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Relationship between serum leptin immunoreactivity and body fat mass as estimated by use of a novel gas-phase Fourier transform infrared spectroscopy deuterium dilution method in cats

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Objective—To validate a recently developed commercially available leptin radioimmunoassay (RIA) for use with feline serum and evaluate the relationship between serum leptin concentrations and body fat mass in domestic cats.

Animals—19 sexually intact male specific–pathogen-free domestic cats that weighed 3.8 to 7.1 kg and were 1.1 to 3.5 years old.

Procedure—Specificity for feline leptin was evaluated by use of gel filtration chromatography and reverse-phase high-performance liquid chromatography fractionation of serum. Body fat mass was determined by use of the deuterium oxide (D_2O) dilution method. Serum water D_2O enrichment was measured by use of gas-phase Fourier transform infrared spectroscopy.

Results—Body fat mass and percentage body fat ranged from 0.3 to 2.3 kg and 7.5 to 34.9%, respectively. Serum leptin concentrations were lower in the unfed versus the fed state and ranged between 1.6 and 4.9 ng/ml human equivalent (HE); mean ± SD value was 2.9 ± 0.2 ng/ml HE. Leptin concentrations increased with increasing body fat mass and percentage of body fat.

Conclusions—Leptin is in the serum of domestic cats in free (> 78%) and apparently bound forms. The relationship between body fat and serum leptin concentration was similar to that observed in humans and rodents and indicative of a lipostatic role for leptin in cats. Cats that have an overabundance of body fat appear to be less sensitive to the weight-normalizing action of leptin than cats of ideal body condition. (*Am J Vet Res* 2000;61:796–801)

Obesity is common in cats and, as in other species, is reported to be an unhealthy condition. Compared with cats of normal body fat mass, obese cats are at greater risk for developing nonallergic skin conditions, hepatic lipidosis, and musculoskeletal disorders.[1] Obesity in cats also reduces tolerance of intravenously administered glucose loads and is suggested to predispose cats to development of diabetes mellitus.[2] Recently, researchers investigating the molecular basis of obesity in a mouse model, C57BL/6J *ob*/*ob*, identified and characterized a gene expressed in adipose tissue that, when mutated, results in profound obesity and type-II diabetes mellitus.[3] The gene, called the *ob* gene after the mouse model, was not unique to mice. Results of cDNA hybridization analysis indicated that *ob*-like genes are present in several vertebrate species, including cats. Daily intraperitoneal injections of the *ob* protein induced a dose-dependent reduction of food intake, body weight, and fat mass, and additionally in *ob*/*ob* mice, return of glucose and insulin concentrations to reference ranges.[4-6] For its weight-reducing effect, the *ob* product was named leptin, after the Greek word for thin, leptós.[4]

Since the discovery of the *ob* gene, scientific interest in the control of body energy balance and the prospect of developing effective anti-obesity treatments have driven an intensive research effort to determine the function of leptin. Following the determination of the *ob* nucleotide sequence, recombinant and chemically synthesized peptide sequences of leptin were produced and used to generate sensitive and specific immunoassays for leptin.[7-11] These assays have been important tools for determining the function of leptin. The purpose of the study reported here was to validate a **radioimmunoassay (RIA)** based on antisera with crossreactivity to leptins of several species for measurement of serum leptin concentrations in domestic cats, and evaluate the relationship between leptin concentrations and body fat mass as estimated by **deuterium oxide (D_2O)** dilution.[12]

Materials and Methods

Cats—Nineteen adult male specific–pathogen-free cats (mean ± SD; age, 3.0 ± 1.0 years; range, 1.1 to 3.5 years) that weighed 4.9 ± 0.8 kg (range, 3.8 to 7.1 kg) were housed at the Feline Nutrition and Pet Care Center, University of California, Davis. Cats were kept in group cages in which food was continuously available. Eleven cats received a complete and balanced commercial extruded (dry-type) diet that was labeled to contain 31% (wt/wt) crude protein, 10% crude fat, 7% ash, 8% moisture, and 1.8% crude fiber. The remaining 8 cats received a complete and balanced purified diet that contained lactic casein[a] (200 g/kg), soybean protein isolate[b] (200 g/kg), sucrose[c] (43 g/kg), animal tallow[d] (250 g/kg), hydrogenated beef tallow flakes[e] (100 g/kg), food-grade corn starch[f] (142 g/kg), mineral mixture[13] (50 g/kg), vitamin mixture[13] (10 g/kg), choline chloride[g] (3 g/kg), taurine[h] (1.5 g/kg), and dl-α-tocopherol acetate[i] (0.12 g/kg).

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Husbandry and experimental procedures were approved by the university animal use and care committee and were in compliance with the *Guide for the Care and Use of Laboratory Animals*.[14]

Body fat determination—Body fat mass was estimated by use of the isotopic dilution method of Lukaski et al.[12] This method was selected for practicality of use in a specific-pathogen-free colony. A tracer dose (0.4 g/kg of body weight) of D_2O[j] was administered IV after food and water were withheld for 24 hours. Total body water mass was estimated by detection of D_2O in serum harvested from a blood sample obtained from the jugular vein after a 2-hour equilibration period. Size of the pool in which the tracer was diluted was assumed to be the total body water mass. Overestimation of total body water mass from deuterium exchange with labile hydrogen was corrected by reducing the total body water mass estimate by 2% of body weight.[12] Body fat mass was determined as the difference between body weight and lean body mass, where lean body mass was estimated as total body water mass divided by 0.744, a reported fractional moisture content of lean body mass in cats.[15]

For D_2O detection, water was extracted from 100 µl of serum by a novel method. Serum was placed in a 2-ml screw-cap tube[k] and held in a well that was formed on the inner surface of the cap. A clean screw-cap tube base was inverted, threaded on the cap, and carefully tightened so that the serum in the cap would not contact the wall of the tube. The inverted and capped tube was then placed, cap down, in glass beads (approximately 2 mm in diameter) of a dry heated bath[l] set at 45 C. The cap portion was buried so that the tube stood vertically, exposed to air at 21 to 23 C. After 14 to 16 hours, approximately 40 µl of serum water typically would condense on the walls of the tube. While still inverted, the tube cap was replaced with a clean cap. The capped tube was then centrifuged to collect the condensed water and stored at –20 C until D_2O detection.

Detection of D_2O in the condensed water was accomplished by a modification of the gas-phase **Fourier transform infrared (FTIR)** spectroscopy method described by Khaled et al.[16] In this method, 20 µl of condensed water was injected into an evacuated (approx 20 mm Hg), heated (124 C) flash injector[m] fitted to a gas cell[n] placed in the sample chamber of a FTIR spectrometer.[o] An absorbance spectrum relative to native water was obtained in 6 minutes by use of 200 scans at a resolution of 4 **wave numbers/cm** (cm^{-1}). Background native water scans were of 800 iterations. Difference in absorbance between native water and samples from cats between 2,720 and 2,735 cm^{-1} indicated the presence of D_2O. This difference increased linearly ($r \geq 0.99$) with enrichments up to 0.067 **atom percent excess (APE)**. Significant ($P < 0.05$) D_2O depletion was not observed in the water extraction procedure. Serum D_2O enrichments were 102 ± 4% (n = 6) and 102 ± 2% (10) of those determined by mass spectroscopy[p] and single frequency infrared[17] methods, respectively **(Fig 1)**. Intra- and interassay coefficients of variation were 1.0 and 1.7%, respectively.

Gel filtration fractionation of serum—The RIA[q] is based on guinea pig antisera raised against recombinant human leptin. To evaluate specificity of the antiserum for feline leptin, leptin immunoreactivity was determined in 3 serum samples fractionated by use of gel filtration chromatography. Each serum sample was from a different cat. One to 2 ml of serum were loaded on an allyl dextran-N,N'-methylenebisacrylamide[r] column equilibrated with 25-m*M* phosphate-buffered saline solution, pH 7.4, at a flow rate of 35 ml/h. Before loading, the serum was centrifuged in an 18 × 102-mm glass tube[s] at 8,000 × g for 30 minutes at 4 C to remove particulate matter. Fractions were collected from

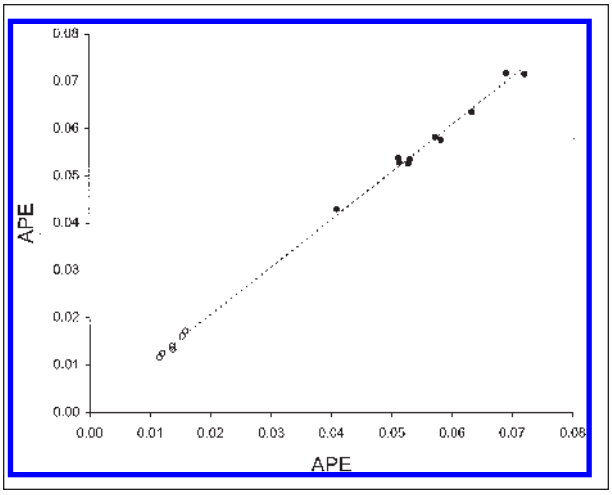

Figure 1—Correlation between serum deuterium oxide enrichment (atom percentage excess [APE]) determined by use of gas-phase Fourier transform infrared spectrometry (y-axis) with that determined by use of mass spectrometry (○) and infrared absorbance at a single frequency (●) (x-axis). The plotted line represents the linear function y = 1.01x + < 0.01, derived from least-squares analysis of the plotted points (r = 0.999).

the column at 2-minute intervals in 12 × 75-mm borosilicate glass tubes for 150 minutes. The fractionation procedure was conducted at 4 C. Fractions were dried in the glass tubes by centrifugal evaporation[t] at 21 to 23 C for 3 hours. The tube contents were reconstituted by addition of 100 µl of human blood bank plasma made leptin-free by use of charcoal extraction. Leptin immunoreactivity in each tube was subsequently determined by use of the RIA kit; each fraction was treated as a serum sample. Immediately before serum fractionation, elution volumes of molecular weight markers, albumin (67 kd), ovalbumin (43 kd), chymotrypsinogen A (25 kd), and ribonuclease A (13.7 kd) were determined from the ultraviolet absorbance at 280 nm of column eluent. Leptin immunoreactivity was not found in eluent collected during fractionation of the molecular markers.

Reverse-phase high-performance liquid chromatography (HPLC) of serum—A modification of the HPLC method of McGregor et al[10] was used in fractionation of serum samples from 3 cats. Briefly, 0.5 ml of serum was loaded on a cartridge[u] C^{18}-conditioned sequentially with 5 ml of 86% ethanol in 4% acetic acid followed by methanol, water, and 4% acetic acid. The loaded cartridge was washed with 5 ml of water and 20% acetonitrile in 0.1% **trifluroacetic acid (TFA)**. Adsorbed leptin was eluted into 8 ml of 0.1% TFA with 3 ml of 80% acetonitrile in 0.1% TFA. The diluted eluent (10 ml) was injected onto a 3.9 × 300-mm C^{18}-column[v] equilibrated with 20% acetonitrile in 0.1% TFA with flow rate of 1.0 ml/min. Acetonitrile in the mobile phase was linearly increased to 70% during a 65-minute period, beginning 15 minutes after extract injection. Column eluent was collected at 1-minute intervals in 12 × 75-mm glass tubes and dried by use of centrifugal evaporation. Leptin immunoreactivity in the fractions was determined by use of the procedure described in gel filtration methods.

Leptin radioimmunoassay—Measurement of serum leptin concentration was conducted as directed in the RIA kit instructions.[q] Briefly, this involved incubation of 100 µl of serum with 100 µl of an assay buffer and 100 µl antiserum in 12 × 75-mm glass tubes for 24 hours at 4 C. After incubation, 100 µl of solution that contained approximately 10,000 disintegrations/min of human recombinant leptin labeled with

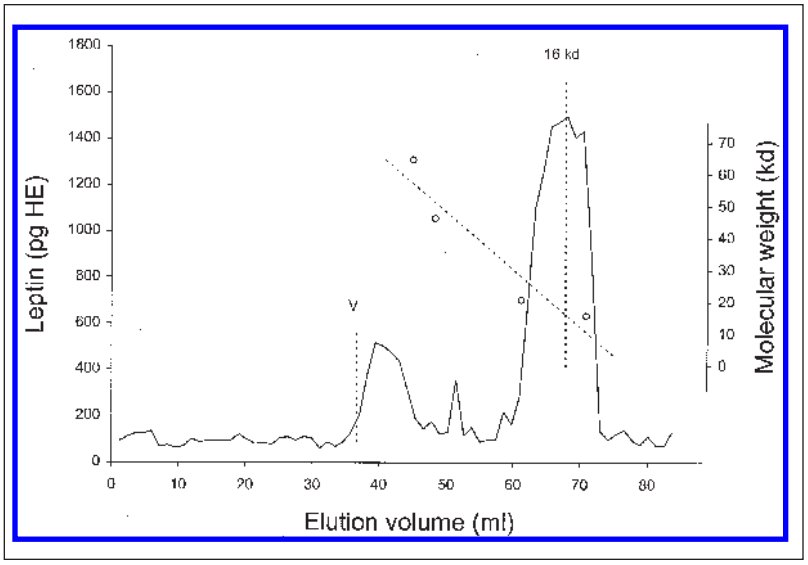

Figure 2—Leptin immunoreactivity (pg of human equivalents [HE]) of 2 ml of feline serum fractionated by use of gel filtration chromatography. Open circles from left to right are plots of the elution volumes of the molecular weight markers, bovine serum albumin (64.5 kd), ovalbumin (46.3 kd), chymotrypsin A (20.7 kd), and ribonuclease A (15.7 kd), respectively. The dashed line is a plot of the linear function estimated from least-squares analysis of volume of elution as a function of relative molecular weight (y = –0.493x + 76.6; r = 0.947). V = Void volume. 16 kd = Volume expected to contain leptin.

radioactive iodide (^{125}I) was added and incubated for 24 hours at 4 C. The label bound to the RIA antiserum was precipitated by adding 1 ml of diluted rabbit anti-guinea pig antisera solution to each tube and incubating for 20 minutes at 4 C. The mixture was centrifuged at 2,000 × g for 20 minutes at 4 C. The precipitant was counted for 1 minute after separation from the supernatant by pouring. Serum leptin concentration was determined from the percentage of total counts that were bound in the precipitant.

Because the serum used in this determination was obtained from blood collected from food-deprived cats, the effectof food deprivation was investigated by determination of leptin immunoreactivity in 11 cats before and after withholding of food for 24 hours.

Statistical analyses—Linear regression and correlation analyses involving serum leptin immunoreactivity, body fat mass, percentage body fat mass, and age were conducted with a computer software program.[w] The effects of diet and withholding food on serum leptin immunoreactivity and body fat mass were evaluated by use of Mann-Whitney tests. Differences were considered significant at $P \leq 0.05$.

Results

Fractionation of serum by use of gel filtration chromatography revealed that most of the leptin immunoreactivity was concentrated in 2 peaks (**Fig 2**). As indicated by molecular weight markers, the highest peak eluted in fractions expected to contain proteins that weighed between 15.7 and 20.7 kd. Linear interpolation indicated that the most immunoreactive fraction could be expected to contain a 16-kd protein. Leptin immunoreactivity in the smaller of the 2 peaks was consistent with the presence of a protein in a molecular weight range greater than that of bovine serum albumin. Total immunoreactivity (n = 3) was equivalent to 6 to 26 ng of recombinant human leptin, and the 2 immunoreactive peaks contained 75 ± 19% and 17 ± 6% of this total, respectively.

Reverse-phase HPLC fractionation of serum revealed that virtually all leptin immunoreactivity was within a single peak (**Fig 3**). Retention time of the peak occurred slightly before the retention time of the ^{125}I-labeled human recombinant leptin used in the RIA. Mean (± SEM) recovery of immunoreactivity in the peak was 16.8 ± 5.6% (n = 3). In some specimens, a portion of the pelleted bound leptin was noticed to be lost during the separation of bound leptin from free leptin, which resulted in a few small peaks in the HPLC

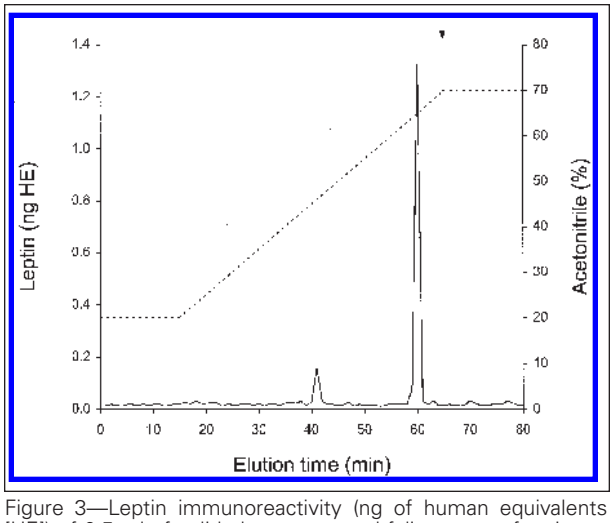

Figure 3—Leptin immunoreactivity (ng of human equivalents [HE]) of 0.5 ml of solid phase-extracted feline serum fractionated by use of reverse-phase high-performance liquid chromatography. Arrow indicates elution time for iodine 125-labeled recombinant human leptin. Dotted line indicates percentage of acetonitrile in the mobile phase.

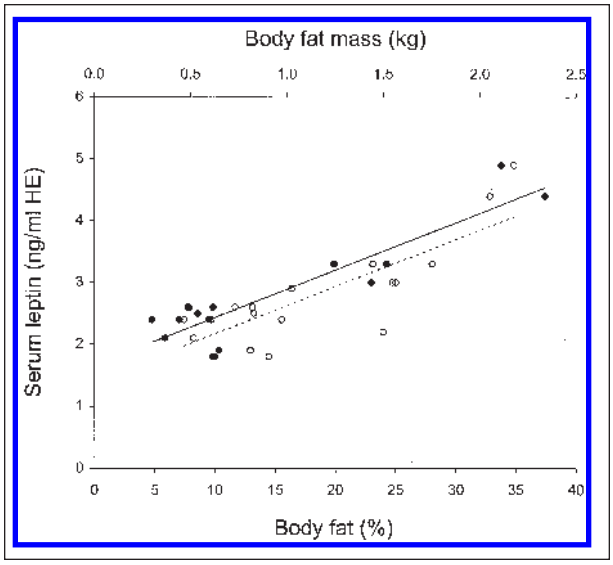

Figure 4—Relationship among serum leptin concentration, body fat mass (●), and percentage body fat (○) of adult male cats. Linear regression functions of leptin concentration on body fat mass (y = 1.20x – 1.67; r = 0.88; $P < 0.001$) and percentage body fat (y = 0.08x + 1.40; r = 0.81; $P < 0.001$) are plotted as solid and dashed lines, respectively.

profile. A peak at the 41-minute elution time was believed to be such an artifact, because it was not consistently observed.

Body fat mass and percentage body fat among cats ranged from 0.3 to 2.3 kg and 7.5 to 34.9%, respectively. Serum leptin immunoreactivity among cats ranged between 1.7 and 5.0 ng/ml **human equivalent (HE)** and was positively correlated with body fat mass and percentage body fat. Leptin immunoreactivity increased linearly with increasing body fat mass (**Fig 4**). A similar relationship was observed between leptin immunoreactivity and percentage of body fat. Serum leptin immunoreactivity was not significantly (P = 0.19) correlated with lean body mass (r = 0.31).

Leptin immunoreactivity was repeatedly measured in serum samples obtained from blood collected from 2 cats to assess intra- and interassay variance. Mean (± [SD/mean] × 100) leptin immunoreactivity of 8 replicates from 1 cat was 2.3 ng/ml ± 12.3% and from the other cat was 11.4 ng/ml ± 3.9%. Mean leptin immunoreactivities determined in assays conducted on 3 different days for these 2 cats were 2.6 ng/ml ± 11.3% and 11.3 ng/ml ± 6.3%, respectively.

The 24-hour withholding of food may have affected serum leptin immunoreactivity. Mean (± SEM) leptin immunoreactivity before food deprivation (2.0 ± 0.2 ng/ml HE) was greater than mean immunoreactivity after food deprivation (1.7 ± 0.2 ng/ml HE), but not significantly (P = 0.051).

Cats given the commercial diet had greater (P = 0.02) leptin immunoreactivity (3.4 ± 0.3 ng/ml HE) than cats given the purified diet (2.4 ± 0.1 ng/ml HE), but an effect of diet could not be clearly discerned from that of body fat mass. The mean body fat mass of cats given the commercial diet was nearly significantly greater (P = 0.055) than that of cats given the commercial purified diet. Percentage body fat of cats given the commercial diet was not significantly different from that of cats given the purified diet.

Serum leptin immunoreactivity was positively correlated (r = 0.66; P = 0.01) with age. Because body fat mass and percentage body fat mass were also positively correlated with age (r = 0.80; $P < 0.001$ and r = 0.76; $P < 0.001$, respectively), an age effect could not be distinguished.

Discussion

The finding of a weak hybridization signal in murine *ob* cDNA probing of feline genomic DNA was an initial indication that a leptin homolog occurs in cats.[3] We presumed that this leptin was bloodborne in cats and attempted to measure it in the serum of lean and overweight cats with a RIA based on antisera raised against mouse, human, primate, and rat leptins. Unfortunately, in initial trials, substantial immunoreactivity was not observed. The cause for the negative result was suspected to be a structural uniqueness of feline leptin, which precluded binding to the tested antisera. Evaluation of reported nucleotide sequences of the *ob* genes of mice, humans, rats, chickens,[18] and pigs[19] indicates that there are species differences in leptin structure. Apparently, the antisera used in the study reported here has affinity for a structural domain conserved among many species, including cats.

Gel filtration fractionation of serum was used in our study to validate that leptin is a circulating factor in cats. Indeed, substantial leptin immunoreactivity was found in serum (Fig 2). Most of the immunoreactivity was in fractions that were expected to contain protein of the molecular weight of leptin (16 kd). Similar results have been observed in gel filtration fractionation of human serum.[9,10,20] Some of the leptin immunoreactivity was observed in fractions expected to contain proteins of much greater molecular weight than leptin. Nonspecific immunoreactivity of the RIA antiserum with serum proteins could explain this result. However, another possibility is that certain serum proteins specifically bound the ^{125}I-leptin label of the RIA. Human serum proteins that weigh 66 to 280 kd bind leptin, and some of the binding is quite specific for leptin.[20] Circulating leptin has been suggested to be the ligand for a splice variant of the leptin receptor that has no apparent transmembrane domain.[21] This soluble receptor, which has a predicted molecular weight of 92 kd, may form circulating complexes with leptin.[22] Such a protein could account for the high molecular weight leptin immunoreactivity detected in our study. Presently, the function of a circulating leptin receptor is speculative. Tartaglia[21] has suggested that such a receptor may mediate transport, modulate potency, or facilitate clearance of leptin. The fraction of leptin bound to the serum proteins of humans is reportedly high in lean individuals and low in obese subjects.[20] This may also be true in cats.

Results of HPLC fractionation of plasma further indicated that the RIA antiserum was specific for feline leptin. One immunoreactive peak, rather than 2, was observed in this procedure (Fig 3). Similar results with reverse-phase HPLC and gel chromatography have

been reported for human serum.[10] The peak eluted at approximately the same time as the recombinant human leptin standard, indicating that the serum contained a leptin chemically similar to the standard. The slight difference observed in retention time between the feline samples and the human standard may indicate a structural difference that affects hydrophobicity. The low recovery of leptin immunoreactivity was suspected to have resulted from loss in the C^{18}-cartridge extraction. To investigate this possibility, serum was extracted after being spiked with the ^{125}I-leptin label used in the assay. The amount of label recovered (22%) was similar to that recovered after HPLC fractionation (20.8%). Some radiolabel (17.5%) passed though the C^{18}-cartridge during loading and washing. Most of the label appeared to be retained on the C^{18}-cartridge, with a substantial amount adsorbed to the frits that retain the silica gel packing. The extraction method used in our study was developed for use on human plasma, but recovery results were not reported.[10]

Serum leptin immunoreactivity among cats increased with increasing percentage of body fat (Fig 4). Percentage of body fat ranged from a high value of 35% to a low value of 7.5%. Results of previous research on subjective assessment of body fat has indicated that approximately 22% body fat is ideal in cats,[23] and that body conditions among cats of our study would be classified as thin to "heavy." For those cats with percentage of body fat near ideal in our study, serum leptin immunoreactivity was approximately 3 ng/ml HE. This concentration is considerably less than leptin concentrations found in serum of normal-weight human subjects.[24-26] The low values probably resulted from lower affinity of the RIA antiserum for feline leptin than its immunogen, recombinant human leptin.

If leptin functions in cats as suggested in other species, the high leptin concentrations detected in our study would indicate an overabundance of body fat, and appropriate changes in food intake, energy expenditure, and metabolism would be orchestrated by hypothalamic nuclei to regulate body fat mass. If this feedback mechanism completely corrected deviations in fat mass, only transient changes in serum leptin concentration would be expected. In the study reported here, cats that had an undesirably large amount of body fat also had high serum leptin immunoreactivity. Similar findings in humans and rodents have led to postulation that most obese humans are resistant to leptin.[7,27] Many causes for this resistance have been suggested, including intravascular defects that limit free leptin from reaching the brain, impaired transport of leptin across the blood-brain barrier, and faulty cellular signal transduction at target tissues.[28-30] Although none of the cats in our study would be considered obese,[23] it would appear that resistance to the weight-regulating effects of leptin may occur in obese cats.

Among cats of our study, a small (20%) but notable decrease was observed in serum leptin immunoreactivity with food deprivation, probably as a manifestation of short-term changes in leptin secretion by adipose tissue. In humans and rodents, circulating concentrations of leptin decrease with brief periods of fasting or energy restriction, and refeeding restores the decrements in leptin concentration.[28-30] Because insulin and glucose appear to be secretogogues of leptin, such short-term changes in energy balance may result from changes in insulin and glucose concentrations.[31] In the fasted state, insulin and glucose concentrations are low and, therefore, leptin secretion should be low. The magnitude of reduction in circulating leptin immunoreactivity observed among the cats of our study was less substantial than that reported in rats and humans.[24,26] Because blood glucose concentration of cats is not profoundly reduced by withholding of food,[32] the small change in serum leptin immunoreactivity observed in cats of our study may reflect a small change in glucose availability. Also, adipose glucose use in cats appears to differ from that in humans and rats. Acetate is preferred to glucose in lipogenesis in cats, whereas glucose is the preferred substrate in humans and rats.[33]

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[a]New Zealand Milk Products, Petaluma, Calif.
[b]Ardex F-dispersible, SPI Group, San Leandro, Calif.
[c]Holly Sugar, Colorado Springs, Colo.
[d]Florin Tallow, Dixon, Calif.
[e]Morgan Specialties, Paris, Ill.
[f]Melojel, National Starch and Chemical, Bridgewater, NJ.
[g]International Mineral and Chemical, Terre Haute, Ind.
[h]Taisho Pharmaceutical, Torrance, Calif.
[i]Sigma Chemical Co, St Louis, Mo.
[j]99.96%, Cambridge Isotope Laboratories Inc, Andover, Mass.
[k]Micro Tube, Sarstedt, Newton, NC.
[l]Model 145, Isotemp Dry Bath, Fisher Scientific, Pittsburgh, Pa.
[m]Model 1060 with oven, Varian Associates, Sugar Land, Tex.
[n]LFT-210, Axiom Analytical, Irvine, Calif.
[o]60Mi Infinity, ATI Mattson, Madison, Wis.
[p]Metabolic Solutions, Merrimack, NH.
[q]Multi-species Leptin RIA kit, Linco Research, St Louis, Mo.
[r]Sephacryl S-100 Hiprep 16/60, Pharmacia Biotech, Alameda, Calif.
[s]Corex, Sorvall, Newtown, Conn.
[t]SS3 SpeedVac Concentrator System, Savant Instruments, Farmingdale, NY.
[u]Sep-Pak Classic, Waters Corp, Milford, Mass.
[v]μBondapak, Waters Corp, Milford, Mass.
[w]Release 10, Minitab Statistical Software, Minitab Inc, State College, Pa.

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