Development of a specific radioimmunoassay to measure physiological changes of circulating leptin in cattle and sheep

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Abstract

Studies of leptin in large domestic ruminants have been limited to measurements of gene expression because methods to measure circulating levels are not available. To develop a bovine leptin radioimmunoassay, we produced recombinant bovine leptin and used it to immunize rabbits, and to prepare bovine leptin tracer and standards. A single antiserum with sufficient affinity and titer was identified. Using this antiserum, logit-transformed binding of ^{125}I -labeled bovine leptin was linearly related ($R^2 = 0.99$) to the log of added bovine or ovine leptin between 0.1 to 2.0 ng. Serial dilution of bovine and ovine plasma, chicken serum and bovine milk gave displacement curves that were parallel to those of bovine or ovine leptin. Recoveries of external addition of bovine leptin in ewe

and cow plasma ranged between 94 and 104%. Plasma leptin concentration measured by this assay was directly related to the plane of nutrition in growing calves and lambs. At 11–14 weeks of age, ewe lambs had a higher circulating leptin concentration than ram lambs. Finally, plasma leptin concentration was linearly related to the fat content of the empty carcass in growing cattle and to body condition score in lactating dairy cows. We conclude that circulating leptin in sheep and cattle is increased by fatness and plane of nutrition, consistent with results in humans and rodents. This assay provides an important tool to investigate mechanisms that regulate plasma leptin in cattle and sheep.

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Introduction

Leptin, the product of the *OB* gene, is a protein secreted predominantly by white adipose tissue (WAT) (Flier 1997, Friedman & Halaas 1998, Houseknecht & Portocarrero 1998). Leptin acts on the central nervous system and perhaps on peripheral tissues to regulate food intake, the storage and dissipation of energy and the coordination of metabolism during periods of suboptimal nutrition (Flier 1997, Friedman & Halaas 1998, Houseknecht & Portocarrero 1998). Leptin also plays important roles in regulating processes critically dependent on energy supply such as reproductive and immune functions (Ahima *et al.* 1997, Finn *et al.* 1998, Lord *et al.* 1998, Cunningham *et al.* 1999).

Thus far, actions and regulation of leptin have been studied most extensively in rodents and humans (Caro *et al.* 1996, Flier 1997, Friedman & Halaas 1998). Leptin mRNA in WAT and plasma leptin are increased chronically by body fatness and by short term cues indicative of abundant nutrient supply such as insulin, and decreased by underfeeding, sympathetic activation of WAT and by cold exposure (Caro *et al.* 1996, Flier 1997, Trayhurn *et al.* 1998). Leptin cDNAs have been cloned in most domestic

animals including cattle, sheep, pigs and chickens (Bidwell *et al.* 1997, Dyer *et al.* 1997, Mendiola *et al.* 1997, Ji *et al.* 1998, Taouis *et al.* 1998). In these animals, leptin mRNA is also expressed predominantly in WAT, with the exception of the chicken which has high expression in liver (Taouis *et al.* 1998, Ashwell *et al.* 1999a). Leptin gene expression in WAT is increased by adiposity in pigs (Bidwell *et al.* 1997, Ramsay *et al.* 1998) and decreased by feed restriction in pigs and sheep (Bocquier *et al.* 1998, Spurlock *et al.* 1998). In chickens, feed restriction decreases leptin expression in both WAT and liver (Ashwell *et al.* 1999b).

Research on the biology of leptin in domestic animals has been seriously hampered by the lack of homologous leptin radioimmunoassays (RIAs). Estimates of plasma leptin have been reported in pigs (Qian *et al.* 1999), sheep (Bocquier *et al.* 1998) and cattle (Chilliard *et al.* 1998) using a commercial assay originally designed to measure human leptin (Ma *et al.* 1996). Essentially, no validation was reported for the use of this RIA in sheep or cattle samples. In preliminary work, we found that this RIA provided estimates of plasma leptin in sheep and cattle that were low and unresponsive to changes in nutrition or adiposity. Therefore, the aim of the present work was to

develop a specific RIA for bovine leptin and to provide evidence for regulation of circulating leptin by nutrition and fatness in cattle and sheep.

Materials and Methods

General reagents and methods

Recombinant human (lot #AFP496C) and mouse leptin (lot #AFP341C) were obtained through the National Hormone and Pituitary Program and Dr Parlow (Torrance, CA, USA). Recombinant ovine leptin was purchased from Diagnostic Service Laboratory (Webster, TX, USA). The Multi-Species Leptin RIA was obtained from Linco Research Inc. (St Charles, MO, USA). The polymerase chain reaction (PCR) was performed using a Thermal cycler (GeneAmp PCR System 2400, Perkin Elmer, Norwalk, CT, USA). DNA sequencing, quantitative amino acid analysis and oligonucleotide synthesis were performed by the Biotechnology Resource Center at Cornell University (Ithaca, NY, USA). Restriction endonucleases and DNA modifying enzymes were purchased from Life Technologies (Gaithersburg, MD, USA) or New England Biolabs (Beverly, MA, USA). A Micromedic 4/200 plus Automatic Gamma Counter (Micromedic, Horsham, PA, USA) was used for γ -counting. Densitometry was performed using an IS-1000 Digital Imaging System (Alpha Innotech, San Leandro, CA, USA).

Production of recombinant bovine leptin

Total RNA from subcutaneous WAT of an adult Holstein cow was reversed transcribed using random hexamers and AMV reverse transcriptase (Boisclair *et al.* 1996). The resulting cDNAs were used in PCR to amplify a fragment corresponding to nt +64 to nt +518 relative to (A₊₁TG) of the bovine leptin cDNA (GenBank accession #U50365). PCR was performed with the forward primer 5'-GT-GCCCATCCGCAAGGTCCAGGAT 3', the reverse primer 5'-AGGCCTTCAAGGCTTCAGCA 3' and KlenTaq DNA polymerase as recommended by the manufacturer (Clontech, Palo Alto, CA, USA).

Using a second PCR, a unique BamHI site and the sequence coding for an enterokinase digestion site was added with forward primer 5'-gccgatccgatgacgatgaca aaGTGCCCATCCGCAAGGTCCAGGAT 3' (added sequence is shown in lower case), and an SalI site was added with reverse primer 5'-ggccgtgacttaTCAGCAC CCGGACTGAGGTCCAG 3'. The expression plasmid bleptinA was obtained by inserting the resulting PCR product between the BamHI and SalI sites of pQE31 (Qiagen, Valencia, CA, USA). A second expression plasmid (bleptinB) was created by PCR using forward primer 5'-ggccgatccgatgacgatgacaaagccGTGCCCATCCGCAAG GT 3' and the same reverse primer, followed by insertion

of this product between the BamHI and SalI sites of pQE30 (Qiagen). Expression vectors were shown by DNA sequencing to encode mature bovine leptin (amino acid residues 22 to 167 of unprocessed bovine leptin) with an amino acid sequence identical to that of Kawakita (GenBank accession #AB003143). Leptin was preceded by the purification tag MRGSHHHHHHTDPDDDDDK in bleptinA and by the purification tag MRGSHHHHHH HGSDDDDDKA in bleptinB.

Expression vectors were transformed into the *Escherichia coli* strain M15, and leptin expression and purification were performed according to the instructions of the manufacturer (Qiagen). Briefly, leptin synthesis was induced by addition of isopropyl- β -D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. Cell pellets were lysed in 6 M guanidinium hydrochloride, 100 mM NaH₂PO₄, 10 mM Tris-HCl, pH 8 (lysis buffer). His-tagged bovine leptin was adsorbed to Ni-NTA resin for 45 min at 20 °C. The resin was washed with step gradients of 20 and 50 mM imidazole in lysis buffer, followed by elution of bovine leptin with 250 mM imidazole in lysis buffer (elution buffer). Yield was typically 35–40 mg/l of *E. coli* culture.

Bovine leptin was refolded according to a procedure described by Fawzi *et al.* (1996) for human leptin. Six milliliters elution buffer, containing approximately 12 mg bovine leptin, were slowly infused over a 20-h period into 750 ml refolding buffer (50 mM Tris-HCl, pH 8.8, 150 mM NaCl, 5 mM EDTA, 2 mM reduced and 0.2 mM oxidized glutathione) and then gently mixed for an additional 24 h at 20 °C. The solution was concentrated approximately 50-fold at 4 °C using an ultrafiltration unit (Amicon, Beverly, MA, USA) fitted with a YM10 regenerated cellulose membrane (Millipore, Bedford, MA, USA). The concentrated solution was dialyzed 4 times against a 200-fold excess of 5 mM phosphate buffer, pH 8 and 50 mM NaCl using a Spectra/Por membrane (8 kDa molecular mass cut-off, Spectrum, Houston, TX, USA).

The purification tag was removed from recombinant bovine (rb) leptinB by incubation with recombinant enterokinase (1 U/mg protein at 20 °C for 16 h) (Novagen, Madison, WI, USA). The reaction was terminated by addition of 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (ICN, Costa Mesa, CA, USA) to a final concentration of 2 mM. The purification tag was adsorbed to Ni-NTA resin in 50 mM NaH₂PO₄, pH 8, 300 mM NaCl, 0.1% Triton X-100 (1 h at 20 °C). After removing the resin by centrifugation, mature bovine leptin (rbleptin) was concentrated by ultrafiltration and then buffer exchanged as above. The concentration of rbleptin was determined by quantitative amino acid analysis (Bidlingmeyer *et al.* 1984).

Production of antisera against bovine leptin

Refolded rbleptinA (100 μ g in 0.5 ml 12 mM phosphate buffer containing 150 mM NaCl, pH 8 (PBS)) was

emulsified with an equal volume of complete Freund's adjuvant for the primary immunizations or with incomplete Freund's adjuvant for the secondary immunizations. Emulsions were injected into 10 subcutaneous sites of 5 Flemish Giant $\times$ Chinchilla rabbits. Secondary immunizations were performed 2 weeks after the primary immunization and at 4-week intervals thereafter. Blood samples (5–10 ml) were collected 10 days following all secondary immunizations by venipuncture of the ear. Serum was stored at -20°C until further analysis.

RIA procedures

rb leptin (2.5 μg) was dissolved in 50 μl 50 mM Na_2PO_4 , pH 8, 0.1% Triton X-100 and iodinated with 0.5 mCi Na^{125}I (Amersham Pharmacia Biotech, Piscataway, NJ, USA) using 2 μg Iodo-gen (Pierce, Rockford, IL, USA). After 2 min, the reaction was stopped by addition of 50 μl PBS containing 10 mg/ml tyrosine, 10% glycerol and 0.1% xylene cyanol. The reaction was applied to a Sephadex G-50 column equilibrated with PBS containing 0.01% NaN_3 . Fractions (≈ 300 μl) were eluted into tubes containing 1 ml assay buffer (PBS, pH 8, containing 0.01% NaN_3 and 1% bovine serum albumin (BSA, RIA grade, Sigma, St Louis, MO, USA)). Radioactivity of each fraction was determined by γ -counting. Incorporation of radioactivity into bovine leptin was 55–60%, yielding a tracer with a specific radioactivity of ≈ 200 $\mu\text{Ci}/\mu\text{g}$.

A disequilibrium, double antibody RIA was established. Unknown samples (25–200 μl , 100 μl in routine use) or mature rb leptin standards were diluted to 400 μl with assay buffer in glass tubes and pre-incubated for 2 h at 20°C in the presence of 0.375% (w/v) sodium dodecyl sulfate. When milk was analyzed, it was first sonicated and mixed thoroughly prior to addition to assay sample buffer (Houseknecht *et al.* 1997). This was followed by the addition of 100 μl of the primary antiserum (diluted 1:300 in assay buffer supplemented with 0.2% non-immunized rabbit serum). After 16 h incubation at 4°C , ^{125}I -labeled rb leptin (20 000 c.p.m. in 100 μl assay buffer) was added and allowed to equilibrate for 8 h at 4°C . Separation of bound and free ligand was initiated by the addition of 200 μl 50 mM phosphate buffer, pH 8, containing 5% (v/v) ovine anti-rabbit γ -globulin (kind gift of W R Butler, Cornell University). After 16 h incubation at 4°C , 1 ml ice-cold 50 mM phosphate buffer, pH 8, containing 3% (w/v) polyethylene glycol (PEG 8000) was added. The tubes were centrifuged immediately ($1670 \times g$, 30 min at 4°C) and the supernatant decanted. Precipitated radioactivity was quantified by γ -counting.

The Multi-Species Leptin RIA is also a disequilibrium, double antibody assay and was performed exactly as recommended by the supplier (Linco Research Inc.). Briefly, plasma or recombinant human leptin standard (100 μl) was combined with assay buffer (200 μl ; 50 mM phosphate buffer, pH 7.4, containing 150 mM NaCl,

0.1% NaN_3 , 0.05% Triton X-100, and 1% RIA grade BSA). After addition of guinea pig anti-human leptin antibody (100 μl), tubes were incubated for 20 h at 4°C . Next, ^{125}I -labeled human leptin tracer was added (100 μl , ≈ 20 000 c.p.m.). After 20 h at 4°C , the supplier's precipitating reagent was added (1.0 ml) to precipitate bound leptin. After centrifugation, bound radioactivity was quantified exactly as for the bovine leptin RIA. Sensitivity of this assay is 100 pg or 1.0 ng/ml. Intra- and interassay coefficients of variation for ovine and bovine samples were less than 8% and 15% respectively.

Animal studies

Experimental procedures were conducted with the approval of the Cornell University Animal Use and Care Committee. Human serum was obtained from an adult male in accordance with the regulations of the Cornell University Committee on Human Subjects. Unless otherwise noted, all plasma samples were prepared using sodium heparin (15 IU/ml). Bovine milk was obtained from a Holstein cow and stored at -20°C until analysis.

To study the effects of nutrition in growing cattle, Holstein bull calves were fed a specially formulated milk replacer so as to grow at target rates of 500 or 1400 g/day from day 3 of postnatal life until they reached 105 kg body weight (5 calves per feeding level). The milk replacer was offered 3 times per day and contained 31 g crude protein, 20 g fat and 5.0 Mcal gross energy per kg dry matter. Four days before slaughter, blood samples were obtained within 4 h of the first feeding by jugular venipuncture. Fat content of the empty whole body (i.e. after removal of gastrointestinal contents) was determined by ether extraction as applied by Diaz *et al.* (1998).

Blood samples were obtained via coccygeal venipuncture from 22 lactating Holstein cows (4.1 ± 0.3 years old, parity number = 2.3 ± 0.3). They were sampled at day 261 ± 16 of lactation when they produced 30 ± 2.2 kg milk. The cows were offered a single diet (18% crude protein, 2.2 Mcal metabolizable energy (ME)/kg dry matter) and were assigned a body condition score according to the method of Wildman *et al.* (1982) (1=thin, 5=fat).

Effects of nutrition were also studied in weaned 11 to 14-week-old Finn $\times$ Dorset lambs fed a moderate quality diet (12% crude protein, 2.17 Mcal ME/kg dry matter) or a high quality diet (19% crude protein, 2.76 Mcal ME/kg dry matter) available *ad libitum* (4 rams and 4 ewes per treatment). Lambs were housed in floor pens at 18°C and ambient light (11 h light : 13 h darkness). After 6 weeks, blood samples were obtained by jugular venipuncture 6 h after feeding and processed to plasma. In addition, blood from 4 of these lambs was processed to serum, or to plasma using K_3EDTA (1.7 mg/ml) or sodium heparin (15 IU/ml). Finally, blood samples were obtained from 28 adult

Figure 1 Expression and purification of bovine leptin. Fractions collected during purification were electrophoresed on a 12% SDS-polyacrylamide gel. Proteins were visualized by Coomassie Brilliant Blue R-250 staining. *E. coli* cells transformed with the expression vector bleptinA were grown in the absence (– IPTG, lane 2) or presence of IPTG (+IPTG, lane 3). Induced cells were lysed with 6 M guanidinium-HCl and incubated with Ni-NTA resin. Other lanes correspond to the flow-through following resin loading into a column (Flow through, lane 4), column wash with 6 M guanidinium-HCl and 20 mM imidazole (Wash, lane 5), elution with 6 M guanidinium-HCl and 250 mM imidazole (Elution, lane 6) and 3 µg refolded rbleptinA (Refolded, lane 7). Artifacts present in lanes 5 and 6 represent cracks created when drying the gel. The molecular weight markers are shown in lane 1.

Karakul ewes by jugular venipuncture to compare estimates of plasma leptin obtained by the bovine leptin and the Multi-Species RIAs.

Statistics

The effect of nutrition in calves was analyzed by one-way analysis of variance. The relation between plasma leptin concentration and fatness (body condition score in lactating cows, percentage body fat in calves) was assessed by linear regression. Estimates for plasma leptin concentration in lambs were analyzed by a model accounting for the effects of sex and diet. Leptin concentrations in ovine plasma and serum were compared by a model accounting for the effects of lamb and sample preparation. The slopes of the relationships between logit transformed ^{125}I -labeled rbleptinB binding and the log of added sample or standard were compared by analysis of variance. All statistics were performed using the Statistical Analysis System (SAS, Cary, NC, USA).

Results

Expression of recombinant bovine leptin

In the presence of IPTG, *E. coli* M15 cells transformed with plasmid bleptinA overexpressed a protein having an

apparent molecular mass of 16 kDa (Fig. 1). After metal affinity chromatography, this his-tag containing protein accounted for more than 95% of total protein by densitometric analysis of SDS-PAGE gels stained with Coomassie Brilliant Blue R-250 (Fig. 1). Following refolding, the purity of the 16 kDa protein, referred to as rbleptinA, remained greater than 95% (Fig. 1). This preparation of rbleptinA was used to immunize rabbits.

We attempted to prepare native bovine leptin from rbleptinA by removing the purification tag with recombinant bovine enterokinase. Enterokinase was unable to cleave the purification tag due to the presence of a proline residue next to the enterokinase digestion site (see Materials and Methods). To correct this problem, we produced rbleptinB which is devoid of proline residues in the purification tag and contains an alanine spacer between the enterokinase site (DDDDK) and valine 22, the first amino acid residue of mature bovine leptin. Incubation of purified, refolded rbleptinB with enterokinase reduced its apparent molecular mass from 16 to 14.3 kDa, indicating removal of the purification tag (Fig. 2). Identity of the 14.3 kDa protein with native bovine leptin was confirmed by quantitative amino acid analysis. This refolded 14.3 kDa protein was used as tracer and standard in the RIA and is referred to as rleptin.

Figure 2 Removal of the purification tag from recombinant bovine leptin. Refolded rbleptinB was incubated in the presence (+, lane 2) or absence (–, lane 3) of enterokinase (1 IU/mg protein, 16 h at 20 °C) and analyzed by electrophoresis on a 15% SDS-polyacrylamide gel. Proteins were visualized by staining the gel with Coomassie Brilliant Blue R-250. Molecular weight markers are shown in lane 1.

Bovine leptin RIA

After 4 rounds of secondary immunization, an antiserum from a single rabbit had acceptable characteristics for use in RIA. At a final dilution of 1:1800, this antiserum bound 35–40% of ^{125}I -labeled rbleptin radioactivity in the absence of unlabeled rbleptin (B_0). Addition of increasing amounts of unlabeled rbleptin or recombinant ovine leptin inhibited the binding of ^{125}I -labeled rbleptin in a dose-dependent manner (Fig. 3, left panel). Logit-transformed binding was linearly related to the log of added rbleptin between 0.1 and 2.0 ng/tube ($R^2 > 0.99$). Non-specific binding of ^{125}I -labeled rbleptin, measured by omission of the primary antiserum, was 2.5–3.5% of the total counts for standards and unknown samples. In contrast, recombinant mouse or human leptin displaced ^{125}I -labeled rbleptin binding poorly, even at the maximal mass of 12.8 ng.

To evaluate this RIA further, we measured displacement of ^{125}I -labeled rbleptin by bovine and ovine plasma (Fig. 3, left panel). Serial dilution of cow or ewe plasma gave displacement curves that were parallel to those of the recombinant bovine or ovine leptin. Recoveries of added rbleptin (0.2 and 0.4 ng) were assessed in plasma obtained from either 3 pregnant (270–275 days post-insemination) or 3 non-pregnant cows (Table 1). Recoveries varied from 94% to 104%, with no effects of pregnancy status or mass of added rbleptin. Recovery of rbleptin was also evaluated

Figure 3 Competitive displacement of rbleptin. Left panel: in the absence of any additions, binding of ^{125}I -labeled rbleptin (B_0) was 38% of the total tracer and non-specific binding was 3.0%. ^{125}I -labeled rbleptin was displaced by addition of unlabeled recombinant leptin (bovine ●, ovine ○, mouse ▲ and human △), or by addition of plasma (bovine ■ and ovine □). For each addition, ^{125}I -labeled rbleptin bound to the antisera is expressed as a fraction of B_0 (B/B_0). Identical displacement curves were obtained with additional plasma obtained from non-pregnant ewes ($n=2$) or non-pregnant (day 270–275 post insemination, $n=3$) dairy cows. Right panel: specificity of the bovine RIA. Competition for ^{125}I -labeled rbleptin binding, expressed as a fraction of B_0 , is shown for unlabeled rbleptin (●), serum from various species (chicken ▲, pig ○, mouse ■ and human △) and bovine milk (□). B_0 was 36% and non-specific binding was 2.9% of the total tracer added.

Table 1 Bovine leptin is quantitatively recovered in bovine and ovine plasma. Recoveries of added rleptin are expressed as recovered/expected × 100 (mean ± standard error)

	Leptin ² (ng)	% recovered
Plasma¹		
Sheep	0.2	98 ± 3.6
	0.4	96 ± 4.2
Cattle		
Non-pregnant	0.2	104 ± 6.6
	0.4	97 ± 7.0
Pregnant	0.2	96 ± 2.1
	0.4	94 ± 3.5

¹Plasma was obtained from four non-pregnant, non-lactating sheep or six dairy cows (3 non-lactating cows on day 270–275 post-insemination and 3 lactating, non-pregnant cows). ²Additions of rleptin were performed in either 50 or 100 µl of plasma. These data were pooled because volume did not alter recovery.

in plasma pools obtained from non-pregnant ewes. Again, quantitative recoveries ranging from 96% to 98% were obtained (Table 1).

Bovine milk and serum from other species were also evaluated for their ability to displace rleptin tracer binding from the antiserum (Fig. 3, right panel). The slopes of the relationship between logit transformed ¹²⁵I-labeled rleptin binding and the log of added bovine milk and hen serum were parallel to those of the rleptin standards whereas the slope produced by sow serum was not ($P<0.05$). Human and mouse serum produced little dose-dependent displacement of ¹²⁵I-labeled rleptin,

which is consistent with results obtained above with recombinant mouse and human leptin (Fig. 3, left panel).

To determine if the method of sample preparation affected the estimation of circulating leptin, a single blood sample was obtained from each of 4 lambs and processed to serum, to plasma using K₃EDTA and to plasma using heparin. The concentration of leptin in plasma prepared with EDTA (6.8 ± 0.5 ng/ml) or heparin (7.0 ± 0.4 ng/ml) did not differ. In addition, leptin concentration in plasma did not differ from that in serum (7.3 ± 0.5 ng/ml).

Overall, these data indicate that this RIA provides reliable estimates of leptin in bovine and ovine plasma. Under routine use, the sensitivity of this assay, defined as the lowest standard mass distinguishable from the zero standard, was 50 pg or 0.5 ng/ml. Half-maximal displacement (ED₅₀) of tracer binding to the antiserum occurred at 0.61 ± 0.03 ng or 6.1 ± 0.3 ng/ml (mean ± standard error; $n=6$ standard curves). Finally, intra- and interassay coefficients of variation in bovine plasma were less than 4% and 10% respectively, and in ovine plasma were less than 4% and 8% respectively.

Measurement of leptin in cattle and sheep

Next, we assessed the ability of the bovine RIA to detect changes in plasma leptin related to plane of nutrition and body fatness. As expected, calves offered the highest plane of nutrition grew the fastest (1200 ± 190 vs 597 ± 63 g/day, $P<0.05$). Plasma leptin concentration was elevated by 46% in the fast-growing calves compared with the slower-growing calves (6.7 ± 0.5 vs 4.6 ± 0.2 ng/ml, $P<0.01$). In

Figure 4 Relationship between plasma leptin concentration and fatness in cattle. Left panel: from day 3 of postnatal life, calves were fed a milk replacer at levels sustaining growth rates of 597 (○) or 1200 (●) g/day. Plasma leptin concentration was measured in samples obtained 4 days before slaughter at 105 kg live body weight. Fat content of the empty body was measured by ether extraction. Right panel: plasma leptin concentration was measured in dairy cows offered a single diet available *ad libitum* during late lactation (day 261 ± 16 of lactation), and fatness was estimated by body condition scoring (Wildman *et al.* 1982).

Figure 5 Effect of plane of nutrition (left panel) and sex (right panel) on circulating plasma leptin in growing lambs. Ram or ewe lambs were fed a diet of moderate or high quality available *ad libitum* for 6 weeks. Plasma leptin concentration was analyzed by the bovine leptin RIA. Means $\pm$ standard errors (8 lambs per diet or sex) differ at $P < 0.005$ (a, b) or $P < 0.05$ (c, d).

these animals, plasma leptin was linearly related to the fat content of the empty carcass ($r = 0.91$, $P < 0.001$; Fig. 4, left panel).

To evaluate the possibility that circulating leptin and fatness are also related in adult cattle, plasma leptin concentration was measured in well-fed cows over the last third of lactation. All animals were estimated to be in positive energy balance at that time (NRC 1988). Body condition score, an indirect appraisal of body energy and protein reserves, explained 37% of the variation in plasma leptin concentration in these animals ($P < 0.005$; Fig. 4, right panel).

Finally, we asked whether nutrition also affected circulating leptin concentration in growing lambs. Lambs consumed similar quantities of a moderate quality or a high quality diet (1330 ± 40 vs 1176 ± 77 g/day, moderate vs high quality). However, lambs receiving the higher quality diet grew faster (400 ± 21 vs 306 ± 15 g/day, high vs moderate, $P < 0.001$) and had plasma leptin concentrations that were elevated by 51% (Fig. 5, left panel). In addition, concentration of leptin in plasma was higher in ewe lambs than in ram lambs regardless of dietary treatment (Fig. 5, right panel). This difference occurred in the absence of significant sex differences in dry matter intake (1311 ± 56 vs 1194 ± 118 g/day, rams vs ewes) or in average body weight gain (379 ± 23 vs 328 ± 29 g/day, rams vs ewes).

Comparisons to Multi-Species RIA

Others have used the Multi-Species Leptin RIA to measure plasma leptin concentration in sheep and cattle (Bocquier *et al.* 1998, Chilliard *et al.* 1998). This commercial RIA is based on an antiserum raised against human leptin, and uses human leptin as tracer and standards. Estimates obtained with the bovine leptin RIA and the commercial RIA were compared for both cattle and sheep. In growing cattle ($n = 10$), plasma leptin concentrations

measured by the bovine RIA were higher (5.4 ± 0.3 ng/ml) than those measured by the Multi-Species RIA (2.8 ± 0.3 ng/ml), but no relationship existed between them (results not shown). In adult sheep, the bovine RIA measured plasma leptin concentrations that were consistently greater than those of the commercial assay and estimates from both assays were positively correlated ($r = 0.79$, $P < 0.001$, Fig. 6).

Figure 6 Relationship between leptin concentration measured by the bovine RIA and the Multi-Species RIA in sheep plasma. Plasma was obtained from 28 adult sheep. The relationship between plasma leptin concentration measured by both assays was modestly altered by removing the animal with the highest plasma leptin concentration ($y = 2.88x - 0.71$, $R^2 = 0.52$).

Discussion

The primary objective of this work was to develop an RIA for measuring leptin in bovine plasma. This required production of rbleptin as a source of antigen and for the preparation of radioiodinated tracer and standards. Using one of many antisera that we produced, an RIA with sufficient sensitivity to detect concentrations of circulating leptin in plasma was established. Serial dilution of bovine plasma and rbleptin produced parallel displacement curves indicating that the antiserum recognized recombinant and plasma leptin in an identical manner. Moreover, additions of rbleptin were quantitatively recovered in plasma. Both of these validations were successful with plasma originating from pregnant and non-pregnant dairy cows. The effect of pregnancy was evaluated because a soluble form of the mouse leptin receptor has been shown to be markedly elevated in plasma during late pregnancy in the mouse (Gavrilova *et al.* 1997). Our recovery and parallelism studies suggest that if this secreted receptor is induced during pregnancy in cattle, it does not introduce a significant bias in estimating leptin concentration in bovine plasma.

Tracer displacement studies suggested additional applications for this RIA. First, bovine milk displaced tracer binding in a manner parallel to that of rbleptin, indicating that our assay detects leptin in milk. This finding is not surprising as leptin has been shown to be present in both whole and skimmed human milk (Casabiell *et al.* 1997, Houseknecht *et al.* 1997) and human mammary epithelial cells have been reported to synthesize leptin (Smith-Kirwin *et al.* 1998). Secondly, recombinant ovine leptin and ovine plasma yielded displacement curves that were essentially identical to those of bovine materials. This was expected given the near identity of sheep and bovine leptin (144 out of 146 amino acids; Dyer *et al.* 1997, GenBank accession #U50365). Our results also suggest that the bovine antisera could serve to develop an RIA for chicken leptin, particularly if recombinant chicken leptin can be used as tracer and standard. In contrast, the bovine RIA has limited usefulness in the pig, and is unable to quantitatively detect mouse and human leptin.

Using this RIA, we have shown that factors known to regulate plasma leptin in rodents and humans have similar effects in cattle and sheep. Plasma leptin concentration was elevated in growing calves and lambs on a high plane of nutrition. Moreover, in the calf study, the concentration of leptin in plasma was found to be positively correlated with body fatness ($R^2=0.83$, $P<0.001$). Body composition was not determined in the growing lambs, but subjective assessment suggested that the better fed lambs were fatter when plasma leptin was measured. Because plasma leptin was measured after an extended period of treatment in both of these studies, both level of nutrition and body composition probably influenced the changes in plasma

leptin concentration. Others have reported that better nutrition and adiposity increase circulating leptin concentration in both rodents (Ahren *et al.* 1997, Cha & Jones 1998, Landt *et al.* 1998) and humans (Maffei *et al.* 1995, Considine *et al.* 1996, Havel *et al.* 1996). Studies in which plasma leptin is measured before body composition is significantly altered are needed to resolve this issue.

We also demonstrated that circulating leptin and body condition score are positively correlated in lactating dairy cows. The high producing cows used in this study ($\approx 11\,000$ kg milk/305 day lactation) were relatively lean, with condition scores ranging from 1.75 to 3.75. According to the prediction equations of Waltner *et al.* (1994), these scores correspond to a range of body fat content of 10 to 22%. Despite being an indirect measure of fatness, body condition score explained 37% of the variation in plasma leptin concentration in these lactating cows. These data in lactating and growing cattle agree with the variation in leptin concentration explained by body fatness (25–78%) in populations of lean and obese adult humans (Considine *et al.* 1996, Havel *et al.* 1996, Hosoda *et al.* 1996, Kennedy *et al.* 1997, Saad *et al.* 1997).

In growing lambs, plasma leptin concentration was higher in ewes than in rams regardless of dietary treatment. A similar sexual dimorphism in circulating leptin has been demonstrated in both adolescent (Roemmich *et al.* 1998) and adult (Kennedy *et al.* 1997, Saad *et al.* 1997) humans, but is reversed in the rat (Landt *et al.* 1998). In humans, this dimorphism has been attributed to differences in overall adiposity, distribution of WAT, and variation in leptin expression across depots (Montague *et al.* 1997, Roemmich *et al.* 1998). In this study, the lambs were approaching puberty when sampled, but the precise timing and basis for the sexual dimorphism in plasma leptin will require further study.

Leptin concentrations measured by the Multi-Species assay in adult sheep and growing cattle plasma were considerably lower than those obtained by the bovine RIA. This discrepancy may be explained by the limited ability of the antisera used in the Multi-Species assay to bind rbleptin (results not shown). Estimates of leptin concentrations obtained by both assays were positively correlated in adult sheep plasma, but not in cow plasma. More importantly, when plasma from growing cattle were analyzed for leptin concentration by the Multi-Species assay, effects of nutrition were not detected, and circulating leptin was not related to body fat (results not shown). These findings suggest that the Multi-Species assay will detect differences only when effects of treatments are large and experimental errors are low (Bocquier *et al.* 1998, Chilliard *et al.* 1998).

In summary, these results demonstrate that we have developed a sensitive RIA for the measurement of leptin in both cattle and sheep. This assay measures plasma leptin concentration as low as 0.5 ng/ml and is able to detect

differences in plasma leptin caused by nutrition, adiposity and gender. This RIA provides an important tool to study factors and mechanisms regulating leptin in sheep and cattle.

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