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Plasma Leptin Turnover Rates in Lean and Obese Zucker Rats*

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ABSTRACT

Conscious female adult lean and obese Zucker rats were injected through the jugular vein with radioactive iodine-labeled murine leptin; in the ensuing 8 min, four blood samples were sequentially extracted from the carotid artery. The samples were used in a modified RIA for leptin, in which paired tubes received the same amount of either labeled or unlabeled leptin, thus allowing us to estimate both leptin levels and specific radioactivity. The data were used to determine the decay curve parameters from which the half-life of leptin (5.46 ± 0.23 min for lean rats and 6.99 ± 0.75 min for obese rats) as well as the size of its circulating pool (32 pmol/kg for lean rats and 267 pmol/kg for obese rats) and the overall degradation rate (96 fkat/kg for lean rats and 645 fkat/kg for obese rats) were estimated. These values are consistent with the hormonal role of leptin and the need for speedy changes in its levels in response to metabolic challenge. (*Endocrinology* **139:** 4466–4469, 1998)

LEPTIN, the product of the *ob* gene (1), is a key factor in the regulation of body weight. Its precise role, however, has been subject to open controversy and discussion (2) despite earlier claims that its main role was that of a ponderostat signal, able in itself to modify the energy partition in some strains of rodents (3). A large amount of research has been carried out to establish the functions of leptin (4, 5) since its discovery in 1994 (1), but our knowledge of its *in vivo* dynamics is sketchy, in part because of methodological difficulty and in part because of the priority given to eventual pharmacological use and the need to characterize the mechanisms regulating its expression and signaling role.

The kinetic analysis of circulating hormones is a good source of information about the theoretical speed of response to change as well as on the demand for synthesis and/or degradation that the living organism devotes to the maintenance of a fully functional and responsive system. Short half-lives represent a higher energy expenditure but allow for faster responses and more immediate adaptation; longer turnover rates are usually correlated with longer term regulatory activity. Leptin turnover has been estimated in humans (half-life of about 25 min) (6) using arterio-venous differences and in mice (half-lives of 1.5–3 h) (7, 8) by measuring the decay of circulating label after the injection of labeled leptin.

Our method for the estimation of circulating peptide hormone turnover rates *in vivo*, which we have applied to insulin (9), has the advantages that only the label in the immunoreactive peptide hormone is taken into account, and all the measurements are performed in a single animal.

We chose the hyperleptinemic Zucker *fa/fa* rat, because of overexpression of the *ob* gene (10), to test whether this overexpression affects the turnover rate of leptin. The main objective, however, was to determine the range of the half-life of circulating leptin to elucidate the predominance of its short or long term metabolic actions.

Materials and Methods

Materials and animals

Pure recombinant murine leptin (Biotrend, Köln, Germany) and ^{125}I-labeled murine leptin (specific radioactivity, 69.2 GBq/μmol; Anawa, Zurich, Switzerland) were used. The labeled leptin used has a chromatographic purity of 98.6%.

Zucker lean (Fa/?) and obese (*fa/fa*) female adult rats, weighing 224 ± 4 and 398 ± 19 g, respectively, bred at the Animal Service of the University of Barcelona (Barcelona, Spain) from heterozygous stock obtained from Charles River Laboratories, Inc. (Wilmington, MA), were used. The animals were housed in individual polypropylene-bottomed cages under standard conditions (lights on from 0800–2000 h; 22–23 C; 70–75% relative humidity), and were fed standard chow pellets (type A04, Panlab, Barcelona, Spain). A series of four rats of each phenotype was cannulated, under ethyl ether anesthesia, in the left carotid artery (bringing the tip of the cannula just to the heart) with P-50 and in the right jugular vein with P-10 polyethylene tubes (Clay-Adams, Parsippany, NJ). The cannulas were filled with heparinized saline and sealed; they were threaded sc, exiting the rat through the back, where they were coiled and held in place with surgical tape. The rats were used for the experiments 5 h later. At the end of the experiment, the rats were killed by decapitation. This study was conducted in accordance with European Community principles, guidelines, and procedures for animal experimentation.

Leptin turnover measurement

The experiment was begun (6 h after the beginning of the light cycle) by injecting each rat (within 5–8 sec) through the jugular venous cannula with 80 kBq (1.16 pmol) labeled leptin in 0.2 ml isotonic saline solution. The radioactivity initially present (and that remaining after the injection)

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in the syringe was measured with a γ-counter. At timed intervals of 1, 2, 5, and 8 min, aliquots of 0.4 ml blood were extracted through the carotid cannula and stored at 4 C in heparinized plastic vials. The blood samples were immediately centrifuged at 4 C to separate the plasma samples, which were used directly for labeled leptin estimation and leptin turnover according to our method for insulin turnover (9) modified for use with leptin. The rat was maintained conscious and unaware of the manipulations (except for the uncoiling of the cannulas), as it remained in its cage out of sight of the researchers throughout the experiment.

Plasma leptin levels were estimated using a standard RIA procedure (Linco Research, Inc., St. Charles, MO) with some modifications: each plasma sample was distributed in two tubes; in the first, in addition to the plasma sample [100 μl (lean) or 40 μl (obese)], 100–160 μl buffer, 100 μl ^{125}I-labeled leptin solution containing 0.43 kBq (*i.e.* 6.25 fmol), and 100 μl diluted specific leptin antibody (Linco Research) were added. In the remaining tube, the plasma samples [100 μl (lean) or 40 μl (obese)] received 100–160 μl buffer, 100 μl unlabeled murine leptin (6.25 fmol), and 100 μl specific leptin antibody. Thus, the second tube finally contained the same amount of leptin as the first (the amount initially present and that added were the same as those in the first tube), but the amount of labeled leptin present in either was different; the second tube lacked the added labeled leptin used for the standard RIA procedure. Since the total amount of leptin was the same in both series of tubes, the labeled leptin initially present in plasma bound in the same proportion to the antibody preparation; thus, the second tube could be used as a blank for the first as in a standard RIA procedure. This allowed estimation of the apparent leptin concentration regardless of the amount and distribution in molecular species of radioactivity initially present in the plasma. This approach circumvented the problems posed by the presence of radioactive sources (leptin and other) in the samples. The RIA was completed with a series of standards of murine leptin, blanks, and several tubes for the estimation of nonspecific binding. All measurements were carried out in duplicate for each animal and time point.

Calculations

The leptin label present in a given sample of plasma was estimated assuming that labeled leptin was bound by the antibody in the same proportion as unlabeled leptin from the same source. From the RIA data, a plot of leptin bound to the antibody *vs.* the concentration of leptin in the tube was drawn using murine leptin as a standard. The data were fitted to an asymmetric sigmoid curve using the FiG-P program (Biosoft, Cambridge, UK); the calculated parameters of the curve were used to estimate the percentage of leptin bound to each of the blood samples obtained in the experiment. This percentage also reflected the proportion of leptin radioactivity bound to the antibody; thus, the total amount of leptin radioactivity, r_t, per ml blood at a given time, t, was established for each sample. The r_t values were plotted against time t and fitted to a standard decay graph using the FiG-P program: $r_t = r_0 \times e^{-K \times dt}$, from which, K, the decay constant, and r_0, the initial radioactivity per ml blood, were obtained. The half-life, $t_{1/2}$, of leptin was calculated from K, since $t_{1/2} = 1/K$. The ratio of total radioactivity injected, R_0/r_0, was used to establish the volume, V, of distribution of the injected label (*i.e.* the virtual or practical leptin space): $V = R_0/r_0$. The content of leptin was calculated from total leptin radioactivity and the specific activity of the labeled leptin injected. Injected leptin was a maximum of 16% (lean) to 1% (obese) of the total body leptin. As this proportion was small, its influence on circulating leptin was minimal. The rat leptin concentration in plasma did not vary during the experiment. The concentration *vs.* time graphs were used to obtain a mean leptin concentration value, l_M, and to check whether there were significant variations in leptin concentration. As we knew both the virtual distribution volume and the concentrations, we could derive the whole mass of circulating leptin L_0 at time zero: $L_0 = l_M \times V$. The rate of loss of leptin (rate of degradation, δ) from this circulating pool could be derived from the decay curve and the mass of leptin: $\delta = K \times L_0$. Indeed, as the virtual distribution volume, V, did not change, the leptin mass at a given time, L_t, can be estimated from the plasma concentrations, l_t, and the degradation rates for different times, δ_t, may be calculated. The values obtained in all cases were very similar, because the changes in leptin concentration during the 8-min analysis were insignificant.

The loss of radioactivity from the labeled leptin pool was studied by establishing the total leptin label values, R_t, at a given time from the radioactivity per ml plasma and the virtual volume of distribution: $R_t = r_t \times V$.

Statistical comparison between groups was established with standard ANOVA programs and Student's *t* test.

Results

Plasma leptin levels were maintained within a maximal range of variation of 6 ± 2% (lean) and 20 ± 7% (obese) of the initial values in the time elapsed between the injection and the last blood extraction. The significances of the effect of time on leptin concentrations were $P = 0.968$ (lean) and $P = 0.250$ (obese; by ANOVA).

Figure 1 presents the decay curves for plasma leptin radioactivity *vs.* time. Lean and obese rats showed a similar pattern over time. The differences between both series of animals were significant, as was the effect of time. These decay curves were used for the calculation of leptin space, turnover rates, and cleavage, shown in Table 1. Leptin levels were higher in the obese rats than in the lean rats. The distribution space of leptin was larger for the larger obese rats, but was comparable for both groups when the data were corrected for body size despite the smaller relative lean body mass of the obese rats.

Obese rats had a higher leptin mass than lean controls; this was maintained even after correcting for body size. The half-life of leptin was in the same range for lean and obese rats; the latter showed somewhat longer (~28%) half-lives. The calculated leptin degradation rates were almost 7-fold higher in obese rats than in the lean (per U body mass).

Discussion

The method used here has been previously successfully used for the analysis of insulin turnover in lean and obese rats (9). It is conceptually simple, but requires a careful development, especially at the critical point of evaluation of leptin radioactivity in the samples. The injection of labeled leptin did not significantly affect the mass of circulating leptin. The levels of leptin did not change as a consequence

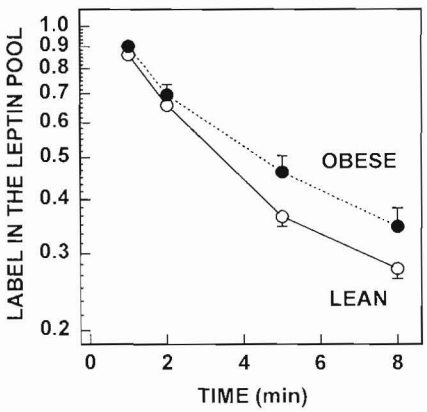

FIG. 1. Leptin radioactivity decay with time in the plasma of Zucker lean and obese rats injected with labeled leptin. The data are the mean ± SEM of four or five different animals and are expressed as the fraction of the injected radioactivity remaining in the whole circulating leptin pool at a given time. Statistical analysis of the differences between groups (by ANOVA): lean *vs.* obese, $P = 0.004$; effect of time on lean, $P = 0.000$; effect of time on obese, $P = 0.000$.

TABLE 1. Comparison of injected labeled leptin turnover in conscious lean and obese Zucker rats

Parameter	Units	Lean rats	P^a	Obese rats	Obese/lean ratio
Plasma leptin	nM	0.42 ± 0.04	<0.05	3.35 ± 0.23	7.98
Leptin virtual vol of distribution	ml	16.88 ± 1.55	<0.05	31.57 ± 2.21	1.87
	% of BW	7.49 ± 0.56	NS	7.92 ± 0.29	1.06
Leptin pool mass	pmol	7.17 ± 1.32	<0.05	107 ± 13	14.9
	pmol/kg BW	31.7 ± 5.4	<0.05	267 ± 23	8.41
Leptin $t_{1/2}$	min	5.46 ± 0.23	NS	6.99 ± 0.75	1.28
Rate of leptin removal/synthesis	fkat	21.5 ± 3.1	<0.05	259 ± 32	12.0
	fkat/kg BW	95.5 ± 12.4	<0.05	645 ± 51	6.76

The data are the mean ± SEM of four or five animals in each group. See *Materials and Methods* for the calculations and derived magnitudes.
[a] Significance of the differences between lean and obese groups was determined by Student's t test. NS, $P > 0.05$.

of the injection of labeled leptin and remained uniform in all of the blood extractions. An additional advantage of this method was its use of relatively undisturbed animals.

A critical point in the investigation of leptin cleavage is the assignment of radioactivity measurements to intact (*i.e.* functional) leptin, without interference by free iodine or other labeled peptide fragments eventually freed by the cleavage of leptin. Leptin turnover estimations calculated from the decay of label in the blood (7, 8) tend to give longer half-lives. The method used here is not affected by this interference, as only the label bound to leptin is measured; free iodine and labeled peptides are removed during the RIA procedure. Only labeled (complete) leptin is bound to the antibody, and thus only this radioactive molecular species is taken into account.

The main difficulty that may arise from a study based on calculated constants taken from calculated values and used to derive the final results is a cumulative effect of residuals in calculations, which may lead to errors. This study has been designed to minimize this effect. The adequacy of decay curve fitting is apparent in Fig. 1, which shows the loss of radioactive leptin in plasma. The low dispersion of data suggests an acceptable degree of precision in the derivation of the virtual volume of diffusion and decay rates shown in Table 1.

The virtual volume of distribution of leptin derived from data of arterial blood may not be real, because we do not know whether the leptin levels are representative; nevertheless, the data are useful and admit comparison between different animals and situations. The leptin pool size, L_v, is more reliable, because it is derived directly from decay curves and leptin levels. Leptin pool size was much higher in obese than in lean rats both in absolute terms and in relation to body weight; in the latter case, the differences diminished, probably because of the dilution effect of the large fat mass in obese rats. The relative uniformity of the virtual volume of distribution *vs.* body weight in lean and obese rats suggested that the distributions of leptin are comparable in lean and fat tissues. As a consequence, the comparisons between both groups were directly referred to body size to establish comparisons despite their different body weights.

The turnover rates found here are much shorter than those found in normal mice (3 h) (7) or in *db/db* and *ob/ob* mice (1.5 h) (8) calculated from label decay curves, and they are also shorter than those estimated in humans from arterio-venous differences (25 min) (6). These differences may be a consequence of the methodology applied; in our case, the circulating levels of leptin were not modified, and only leptin label was taken into account. The timing of sample extraction was also in the range of the measured turnover, which gave higher precision to our estimations.

The Zucker *fa/fa* rats are hyperleptinemic, but this leptin is inoperative because they lack a functional hypothalamic leptin receptor (10, 11). This deficiency has been postulated as the cause of their obesity, because it deprives the rat of a key element in the control of energy partition (12). The white adipose tissue of *fa/fa* rats overexpresses the *ob* gene, thus inducing massive synthesis of leptin (10). The synthesis of leptin in Zucker obese rats is further enhanced by their large fat mass, as leptin production has been found to be related to adipose tissue mass in humans and rodents (13, 14). However, the half-life of leptin is in a similar range in lean and obese rats; the latter show a mere 28% higher mean rate. As the leptin pool size is much larger in the obese rats, the maintenance of similar turnover rates implies a more active degrading process in these animals than in the controls. We have no clear idea where this leptin is degraded. Some reports suggest that the kidney plays a significant role in the clearance of excess leptin from the blood (15, 16). Lean and obese Zucker rats do not show different kidney blood flows (our unpublished results), but the much higher concentration of leptin in the blood of the obese rats may be a key factor facilitating its removal; the mean ratio of plasma concentrations (obese/lean) is 4.12, and that of the pool removal rates is 6.17 (3.49 when corrected for body size).

As the levels of leptin in undisturbed animals are uniform, it may be assumed that the removal rate is essentially identical to the synthesis rate of leptin. This means that a 225-g lean rat synthesizes (and degrades) about 30 μg leptin/day (*i.e.* 260-fold the whole body leptin mass), much less (a mere 8%) than that of a 400-g obese rat (358 μg/day, *i.e.* 210-fold the leptin mass).

The differences between lean and obese rats found for leptin were fairly similar to those for insulin in this same animal model of obesity. Zucker *fa/fa* rats are hyperinsulinemic (17) and show insulin turnover rates similar to those of controls (9). The range of $t_{1/2}$ was also similar between leptin and insulin (only a few minutes). This value is consistent with the hormonal role of leptin and the eventual need for speedy changes in its levels as a response to metabolic challenge, such as that found under starvation (18), diet (19, 20), or hormonal manipulation (21), and may help explain the ultradian rhythms of this hormone (22).

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