*Rapid communication***Free and protein bound leptin are distinct and independently controlled factors in energy regulation**

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Abstract

Aims/hypothesis. Leptin exerts important regulating effects on energy homeostasis and could have a central role in our understanding of obesity, diabetes mellitus and the metabolic syndrome. Leptin circulates in a free and protein bound form. The aim of the present study was to test whether both fractions of the leptin system can be selectively regulated and thus serve independent physiological roles.

Methods. Using specific radioimmunoassays we measured both leptin components in relation to BMI in healthy subjects before and after weight reduction and in hyperthyroid patients during correction of thyrotoxicosis. In the latter group body composition and resting energy expenditure was monitored. In addition, we measured serum and cerebrospinal fluid concentrations of free and bound leptin in patients with neurological disorders.

Results. Under all conditions free leptin concentrations reflected body fat mass. Bound leptin concentrations decreased during weight reduction but also

after treatment of hyperthyroidism despite an increase in fat mass. Direct measurement of resting energy expenditure and bound leptin in hyperthyroid patients and under thyrostatic treatment showed a significant positive correlation of both variables. In contrast to free leptin whose transport into the cerebrospinal fluid appears to be saturated at low physiological concentrations of serum free leptin, bound leptin concentrations in the cerebrospinal fluid increased in parallel to serum concentrations over the whole physiologically relevant range.

Conclusion/interpretation. Our data indicate a distinct role of free and bound leptin in the feedback regulating energy intake and expenditure and could have important implications for our understanding of the physiology and pathophysiology of leptin-dependent signalling. [Diabetologia (2000) 43: 438–442]

Keywords Free leptin, bound leptin, resting energy expenditure, hyperthyroidism, weight reduction, leptin in CSF.

High circulating leptin concentrations observed in many obese subjects suggest dysfunction of the central feedback action on energy intake and expendi-

ture but the underlying cause of this apparent leptin resistance is speculative [1–4]. Leptin circulates in a free and a protein-bound form and at least part of the circulating leptin is bound to a soluble isoform of the leptin receptor [5–12]. According to gel chromatographic studies most types of human obesity are characterised by a predominant increase in serum free over bound leptin concentrations indicating independent regulation of free and bound leptin and suggesting a relative deficiency of the latter in obesity [10].

Using recently developed specific radioimmunoassays by which free and bound leptin can be measured

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Abbreviations: CSF, Cerebrospinal fluid; BIA, bioelectrical impedance analyser; DEXA, dual-energy x-ray absorptiometry; REE, resting energy expenditure; TFM, total fat mass.

selectively in serum [11, 12] we evaluated such differential regulation of free and bound leptin both in obese subjects undergoing weight reduction and after the correction of thyrotoxicosis. During weight reduction body fat mass is lowered and both physiological targets of leptin action, energy intake and energy expenditure decrease [4, 13–17]. When thyrotoxicosis is corrected energy intake and energy expenditure decrease as well but this time body fat mass can increase [18]. Previously published studies on total leptin concentrations in thyrotoxic patients during therapy reflect these dual effects on serum leptin concentrations with an increase due to increased body fat and a potential decrease after decreased energy expenditure and yielded controversial results with a decrease, increase or no change in serum leptin concentrations after treatment [19–23]. None of these studies differentiated, however, between free and bound forms of leptin.

Subjects and methods

Subjects. Blood samples for the assessment of basal free and bound leptin concentrations were obtained from 72 normal weight and obese, otherwise healthy women (age 37.9 ± 12 years, BMI between 18 and 55 kg/m^2). Of these subjects 40 (age 38.3 ± 12.7 years, BMI $37.9 \pm 5.8 \text{ kg/m}^2$) underwent a weight reduction programme (Optifast, Novartis, Celle, Germany) with 800 kcal/day over 7 weeks. All patients were tested at baseline and after 4 and 7 weeks as to their BMI, free and bound leptin concentrations.

We studied 11 thyrotoxic subjects with Graves disease (age 21 to 71 years, all women) every 3 months for 1 year after therapy with antithyroid drugs (methimazole, thycapzol) and compared them with a group of 9 subjects (age 25 to 53 years, 2 men, 7 women) who received similar doses of antithyroid drugs (subgroups of ref. 23). At each time point thyrotropin (TSH), thyroid hormones, free leptin, bound leptin, body weight and body composition were measured. In adherence to the hypothesis that body fat mass is a major determinant of serum leptin concentrations, we analysed only patients with a minimal gain in total fat mass (TFM) of 5% during treatment and compared the time of maximum increase to baseline.

We also followed 6 children (age 9.3 to 13.4 years, all girls) with recently diagnosed Graves disease for 3 to 9 months during correction of thyrotoxicosis. Resting energy expenditure (REE) and body composition were measured at least every month (five to nine measurements for each subject).

In 66 subjects (age 22–77 years; 32 men, 34 women) studied for clinical neurological reasons (polyneuropathy, seizures, brain tumor, multiple sclerosis, headache, psychoneurosis, brain infarct, transitory ischaemic attacks, bacterial and viral meningitis, Guillain-Barré syndrome, amyotrophic lateral sclerosis, dementia) CSF samples were obtained by lumbar puncture. The CSF:serum ratio of albumin varied over a wide range ($\text{CSF} \times 1000/\text{serum}$, range 2.6–112).

The various parts of the study were approved by the respective ethics committees and subjects gave informed consent. All parts of the study were done according to the principles of Helsinki.

Fig. 1. Relation between BMI and serum bound leptin ($\square$, $r^2 = 0.36$) and serum free leptin ($\bullet$, $r^2 = 0.53$) concentrations in 72 otherwise healthy, normal-weight and obese women

Analytical methods

Hormone measurements. Free leptin and bound leptin concentrations were measured by specific radioimmunoassay systems as described previously [11, 12]. The assays were adapted to the different concentrations in serum and CSF.

Serum concentrations of TSH and thyroid hormones were measured by commercially available IRMA systems and albumin in serum and CSF by kinetic nephelometry.

Measurement of resting energy expenditure and body composition. Resting energy expenditure (REE) was assessed by indirect calorimetry (MBM-200, Deltatrac II, Datex Instrumentarium, Helsinki, Finland). Body composition was measured either by bioelectrical impedance analyser (BIA 101/S, RJL systems, Detroit, Mich. USA; children study) or by dual-energy x-ray absorptiometry (DEXA, Norland XR-26 MarkII/HS, Norland Scientific Instruments, LS Barn, The Netherlands; adult study). All measurements were done between 0800 hours and 0900 hours after an overnight fast and before medication.

Statistical analysis. The significance of regression analyses was evaluated by *t* test (slope of the regression line) and *F* Test (r^2) after $\log_{10}$ transformation of free and bound leptin values, the *p* value given is that of the less significant tests.

Paired data were analysed by two-tailed paired Student's *t* test. Calculations were done with NCSS software (NCSS, Kaysville, Utah, USA). A *p* value less than 0.05 was considered significant.

Results

Correlation of bound and free leptin to BMI and changes during weight loss. In lean and obese subjects BMI was positively related to serum free leptin ($r^2 = 0.53$; $p < 0.0001$) as was serum protein-bound leptin ($r^2 = 0.32$; $p < 0.0001$; Fig. 1). Both variables responded greatly to a standardised weight reduction programme. After a diet of 800 kcal/day over 7 weeks, serum free leptin concentrations and BMI decreased ($p < 0.0001$ at week 4 and at week 7) as did serum bound leptin concentrations ($p < 0.02$ at week 4 and $p < 0.004$ at week 7; data not shown).

Fig. 2. A Changes in mean body weight, total fat mass (TFM), free leptin (FL) bound leptin (BL) during correction of thyrotoxicosis in 11 patients at the time of maximum weight gain compared with baseline. T4 changed from a mean of 248 nmol/l to 96 nmol/l, TSH from undetectable concentrations to a mean of 1.6 mU/l. **B** Relation between resting energy expenditure (REE) and protein-bound leptin concentrations in six patients during treatment for hyperthyroidism (multiple readings during treatment of individual patients; the regression line was calculated using all readings; the symbols represent the individual patients). $r^2 = 0.30$

$p < 0.003$; Fig. 3). Serum free leptin concentrations were not correlated to concentrations of bound leptin in the CSF ($r^2 = 0.037$, NS) nor were protein-bound leptin to CSF free leptin concentrations ($r^2 = 0.051$, NS). In contrast, protein-bound leptin concentrations had a strong positive correlation with bound leptin concentrations in CSF with a steep slope over the whole range of protein-bound leptin concentrations expected for lean and obese healthy subjects ($r^2 = 0.50$; $p < 0.0001$; Fig. 3).

Changes during weight gain and correlation to REE. Correction of thyrotoxicosis induced a statistically significant increase in serum free leptin concentrations which paralleled the gain in body weight and total fat mass. Under these conditions serum bound leptin concentrations significantly decreased (Fig. 2) whereas none of these variables changed statistically significantly in the control group (data not shown).

To further substantiate a potential association of bound leptin with energy expenditure we repeatedly measured bound leptin and REE in another six thyrotoxic patients during thyrostatic therapy. Resting energy expenditure and bound leptin decreased ($p < 0.05$ and < 0.04 , data not shown) with a positive correlation between them ($r^2 = 0.30$, $p < 0.0001$ when all subjects and readings are included; Fig. 2) indicating that bound leptin is correlated with energy expenditure independent of body fat stores.

Correlation of serum bound leptin and free leptin concentrations to CSF concentrations. To further evaluate a distinct role for bound and free leptin we measured both components in the serum and CSF of 66 subjects. None of the components of the leptin system was related to alterations in the blood-brain-barrier judging by the CSF:serum ratio of albumin (data not shown). Free leptin concentrations in the lower serum range correlated well with the corresponding CSF free leptin concentrations but high serum concentrations were only reflected in a small further increment in the CSF compartment ($r^2 = 0.13$;

Discussion

Our data in lean and obese healthy subjects show a positive correlation by both components of the leptin system with the degree of obesity but with a steeper slope for the free hormone. Serum concentrations of both components were statistically significantly lowered by a decrease in body fat during a weight reduction programme. When thyrotoxic patients increased their body fat mass by successful thyrostatic therapy only serum free leptin concentrations reflected this increment, supporting the hypothesis that the free leptin fraction selectively represents body fat mass. Bound leptin in contrast decreases which indicates a regulatory role distinct from free leptin and not related to body fat mass. Because bound leptin decreases both during fasting and after recovery from thyrotoxicosis (two conditions associated with a reduced resting energy expenditure), we directly tested the hypothesis that bound leptin is correlated with energy expenditure. Our data in a limited number of subjects suggest a close correlation between these variables.

One of the most important determinants of the REE is the activity of the sympathetic nervous system. Data in Pima Indians suggest low resting sympathetic nervous system activity and its apparent dissociation from metabolic rate is a causative factor in the development of obesity [24]. Leptin directly stimulates central sympathetic outflow in rodents and primates [25–27] and leptin deficiency in humans is associated with a lowered sympathetic tone [28].

Fig. 3. Relation between serum and CSF concentrations of bound leptin ($\square$, $r^2 = 0.05$) and free leptin ($\bullet$, $r^2 = 0.13$) in 66 patients

In contrast to leptin-deficient mice, where application of recombinant leptin is effective in both decreasing appetite and stimulating REE by the action on hypothalamic centres [1], small doses of recombinant leptin in humans seems, however, to selectively affect appetite [29]. About 10 % of the serum concentrations observed in normal subjects is sufficient to lower body weight in leptin-deficient children by reducing energy intake without any measurable effect on energy expenditure [29]. Normal and obese subjects without leptin deficiency need much higher, supraphysiological doses of recombinant leptin to elicit any response [30].

Our findings on the serum:CSF ratio of free and bound leptin could help to explain this discrepancy. Free leptin seems to be transported into the CSF by a high affinity transport system of low capacity which is already saturated at low circulating serum concentrations. This finding supports previous data with non-selective leptin assays and experimental data on saturation and desensitization of the putative leptin transport receptor [7, 31]. Transport of bound leptin not selectively tested previously seems not to be saturated as serum and CSF concentrations were closely correlated over the whole range of serum concentrations observed in lean and obese subjects. These observations suggest an independent physiological role of bound leptin and implicate regulation by a receptor bound hormone. This is a new concept but has recently been discussed for IL-6, a cytokine closely related to leptin. Cellular response to IL-6 is greatly enhanced up to 1000-fold when a complex is formed between this cytokine and its soluble receptor [32–34]. A similar mechanism could hold true for leptin complexed to the soluble form of its receptor and could explain why peripheral application of recombinant leptin elicits only small effects in obese subjects with high circulating (free) leptin serum concentrations.

Our data suggest that free leptin selectively reflects body fat mass whereas bound leptin is closely

associated with energy expenditure. The serum:CSF ratio of free leptin indicates a high affinity transport system of low capacity in contrast to bound leptin whose transport seems not to be rapidly saturated. These findings of a distinct role of bound leptin add a new component to our understanding of leptin-dependent regulation and could have important diagnostic and potential therapeutic implications for pathophysiological conditions where energy expenditure is reduced.

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