* 1995, 1998, Boukhliq & Martin 1997). Insulin stimulates the secretion of LH as effectively as an increase in the level of nutrition, but only when it is infused into the cerebroventricular system (Miller *et al.* 1995). However, it doesn't seem to act alone to control GnRH neural activity, because feeding a supplement of fatty acids can stimulate GnRH secretion without affecting peripheral insulin levels, and intravenous infusion of glucose can stimulate insulin secretion without affecting GnRH activity (Chagas *et al.* 1997, 1999). It is therefore most likely that insulin needs to interact with other factors in order to affect the hypothalamic-pituitary axis.

Another candidate for an endocrine link between nutrition and reproduction is leptin, a hormone that has been suggested as playing a key role in the control of nutrition, metabolism and reproductive endocrinology in both humans and laboratory rodents (for reviews see Houseknecht & Portocarrero 1998, Messinis & Milingos 1999). The leptin gene, its protein product and the receptor have been identified in sheep (Dyer *et al.* 1997*a*,b), and the expression of mRNA for the receptor is higher in hypothalamic and pituitary tissue in feed-restricted ewes than in well-fed ewes (Dyer *et al.* 1997*a*). In female sheep, plasma concentrations of leptin, measured using a method derived from a human leptin assay, appear to be correlated with body fatness (Chilliard *et al.* 1998). However, intracerebroventricular infusion of recombinant human leptin does not affect LH secretion in ovariectomised ewes (Henry *et al.* 1999). The results from these studies, all done in mature or

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ovariectomised female sheep, might not be applicable to the mature male because, in contrast with ewes, GnRH secretion is acutely affected by nutrition in rams (Martin *et al.* 1994*a*,*b*).

To study these relationships, we have developed a radioimmunoassay based on recombinant bovine–ovine leptin (b/o-leptin) so we can observe the short- and long-term effects of nutrition on circulating leptin concentrations in sheep and compare the responses in females, castrated males and intact males. In intact males, we have also compared leptin concentrations in blood plasma and cerebrospinal fluid (CSF). Finally, we have described minute-by-minute changes in leptin concentrations because, in women, leptin secretion is thought to be episodic and synchronised with the pulsatile secretion of LH (Licinio *et al.* 1998).

Materials and Methods

Production of recombinant b/o-leptin

Adipose tissue was removed from the retroperitoneal area of *Bos taurus* cattle at slaughter. Poly A^+ RNA was prepared from this tissue using an Oligotex mRNA kit (Qiagen, Clifton Hill, Australia) and subsequently cDNA was produced using the RiboClone cDNA synthesis system (Promega, Sydney, Australia). Two oligonucleotide primers were synthesised based on the genomic sequence of the bovine obese gene (GeneBank accession number U50365). The first was a sense primer and corresponded to the 5′ end of the mature protein sequence (i.e. 5′-GTGCCCATCCGCAAGGTCC-3′). The second was a reverse sense primer based in the immediate 3′ UTR following the stop codon (i.e. 5′-TCAGCACCCGG GACTGAGG-3′). PCR was performed on 5 ng cDNA in the presence of 100 µl of 10 mM Tris–HCl, pH 8·3, 50 mM KCl, 2·0 mM $MgCl_2$, 200 µM of each dNTP, 500 ng of each oligonucleotide primer and 2·5 U AmpliTaq (Promega, Sydney, Australia). Each reaction was overlaid with 100 µl mineral oil. Amplification was performed for 35 cycles, each of which consisted of denaturation for 60 s at 95 °C, annealing for 60 s at 50 °C and extension for 120 s at 72 °C. A unique DNA band of about 440 bp was produced and authenticated by sequencing on both strands using standard procedures (Sanger *et al.* 1977). The amplified DNA was then cloned into the expression vector pQE9 (Qiagen, Clifton Hill, Australia) and expressed essentially according to the manufacturer's instructions. This vector adds a hexaHis-affinity tag to the amino-terminus of the recombinant protein.

There are only two conservative amino acid differences between the mature forms of bovine and ovine leptins. Consequently, the use of recombinant bovine leptin as an immunogen would be expected to induce antibodies that recognise endogenously produced circulating ovine leptin.

Recombinant hexaHis–leptin was expressed in high yield but was sequestered into inclusion bodies. The following procedure was used to solubilise, purify and refold the hexaHis–leptin. The bacterial cell pellet from a 750 ml cell culture was resuspended in 30 ml cold digestion buffer (50 mM Tris–HCl, 50 mM NaCl, 1 mM EDTA, pH 7·5, 1 mM benzamidine, 12 mM 2-mercaptoethanol, 10 µM 4-(2-aminoethyl)-benzenesulfonylfluoride containing 5 g/l lysozyme) and homogenised with a glass tissue grinder for 2 min at 4 °C after which the suspension was made to 0·1% in Triton X-100 and sonicated 3 times each for 30 s. The suspension was centrifuged (12 000 ***g***, 30 min, 4 °C) and the pellet washed in the same buffer. The pellet obtained after subsequent centrifugation was suspended in digestion buffer containing 0·5% Triton X-100 and 10 µg/ml DNase-I and then sonicated 3 times each for 30 s. After an incubation period of 30 min at 37 °C, the suspension was centrifuged and washed as described above. The resulting pellet was resuspended in 30 ml of 6 M guanidine-HCl, 0·1 M NaH_2PO_4, 0·01 M Tris–HCl, pH 8·0, 5 mM 2-mercaptoethanol, 1 mM dithiothreitol (DTT) for 60 min at room temperature. The final suspension was centrifuged at 100 000 ***g*** for 30 min at 20 °C and the supernatant retained.

The solubilised hexaHis–leptin was purified by nickel nitrilotriacetic acid (Ni-NTA) affinity chromatography essentially according to the manufacturer's instructions (Qiagen, Clifton Hill, Australia). The purified hormone was dialysed against 20 mM Tris–HCl, 500 mM NaCl, 8 M urea, pH 8·5 and then diluted dropwise to a final protein concentration of 50 µg/ml with 20 mM Tris–HCl, 500 mM NaCl, 2 M urea, pH 8·0. The protein was allowed to refold in this buffer for 18 h at 4 °C and afterwards dialysed into 40 mM Tris–HCl, 140 mM NaCl, pH 8·0. The authenticity of the purified hexaHis–leptin was verified by its relative size using SDS-PAGE and amino-terminal amino acid sequencing (Applied Biosystems 471A protein sequencer). Protein samples were analysed by SDS-PAGE and immunoblots according to previously described procedures (East *et al.* 1993, Richardson *et al.* 1993).

Determination of the biological activity of hexaHis–leptin

The biological activity of the refolded hexaHis–leptin was determined using the procedure described by Gainsford *et al.* (1996). Briefly, the proliferative response of murine Ba/F3 cells transfected with the full-length human leptin receptor was measured as a function of leptin concentration. Viable cells in each well were counted using an inverted microscope after the cells were incubated at 37 °C in a humidified incubator containing 10% CO_2/90% air for 2 days. The ability of bovine hexaHis–leptin to stimulate the proliferation of these cells was compared with recombinant human leptin.

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B/o-leptin radioimmunoassay

Immunisation and antibodies Attempts to raise high titre antibodies against b/o-leptin in rabbits were not successful, so we used a male emu (*Dromaius novaehollandiae*) because that species has a large body mass and is phylogenically distant from mammals. The b/o-leptin was conjugated to maleimide-activated keyhole limpet haemacyanin (KLH) according to the manufacturer's specifications (Pierce, Rockford, IL, USA). The primary immunisation used 100 µg b/o-leptin–KLH in non-ulcerative complete Freund's adjuvant (Sigma, St Louis, MO, USA). Two booster immunisations, each containing 200 µg b/o-leptin-KLH in incomplete Freund's adjuvant (Sigma, St Louis, MO, USA), were given at 4 week intervals after the initial injection.

Assay protocol for plasma samples The assay was carried out in 11 × 75 mm polystyrene test tubes. The assay buffer was 0·05 M phosphate (pH 7·4) containing 0·154 M NaCl, 0·025 M EDTA, 0·5% bovine serum albumin (BSA, RIA grade), 0·025% sodium azide and 0·05% Triton X-100. B/o-leptin (3 µg in 10 µl of 0·1 M phosphate buffer pH 7·5) was labelled by mixing 10 µl of 0·3 M phosphate buffer pH 7·5, 0·5 mCi $Na^{125}I$ and 10 µl chloramine-T (200 µg/ml in 0·3 M phosphate buffer pH 7·5). After 90 s, the reaction was stopped by adding 200 µl of 0·3 M phosphate buffer pH 7·5. Labelled hormones were separated from free iodine on a Sephadex G25 column (Pharmacia, Sydney, NSW, Australia) eluted with 1% BSA in 0·1 M phosphate buffer pH 7·5. Peak fractions were added to an equal volume of 2% BSA in 0·1 M phosphate buffer (pH 7·5) and stored at 4 °C. Two triplicates of standard (b/o-leptin) and 100 µl duplicates of unknown samples, 50 µl anti-b/o-leptin (1:5000) and 50 µl normal emu serum (1:500) were added. After incubation overnight at 4 °C, 50 µl ^{125}I-b/o-leptin (∼10 000 c.p.m.) was added and the mixture was incubated for 48 h at 4 °C. To precipitate the antibody–antigen complex, 100 µl sheep anti-emu immunoglobulin serum (diluted 1:12) was added and tubes were incubated for 48 h at 4 °C. To improve the stability of the pellet, 1 ml of 3% (w/v) polyethylene glycol 6000 (Sigma, St Louis, MO, USA) in 0·01 M phosphate buffered saline was added before centrifugation at 2000 ***g*** for 30 min. The supernatants were decanted and the pellets were left to dry overnight before the radioactivity was counted. The limit of detection was 100 pg/ml, and the intra-assay and inter-assay coefficients of variation were 5·7% and 4·8%, respectively.

Assay protocol for CSF samples The same procedure was used to make the tracer, but we used duplicate 500 µl samples of CSF (or standards), anti-b/o-leptin at 1:3000 and normal emu serum at 1:200. To cope with the increased volume of reaction, the tubes were incubated for 48 h before tracer was added and then for another 48 h before the second antibody was added. The anti-emu immunoglobulin serum was diluted 1:5 and was left with the reaction mixture for a further 48 h. Increasing the volume of sample made the assay 5-fold more sensitive, the limit of detection was 25 pg/ml. The within-assay coefficient of variation was 4·8%.

Validation of the assay *Linearity and recovery* Increasing amounts of b/o-leptin were added to plasma containing low concentrations of leptin, and the samples were assayed. Plasma samples containing a high concentration of leptin were diluted in leptin-free plasma and assayed. The leptin-free plasma was obtained by incubation of sheep plasma with activated charcoal (25 mg/ml) under continuous mixing for 24 h at 4 °C. The charcoal was removed by centrifugation.

Comparison with the Linco Multispecies Leptin Kit Single venepuncture samples taken from 50 mature Merino ewes were processed using both the assay described here and the Multispecies Leptin Kit from Linco Research (St Charles, MO, USA) following the manufacturer's specifications. We used aliquots of 100 µl plasma in both assays and compared the two sets of data.

Comparison of serum and plasma samples Single blood samples (30 ml) were taken by venepuncture from 10 rams. Aliquots of 10 ml were collected either in heparin-coated plastic tubes (150 IU lithium-heparin/tube, Sarstedt, SA, Australia), in glass tubes containing EDTA (15 mg EDTA/tube, Becton Dickinson, NJ, USA) or in plain glass tubes. Samples for serum (in plain glass tubes) were left at room temperature for 2 h and overnight at 4 °C before centrifugation. Plasma or serum was separated by centrifugation at 2000 ***g*** for 15 min at 4 °C, and then assayed.

LH radioimmunoassay

LH was measured in duplicate by double antibody radioimmunoassay as described by Tjondronegoro *et al.* (1996). The preparation CNRS-M3 (biopotency 1·8 IU NIH-LH-S1 per mg) used for reference was kindly supplied by Dr M Jutisz (Collège de France, Paris, France). The tracer was prepared using NIDDK-oLH-I-3 donated by the National Institute of Diabetes, Digestive and Kidney Disease (Baltimore, MD, USA). The anti-LH serum R1 was raised in a rabbit in our laboratory and had the following cross-reactions; 100% with NIH-LH-S1, 97% with NIH-LH-S20, 18% with NIAMDD-0FSH-RP1, 0·93% with NIH-FSH-S12, 8·2% with oGH and 5% with NIH-TSH-S8. The intra-assay coefficients of variation were estimated using three pooled samples containing 1 ng/ml (11·5%), 2·6 ng/ml (5·5%) and 4·6 ng/ml (5·1%). The limit of detection was 0·1 ng/ml. All the samples

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Cow     MRCGPLYRFLWLWPYLSYVEAVPIRKVQDDTKTLIKTIVTRINDISHTQSVSSKQRVTGL
Sheep   ---------------------VPIRKVQDDTKTLIKTIVTRINDISHTQSVSSKQRVTGL
Human   MHWGTLCGFLWLWPYLFYVQAVPIQKVQDDTKTLIKTIVTRINDISHTQSVSSKQKVTGL
                             *** ****************************** ****

Cow     DFIPGLHPLLSLSKMDQTLAIYQQILTSLPSRNVVQISNDLENLRDLLHLLAASKSCPLP
Sheep   DFIPGLHPLLSLSKMDQTLAIYQQILASLPSRNVIQISNDLENLRDLLHLLAASKSCPLP
Human   DFIPGLHPILTLSKMDQTLAVYQQILTSMPSRNVIQISNDLENLRDLLHVLAFSKSCHLP
        ******** * ********* *****  * *****  ************** ** **** **

Cow     QVRALESLESLGVVLEASLYSTEVVALSRLQGSLQDMLRQLDLSPGC
Sheep   QVRALESLESLGVVLEASLYSTEVVALSRLQGSLQDMLRQLDLSPGC
Human   WASGLETLDSLGGVLEASGYSTEVVALSRLQGSLQDMLWQLDLSPGC
            ** * *** ***** ******************* ********
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Figure 1 The amino acid sequence of bovine leptin (Genbank accession no U43943) aligned with those from human (Genbank accession no U18915; Zhang *et al.* 1994) and sheep (Genbank accession no U84247; Dyer *et al.* 1997*b*). The signal sequence is underlined. Asterisks indicate amino acids conserved between the three sequences. The differences between the bovine and ovine leptins are boxed.

were measured in the same assay to avoid the effects of between-assay variation on pulse analysis.

Experimental protocols for animal studies

Merino sheep of both sexes were purchased from either the CSIRO Yalanbee Research Farm (Bakers Hill, Western Australia) or from registered breeders of the Australian Merino Society. They were at least 2 years old and in good health. The experimental protocol was approved by the Animal Ethics Committee of the University of Western Australia according to the recommendations of the Australian National Health & Medical Research Council. Blood was sampled by jugular venepuncture for infrequent samples, or jugular catheter for frequent samples (e.g. removal of 3 ml blood every 20 min). Catheters were inserted without anaesthesia about 24 h before sampling and the site of insertion was treated with topical antibiotic (Tricin, Jurox, Auckland, New Zealand).

Long-term effect of nutrition on leptin concentrations Adult Merino sheep, 68 females and 67 wethers (males castrated at about 8 weeks of age), were allocated into one of three groups of same average body condition score (1·75 ± 0·04) and live weight (43·6 ± 0·3 kg). For 2 months, they were fed either a low diet (natural summer pasture), a medium diet (the same pasture plus a supplement of lupin grain, 350 g per head per day), or a high diet (the same pasture and 800 g lupin grain per head per day). Before and after the dietary treatment period, body condition was scored using the method described by Jefferies (1961). At the end of the dietary treatment period, subcutaneous back-fat thickness was measured at site 'C' on the 12th rib according to the method described by Gooden *et al.* (1980). This non-invasive measurement is correlated with body condition score on a scale of 0–5 (r=0·82) and is a good predictor of carcass fat percentage (r^2=0·58; Gooden *et al.* 1980). Blood (10 µl) was sampled by venepuncture before and after the dietary treatment and serum was separated and stored at –20 °C until assayed for leptin.

Short-term effects of nutrition and 24 h patterns of leptin concentration The daily pattern of secretion of leptin was measured in six adult rams (live weight 55·4 ± 2·8 kg) that were kept indoors under natural light. They were fed 800 g wheaten hay per day for 2 weeks and then fed on a high plane of nutrition (1 kg wheaten hay supplemented with 800 g lupin grain). Feeding time was 0700 h. Blood (3 ml) was sampled via in-dwelling catheter every 20 min for 24 h (starting at 0700 h), before and 5 days after the change of diet. During the hours of darkness, samples were taken under a 3 W red lamp to prevent any disturbance of the natural daily light cycle. Plasma samples were stored at –20 °C until assayed for leptin and LH.

Episodes in the pattern of leptin concentration The possibility of episodic secretion of leptin was investigated in five adult rams (live weight 61·5 ± 5·9 kg) that were maintained under the same conditions and receiving the same dietary treatments described above. Starting at 0700 h, blood (3 ml) was sampled via in-dwelling catheter every 5 min for 4 h, before and 5 days after the change to the high plane of nutrition. On the sampling day, animals were fed after sampling to limit interferences. Serum samples were stored at –20 °C until assayed for leptin.

Effects of nutrition on leptin concentrations in blood and CSF We used eight mature Merino rams (2 years old) of similar body weight (59·2 ± 2·5 kg) that were each

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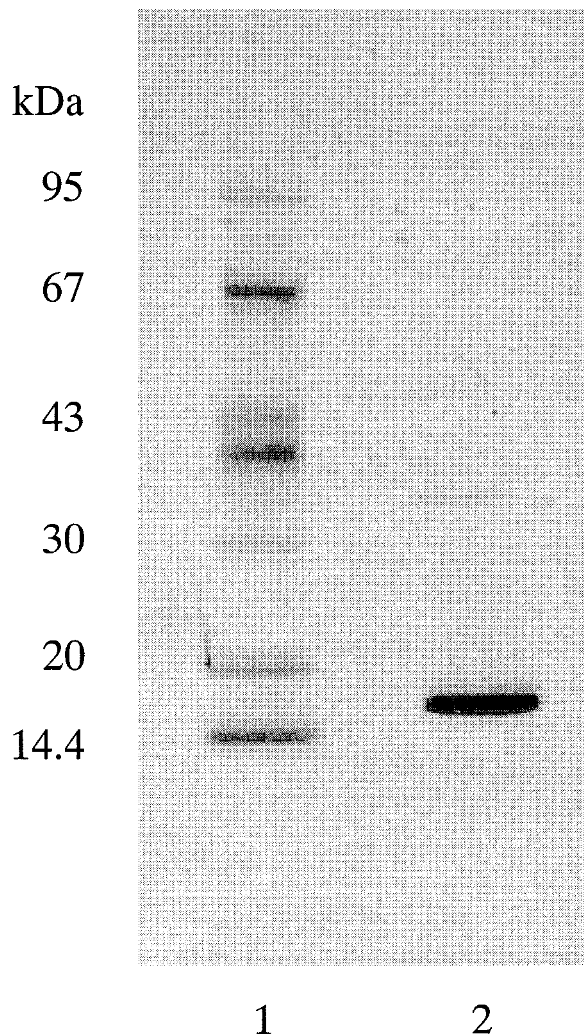

Figure 2 Silver-stained gradient SDS-PAGE of purified recombinant bovine hexaHis–leptin (lane 2; 1 µg). Molecular weight standards are in lane 1.

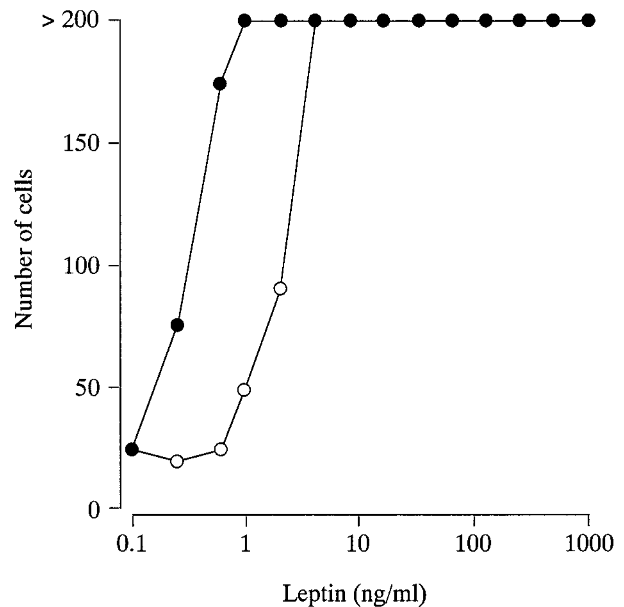

Figure 3 Proliferation of Ba/F3 cells transfected with the 'long' form of the human leptin receptor after stimulation by recombinant bovine hexaHis–leptin (○) or recombinant human leptin (●). Cells were counted after 48 h of incubation. Note the logarithmic scale of the *x* axis.

bearing a cannulae in a lateral cerebral ventricle. They were housed in individual indoor pens, under natural photoperiod, and acclimatised for 2 weeks to a maintenance diet consisting of 800 g wheaten chaff and 100 g lupin grain, supplemented with 16 g mineral mix (Siromin, Narrogin Mineral Stockmix, Narrogin, Australia). Animals were then allocated to either a low diet (300 g wheaten chaff plus 10 g lupin grain) or a high diet (800 g wheaten chaff plus 800 g lupin grain). They were fed daily at 0800 h for 5 days. The day before sampling, indwelling catheters were placed in both jugular veins. On Day 5 of the nutritional treatment period, blood (3 ml) and CSF (0·8 ml) were sampled every 20 min for 24 h beginning at 0700 h. Plasma and CSF were stored at − 20 °C until assayed. All plasma samples were assayed for LH. Leptin concentrations in both plasma and CSF were measured in the pooled samples from each 24 h period so that sufficient sample was available for the CSF assay.

Data analysis

Groups were compared by ANOVA using the Super-ANOVA package (Abacus Concepts, USA), and Student's paired *t*-test was used for comparisons within groups. LH pulse frequencies were analysed using the Mann–Whitney *U* test for independent groups and Wilcoxon's test for related groups (Siegel 1956). Linear regression and Pearson product–moment correlations were used to evaluate the relations between variables. Probability less than 0·05 was accepted as significant. Serial samples for LH and leptin were analysed with a modified version of the 'Pulsar' algorithm developed by Merriam & Wachter (1982) and modified for the Apple Macintosh computer ('Munro', Zaristow Software, West Morham, Haddington, East Lothian, UK). The '*G*' parameters (the number of standard deviations by which a peak must exceed the baseline in order to be accepted) were 3·98, 2·4, 1·68, 1·24, and 0·93 for *G*1–*G*5, these being the requirements for pulses composed of one to five samples that exceed the baseline, respectively. The Baxter parameters describing the parabolic relationship between the concentration of a hormone in a sample and the standard deviation (assay variation)

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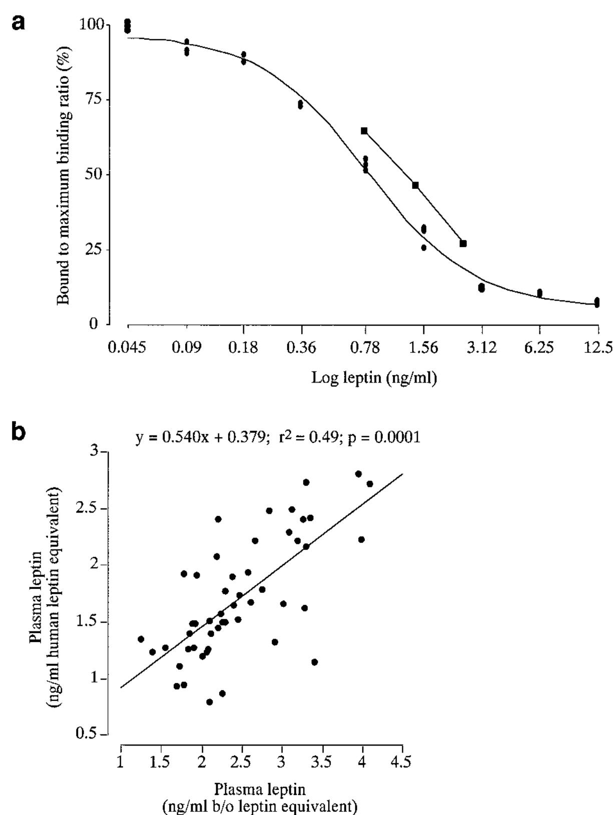

Figure 4 (a) A typical standard curve for b/o-leptin (●) with a serial dilution of a pooled sheep sample in leptin-free plasma (■). (b) Correlation between plasma leptin concentrations in adult male sheep (n=50) measured in the b/o-leptin assay and in a human leptin assay (Linco Leptin Multispecies Kit).

about that concentration were 0·30853 ($b1$, the y intercept), 0·00213 ($b2$, the x coefficient) and 0·00268 ($b3$, $x2$ coefficient) for LH, and 0·48234 ($b1$), 0·00312 ($b2$) and 0·00208 ($b3$) for leptin.

Results

Production of recombinant hexaHis–leptin

Recombinant bovine leptin with an amino-terminal hexa-His tag was produced in bacteria (Fig. 1). The identity of the recombinant protein was confirmed using the following criteria: (1) the size of the protein as measured by SDS-PAGE was consistent with its predicted size (i.e. 16 kDa; Fig. 2); (2) the hexaHis–leptin could be affinity purified on a Ni-NTA resin, as would be expected for a protein containing the designed hexaHis-affinity tag; (3) the amino-terminal amino acid sequence of the hexaHis–leptin was MRGS(H)6 GSVPIRK which is consistent with the design of this recombinant fusion protein; the five carboxy-terminal amino acids of this sequence (underlined) correspond to those of the mature amino-terminus of bovine leptin (Fig. 1).

The relatively weakly stained 33 kDa band (Fig. 2) is probably a dimer of the hexaHis–leptin as its amino-terminal sequence is the same as the sequence of the major 16 kDa band. Less than 5% of the hexaHis–leptin was present as this putative dimer. The hexaHis–leptin was purified from solubilised inclusion bodies and refolded in a manner that resulted in a fully soluble protein in the absence of denaturating agents. The solubility of this recombinant protein is consistent with a correctly folded protein.

In vitro *bioassay*

The refolded hexaHis–leptin was biologically active in the cell proliferation assay system with a half-maximal effect at 1·5 ng/ml, compared with a value of 0·4 ng/ml for recombinant human leptin (Fig. 3). This difference in specific activities in an assay that uses the human leptin receptor may be attributed to the 18 amino acid differences between the mature forms of human and bovine leptins (Fig. 3). The hexaHis-affinity tag and the efficiency of refolding may also have contributed. Since the murine cell line had been transfected with a human leptin receptor, the minor sequence differences (two conservative substitutions) between bovine and ovine leptins are not likely to be significant in the context of either biological activity or immunogenicity.

Radioimmunoassay validation

In the linear part of the standard curve, the amount of leptin added to samples was recovered within 2·5% accuracy. Diluted samples were measured to within 2·5% accuracy (Fig. 4a). The radioimmunoassay using b/o-leptin was a more sensitive and read samples over a wider range than the Linco Multispecies Kit (Fig. 4b). Consequently, the correlation between the two assays was poor, although highly significant. Leptin concentrations were similar between the heparin–plasma and serum samples, but EDTA–plasma gave unreliable readings (data not shown).

Long-term effects of nutrition on plasma leptin concentrations

The dietary treatments led to large differences in both body condition score (high diet 2·54 ± 0·06; medium 2·00 ± 0·06; low 1·21 ± 0·04; P=0·0001) and live weight (high diet 50·6 ± 0·5; medium 46·2 ± 0·6; low 40·6 ± 0·6; P=0·0001). The back-fat thickness and the ratio of back-fat thickness to live weight were higher in the high diet group than in the medium or low diet groups (Fig. 5a,b). Plasma leptin concentrations were similar before and after dietary treatment in the low diet group but increased in parallel with the changes in body condition in both high

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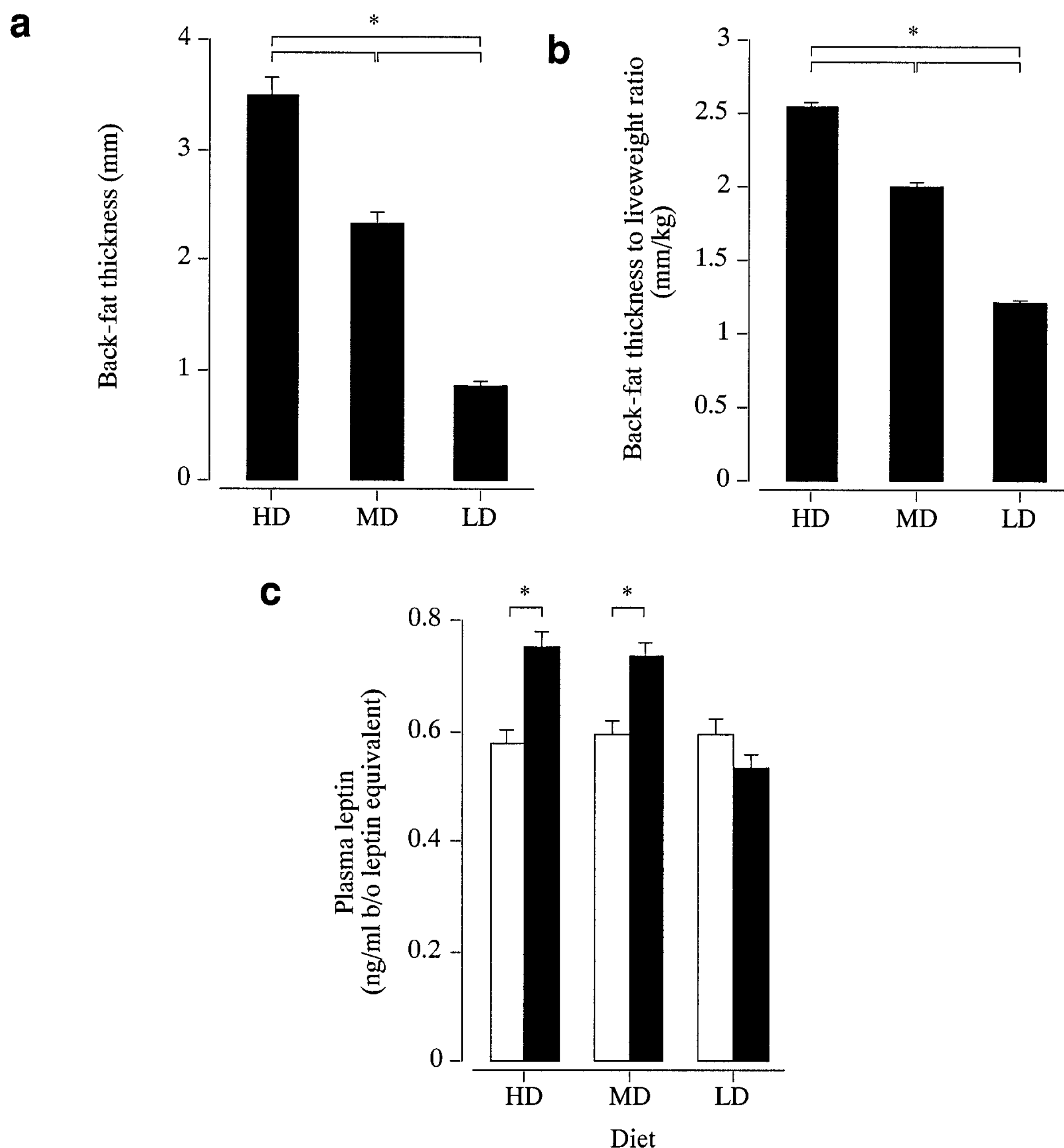

Figure 5 Effect of 2 months of high diet (HD), medium diet (MD) or low diet (LD) in sheep on back-fat thickness (a), the ratio of back-fat thickness to liveweight (b), and plasma leptin concentrations, before (□) and after (■) the dietary treatments (c). * $P<0·05$ ($n=135$).

and medium diet groups (Fig. 5c). The average leptin value in the total flock was higher for females than for males before (0·67 ± 0·02 vs 0·52 ± 0·01 ng/ml; $P=0·0001$) and after the dietary treatment period (0·63 ± 0·02 vs 0·71 ± 0·03 ng/ml; $P=0·01$). Plasma leptin concentrations were highly correlated with the ratio of back-fat thickness to live weight, with similar correlation coefficients for the two sexes (Fig. 6).

Short-term effects of nutrition on leptin concentrations in plasma and CSF

Plasma leptin concentrations and LH pulse frequency had both increased by 5 days after the change from a low to a high diet (Fig. 7a). The concentrations of leptin did not vary over the 24 h sampling period (Fig. 8). Eleven significant 'episodic increases' were detected in samples that were taken every 20 min (Fig. 8) or every 5 min (Fig. 9).

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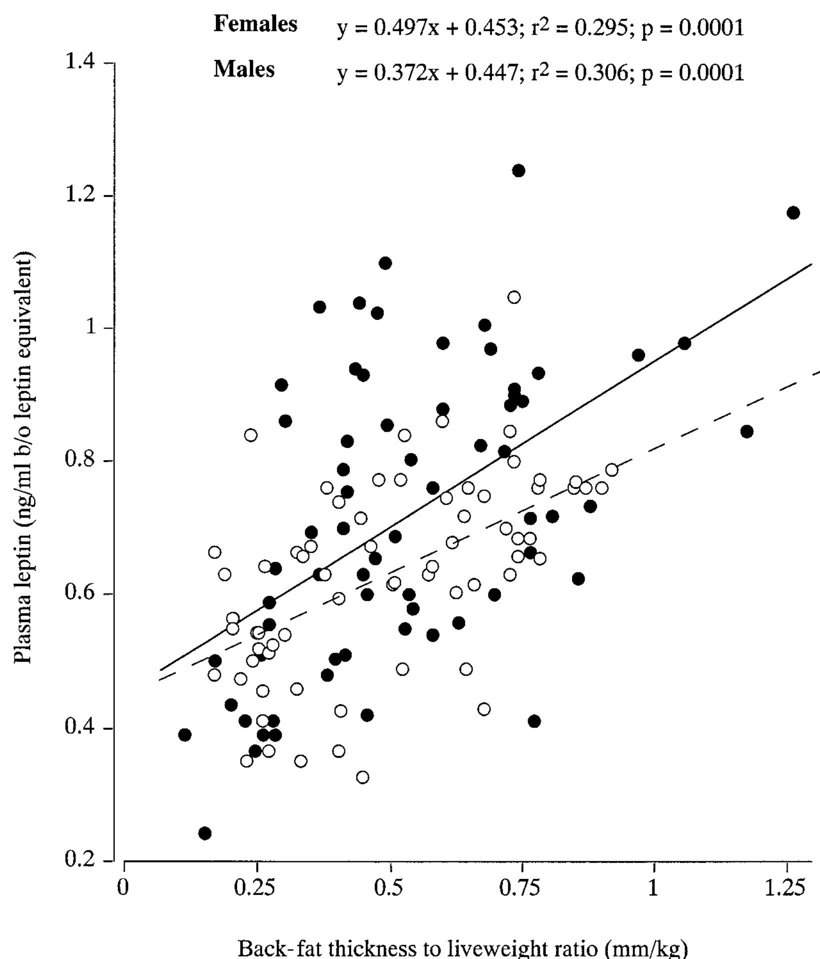

Figure 6 Correlation between ratio of back-fat thickness to live weight and plasma leptin concentrations in female Merino sheep (n=68, continuous line) and prepubertally castrated male Merino sheep (n=67, dashed line). ○, males; ●, females.

They were scattered among the 22 profiles analysed, although six such 'episodes' were observed in a single 20 min profile. The occurrence of 'episodes' was not modified by 5 days of high diet, yet this treatment increased the baseline concentrations of leptin from 0·65 ± 0·06 to 1·27 ± 0·14 ng/ml (P=0·008; n=6). There was no temporal relationship between leptin 'episodes' and LH pulses within a series of samples.

In the rams with cerebroventricular cannulae, LH pulse frequency was increased by the acute change to a high diet (Fig. 7b). Animals fed the high diet had higher leptin concentrations in both plasma and CSF than animals fed the low diet (Fig. 7b). Plasma and CSF concentrations of leptin were related (r^2=0·50, P<0·05, n=8).

Discussion

Our radioimmunoassay based on recombinant bovine–ovine leptin has allowed us to measure leptin in blood plasma and third ventricular CSF from sheep. Circulating concentrations of leptin are sexually dimorphic and highly correlated with body fat content in female and castrated male sheep. Frequent serial samples indicate occasional 'episodes' of secretion, but this probably does not reflect controlled pulsatile secretion such as that seen with LH. Finally, acute increases in the level of nutrition evoke increases in leptin concentrations in both blood plasma and cerebrospinal fluid that are correlated with stimulation of GnRH activity. These observations are consistent with current hypotheses suggesting that an increase in metabolic status stimulates adipose tissue to secrete leptin which is carried to the central nervous system, where it is transported into the cerebrospinal fluid and thus affects the centres controlling appetite and perhaps reproduction (Houseknecht & Portocarrero 1998).

Several observations support the validity of the assay. First, we used a recombinant preparation of bovine–ovine leptin that is biologically active, as demonstrated by the

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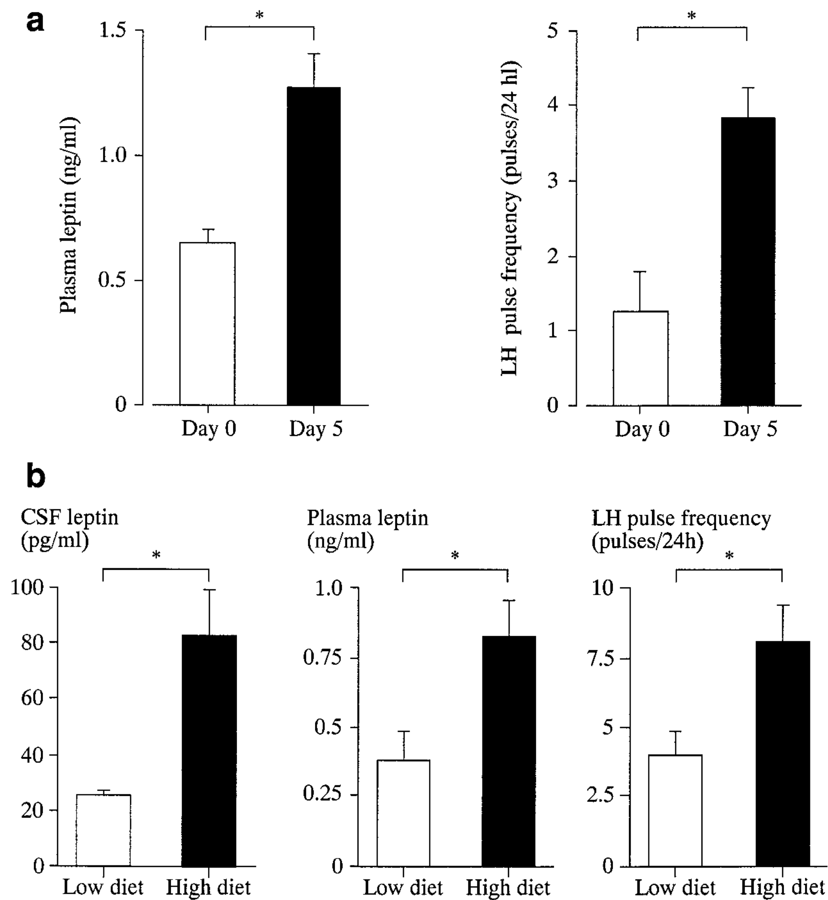

Figure 7 (a) Mean plasma concentrations of leptin and LH pulse frequency in intact male sheep before (Day 0) and 5 days after (Day 5) a change from a low to a high diet. $^{*}P<0{\cdot}05$ ($n=6$). (b) Leptin concentrations in plasma and CSF, and LH pulse frequencies, in rams fed low or high diets for 5 days. Note the difference in the scales of the *y* axes for plasma and CSF leptin. $^{*}P<0{\cdot}05$ ($n=4$).

results of the *in vitro* bioassay, and also by the decrease in food intake that it induces when infused into the third cerebral ventricle of mature male sheep (Celi *et al.* 1998). Second, despite the great deal of homology between the human and bovine–ovine forms of the hormone, the recombinant human leptin from Linco Research cross-reacts very little in our assay (<1%). Third, plasma concentrations of leptin measured with our assay are always higher than the concentrations measured using the Linco Multispecies Kit, yet our limit of detection (100 pg/ml) is lower than that of the Multispecies Kit (500 pg/ml) probably because the kit uses antibodies raised against a recombinant human leptin. Thus, this assay appears to be specific for biologically active bovine–ovine leptin and sufficiently sensitive to reveal important physiological changes.

Generally, the data suggest considerable similarity between humans and sheep, and thus monogastrics and ruminants, in the control of leptin secretion: first, plasma leptin concentrations range from 1 to 9 ng/ml in sheep, similar to the range observed in healthy humans (Considine *et al.* 1996); second, in both male and female sheep, leptin concentrations depend on body fat content, as previously described for humans (Considine *et al.* 1996), although we found that only 30% of the variation in leptin concentrations is explained by back-fat thickness, suggesting that other factors exert major effects on leptin secretion; and third, the difference between sexes in sheep is similar to that seen in other species, including human and rat (Rosenbaum *et al.* 1996, Landt *et al.* 1998). The role of the gonadal steroids in determining this gender difference is controversial. Testosterone and leptin concentrations are inversely correlated in men, and testosterone treatment decreases leptin concentrations in male humans and rats (Luukkaa *et al.* 1998, Pinilla *et al.* 1999). On the other hand, testosterone does not stimulate leptin production from

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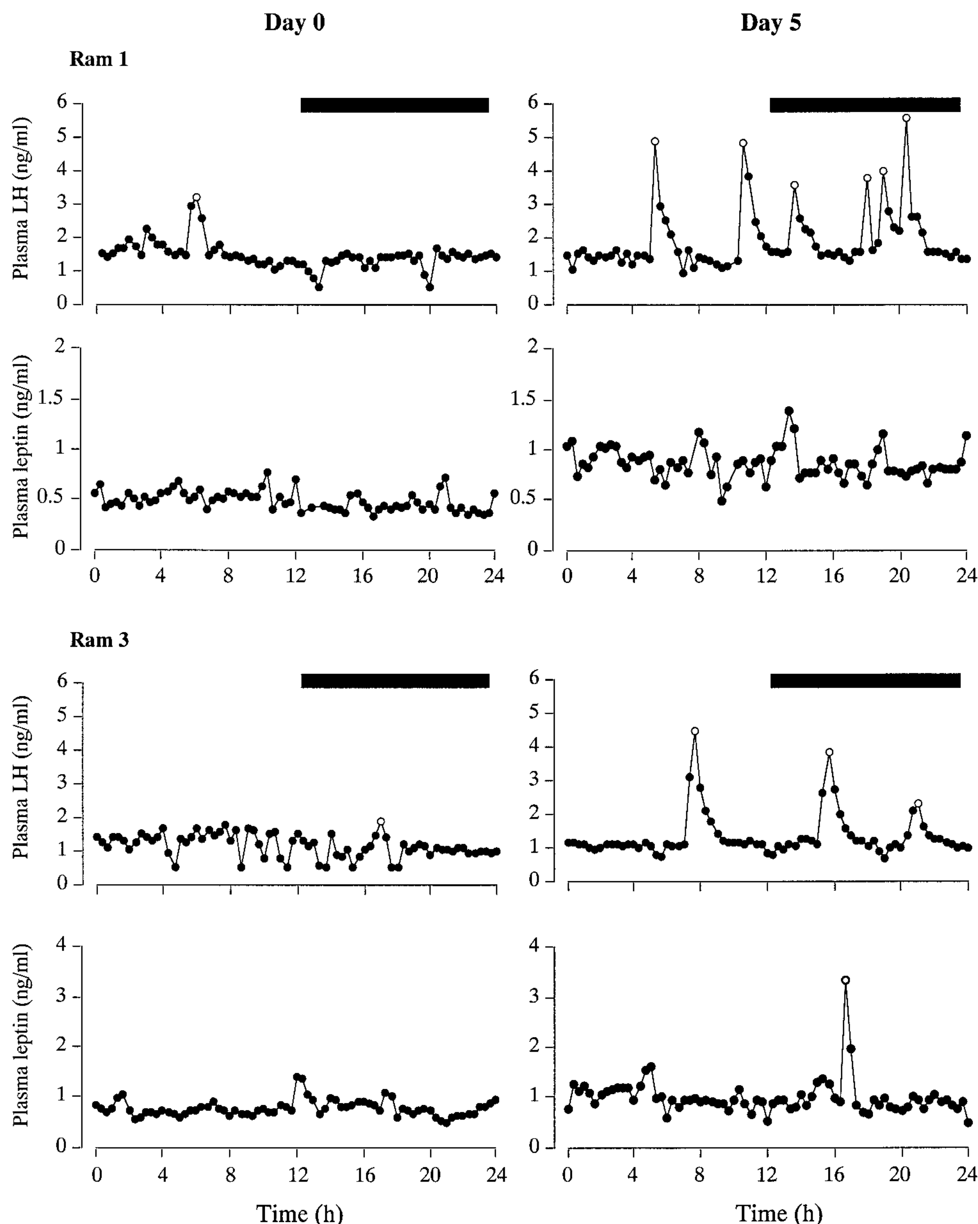

Figure 8 Profiles of secretion of LH and leptin in two intact male sheep before (left panel) and 5 days after (right panel) change from a low to a high diet. The horizontal bar indicates the dark period of the day.

cultured human adipose tissue (Pineiro *et al*. 1999), suggesting that an intermediary factor is involved. The present studies suggest that plasma concentrations of leptin are similar for wethers and intact rams, so it seems unlikely that testicular steroids are solely responsible for the sex difference. Moreover, leptin concentrations for castrated males were also lower than those in intact females, even when corrected for back-fat thickness. The females were sampled at random times in the oestrous cycle, so the sex difference might be accentuated if the stage of cycle were taken into account. This remains to be tested, as do the effects of oestrogen, progestagen and androgen on leptin production.

The secretion of leptin might be episodic, but controlled pulsatile secretion, as described for other hormones such as LH (Veldhuis 1998), is not likely in sheep and does not seem to be involved here. Importantly, a higher frequency of sampling did not improve the definition of the episodes, suggesting that they are uncontrolled, arguably

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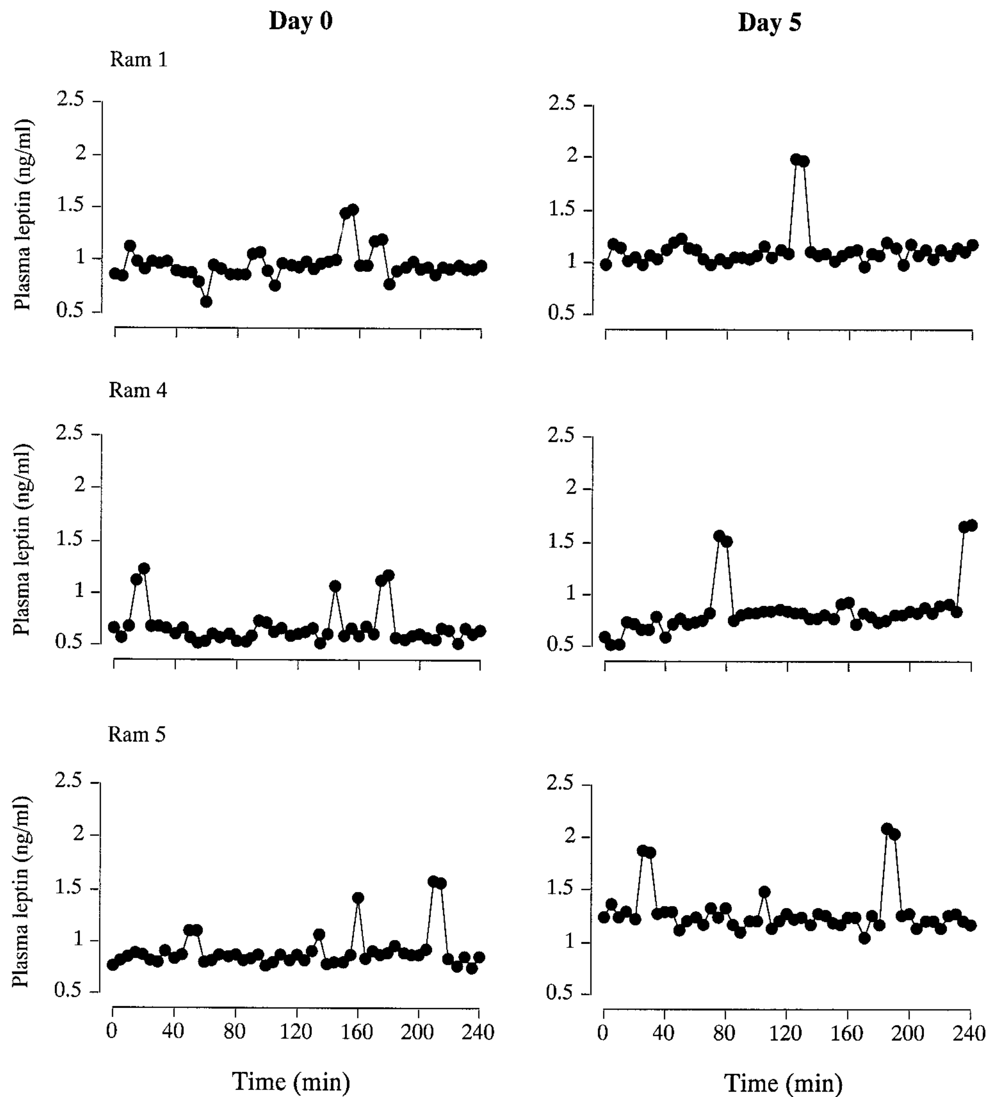

Figure 9 Representative leptin profiles observed in serial (every 5 min) samples in three intact male sheep before (left panel) and 5 days after (right panel) change from low to high diet.

random, events. On the other hand, the pattern of leptin secretion might be sexually differentiated because it appears to be pulsatile in women (Licinio *et al.* 1998), and we have yet to study frequent serial samples in female sheep.

There was no increase in the baseline values for plasma leptin during the night in our male sheep, in contrast to reported diurnal patterns for healthy women and lean or obese women and men (Sinha *et al.* 1996, Licinio *et al.* 1998). Schoeller *et al.* (1997) showed that this diurnal rhythm in leptin secretion in humans is entrained by the meal pattern and, in rats, food intake increases the production of ob mRNA in adipose tissue (Saladin *et al.* 1995). In the present study, however, leptin concentrations were not affected by feeding time and baseline values did not vary during the 24 h after feeding or during frequent blood sampling (every 5 min) which was carried out after 24 h fasting. In this respect, ruminants may differ from monogastric species because the process of rumen fermentation buffers the entry of nutrients into the blood stream. For whatever reason, biological rhythms that regulate leptin secretion seem to differ between humans, rats and sheep.

Leptin concentrations in plasma and cerebrospinal fluid were correlated and increased in parallel with the increase in LH pulse frequency when rams were moved to a high level of nutrition. Leptin in the circulation and CSF has been suggested to link nutrition and reproduction in

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humans, monkeys and rats (Mantzoros *et al.* 1997, Finn *et al.* 1998, Nagatani *et al.* 1998) and our observations seem to support this hypothesis. However, it is only a correlation and the relative importance of leptin in the control of the activity of the hypothalamo–pituitary–gonadal axis of sheep requires confirmation using a more direct approach (Cunningham *et al.* 1999, Foster & Nagatani 1999). This is illustrated by the work of Henry *et al.* (1999), who found that intracerebroventricular infusion of human recombinant leptin did not affect LH secretion in ovariectomised ewes. The use of human hormone and the absence of steroid feedback on the hypothalamo–pituitary axis are problematical, but our preliminary study with i.c.v. infusion of b/o-leptin into intact rams also shows no effect on LH pulse frequency (Celi *et al.* 1998).

The increases in leptin concentrations that we observed in sheep within 5 days after an increase in the level of nutrition are not likely to be mediated by increases in live weight or fat mass because such endpoints change slowly. The supply of nutrients, such as glucose and volatile fatty acids, are also affected by this change of diet (Harman 1991, Boukhliq *et al.* 1996) but, in humans, acute hyperglycaemia does not stimulate leptin secretion (Ryan & Elahi 1996) and the levels of fatty acids in blood are not correlated with leptin concentrations (Kolaczynski *et al.* 1996). Alternatively, metabolic hormones that are affected by acute changes in nutrition, such as insulin (Miller *et al.* 1998), may affect *ob* gene expression and leptin secretion in ovine adipose tissue, as has been demonstrated for rats (Saladin *et al.* 1996).

In summary, leptin concentrations in blood and CSF in sheep are influenced by both acute and chronic changes in nutrition, and the response appears to be sexually differentiated. The regulation of leptin production by gonadal steroids and interactions between leptin production and other metabolic hormones that are affected by nutrition, such as insulin, now need to be investigated. The techniques described in this paper should help us to answer these questions.

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