Plasma leptin determination in ruminants: effect of nutritional status and body fatness on plasma leptin concentration assessed by a specific RIA in sheep

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Abstract

A specific leptin RIA was developed to assess concentrations of leptin in ovine plasma, and was shown to be efficient with bovine and caprine plasma. A specific, high-affinity antibody was generated against recombinant ovine leptin which, when used in a competitive leptin RIA, provided valid estimates of linearity ($r=+0{\cdot}989$–$0{\cdot}998$), recovery (102%), repeatability (13%) and limit of sensitivity (0·83 ng/ml for 100 µl sample size). Serial dilutions of five ovine, bovine or caprine plasma samples showed good linearity and parallelism with the recombinant ovine leptin standard curve. A comparison of this RIA was made with a commercial 'multi-species' RIA kit using 56 ovine plasma samples. Major differences were found in assay sensitivity. Non-lactating, non-pregnant, ovariectomized ewes were fed a ration for 65 days which provided 90 ± 9% (control; $n=12$) or 39 ± 2% of maintenance energy requirements (underfed; $n=16$) in order to analyse the respective effects of body fatness (estimated by either an *in vivo* dilution technique or body condition scoring) and of nutritional status on plasma leptin concentration. There was a significant positive correlation between body fatness or body condition score and plasma leptin levels ($r=+0{\cdot}68$, $P<0{\cdot}001$ or $r=+0{\cdot}72$, $P<0{\cdot}001$ respectively). When concentrations of leptin were assessed over time, underfed ewes exhibited a dramatic reduction in plasma leptin values (−56%, $P<0{\cdot}001$). These data provide strong evidence that, in sheep, the variations in plasma concentrations of leptin are related to variations in body fatness (35%) and, to a lesser extent, in nutritional status (17%).

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Introduction

Kennedy (1953) proposed the lipostatic theory whereby control of mammalian energy balance, body weight (BW) and body composition involved a hypothalamic feedback loop in which body fat reserves modulated food intake and energy expenditure. Zhang *et al.* (1994) identified the *ob* gene, which encodes the 16 kDa protein leptin that is secreted by adipose tissue and is probably a major component of this homeostatic system. Within the last 5 years numerous studies have confirmed leptin's involvement in metabolic homeostasis in human and rodent species (Friedman & Halaas 1998). In the livestock species such evidence is incomplete; however, a similar role has been suggested. Dyer *et al.* (1997*a*) reported that hypothalamic expression of the long form of the leptin receptor increased in feed-restricted ewes, presumably the result of lower circulating concentrations of leptin. Moreover, it was recently reported that central injection of ovine (Morrison *et al.* 1998) or human (Henry *et al.* 1999) recombinant leptin into ewes induced a reduction in appetite.

The cloning and sequencing of ovine and bovine leptin cDNA (Dyer *et al.* 1997*b*, Ji *et al.* 1998) revealed that their coding region has 86 and 87% homology, respectively, with the human sequence. Furthermore, mRNA studies demonstrated that the ruminant leptin gene is specifically expressed in adipose tissues (Dyer *et al.* 1997*b*, Ji *et al.* 1998, Tsuchiya *et al.* 1998) and is related to feeding level

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(Bocquier *et al.* 1998, Kumar *et al.* 1998). Leptin gene expression is also modulated by daylength in sheep, independently of food intake, adiposity and reproductive activity (Bocquier *et al.* 1998). The complexity of peripheral systems involved in the physiological regulation of plasma leptin has been described in rodents and humans (Trayhurn *et al.* 1998). However, in order to better understand this regulation in ruminants, peripheral concentrations of leptin must be assessed in these species. Several leptin RIAs and ELISAs have been developed for use with human or rodent serum or plasma samples (e.g. Considine *et al.* 1996, Hardie *et al.* 1996, Blum *et al.* 1997, Imagawa *et al.* 1998). In ruminants, preliminary results were obtained in our laboratory (Bocquier *et al.* 1998, Chilliard *et al.* 1998*a*, Delavaud *et al.* 1999) with a commercially available 'multi-species' RIA kit, developed according to Ma *et al.* (1996). However, the weak antibody specificity for ruminant leptin may not have been sufficient to determine reliable values for leptin, despite the apparent physiological significance of their variations. The aim of the present study was to develop a specific ovine leptin RIA and to apply this assay to the assessment of plasma samples obtained from ewes of known body fatness receiving different levels of energy intake.

Materials and Methods

Leptin RIA

Ovine-specific RIA *Recombinant ovine leptin* For all assays, ovine leptin was produced and purified as described by Gertler *et al.* (1998). It was >98% pure by SDS-PAGE under non-denaturating conditions. On gel filtration at pH 8 it showed about 10% dimers and 90% monomers.

Antibody production Antibodies were raised in six New Zealand rabbits as follows: 125 μg recombinant ovine leptin (Gertler *et al.* 1998) were dissolved in physiological saline (0·5 ml) and emulsified at 4 °C in Freund's complete adjuvant (Sigma, St Louis, MO, USA). The antigen was administered s.c. twice each month and blood samples (5 ml) were collected on alternate weeks by ear vein venepuncture into evacuated blood collection tubes containing 1·8 mg EDTA/ml (Greiner, Labortechnik, Kremsmünster, Austria). After centrifugation, rabbit plasma was collected and stored frozen at − 20 °C.

Recombinant ovine leptin labelling Ten micrograms ovine leptin were dissolved in 10 μl phosphate buffer (0·05 M, pH 7·5) and radioiodinated in the presence of 1 mCi (37 MBq) $Na^{125}I$ (IMS 300, Amersham-Pharmacia Biotech, Orsay, France) by the chloramine-T method (Hunter & Greenwood 1962) modified as previously described by Kann (1971). Briefly, the reaction was initiated by 30 μg chloramine-T (3 mg/ml), then stopped after 30 s by adding 50 μg sodium metabisulphite (5 mg/ml). Finally, 10 μg potassium iodide (KI, 10 mg/ml) were added to the reaction vial. All reagents were purchased from Merck (Darmstadt, Germany) and were diluted in phosphate buffer (0·05 M, pH 7·5). The reaction mixture was applied to a 0·9 × 30 cm column (K9–30, Pharmacia Biotech, Orsay, France) containing AcA 54 Ultragel (Sepracor-IBF; Biotechnics, Villeneuve la Garenne, France) pre-equilibrated with a 0·01 M phosphate buffer, 0·15 M NaCl, pH 7·2 containing 0·1% gelatine, 0·02% sodium azide and Tween 20 (100 μl/l). Ultimately, the reaction vial was rinsed with 100 μl of the KI solution and was applied to the separation column. Elution was performed with the same buffer and 1 ml fractions were collected. Radioactivity of each fraction was measured in an ionization chamber (Merlin-Gerin, Grenoble, France) and the leptin fractions that were in the first radioactive peak were immediately diluted (v/v) with pure glycerol (Prolabo, Paris, France) and stored at − 20 °C. Iodination yield accounted for 60–80%, resulting in a specific activity of 60–80 μCi/μg.

RIA procedure A specific, double-antibody, non-equilibrium RIA was established employing the previously described column elution buffer as the assay and reagent dilution buffers. Initially, the most suitable antibody (across rabbits), antibody dilution, and radioiodinated fraction of leptin were identified by binding studies. Subsequently, the assay was performed as follows. Standard concentrations of recombinant ovine leptin were prepared for each standard curve from a stock preparation of leptin (1 μg/ml) diluted in buffer/glycerol (v/v) and stored at − 80 °C. Triplicate standards (0·0833, 0·125, 0·25, 0·4, 0·75, 2·0, 2·5 and 4 ng/tube) were added in a volume of 50 μl. For the sample tubes, duplicate aliquots of 100 μl plasma were assayed. Both standard and sample tubes were then incubated for 24 h at 4 °C with 50 μl of a 1:1500 working dilution of leptin antisera (Ab 7137 diluted in buffer containing 1:100 normal rabbit serum) to achieve a total volume of 400 μl in incubation buffer. After the initial incubation, 100 μl ^{125}I-ovine leptin (20 000 c.p.m.) were added to each tube and the incubation continued for an additional 20 h at 4 °C. The final dilution of leptin antisera was 1:15 000. Bound and free ligands were then separated by adding 100 μl of a specific anti-rabbit ram plasma which was diluted either 1:5 in horse serum for standard curves or 1:5 in incubation buffer for the unknown plasma samples. The addition of horse serum was to equalize the protein content in all tubes (standards and samples) by the end of the double-antibody precipitation procedure, which was allowed to proceed at room temperature for 1 h. Precipitation of the antibody–antigen complexes was then accomplished by the addition of 2·0 ml 4·4% polyethylene glycol 6000 (Prolabo), immediate centrifugation (3000 ***g***, 25 min, 4 °C), and the unbound ^{125}I-ovine leptin was removed by

aspiration of the supernatant. The remaining radioactivity in the precipitate was counted with a Cobra II gamma counter (Packard Inc., Downers Grove, Australia).

Multi-species RIA kit Plasma immunoreactive leptin was independently determined in duplicate 100 µl aliquots of plasma samples utilizing a 'multi-species' RIA kit (XL-85K; Linco, St Louis, MO, USA) according to the manufacturer's recommendations. Briefly this double-antibody assay utilized a guinea pig leptin antibody, ^{125}I-labelled human leptin, and human leptin as standards. The limit of sensitivity was 1·0 ng/ml (for 100 µl sample size) and the intra- and interassay coefficients of variation were 6·5 and 7·4% respectively.

Animals and measurements

Parallelism studies for RIA validation were conducted on ovine and bovine plasma from dry, non-pregnant ewes and cows that were either fat and overfed, or lean and underfed, in order to increase or decrease, respectively, the levels of endogenous leptin, as could be predicted from previous results with the commercial kit (Chilliard *et al.* 1998*a*). One fat and one lean ewe (body condition score (BCS) 4 and 1·5, estimated on a 0 to 5 scale) were respectively either overfed (hay freely available, concentrate increased from 250 g to 1 kg/day on 5 days) or underfed (straw 0·15 kg/day during 3 days). In the same way, one fat and one lean cow (BCS: 4 and 2·5) were respectively either overfed (hay freely available, concentrate increased from 2 to 8 kg/day on 5 days) or underfed (straw 1·50 kg/day during 3 days). At the end of each nutritional treatment, jugular blood samples were collected into tubes containing 0·24% EDTA. After centrifugation, plasma was removed and immediately frozen at −20 °C until RIA analysis. A fifth plasma sample was prepared from a late lactation (8 months) non-pregnant goat in medium condition score (BCS: 3·0) which was fed with freely available hay and concentrates.

On the other hand, 28 non-lactating, non-pregnant, multiparous Lacaune ewes (2–7 years old) were ovariectomized approximately 50 days before the start of experimentation and treated with oestradiol implants (25 days later) in order to maintain a constant sexual status during the course of the feeding trial. During the pre-experimental period (25 days), all the animals were fed hay and straw (mixture 50/50) freely. Thereafter, ewes were randomly allocated into two groups according to age, BW and BCS. In the control group, 12 ewes were fed 90 ± 9% of their theoretical energy requirements and 119 ± 6% of their theoretical protein requirements (hay/straw, 50/50; 19·2 g/kg BW per day), in order to maintain a constant BW during the course of the experimental period, which lasted 65 days. In the second group, 16 ewes were underfed at 39 ± 2% of their theoretical energy requirements and 46 ± 6% of their theoretical protein requirements (hay/straw, 50/50; 7·6 g/kg BW per day). Individual energy balances were assessed by assuming that maintenance energy requirement was 0·40 MJ metabolizable energy/kg of metabolic BW ($BW^{0·75}$), and energy intake was evaluated through chemical analysis and INRA feed tables (Bocquier & Thériez 1989). Ewes were individually penned and food supplied in a limited amount according to experimental diets. Ewes were weighed weekly and *in vivo* body composition, especially fat mass, was estimated by the deuterium oxide dilution technique (Bocquier *et al.* 1999) at the end of the pre-experimental period and at the 65th day of nutritional treatment. Concomitantly, pre-feeding jugular blood samples were collected and plasma prepared as previously described, before RIA measurement. All experimental procedures were conducted in accordance with French Guidelines (19 April 1988) concerning the use of experimental animals including animal welfare.

Statistical analysis

Results are expressed as mean and standard deviation (S.D.). Comparisons between feeding levels (control vs underfed) were performed by variance–covariance analysis, using the GLM procedure of SAS (1987) and initial measurements as covariates. Intra-individual changes between initial and final values were analysed by the paired Student's *t*-test.

Results

Ovine-specific RIA characteristics

Six rabbits were immunized multiple times with 125 µg recombinant ovine leptin in order to obtain an acceptable antibody. After 9 months of injections, an antibody was obtained in one rabbit which at a final dilution of 1:15 000 provided 39·5% specific binding. Non-specific binding with normal rabbit serum was 3·4%. The addition of recombinant ovine leptin standards (from 0·0833 to 4 ng/tube) competitively displaced the ^{125}I-ovine leptin as shown in Fig. 1a. Attempts to duplicate the displacement curve with recombinant human leptin revealed an apparent lack of cross-reactivity at a mass of up to 2·5 ng human leptin (Fig. 1a). The log-logit transformation (Rodbard *et al.* 1969) of the standard ovine curve exhibited a linear relationship (Fig. 1b) which allowed the determination of plasma leptin concentrations. Five plasma samples obtained from either two ewes and two cows, with high or low levels of leptin when determined in a 100 µl volume (ewes: 3·9 and 14·8 ng/ml; cows: 3·2 and 12·2 ng/ml), or one goat (4·7 ng/ml) were used for the parallelism study. Serial volumes (10–150 µl) of these five plasma samples introduced into the same RIA trial resulted in logit B/Bo values linearly related (r=+0·989–0·998; Fig. 1b) to

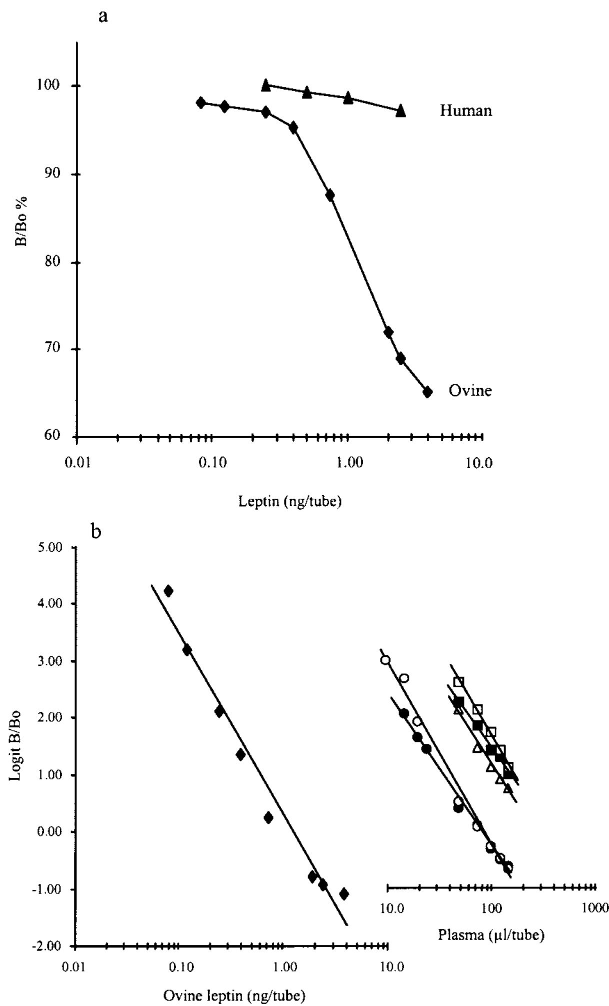

Figure 1 (a) Standard curve of recombinant ovine leptin (◆: 0·0833, 0·125, 0·25, 0·4, 0·75, 2·0, 2·5 and 4·0 ng/tube). In this ovine RIA system, the addition of recombinant human leptin (from the Linco kit) in known amounts (▲: 0·25, 0·5, 1·0 and 2·5 ng/tube) did not alter the bond between labelled ovine leptin and the antibody. (b) Linear log-logit representation (Rodbard *et al.* 1969) of recombinant ovine leptin standard curve (◆, r=+0·986), and of dilution curves of two ovine (■ and ●), two bovine (□ and ○) and one caprine (△) plasma samples (10–150 µl per tube, r=+0·989–0·998 for the five plasma samples).

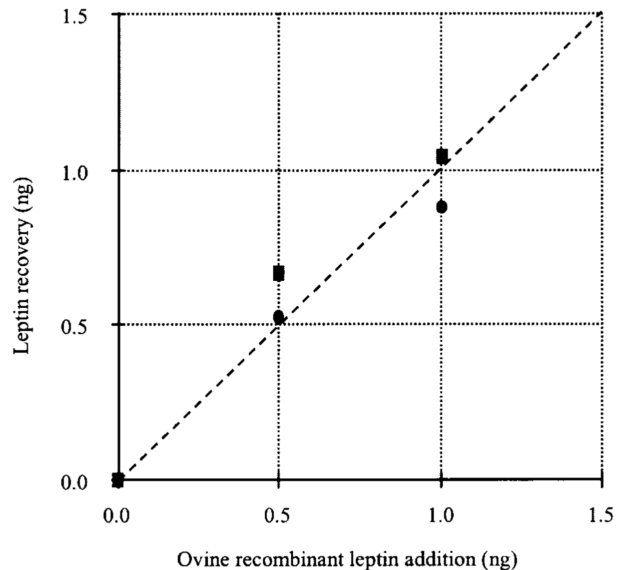

Figure 2 Recovery of known amounts (0·5 and 1·0 ng) of exogenous ovine leptin, added to two ovine plasma samples (9·0 (■) or 6·0 (●) ng/ml of endogenous leptin respectively), in the ovine RIA system was 102 ± 8% (n=4).

The relationship between leptin levels measured in 56 plasma samples either with the commercial RIA kit or with the ovine-specific RIA is presented in Fig. 3. Four

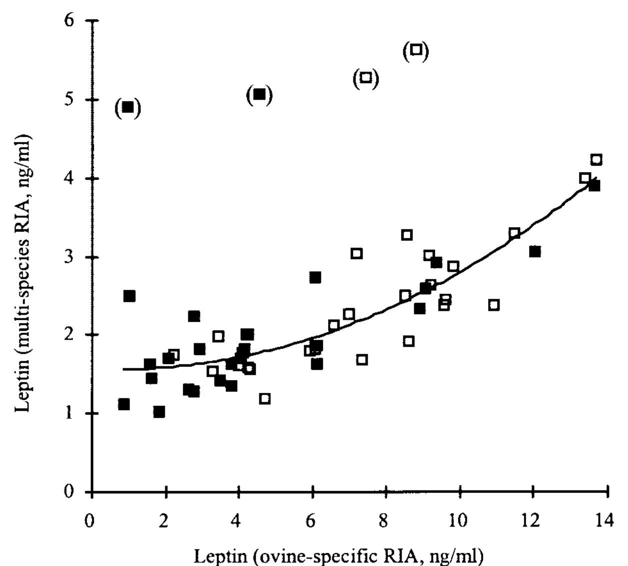

Figure 3 Curvilinear relationship between plasma leptin concentrations determined either by the ovine RIA system (x axis) or by the multi-species RIA kit (y axis). All the samples (n=56) taken before (□) and by the end (■) of nutritional treatments are represented. Four abnormally high plasma leptin levels in the 'multi-species' RIA kit system, and presented in parentheses, were not used in the following regression: $y=0{\cdot}015x^2-0{\cdot}024x+1{\cdot}578$, r=+0·87, n=52.

plasma volume and exhibiting a good parallelism with the standard curve. The recovery of exogenous recombinant ovine leptin added in known amounts (0·5 and 1·0 ng) to two ovine plasma samples (9·0 or 6·0 ng endogenous leptin per ml) was 102 ± 8% (Fig. 2). The limit of sensitivity, determined as the lower leptin quantity able to generate a diminution of 5% of the B/Bo ratio was 0·83 ng/ml for 100 µl sample size. The pooled intra-sample standard deviation and coefficient of variation calculated from duplicate analysis of 180 ovine plasma samples were 1·3 ng/ml and 13% respectively.

Table 1 Mean characteristics of ewes and their plasma leptin level by the end of the pre-experimental period (*ad libitum* diet), and comparison between control and underfed ewes after 65 days of nutritional treatment

	Initial[2]		**Final**[2]		
	Control *ad libitum* (n=12)	Underfed *ad libitum* (n=16)	Control 90% ER[3] (n=12)	Underfed 39% ER[3] (n=16)	P values[4]
Body weight (kg)	72·2 ± 6·7[a]	71·6 ± 5·2[a]	71·1 ± 7·2[a]	58·2 ± 5·7[b]	0·001
BCS[1]	3·5 ± 0·6[a]	3·6 ± 0·5[a]	2·9 ± 0·9[b]	2·1 ± 0·9[b]	0·001
Body fat mass (kg)	22·0 ± 6·1[a]	21·0 ± 3·9[a]	19·5 ± 6·2[b]	15·1 ± 4·0[b]	0·001
Body fatness (% BW)	30·1 ± 6·3[a]	29·1 ± 4·0[a]	27·0 ± 6·5[b]	25·6 ± 4·9[b]	0·664
Leptin (ovine RIA, ng/ml)	7·48 ± 3·32[a]	7·58 ± 2·77[a]	6·30 ± 3·86[a]	3·30 ± 2·14[b]	0·002
Leptin (multi-species RIA, ng/ml)	2·36 ± 0·87[a]	2·71 ± 1·26[a]	2·27 ± 0·78[a]	2·09 ± 1·19[b]	0·003

[1]Estimated on a 0 to 5 scale.
[2]Results are expressed as means ± S.D.
[3]Energy requirement.
[4]Variance–covariance analysis testing underfed vs control groups (final stage) with initial measurements used as covariates.
[a,b]: final values with different superscripts are significantly different (P<0·001, paired Student's t-test) from the corresponding initial values.

points measured with the 'multi-species' RIA kit were clearly out of the general relationship and above the values measured in the other plasma samples. These points were, however, in the normal range of values when determined with the ovine RIA system. When these four 'high' values were omitted from consideration, a coefficient of correlation of +0·87 between the two RIAs was obtained using a curvilinear adjustment (Fig. 3). On these 52 plasma samples, the ovine-specific RIA system resulted in a much greater range of detection of leptin concentration (0·85–13·7 ng/ml) than the 'multi-species' RIA (1·04–4·24 ng/ml). Furthermore, the shape of the regression curve (Fig. 3) suggests that some insensitivity exists within the 'multi-species' RIA in the lower range (<5 ng/ml by the ovine RIA) of leptin concentrations.

Influence of body fatness and nutritional status on plasma leptin concentrations

The mean characteristics of the ewes are presented in Table 1. During the pre-experimental period, plasma leptin measured by the ovine-specific RIA in the 28 ewes was 7·54 ± 2·96 ng/ml (coefficient of variation=39%) and was positively related to BW (r=+0·47, P<0·05), and more strongly to BCS (r=+0·72, P<0·001; Fig. 4a) and body fatness (lipids as per cent BW (lip%BW), r=+0·68, P<0·001; Fig. 4b). Furthermore, the control and underfed groups had similar initial BWs, BCS, body fat mass and plasma leptin levels (Table 1). The same trends were observed with the commercial RIA kit.

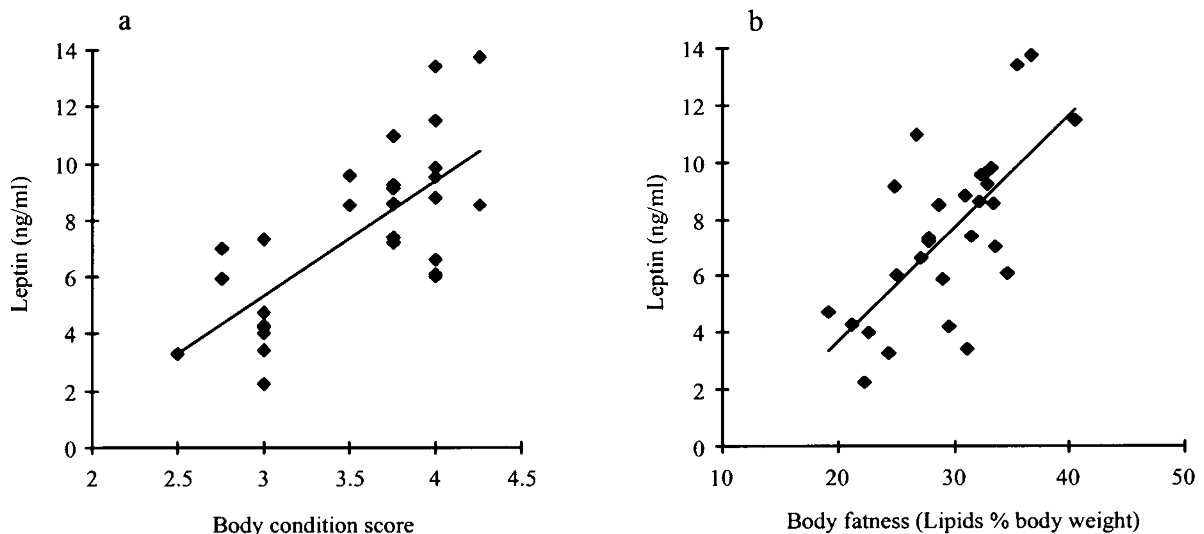

Figure 4 Relationship between plasma leptin and either (a) BCS (scale 0 to 5): y=4·09x − 6·94, r=+0·72, P<0·001, or (b) body fatness: y=0·40x − 4·30, r=+0·68, P<0·001, for the 28 ewes at the end of the pre-experimental period.

Ewes which received the control ration (90% of their maintenance energy requirements) for 65 days did not exhibit an alteration in either BW or plasma leptin level, despite small but significant ($P<0{\cdot}001$) decreases in BCS (− 17%), body fat mass (− 11%) and body fatness (− 10%) (Table 1). However, in the group of ewes which were underfed (39% of maintenance energy requirements), significant ($P<0{\cdot}001$) decreases in BW (− 19%), BCS (− 42%), body fat mass (− 28%), body fatness (− 12%) and plasma leptin levels (− 56% and − 23% using ovine-specific and 'multi-species' RIA determinations respectively; Table 1) were observed. After 65 days of dietary treatment, when initial leptin values were used as covariates, the analysis of leptin variance testing underfed vs control groups allowed similar conclusions (Table 1).

The respective effects of body fatness (lip%BW) and feeding level (control vs underfed) on plasma leptin concentrations at the end of the experimental period were analysed through multiple regression. When plasma leptin was determined by the ovine-specific RIA, the relationship between these three parameters was found to be: plasma leptin (ng/ml) = 0·33 × lip%BW − 2·63 (control) or − 5·17 (underfed, $P<0{\cdot}02$) ($r^2=0{\cdot}52$, $n=28$, $P<0{\cdot}001$). This model explains 52% of the variation in leptin concentrations, with the respective contributions of body fatness and feeding level being 35 and 17%. When plasma leptin was measured by the commercial RIA kit, and if the two ewes that were out of the normal range were excluded, the relationship was: plasma leptin (ng/ml) = 0·077 × lip%BW + 0·199 (control) or − 0·258 (underfed, $P<0{\cdot}02$) ($r^2=0{\cdot}62$, $n=26$, $P<0{\cdot}001$). Thus, 62% of the variance of the data was explained, the respective contributions of body fatness and feeding level being 48 and 14%.

Discussion

This work is the first to present a homologous specific RIA for the determination of ovine plasma leptin concentrations. This RIA is also applicable to bovine and caprine plasma samples. However, in this system, poor cross-reactivity was observed with human leptin, despite an 87% homology in amino acid sequence between human and ovine protein (Dyer *et al.* 1997*b*). This suggests that ovine leptin epitopes recognized for antibody production are located in regions with a high heterogeneity between species in the protein sequence. Finally, this may explain why the production of a specific polyclonal antibody with high affinity for ovine leptin has greatly lagged behind the availability of the recombinant protein (Gertler *et al.* 1998).

The absolute values of the limit of sensitivity of the ovine-specific RIA (0·83 ng/ml) and the 'multi-species' RIA kit (1·0 ng/ml) were similar. However, when these values were expressed as per cent of the average leptin concentrations measured in the two systems, the ovine RIA sensitivity (11%) was 3·8 times better than that of the commercial RIA kit (42%). Plasma leptin concentrations determined by the commercial RIA kit were not well related to the values obtained in the homologous ovine-specific system. These differences could be due to an insufficient sensitivity of determination together with plasma interference in the low range or conversely to the weak specificity of the 'multi-species' antibody in the high-range values. The same observation was reported by Imagawa *et al.* (1998) for low-range values determined by the kit produced commercially by Linco for human leptin when compared with results obtained with a human-specific leptin ELISA performed with monoclonal antibody. However, despite the differences in absolute values observed between the two systems (homologous and heterologous), the physiological variation in plasma leptin were generally similar (Table 1). The four abnormally high concentrations determined by the commercial RIA kit (Fig. 3) corresponded to plasma samples from two ewes, collected twice at the beginning and the end of the experimental period. It is notable that these values were highly repeatable, although abnormally high. Hence, this reinforces the hypothesis that the commercial RIA kit may cross-react with plasma components other than leptin.

The present study also allows an evaluation of the respective effects of body fatness and underfeeding on plasma leptin in sheep, thanks to the use of an experimental design in which the diet composition and intake, as well as the initial body fatness and the ovarian activity of the ewes were strictly controlled. The ovine-specific leptin RIA shows a good correlation between adiposity and leptin values ($r=+0{\cdot}68$). This result is in agreement with that obtained by Havel *et al.* (1996) in two groups of lean or obese human subjects, showing positive correlations between body fatness and leptin concentration ($r=+0{\cdot}64$, $n=19$, $P<0{\cdot}01$ or $r=+0{\cdot}69$, $n=19$, $P<0{\cdot}01$, for lean or obese group respectively). These results were confirmed by other studies in humans (Bauman *et al.* 1996, Considine *et al.* 1996). It was suggested that the increase in plasma leptin concentration in obese humans is due to an up-regulation of leptin gene expression, linked to an increase in the number and size of the fat cells, rather than to an alteration in leptin clearance (Considine *et al.* 1996, Klein *et al.* 1996). The fact that an increase in body fat could be translated into an increase in plasma leptin through adipocyte hypertrophy is in agreement with the positive relationship ($r=+0{\cdot}73$, $n=18$, $P<0{\cdot}01$) between plasma leptin and adipocyte volume in the cow (Chilliard *et al.* 1998*b*). Thus, plasma leptin content is a good indicator of body fatness in ruminants (sheep, present work; cattle, Chilliard *et al.* 1998*b*) as in humans and rodents (Maffei *et al.* 1995) fed near their maintenance requirements.

In this work, variations in body fatness explained 35–48% of the total plasma leptin inter-individual

variations. The plasma leptin level was also significantly modulated by the feeding level (Table 1) and this factor explained 14–17% of the variation in leptin values. A similar nutritional effect was reported in cattle (Chilliard *et al.* 1998*a*, Delavaud *et al.* 1999), humans (Dubuc *et al.* 1998) and rodents (Ahima *et al.* 1996). Moreover, recent studies in ruminants showed that 7 days of underfeeding on a diet providing 22% of energy requirements (Bocquier *et al.* 1998) or a 48 h fast (Kumar *et al.* 1998, Tsuchiya *et al.* 1998) had negative effects on adipose tissue leptin mRNA amount, thus suggesting a pre-translational regulation. The mechanism of this regulation is unknown in ruminant species, but could be due to an increase in the sympathetic nervous system activity in white adipose tissue, as was shown in mice (Trayhurn *et al.* 1998) and is suggested by the negative effect of a beta-agonist infusion on cattle plasma leptin (Chilliard *et al.* 1998*a*). An alternative explanation could be that the stimulation of leptin expression by insulin (Houseknecht *et al.* 1998*a*) was decreased during underfeeding. This reduction in leptin production is observed whatever the body fatness of the ewes, which shows that the nutritional status *per se* could modulate plasma leptin in ruminants, as in monogastric animals. However, there remains a large unexplained source of variation in plasma leptin levels between ewes exhibiting the same body fatness and feeding level.

In conclusion, the development of a specific RIA for ovine leptin determinations has been reported in this work, together with a comparison with a commercially available leptin RIA kit. The observation is indeed that the specificity of the antibody is critical in determining reliable values of ovine plasma leptin. Even if the physiological variation observed using the 'multi-species' commercial RIA kit (Bocquier *et al.* 1998, Chilliard *et al.* 1998*a*) are in agreement with those obtained in the homologous system (present study), some relative values can be greatly biased and could cause inaccuracy in the interpretation of the results. Thus, an ovine-specific leptin RIA determination is now available to analyse different aspects of the physiological regulation and variation of leptin in ruminants, and its putative roles (Houseknecht *et al.* 1998*b*) in the control of appetite, carcass composition, meat quality, reproduction and health of these species.

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References

Ahima RS, Prabakaran D, Mantzoros C, Qu D, Lowell B, Maratos-Flier E & Flier JS 1996 Role of leptin in the neuroendocrine response to fasting. *Nature* **382** 250–252.

Bauman WA, Spungen AM, Zhong YG & Mobbs CV 1996 Plasma leptin is directly related to body adiposity in subjects with spinal cord injury. *Hormone and Metabolic Research* **28** 732–736.

Blum WF, Englaro P, Hanitsch S, Juul A, Hertel NT, Muller J, Skakkebæk NE, Heiman ML, Birkett M, Attanasio AM, Kiess W & Rascher W 1997 Plasma leptin levels in healthy children and adolescents: dependence on body mass index, body fat mass, gender, pubertal stage, and testosterone. *Journal of Clinical Endocrinology and Metabolism* **82** 2904–2910.

Bocquier F & Thériez M 1989 Sheep in ruminant nutrition. In *Ruminant Nutrition: Recommended Allowances and Feed Tables*, pp 153–165. Ed. R Jarrige. Montrouge: John Libbey Eurotext.

Bocquier F, Bonnet M, Faulconnier Y, Guerre-Millo M, Martin P & Chilliard Y 1998 Effects of photoperiod and feeding level on perirenal adipose tissue metabolic activity and leptin synthesis in the ovariectomized ewe. *Reproduction, Nutrition, Development* **38** 489–498.

Bocquier F, Guillouet P, Barillet F & Chilliard Y 1999 Comparison of three methods for the *in vivo* estimation of body composition in dairy ewes. *Annales de Zootechnie* **48** 1–12.

Chilliard Y, Bocquier F, Delavaud C, Guerre-Millo M, Bonnet M, Martin P, Faulconnier Y & Ferlay A 1998*a* Leptin in ruminants: effects of species, breed, adiposity, photoperiod, β-agonists and nutritional status. In *Proceedings 1998 of the Cornell Nutrition Conference for Feed Manufacturers*, pp 65–74. New York: Cornell University.

Chilliard Y, Ferlay A, Delavaud C & Bocquier F 1998*b* Plasma leptin in underfed or overfed adult Holstein and Charolais cows, and its relationship with adipose tissue cellularity. *International Journal of Obesity* **22** (Suppl 3) S171.

Considine RV, Sinha MK, Heiman ML, Kriauciunas A, Stephens TW, Nyce MR, Ohannesian JP, Marco CC, McKee LJ, Bauer TL & Caro JF 1996 Serum immunoreactive-leptin concentrations in normal-weight and obese humans. *New England Journal of Medicine* **334** 292–295.

Delavaud C, Faulconnier Y, Bocquier F & Chilliard Y 1999 Pre- and postprandial changes in plasma leptin and insulin concentrations during underfeeding and refeeding in dry cows. *Proceedings of the Nutrition Society* **58** 108A.

Dubuc GR, Phinney SD, Stern JS & Havel PJ 1998 Changes of serum leptin and endocrine and metabolic parameters after 7 days of energy restriction in men and women. *Metabolism* **47** 429–434.

Dyer CJ, Simmons JM, Matteri RL & Keisler DH 1997*a* Leptin receptor mRNA is expressed in ewe anterior pituitary and adipose tissues and is differentially expressed in hypothalamic regions of well-fed and feed-restricted ewes. *Domestic Animal Endocrinology* **14** 119–128.

Dyer CJ, Simmons JM, Matteri RL & Keisler DH 1997*b* cDNA cloning and tissue-specific gene expression of ovine leptin, NPY-Y1 receptor, and NPY-Y2 receptor. *Domestic Animal Endocrinology* **14** 295–303.

Friedman JM & Halaas JL 1998 Leptin and the regulation of body weight in mammals. *Nature* **395** 763–770.

Gertler A, Simmons J & Keisler DH 1998 Large-scale preparation of biologically active recombinant ovine obese protein (leptin). *FEBS Letters* **422** 137–140.

Hardie LJ, Rayner DV, Holmes S & Trayhurn P 1996 Circulating leptin levels are modulated by fasting, cold exposure and insulin administration in lean but not Zucker (*fa/fa*) rats as measured by ELISA. *Biochemical and Biophysical Research Communications* **223** 660–665.

Havel PJ, Kasim-Karakas S, Mueller W, Johnson PR, Gingerich RL & Stern JS 1996 Relationship of plasma leptin to plasma insulin and

adiposity in normal weight and overweight women: effects of dietary fat content and sustained weight loss. *Journal of Clinical Endocrinology and Metabolism* **81** 4406–4413.

Henry BA, Goding JW, Alexander WS, Tilbrook AJ, Canny BJ, Dunshea F, Rao A, Mansell A & Clarke IJ 1999 Central administration of leptin to ovariectomized ewes inhibits food intake without affecting the secretion of hormones from the pituitary gland: evidence for a dissociation of effects on appetite and neuroendocrine function. *Endocrinology* **140** 1175–1182.

Houseknecht KL, Portocarrero CP, Ji S, Lemenager RP & Spurlock ME 1998*a* Growth hormone (GH) regulation of leptin gene expression in bovine adipose tissue: *in vitro* and *in vivo* studies. *International Journal of Obesity* **22** (Suppl 3) S166.

Houseknecht KL, Baile CA, Matteri RL & Spurlock ME 1998*b* The biology of leptin: a review. *Journal of Animal Science* **76** 1405–1420.

Hunter WM & Greenwood FC 1962 Preparation of iodine-131 labelled human growth hormone of high specific activity. *Nature* **194** 495.

Imagawa K, Matsumoto Y, Numata Y, Morita A, Kikuoka S, Tamaki M, Higashikubo C, Tsuji T, Sasakura K, Teraoka H, Masuzaki H, Hosoda K, Ogawa Y & Nakao K 1998 Development of a sensitive ELISA for human leptin, using monoclonal antibodies. *Clinical Chemistry* **44** 2165–2171.

Ji S, Willis GM, Scott RR & Spurlock ME 1998 Partial cloning and expression of the bovine leptin gene. *Animal Biotechnology* **9** 1–14.

Kann G 1971 Radioimmunoassay of plasma prolactin in sheep. *Comptes-Rendus Hebdomadaires des Séances de l'Académie des Sciences, Série D* **272** 2808–2811.

Kennedy GC 1953 The role of depot fat in the hypothalamic control of food intake in the rat. *Proceedings of the Royal Society (B)* **140** 578–592.

Klein S, Coppack SW, Mohamed-Ali V & Landt M 1996 Adipose tissue leptin production and plasma leptin kinetics in humans. *Diabetes* **45** 984–987.

Kumar B, Francis SM, Suttie JM & Thompson MP 1998 Expression of obese mRNA in genetically lean and fat selection lines of sheep. *Comparative Biochemistry and Physiology Part B* **120** 543–548.

Ma Z, Gingerich RL, Santiago JV, Klein S, Smith CH & Landt M 1996 Radioimmunoassay of leptin in human plasma. *Clinical Chemistry* **42** 942–946.

Maffei M, Halaas J, Ravussin E, Pratley RE, Lee GH, Zhang Y, Fei H, Kim S, Lallone R, Ranganathan S, Kern PA & Friedman JM 1995 Leptin levels in human and rodent: measurement of plasma leptin and ob RNA in obese and weight-reduced subjects. *Nature Medicine* **1** 1155–1161.

Morrison C, Daniel J, Holmberg B, Bolden O, Raver N, Gertler A & Keisler DH 1998 Effects of lateral cerebroventricular infusion of leptin on ewe lambs. *Journal of Animal Science* **76** (Suppl 1) 225.

Rodbard D, Bridson W & Rayford PL 1969 Rapid calculation of radioimmunoassay results. *Journal of Laboratory and Clinical Medicine* **74** 770–781.

SAS/STAT 1987 *Guide for Personal Computers, version 6*. Cary, NC: SAS Institute Inc.

Trayhurn P, Duncan JS, Hoggard N & Rayner DV 1998 Regulation of leptin production: a dominant role for the sympathetic nervous system? *Proceedings of the Nutrition Society* **57** 413–419.

Tsuchiya T, Nagao Y, Ozawa A, Matsumoto M, Sugahara K, Kubo T & Kato H 1998 Decrease of the obese gene expression in bovine subcutaneous adipose tissue by fasting. *Bioscience, Biotechnology and Biochemistry* **62** 2068–2069.

Zhang Y, Proenca R, Maffei M, Barone M, Leopold L & Friedman JM 1994 Positional cloning of the mouse *obese* gene and its human homologue. *Nature* **372** 425–432.

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