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 ($r=+0.989–0.998$), recovery (102%), repeatability (13%) and limit of sensitivity ($0.83$ ng/ml for $100 \mu$l 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 $90\%$ (control; $n=12$) or $39\%$ 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 ($r=+0.68$, $P<0.001$ or $r=+0.72$, $P<0.001$ respectively). When concentrations of leptin were assessed over time, underfed ewes exhibited a dramatic reduction in plasma leptin values ($−56\%$, $P<0.001$). 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. (1997a) 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. 1997b, 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. 1997b, Ji et al. 1998, Tsujiya et al. 1998) and is related to feeding level
et al. according to Ma et al. (1996) modified by chloramine-T method commercially available by the chloramine-T method (Kann 1971). Brie fi et al. (Bocquier et al. 1998, Chilliard et al. 1998a, 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-denaturing 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) Na125I (IMS 300, Amersham-Pharmacia Bio- tech, 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 (Sepacor-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, 0·25, 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 saline). Bound and free ligands were then separated by adding 100 μg of a specific anti-rabbit ram 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 125I-ovine leptin was removed by
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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, 125I-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. 1998a). 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 ovario-ectomized 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% of their theoretical energy requirements and 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 (BW0·75), 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 0·833 to 4 ng/tube) competitively displaced the 125I-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
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/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 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).

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=0·015x² - 0·024x + 1·578, r=+0·87, n=52.
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

<table>
<thead>
<tr>
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<th>Initial2 (n=12)</th>
<th>Final2 (n=16)</th>
<th>P values4</th>
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<tbody>
<tr>
<td>Body weight (kg)</td>
<td></td>
<td></td>
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<tr>
<td>Control ad libitum</td>
<td>72.2 ± 6.7a</td>
<td>71.6 ± 5.2a</td>
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<tr>
<td>Underfed ad libitum</td>
<td>3.5 ± 0.6b</td>
<td>3.6 ± 0.5b</td>
<td></td>
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<tr>
<td>Body fat mass (kg)</td>
<td>22.0 ± 6.1a</td>
<td>21.0 ± 3.9a</td>
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<tr>
<td>Body fatness (% BW)</td>
<td>30.1 ± 6.3a</td>
<td>29.1 ± 4.0a</td>
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<tr>
<td>Leptin (ovine RIA, ng/ml)</td>
<td>7.48 ± 3.32a</td>
<td>7.58 ± 2.77a</td>
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<tr>
<td>Leptin (multi-species RIA, ng/ml)</td>
<td>2.36 ± 0.87a</td>
<td>2.71 ± 1.26a</td>
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</table>

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.
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. 1997b). 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 ($r=+0.68$). 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 ($r=+0.64$, $n=19$, $P<0.01$ or $r=+0.69$, $n=19$, $P<0.01$, 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 ($r=+0.73$, $n=18$, $P<0.01$) between plasma leptin and adipocyte volume in the cow (Chilliard et al. 1998b). Thus, plasma leptin content is a good indicator of body fatness in ruminants (sheep, present work; cattle, Chilliard et al. 1998b) 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. 1998a, 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) 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 suggested by the negative effect of a beta-agonist infusion on cattle plasma leptin (Chilliard et al. 1998a). An alternative explanation could be that the stimulation of leptin expression by insulin (Houseknecht et al. 1998a) 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. 1998a) 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. 1998b) in the control of appetite, carcass composition, meat quality, reproduction and health of these species.

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