Age-Related Changes in Plasma Leptin Binding Activity in Rats: A Comparison of a Simple Acid-Ethanol Precipitation Technique with Column Chromatography

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Abstract. **A novel assay for measuring the free leptin fraction was developed and validated against a chromatographic technique. The assay used acid-ethanol extraction (AEE) for separation of bound/free leptin moieties. The interassay coefficient of variation was 3.9%. The specificity for leptin binding was confirmed by incubation with 1 μg of unlabeled rat leptin that effectively competed with radiolabeled leptin whereas human growth hormone and interleukin-6 were ineffective in competing with radiolabeled leptin binding. Scatchard analysis of competitive binding experiments with rat plasma demonstrated a linear relationship with a binding affinity of 0.3–0.6 × 10^9 M^{-1}. This novel assay was used to determine if age-related insensitivity to leptin action is secondary to altered serum leptin binding. Rats at various age groups were studied for changes in body adiposity and serum total and free leptin concentrations. Serum free leptin concentrations (ng/ml mean ± SEM) were significantly increased in 24-month-old rats (5.56 ± 0.21) compared with 18-month-old rats (4.76 ± 0.17) ($P < 0.01$) despite similar body weight and adiposity of the two age groups. The increase in plasma free leptin concentrations in 12-month-old rats (3.86 ± 0.28) and 6-month-old rats (2.05 ± 0.06) relative to 3-month-old rats (1.37 ± 0.06) ($P < 0.001$) was out of proportion to the increase in body adiposity in aging rats. It is concluded that aging in rats is associated with relative insensitivity to leptin. This change cannot be attributed to increased plasma binding or to a reduction in the leptin free fraction.**

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Leptin binding in human or rodent plasma has been measured using a variety of techniques (1–5). We have modified an acid-ethanol extraction technique used previously for insulin-like growth factor-1 (IGF-1) binding (6), to measure the fractional binding of leptin in plasma.

Aging is associated with increased adiposity in humans and animals (7). The cause of increasing body fat with age is probably multifactorial. It has been postulated that changes in body composition with age could partially be the result of insensitivity to the action of the *Ob* gene product leptin (8, 9). The mechanisms underlying this insensitivity to leptin are not known. One potential mechanism is reduced leptin transport across the blood-brain barrier (BBB) (10–12). This is supported by previous observations indicating that aging is associated with a host of structural and physiological changes of the BBB (13, 14). One of the potential determinants of the BBB transport of leptin is the plasma leptin binding properties. Using this newly developed assay, we measured plasma leptin binding in postpu-

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bertal rats at different ages to determine if free leptin levels correlate with body composition.

Materials and Methods

Animals. Male Fischer 344 rats at 4, 6, 12, 18, and 24 months of age were purchased from Harlan Industries (Indianapolis, IN). After 5–7 days in our animal facility, body weight and food intake had stabilized. After 1 week of observation, the rats were sacrificed by exsanguination through the abdominal aorta under pentobarbital anesthesia (45 mg/kg ip). The epididymal fat pad and retroperitoneal fat pad were weighed, and plasma was collected for total and free leptin measurements.

Plasma Leptin Measurements. Plasma leptin concentrations were measured in duplicate using a specific rat leptin radioimmunoassay kit (Linco Research, St. Charles, MO). The interassay coefficient of variation was less than 6%, and the detection limit was 0.5 ng/ml.

Acid-Ethanol Extraction (AEE) Technique. A previously described technique used for IGF-1 measurements was used with some modifications (6). To 100 μl plasma sample, 100 μl (10,000–20 000 cpm) of radiolabeled ^{125}I-leptin (specific activity of 135 μCi/μg, purchased from Linco Co., St. Charles, MO) was added along with 20 μl of phosphate-buffered saline (PBS, pH 7.4) containing various amounts of unlabeled rat leptin (purchased from R & D Systems, Minneapolis, MN). After incubating at 4°C for 48 hr, 0.8 ml of a mixture of 87.5% ethanol and 12.5% 2*N* hydrochloric acid (v/v) was added. The mixture was mixed thoroughly, allowed to stand for 15 min at room temperature, and centrifuged at 1860*g* for 30 min at 4°C. Delaying centrifugation beyond 15 min did not alter the results. The pellet was washed with 1 ml of the cold acid ethanol extraction mixture using the centrifugation conditions described above. The supernatant, pellet, and 1 ml of the extraction mixture used for washing the pellet were counted in a gamma counter. The percentage (%) of bound leptin was calculated by determining the ratio of the counts in the pellet to the total counts (pellet, wash, and supernatant) multiplied by 100.

Column Chromatography. A previously described gel filtration technique for measuring free and bound plasma leptin fractions was used with some modifications (1). To 500 μl of rat plasma sample, 500 μl (100,000 cpm) of radiolabeled rat ^{125}I-leptin was added and the mixture was incubated at 4°C for 48 hr. This mixture was eluted at 4°C from a Sephadex G-100 gel filtration column with 25 m*M* phosphate-buffered saline (PBS) (pH 7.4) containing 0.01% sodium azide. The radioactivity eluting in the void volume represents the bound fraction whereas radioactivity eluting between the void and bed volume represents the free fraction. A representative elution profile is shown in Figure 1. The percentage of bound and percentage of free ^{125}I-leptin were determined by dividing the area under peak I (bound) or peak II (free), respectively, by the total areas under the two peaks. The results were identical when the sum of radioactivity eluting in the two peaks was calculated and then percentage bound or free ^{125}I-leptin was determined by dividing the counts in each peak by the total counts.

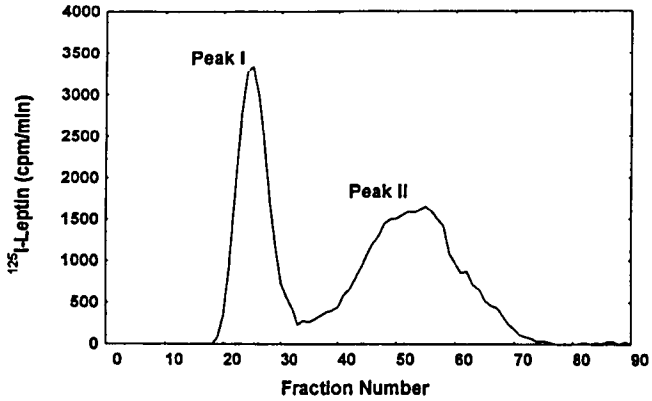

Figure 1. Sephadex G-100 elution profile of ^{125}I-leptin in rat plasma. Peak I represents the bound fraction, and peak II represents the free fraction.

Plasma Leptin Binding Assay. To determine the proportions of leptin binding to plasma proteins, plasma samples stripped of endogenous leptin were incubated with varying amounts (0–2500 ng/ml) of recombinant rat leptin (R & D Systems, Minneapolis, MN), and binding was assayed by acid-ethanol extraction.

The stripping of endogenous leptin from plasma was accomplished by using a previously described method (4). To 200 μl of plasma, 500 μl of prechilled 2% Norit A charcoal (Sigma Chemical Co., St. Louis, MO), 0.2% Dextran 70 (Amersham, Pharmacia Biotech, Piscataway, NJ) in PBS was added, vortexed, and placed on ice for 5 min. The samples were centrifuged at 4°C for 12 min at 10,000*g*. The supernatant (plasma stripped of leptin) was removed and used in binding assays.

Statistical Analysis. The results are reported as mean ± SEM. One-way ANOVA was used to determine the effect of age on plasma leptin binding measurements. The *t* test with Bonferroni correction was used to determine the statistical significance of the differences between the groups ($P < 0.05$ was considered the level of statistical significance). Correlations with the acid-ethanol extraction technique and column chromatography at various dilutions were determined with Pearson's correlation coefficients. The plasma leptin binding data were subjected to nonlinear least square regression analysis using the Statistica Program (Statsoft, Tulsa, OK).

Results

Assay Validation. The results of acid-ethanol extraction (AEE) were compared with those obtained with Sephadex gel filtration chromatography. The percentage of bound leptin measured with the two methods were concordant (Table I). The interassay coefficient of variation for the column method was 2.5% and for the AEE was 3.9%.

Table I. Comparison of Results of Percentage Leptin Bound in Rat Plasma as Measured with Acid-Ethanol Extraction (AEE) Technique and Sephadex Gel Filtration Chromatography Using Seven Independent Samples of Rat Plasma

Samples	% Bound	
	AEE	Chromatography
1	40.40	41.96
2	41.03	41.57
3	40.59	43.62
4	36.76	40.79
5	39.19	43.12
6	38.00	40.98
7	39.31	41.75
Mean ± SEM	39.33 ± 0.58	41.97 ± 0.40
A	35.86	36.11
B	31.85	32.61
C	22.78	25.18

Note. Samples A, B, and C are rat plasma diluted with phosphate buffered saline (PBS) at ratios of 3:1, 1:1, and 1:3 (plasma: PBS), respectively.

To determine the reproducibility of the AEE procedure, it was compared with the column method using rat plasma diluted with incubation buffer (PBS). Various dilutions of plasma with PBS did not alter the concordance between the AEE and the column methods (Table I); however, the change in leptin binding was not proportional to the change in plasma proteins, suggesting that there is some degree of negative cooperativity of leptin binding to plasma. These measurements on serially diluted plasma involved binding to both specific and nonspecific sites. The negative cooperativity of leptin binding occurs at specific sites. To demonstrate this phenomenon conclusively, the leptin binding to serially diluted plasma was carried out in the presence of receptor-saturating concentrations of leptin (10,000 ng/ml). Under these experimental conditions, the changes in leptin binding were proportional to changes in plasma proteins such that the percentage of leptin binding (± SD) in undiluted plasma was 44.7 ± 1.2%, whereas in 3:1, 1:1, or 1:3 diluted plasma (plasma:PBS), the binding was 40.0 ± 0.8%, 29.0 ± 1.2%, and 14.3 ± 0.50%, respectively. The correlation coefficient of the relationship between leptin binding and the degree of plasma dilution was 0.977. When only the last three dilutions were considered, the correlation coefficient increased to 0.996.

The AEE method was used to determine if specific leptin binding to plasma proteins can be demonstrated. Figure 2A is a representative of a competitive binding assay of ^{125}I-leptin to serum from 4-month-old rats. The specific binding is expressed as a percentage of the maximal binding. Values are the mean of duplicate determinations. The specificity for leptin was confirmed by incubation with 1 μg of unlabeled rat leptin, human growth hormone (Eli Lilly Co., Indianapolis, IN), and interleukin-6 fragment (Sigma Chemical Co, St. Louis, MO).

Scatchard analysis of competition experiments with rat

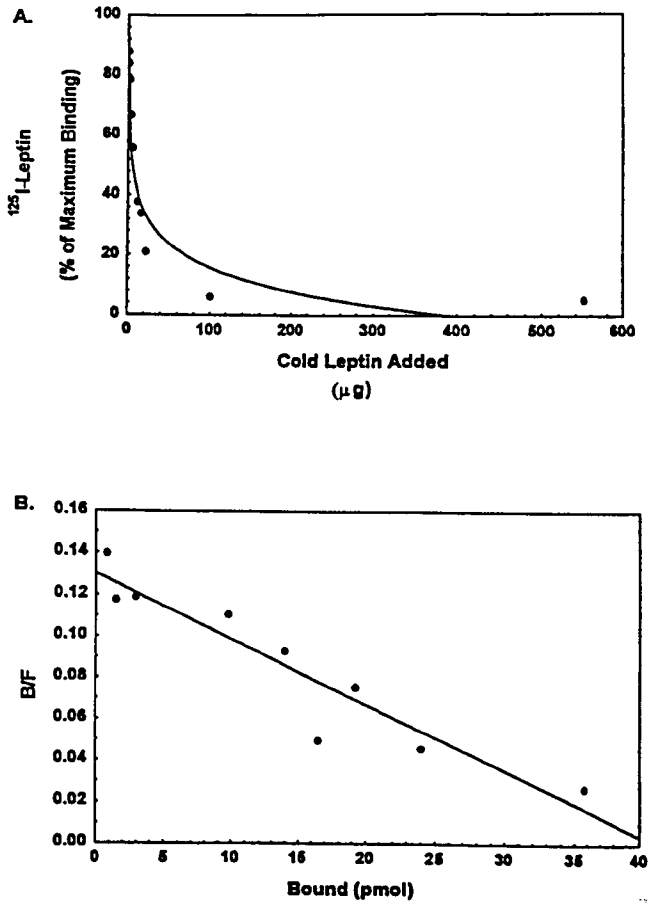

Figure 2. (A) Saturability of plasma binding of ^{125}I-leptin to normal rat plasma. The specific binding is expressed as a percentage of the maximal binding. Values are the mean of duplicate determinations from a representative experiment. The experiment was repeated three times. (B) Scatchard analysis of ^{125}I-leptin binding to rat plasma proteins.

leptin showed a linear plot with a binding affinity of 0.3–0.6 × 10^9 M^{-1} (Fig. 2B).

Age-Related Changes in Plasma Leptin Binding. The body weight, epididymal and retroperitoneal fat weight, and plasma leptin levels are summarized in Figure 3. In this strain of rats, body weight increased modestly after the age of 4 months and reached a near plateau by 12 months of age. In contrast, the epididymal and retroperitoneal fat pad weights increased more rapidly between the ages of 6–12 months, and thereafter the changes were modest until 24 months of age. In these studies, the changes in plasma leptin correlated best with retroperitoneal fat weight (r = 0.997) and epididymal fat weight (r = 0.972). However, there is approximately a 4-fold change in plasma leptin during aging whereas visceral fat weight change during the same period was only 2-fold, and the total body weight increased only 50%. These findings are consistent with observations previously reported in different strains of rats (8, 15).

The age-related changes in total and free serum leptin concentrations and the percentage of bound leptin in serum are summarized in Table II. The percentage of bound leptin

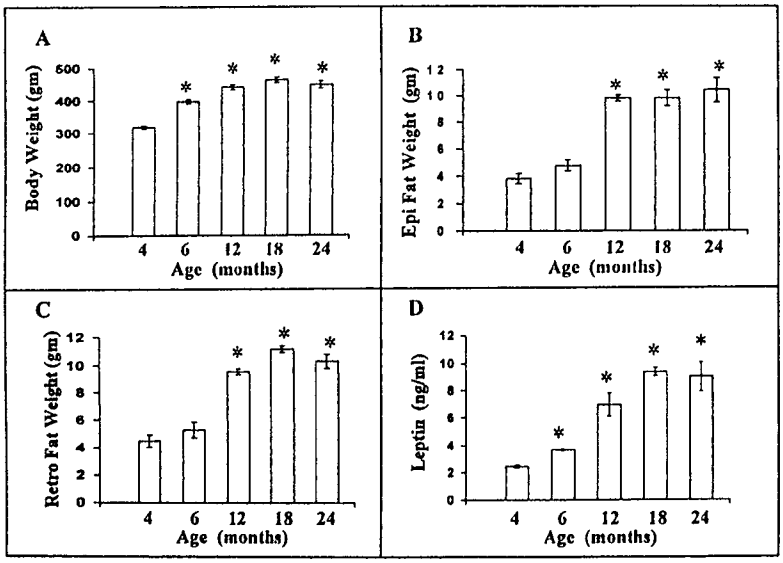

Figure 3. (A) The mean (± SEM) body weight, (B) epididymal (Epi) fat pad weight, (C) retroperitoneal (Retro) fat pad weight, and (D) total plasma leptin concentrations in rats at various ages. *$P < 0.01$ compared with 4-month-old rats. $n = 10$ for 4-month-old rats and $n = 5$ for all the other age groups.

Table II. The Mean (± SEM) of Total and Free Plasma Leptin Concentrations and Percentage Bound Leptin in the Plasma (% bound)

Age (months)	n	Total leptin (ng/ml)	Free leptin (ng/ml)	Percentage bound (%)
4	10	2.44 ± 0.11	1.37 ± 0.06	43.72 ± 1.02
6	5	3.66 ± 0.06[a]	2.05 ± 0.06[a]	44.83 ± 1.75
12	5	6.90 ± 0.86[a]	3.86 ± 0.28[a]	44.02 ± 1.31
18	5	9.35 ± 0.32[a]	4.76 ± 0.17[a]	47.80 ± 1.72
24	5	8.97 ± 1.01[a]	5.56 ± 0.21[a,b]	38.28 ± 1.46[a,b]

[a] $P < 0.01$ compared with 4-month-old.
[b] $P < 0.01$ when 24-month-old rats were compared with younger age groups.

was reduced significantly in 24-month-old rats compared with younger age groups. Consequently, the serum free leptin concentration was significantly increased in 24-month-old rats compared with younger age groups.

Discussion

In this study we have validated an assay for measuring free leptin concentrations in rat plasma. This simple assay is reproducible and specific for leptin, and the results are comparable with chromatographic estimation of free/bound leptin fractions. Scatchard analysis of the binding data measured with this technique reveals a single high-affinity binding site in rat serum with a K_a of 0.3–0.6 × 10^9 M^{-1}. Although a single binding site is suggested by the Scatchard analysis, the limitations of the assay would not allow detection of lower affinity binding sites in plasma.

Unlike some of the previously published observations (1), there was no inverse relationship between the percentage of leptin bound fraction and plasma leptin levels in rats. The lack of such an inverse relationship was also observed in a recent study in human subjects (4). However, free leptin fractions were increased in the oldest animal groups. The age-related changes in plasma leptin levels confirm the observations in previously published studies (8, 15). Although the increasing plasma leptin levels with age correlates with increased body weight and adiposity, the rate of change in leptin levels with age is higher than the rate of change in body adiposity. This has been interpreted in the past as evidence of decreased leptin sensitivity in aging animals (8, 9). The present study extends those observations to indicate that this age-related insensitivity to leptin is not secondary to the reduced free fraction of serum leptin. On the contrary, the free fraction of leptin, presumably the moiety available for BBB transport, was increased in the oldest age group. It is likely that the reduced leptin sensitivity in aged rats occurs at a site distal to BBB uptake of leptin; however, caution should be exercised in interpreting such data since at the present time, there is no conclusive proof that the free fraction is the only fraction available for BBB transport. It is possible that the bound fraction may also be available for tissue uptake and may well have biological activity.

It is noteworthy that an inverse relationship between age and leptin binding of plasma proteins has been observed previously in human subjects (4). However, this change was apparent during early maturation through puberty whereas older men and women with a mean age of 68 years did not have significant changes in serum leptin binding compared with young adults with a mean age of 22 years. It is possible that if subjects older than 75 years were studied, leptin binding may have been reduced. In our study, only 24-

month-old rats, but not those as old as 18 months had reduced serum leptin binding. In this strain of rats, less than 10% of the cohort will survive until the age of 24 months (16) suggesting that it represents a far advanced age. Although possible, it is also unlikely that the changes in leptin binding are secondary to a disease state commonly found in aging rats since rats with near normal caloric consumption were selected, and renal failure and gross pathology were excluded.

Overall, the availability of simple and reproducible assays for plasma free leptin determinations will allow further examination of the role of various physiological modulators of plasma leptin concentrations in human and animal obesity. Using this assay, the present study demonstrates reduced leptin binding and increased plasma free leptin concentrations in aged rats. These observations make it unlikely that the previously reported relative insensitivity of aged rats to leptin is due to altered plasma leptin binding.

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