Evidence of Free and Bound Leptin in Human Circulation
Studies in Lean and Obese Subjects and During Short-Term Fasting

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Abstract

Little is known about leptin's interaction with other circulating proteins which could be important for its biological effects. Sephadex G-100 gel filtration elution profiles of ^{125}I-leptin–serum complex demonstrated ^{125}I-leptin eluting in significant proportion associated with macromolecules. The ^{125}I-leptin binding to circulating macromolecules was specific, reversible, and displaceable with unlabeled leptin (ED_{50}: 0.73 ± 0.09 nM, mean$\pm$SEM, $n = 3$). Several putative leptin binding proteins were detected by leptin-affinity chromatography of which either 80- or 100-kD proteins could be the soluble leptin receptor as $\sim 10\%$ of the bound ^{125}I-leptin was immunoprecipitable with leptin receptor antibodies.

Significantly higher ($P < 0.001$) proportions of total leptin circulate in the bound form in lean ($46.5\pm6.6\%$) compared with obese ($21.4\pm3.4\%$) subjects. In lean subjects with 21% or less body fat, 60–98% of the total leptin was in the bound form. Short-term fasting significantly decreased basal leptin levels in three lean ($P < 0.0005$) and three obese ($P < 0.005$) subjects while refeeding restored it to basal levels. The effects of fasting on free leptin levels were more pronounced in lean subjects (basal vs. 24-h fasting: 19.6 ± 1.9 vs. 1.3 ± 0.4 ng/ml) compared with those in obese subjects (28.3 ± 9.8 vs. 14.7 ± 5.3). No significant ($P > 0.05$) decrease was observed in bound leptin in either group. These studies suggest that in obese individuals the majority of leptin circulates in free form, presumably bioactive protein, and thus obese subjects are resistant to free leptin. In lean subjects with relatively low adipose tissue, the majority of circulating leptin is in the bound form and thus may not be available to brain receptors for its inhibitory effects on food intake both under normal and food deprivation states. (*J. Clin. Invest.* 1996. 98:1277–1282.) Key words: free leptin • bound leptin • leptin binding protein(s) • soluble leptin receptor • fasting

Introduction

Since the isolation of the obesity (ob) gene in ob/ob mouse (1), significant progress has been made in understanding leptin's role in controlling food intake and energy metabolism (2–5). Leptin is primarily secreted by adipose tissue (6, 7) and interacts with its brain receptor (8–10), a new member of the class I cytokine receptor family (11). In humans, circulating leptin levels are increased in obesity and are regulated by fasting, feeding, and body weight changes (12–14). Recently, we demonstrated that leptin secretion shows circadian and ultradian oscillations (15, 16). Besides steroid (17) and thyroid (18) hormones, insulin-like growth factors (IGF) circulate bound to specific IGF binding proteins (19). Cytokine and growth hormone (GH) receptors are also present in human serum and are known to modulate the availability of free hormones for their biological actions (20, 21).

To understand the physiology of circulating leptin, it is important to determine its interaction with other circulatory components. In this study, we demonstrate the presence of leptin binding proteins, one of which appears to be the recently described soluble leptin receptor (Ob-Re) (9). In lean subjects with minimal adipose tissue, the majority of leptin circulates in the bound form, whereas in obese subjects the majority of leptin circulates as free leptin.

Methods

Study design. 46 subjects were studied. Of these, 16 subjects (4 male and 12 female) were lean (age 30.0 ± 2.4 yr, BMI 22.30 ± 0.54 kg/m^2: range 19.0–27.3 and % body fat [BF][1] 24.7 ± 1.6:14.0–34.1) and 30 subjects (9 male and 21 female) were obese (age 37.0 ± 2.4, BMI 39.1 ± 1.53:28.4–60.9 and % BF 42.0 ± 2.0:16.0–59.0) according to the criteria established by the National Institute of Health Consensus Development Panel (22). % BF was determined by measuring bioelectric impedance (RJL Systems, Inc., Mt. Clemens, MI) and skin-fold thickness (23). In six subjects (three lean women, two obese women, and one obese man), % BF was extrapolated from binomial equations for males and females derived from a previous study of 275 subjects using BMI as dependent variable (12). None of the subjects had any disease except obesity and were not in any active weight loss program or taking any drug for the treatment of obesity. In addition, we studied the

Address correspondence to Madhur K. Sinha, Ph.D., Thomas Jefferson University, Jefferson Medical College, 1025 Walnut Street, Room 813 College Building, Philadelphia, PA 19107. Phone: 215-955-1265; FAX: 215-923-7932.

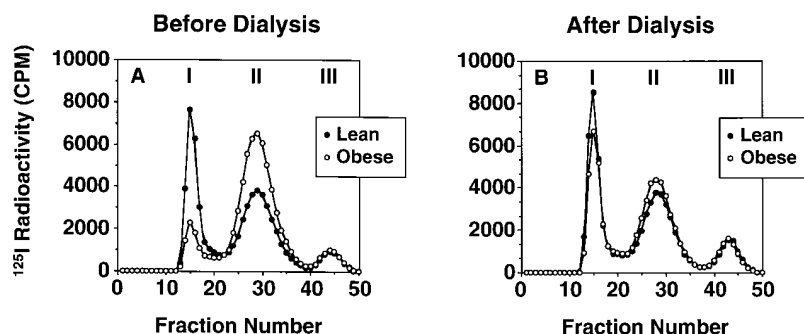1. *Abbreviation used in this paper:* BF, body fat.

Figure 1. Sephadex G-100 elution profiles after incubation of ^{125}I-leptin with serum for 48 h at 4°C from lean and obese subjects. *A* (representative of five different experiments) shows serum samples before dialysis and *B* (representative of two separate experiments) after 72 h of dialysis at 4°C.

effect of short-term fasting and refeeding (14) on leptin levels in six females (three lean, age 36.3±3.2 yr, BMI 22.7±1.3 kg/m²: range 20.2–24.7 and % BF 28.3±2.6:24–33; three obese, age 31.0±3.2 yr, BMI 31.2±2.3 kg/m²:28.4–35.7 and % BF 39.7±2.3:36.0–44.0). During the baseline period, each subject received an isocaloric diet (30 kcal/kg/d; 50% carbohydrates, 35% fat, and 15% protein) distributed among four meals a day. A 24-h fast was initiated in overnight fasted subjects at 9 a.m. during which period they received water only. During the refeeding phase of 24 h, each subject received isocaloric diet similar to that provided during the baseline period. All of the food provided was consumed under the supervision of the nursing staff. The study protocol was approved by the Institutional Review Board and each study subject signed the informed written consent form.

Reagents. Recombinant human leptin and high specific activity ^{125}I-leptin (2,200 Ci/mmol), which was prepared by modified Chloramine-T method (24), were provided by Eli Lilly and Co. Polyclonal antibodies against extracellular domain of leptin receptor were obtained 21 d after immunization with OB-R peptide (236–254)–KLH conjugate (0.5 mg) in complete Freund's adjuvant (BABCO, Inc., Richmond, CA). The antibody titer was 1:6,000 as determined by ELISA using OB-R peptide (236–254).

Gel filtration. A single 2.0-ml aliquot of the serum samples from lean and obese subjects was incubated with ^{125}I-leptin ($\sim$ 130,000 cpm, 45.6 fmol) for 24–48 h at 4°C and then eluted at 4°C from Sephadex G-100 gel filtration column with 25 mM phosphate-buffered saline, pH 7.4, containing 0.01% sodium azide. Bound fraction represented radioactivity eluting in the void volume, whereas free fraction represented radioactivity eluting in ^{125}I-leptin region. After discarding 1–3 nadir radioactive fractions, the sum of radioactivity eluting in the two peaks was calculated and then percent bound and percent free ^{125}I-leptin was determined by dividing with total radioactivity eluted. No appreciable dissociation (< 10%) of bound ^{125}I-leptin was observed when peak I was rechromatographed at 4°C.

Determination of total, bound, and free leptin in human serum. Serum samples (2 ml) were fractionated by Sephadex G-100 gel filtration. Fractions eluting between void and bed volumes were assayed for immunoreactive leptin. Percent bound and percent free leptin eluting in two peaks, devoid of nadir values, were calculated by divid-

ing with total leptin eluted. The recovery of leptin from gel filtration was not influenced ($r = 0.19$) by low or high leptin concentrations of different serum samples (lean: 92.65±3.13% and obese: 89.61±5.17%). Absolute levels of bound and free leptin were then calculated by multiplying percent bound and percent free leptin with total leptin concentrations and dividing by 100. Leptin levels were assayed with Leptin RIA kit (Linco Inc., St. Charles, MO). The detection limit was 0.5 ng/ml (percent buffer control: 91.6±0.4; $n = 22$). The interassay of coefficients of variation (CV) in 22 RIAs were 9.7 at 3.1±0.6 ng/ml and 6.0 at 15.1±0.2 ng/ml. The intraassay CVs were 4.5 at 2.9±0.1 ($n = 8$) and 3.9 at 15.4±0.2 ($n = 8$) ng/ml leptin.

To test if peak I contributes bound leptin that is not measured by RIA, we incubated the peak I at low pH or at 22°C to allow bound leptin to dissociate. If the RIA measures all bound leptin, the concentration of leptin in peak I should be the same, i.e., before and after dissociation. Indeed the initial levels of bound leptin in peak I did not increase after incubation at low pH for 2 h at 4°C (pH 7.5, 7.1, 6.6, 5.5, and 3.6: peak I leptin levels 4.2, 3.9, 4.1, and 4.5 ng/ml, respectively), and after incubation at 22°C for different times (0, 0.5, 1, 2, 4, and 5 h: 5.8, 5.9, 5.4, 5.9, and 5.7 ng/ml, respectively). To demonstrate that bound leptin dissociates, peak I (3.0 ng/ml) of an obese serum after dissociation at 22°C was rechromatographed. Only 60% of the total immunoreactivity eluted as peak I, whereas 40% eluted as peak II (free leptin). Furthermore, the sum of leptin in peak I and peak II was $\sim$ 3 ng/ml, considering that the recovery was 79% of the initially charged leptin.

Affinity chromatography and polyacrylamide gel electrophoresis. Aminolink leptin-agarose column (2.9 mg leptin/2 ml agarose gel) was prepared according to the manufacturer's instructions using AminoLink plus kit (20394; Pierce, Rockford, IL). Serum (1 ml) was diluted 1:4 with 50 mM phosphate 0.5 M saline buffer, pH 7.5, containing 0.2% Tween 20 and passed through affinity column three times at 22°C. After extensive washings, proteins bound to the column were eluted with 0.1 M glycine buffer, pH 2.8. Following Centricon 3 (Amicon, Inc., Beverly, MA) concentration, affinity-purified material was subjected to sodium dodecyl sulfate-polyacrylamide gel (7.5%) electrophoresis under reduced conditions and the protein bands were detected by silver staining (rapid silver staining kit RSK-1;
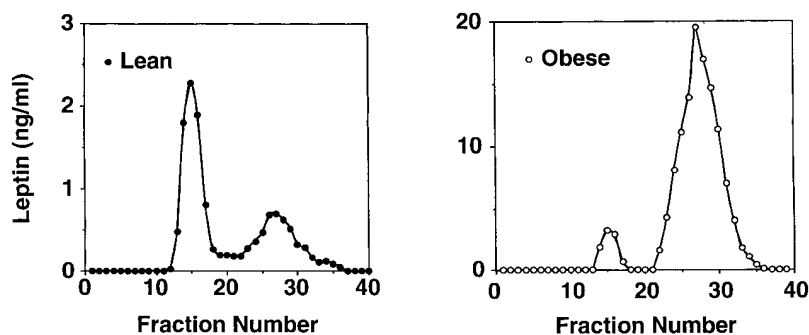

Figure 2. Sephadex G-100 elution profile of immunoreactive leptin in serum samples from a lean subject (7.3 ng/ml leptin level) (*left*) and an obese subject (70.3 ng/ml leptin level) (*right*).
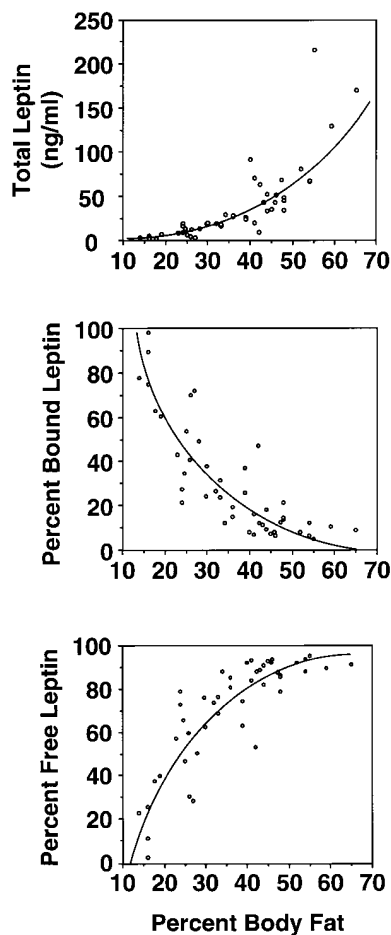
Figure 3. Total leptin (*top*), percent bound (*middle*), and percent free (*bottom*) leptin levels are plotted against % BF from 16 lean and 30 obese subjects.

Results

Fig. 1 *A* shows representative elution profiles of Sephadex G-100 gel filtration after 24 h of incubation at 4°C of ^{125}I-leptin (45.6 fmol) with 2-ml serum samples from one lean subject (7.3 ng/ml leptin) and one obese subject (70.3 ng/ml leptin). Peak I elutes in the void volume, peak II corresponds to ^{125}I-leptin position, and peak III in the bed volume region representing free ^{125}I-radioactivity. Radioactivity in peak I which represents ^{125}I-leptin complex with macromolecule(s) is about two times higher in lean ($n = 6$; $37.1\pm2.0\%$ of the total radioactivity) compared with obese ($n = 5$, $17.8\pm2.9\%$) subjects ($P < 0.0005$). Peak II is higher ($P < 0.0005$) in obese ($82.16\pm2.91\%$) compared with that in lean ($62.91\pm1.99\%$) subjects. These gel filtration results suggest that leptin circulates bound to macromolecule(s) and that the lower peak I in obesity is due to occupied binding sites by the high endogenous leptin levels. To test the latter hypothesis, sera from two lean and two obese subjects were dialyzed for 72 h (25,000 MWCO cellulose ester tubing; Spectrum Medical Industries, Inc., Houston, TX) at 4°C to remove endogenous leptin. Using these sera depleted of endogenous leptin (lean: 0.3 ng/ml and obese: 3.67 ng/ml) the same experiments as in Fig. 1 *A* were performed. The radioactivity associated with peak I and peak II was similar between lean and obese subjects (Fig. 1 *B*). Since in obesity the majority of the binding sites appears to be occupied by endogenous leptin, it is important to determine free leptin to rule out the possibility that hyperleptinemia in obesity (12, 13) is not due to an increase in bound leptin only.

To this end, we fractionated sera from 46 subjects with a wide range of body weight. Fig. 2 shows a typical profile from a lean subject (21% BF, 7.3 ng/ml leptin level), in whom the majority of leptin was associated with peak I (60.10%), i.e., in the bound form, whereas in the obese subject (40% BF, 70.3 ng/ml leptin) the majority of leptin (93.05%) eluted with peak II, i.e., in the free form.

Total leptin levels were significantly higher ($P < 0.0005$) in the group of obese subjects (51.4 ± 8.5 ng/ml; $n = 30$) compared with the group of lean subjects (12.2 ± 2.0; $n = 16$). Bound (6.0 ± 0.5 vs. 4.0 ± 0.5 ng/ml; $P < 0.01$) and free (45.4 ± 8.1 vs. 8.1 ± 1.8 ng/ml; $P < 0.0005$) leptin levels were also significantly increased in the obese group.

The significance of bound and free leptin levels in lean and obese subjects became even more striking when percent bound and percent free leptin were related to the degree of obesity (Fig. 3). In lean subjects, particularly those with 21% BF or less, the majority of leptin (60–98%) circulates in the bound form. Conversely, in obese subjects with leptin levels of 35 ng/ml or more, the majority (85.7–95.4%) of leptin circulates as free leptin.

Sigma Immunochemicals, St. Louis, MO) using silver stain standard markers M 5505 (Sigma Immunochemicals).

Immunoprecipitation of bound ^{125}I-leptin. After 24 h of incubation at 4°C, ^{125}I-leptin–serum complex (440,000 cpm/ml) were fractionated by gel filtration. Thereafter, 250-μl aliquots of peak I representing bound ^{125}I-leptin ($\sim$ 20,000 cpm) in quadruplicate were incubated with either preimmune rabbit serum or leptin receptor antibodies at 1/10 and 1/100 dilutions for 24 h at 4°C. The ^{125}I-leptin receptor complex was then immunoprecipitated with secondary antibody (sheep anti–rabbit IgG serum; Antibodies Inc., Davis, CA) as described previously for leptin RIA (12, 15).

Statistical analysis. The data are expressed as mean±SEM. For statistical analysis, we used Student's *t* test (paired and unpaired), ANOVA, and regression analysis using Microsoft Excel, Statview 512, or Cricket 2 programs for a Macintosh computer.

Table I. Bound and Free Leptin During Short-Term Fasting and Refeeding in Lean and Obese Humans

Leptin (ng/ml)	Subjects	Basal	Fasting 9 h	Fasting 24 h	Refeeding 9 h	Refeeding 24 h
Free leptin	Lean $n = 3$	10.6 ± 1.9	4.3 ± 1.1*	1.3 ± 0.4*	2.7 ± 1.1*	10.2 ± 2.6
	Obese $n = 3$	28.3 ± 9.8	24.7 ± 10.3*	14.7 ± 5.3*‡	22.0 ± 8.3‡	27.1 ± 9.2
Bound leptin	Lean $n = 3$	5.2 ± 1.0	3.9 ± 0.6	3.3 ± 0.2	4.1 ± 0.9	5.6 ± 1.1
	Obese $n = 3$	6.3 ± 1.2	5.8 ± 0.5	5.2 ± 0.4‡	5.4 ± 0.4‡	6.2 ± 0.4

*$P < 0.05$ (against basal, paired *t* test); ‡$P < 0.05$ (between lean and obese groups, unpaired *t* test).

Next, we investigated the effect on free and bound leptin in physiological conditions which are known to alter total leptin levels such as fasting and refeeding (14). Basal total leptin levels in both lean (15.8 ± 1.5 ng/ml; $n = 3$) and obese subjects (34.6 ± 8.9; $n = 3$) decreased significantly after a 24-h fast (lean: 4.6 ± 0.3 and obese: 19.9 ± 4.9) and returned to baseline levels after a 24-h refeeding (lean: 15.8 ± 2.8 and obese 33.3 ± 9.3).

No significant ($P > 0.05$) changes were observed in bound leptin after 24 h of fasting in either lean or obese subjects (Table I). In contrast, significant changes in free leptin (percent of basal) were observed in lean ($F = 34.4$, $P < 0.0001$) and obese ($F = 7.8$, $P < 0.005$) subjects as a result of fasting and refeed-
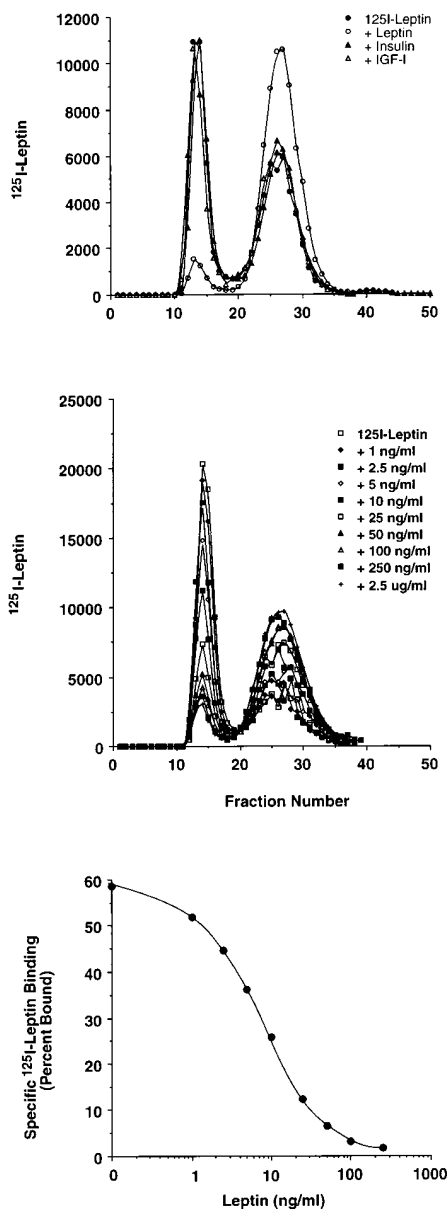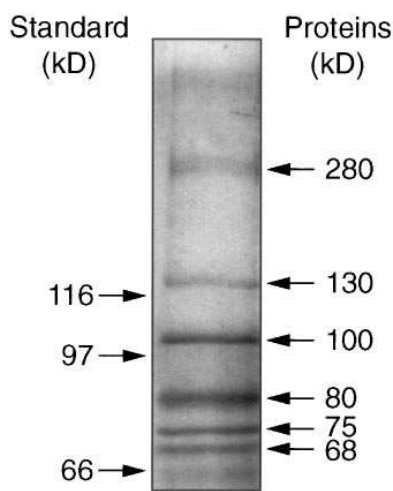

Figure 5. Leptin affinity purification of human serum after leptin-agarose affinity chromatography, SDS-PAGE, and silver staining.

ing when analyzed by ANOVA. The changes in free leptin are rapid and significant. Particularly in lean subjects, fasting had a marked influence on free circulating leptin levels which were reduced to $\sim 1/8$ of the basal free leptin levels in 24 h.

Therefore, it is clear that bound and free leptin behave as different compartments in physiological alterations such as fasting and refeeding or departures from normal nutrition such as obesity. Therefore, we next studied the specificity and properties of the binding sites.

Total ^{125}I-leptin binding in the absence of any hormone was 46.8%, whereas the nonspecific binding in the presence of excess unlabeled leptin was 6.8% of the total radioactivity. The ^{125}I-leptin binding separated as peak I was specific leptin binding as insulin and IGF-I did not appreciably decrease ^{125}I-leptin binding of peak I (Fig. 4, *top*). Unlabeled leptin (1–250 ng/ml) displaced ^{125}I-leptin binding when added to dialyzed serum from a lean subject (1.2 ng/ml leptin level) in a dose-dependent manner (Fig. 4, *middle* and *bottom*). A similar dose-dependent decrease in immunoreactive leptin associated with peak I was observed when unlabeled leptin (0–200 ng/ml) was added to
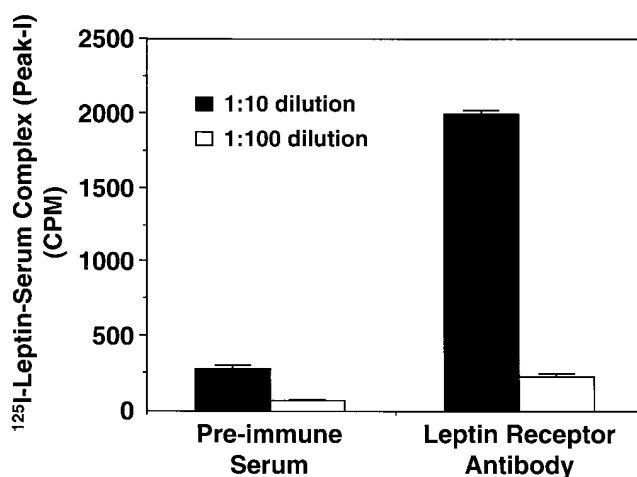

Figure 4. Sephadex G-100 elution profiles after incubation of ^{125}I-leptin with serum from a lean (5.4 ng/ml leptin level) subject for 72 h at 4°C in the absence and presence of 1 μg/ml leptin, 1 μg/ml IGF-I, or 1 μg/ml insulin (*top*). Sephadex G-100 elution profile (*middle*) and specific ^{125}I-leptin binding (*bottom*) in the presence of unlabeled leptin (0–250 ng/ml) with dialyzed serum (1.2 ng/ml leptin level).

Figure 6. Immunoprecipitation of bound ^{125}I-leptin from peak I (20,000 cpm) of Sephadex G-100 gel filtration by leptin receptor antibody raised against synthetic peptide (236–254) of the extracellular domain.

lean serum (7.5 ng/ml leptin level). Specific ^{125}I-leptin binding was $56.0\pm8.6\%$ ($n = 3$) of the total radioactivity (123,000 cpm) and 50% displacement (ED$_{50}$) of the specific ^{125}I-leptin binding was observed at 11.7 ± 1.5 ng/ml ($n = 3$), i.e., 0.73 ± 0.09 nM leptin concentration. Scatchard plots of the leptin binding data were curvilinear, suggesting either negative cooperativity or multiple binding sites.

Several protein bands were identified when human serum samples were subjected to leptin-agarose affinity chromatography, SDS-PAGE, and silver staining (Fig. 5). Elution profiles from Sephadex G-100 and Sephadex G-200 gel chromatography would indicate that 280-kD protein is at least one of the several putative leptin binding proteins. Either of the 80- or 100-kD proteins could be the proposed soluble leptin receptor Ob-Re (9), since they closely resemble the predicted molecular size of $\sim$ 90 kD. To test this hypothesis, we incubated ^{125}I-leptin with serum and separated bound from free by Sephadex G-100 chromatography. Approximately 10% ($n = 2$) of the radioactivity eluting in the bound form (peak I) could be immunoprecipitated by leptin receptor antibody raised against synthetic peptide (236–254) of the extracellular domain of the Ob-R (Fig. 6). Although it is premature to talk about quantitative immunoprecipitation, these latter experiments suggested that other proteins besides the soluble form of the leptin receptor might also bind leptin in human circulation and one of the putative leptin binding proteins is of 280 kD in molecular size.

Discussion

To understand the physiological significance of any hormone, it is important to determine its possible interaction with other circulatory proteins and its bioavailability to target tissue(s). Our gel filtration experiments with exogenously added ^{125}I-leptin and/or unlabeled leptin to serum samples demonstrated the presence of reversible, dose-dependent leptin specific binding (ED$_{50}$ = 0.73 ± 0.09 nM) probably due to multiple binding sites.

The fact that two distinct methodological approaches with different objectives, the present study and those of Tartaglia et al. (8), report similar leptin binding kinetics for the leptin-binding protein(s) (ED$_{50}$ = 0.73 ± 0.09 nM, $n = 3$) and leptin receptor (K_d 0.5 nM) (8) is probably not a coincidence and may be important. The leptin receptor (8, 9) belongs to the class I cytokine receptor family (11), an example of which is the hGH receptor (11, 25). The extracellular domain of the hGH receptor occurs naturally in serum in the form of a hormone binding protein, which binds hGH with approximately the same affinity as the intact receptor (21, 25–28).

Purification of human serum by leptin affinity chromatography demonstrated the presence of several proteins of which either 100- or 80-kD protein is probably the soluble leptin receptor (9). Approximately one-tenth of ^{125}I-leptin eluting in the bound form after gel filtration could be immunoprecipitated with a polyclonal antibody against the extracellular domain of the leptin receptor (9).

At present, it is difficult to ascertain the precise nature of the serum protein(s) interacting with circulating leptin in humans. Furthermore, ^{125}I-leptin binding and separation of bound and free leptin were performed at 4°C, whereas in vivo regulation occurs at a body temperature of 37°C. Irrespective of the fact that leptin binding in human serum is due to the soluble leptin receptor and/or some other distinct circulating pro-

tein(s), evaluation of bound and free leptin levels has important pathophysiological implications for the understanding of leptin action in humans.

The importance of these results is not only that we are showing larger differences of free, presumably bioactive leptin in obese individuals than those previously reported for total leptin (12, 13). We feel that these studies are significant because in lean individuals with minimal adipose tissue mass, a greater proportion of the leptin is in the bound form and only a fraction of the total circulating leptin is in the free form to exert its biological effect on food intake and possibly energy metabolism. To illustrate our point, we cite an example, though extreme, of a lean male subject with a BMI of 20.5 and 16% BF. This subject with a total leptin level of 2.1 ng/ml has 98% leptin in the bound form (2.06 ng/ml) and only 2% leptin in the free form (0.04 ng/ml). There is a possibility that even this little leptin (0.04 ng/ml) is the leptin RIA noise. Thus, "all" circulating leptin in this lean individual is circulating in the bound form.

Our short-term fasting and refeeding experiments also suggest a similar conclusion. After 24 h of fasting, free leptin in lean subjects decreases to 1.34 ± 0.39 ng/ml which is only one-tenth of that in obese subjects (14.7 ± 5.31 ng/ml). Again, we illustrate the extreme example where after a 24-h fast in a lean female with 28% BF and basal leptin level of 13.24 ng/ml, total leptin decreases to 4.0 ng/ml, of which 85% is in the bound form and only 15% is in the free form (0.61 ng/ml). Because lean individuals have fewer energy stores and food deprivation poses a greater threat to their well being, it seems logical that circulating leptin is predominantly in the bound form.

In summary, we demonstrate that in humans leptin circulates both in bound and free forms. It is possible that the fraction of free/total leptin may not be constant and independent of physiological states such as fasting and refeeding, but rather that a dynamic equilibrium probably exists between the circulating binding protein(s) and free leptin and that this balance may be affected by metabolic state. In lean individuals with relatively low adipose tissue depots, the majority of leptin is in the bound form and thus possibly restricts the availability of free leptin to its inhibitory effect on food intake both under normal and food deprivation states.

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