Leptin is a adipocyte-derived hormone with potent weight reducing effects. Genetically obese rodents with mutations of leptin or the leptin receptor are defective in leptin signaling and develop morbid obesity and diabetes. Interestingly, the levels of both leptin mRNA and protein are increased by up to 20-fold in these animals, suggesting the existence of a feedback mechanism controlling the amount of leptin in circulation. In this report, we attempted to determine whether the up-regulation of circulating leptin in Zucker Diabetic Fatty rats, which are nonresponsive to leptin due to a receptor point mutation, is entirely due to increased expression of leptin. We demonstrate that the high level of circulating leptin in these rats is attributable to at least two factors: increased leptin expression by the adipose tissue and delayed clearance of leptin from circulation due to binding to its soluble receptor. The latter conclusion was supported by three lines of evidence: 1) The soluble leptin receptor is up-regulated by about 20-fold in Zucker Diabetic Fatty rats; 2) Adenovirus-mediated overexpression of the soluble leptin receptor results in a similar -fold increase of circulating leptin; 3) In ob/ob mice, which have no endogenous leptin, exogenously administered leptin reaches a higher level when the soluble leptin receptor is overexpressed. The weight-reducing effect of leptin is enhanced in C57Bl/6 ob/ob mice with overexpression of the soluble leptin receptor. Soluble leptin receptor may be a significant factor determining the amount of total leptin in circulation.

Leptin is a adipocyte-derived hormone of 167 amino acids (1). It has potent weight-reducing effects in vivo (2–4). In ob/ob mice, the gene encoding leptin is mutated, resulting in morbid obesity and associated abnormalities, including hyperphagia, hypothermia, diabetes, and infertility.

The leptin receptor, OB-R, is a member of the cytokine receptor family (5). It is encoded by the diabetes (db) gene, mutation of which also results in morbid obesity and other abnormalities similar to that in ob/ob mice. OB-R is alternatively spliced into at least five transcripts from a single gene. These transcripts encode proteins that are called the long (OB-Rb), short (OB-Ra, -c, and -d), and soluble (OB-Re) forms of the leptin receptor. With the exception of the soluble leptin receptor, receptor isoforms differ from each other by the alternative use of a unique terminal coding exon (6). OB-Rb is essential in mediating leptin's weight-reducing and other biological effects (6, 7).

OB-R is expressed in both the nervous system and peripheral tissues. The relative levels of expression of different receptor isoforms vary among different tissues, providing a possible mechanism of regulating leptin's biological activity at various leptin target sites (8). OB-Rb is enriched in the hypothalamus, the site of leptin's action on food intake and body weight. Leptin activation of OB-Rb within this brain region results in the inhibition of neuropeptide Y/agouti-related protein neurons and activation of pro-opiomelanocortin/cocaine- and amphetamine-regulated transcript neurons (9). These neural circuits in turn mediate leptin's biological effect centrally. OB-Rb can also activate signal transduction in a variety of peripheral tissues, including the adipose tissue, T cells, endothelial cells, and the pancreatic β cells (10–14). Direct leptin signaling at these additional sites may contribute to the many biological effects of leptin.

Although OB-Rb is essential in mediating leptin’s biological effects, other receptor isoforms may still be necessary for leptin to exert its full spectrum of in vivo functions. Among the short forms of the leptin receptor, OB-Ra is most abundantly expressed (15). It is enriched at the choroid plexus and brain microvessels, sites of blood-cerebrospinal fluid barrier and blood-brain barrier, suggesting that it may be involved in the transport of leptin across these barriers to reach the hypothalamus. One of the mutant OB-R alleles in rats, fa^k/fa^k, does not have this form of the leptin receptor. They have reduced levels of leptin in the cerebrospinal fluid, supporting a role of the short form leptin receptor in leptin transport to the hypothalamus (16, 17).

Previously, we have shown that the secreted form of the leptin receptor, OB-Re, circulates in mouse plasma and is capable of binding to leptin (18). The level of both OB-Re and leptin increased by up to 40-fold during late stages of mouse pregnancy, suggesting that the soluble leptin receptor may modulate leptin’s biological activity in vivo (19). In this report, we demonstrate that, in leptin nonresponsive ZDF rats, OB-Re expression is increased by more than 20-fold. We also show that adenovirus-mediated overexpression of the soluble leptin receptor causes increases of circulating leptin without affecting leptin expression. The elevation of circulating leptin results from delayed clearance in the presence of overexpressed OB-Re. Finally, we show that in ob/ob mice, leptin’s effect on food intake and body weight is enhanced when its soluble receptor is overexpressed.

**EXPERIMENTAL PROCEDURES**

**Construction of Adenoviruses Encoding the Soluble Leptin Receptor**

The cDNA encoding the soluble leptin receptor was polymerase chain...
reaction-amplified and subcloned into a shuttle vector pAdCMV. The shuttle vector containing the soluble leptin receptor was first transfected into 293T cells to verify the correct expression of the encoded protein. It was then recombined with the adenovirus backbone vector pJM17 by cotransfection into 293T cells. Viruses were plaque-purified on soft agar plates and then amplified to a titer of \(10^{12}\) virus particles/ml before being used for injection. Adenoviruses encoding leptin (Ad-CMV-leptin) (20) and \(\beta\)-galactosidase (Ad-CMV-\(\beta\)-Gal) (21) were kindly provided by Dr. C. B. Newgard (University of Texas Southwestern Medical Center, Dallas, TX).

**Animals**—Male Zucker Diabetic Fatty (ZDF) rats and Zucker lean rats at 9 weeks of age were obtained from Dr. Roger Unger’s laboratory (University of Texas Southwestern Medical Center). Lean rats were divided into two groups. The experimental group received AdCMV-OB-Re (OB-Re) virus, whereas the control group received AdCMV-\(\beta\)-Gal virus. After virus injection, rats were fed with high fat diet containing 20% fat (Teklad, Madison, WI). Female C57Bl/6J ob/ob mice at 5 weeks of age were purchased from The Jackson Laboratories (Bar Harbor, ME). Mice received virus injection similarly as performed in rats. All animals used were housed and cared for in the Animal Resource Center of University of Texas Southwestern.

**Adenovirus Infusion**—Adenoviruses encoding OB-Re or \(\beta\)-galactosidase were injected into the jugular vein of rats weighing between 250 and 300 g. Each rat received \(\sim 1 \times 10^{12}\) total virus particles in 0.5 ml of PBS. For expression in mice, each mouse received \(\sim 1 \times 10^{11}\) virus particles in 0.1 ml of PBS via the tail vein.

**Leptin Treatment of ob/ob Mice**—Three days after adenovirus injection, one group of ob/ob mice was implanted subcutaneously with Alzet osmotic pumps (model 1002D, Alza Corp., Palo Alto, CA). The pumps delivered 12.5 \(\mu\)g of mouse recombinant leptin (Sigma Chemical Co., St. Louis, MO) per day continuously for 12 days. Food intake and body weight were measured daily.

**Plasma Preparation**—Blood samples were collected from the tail vein into Eppendorf tubes coated with EDTA. Plasma was prepared by low speed centrifugation (5000 \(\times g\), 5 min) and used for measurement of glucose, free fatty acids, triglyceride, insulin, the soluble leptin receptor, and leptin.

**Northern Blot Analysis of Leptin Expression**—Rats were sacrificed under sodium pentobarbital anesthesia. Epididymal fat was dissected immediately, washed with phosphate-buffered saline, and snap-frozen in liquid nitrogen. Total RNA was extracted from tissues with TRIzol reagent (Life Technologies Inc., Gaithersburg, MD) following the manufacturer’s instructions. Northern blotting of leptin was performed as described previously. Probes were derived by reverse transcriptase-polymerase chain reaction amplification using primers specific for leptin. Primer sequences were: forward primer, 5'-TGACGTTCAGCAGAATTCG-3'; reverse primer, 5'-GGCCATTCCAGGTCTTCC-3'. Product size was expected to be 190 base pairs. Probe was labeled with \([\alpha-32P]dCTP\) (PerkinElmer Life Sciences) with a random primer labeling kit (New England BioLabs) with a random primer labeling kit (New England BioLabs).

**Soluble Leptin Receptor Assay**—Plasma from ZDF rats and lean rats were diluted in PBS and incubated with leptin-Sepharose resin overnight. Leptin beads were washed with PBS three times. After boiling in 2\(\times\) SDS sample buffer for 5 min, resin suspension was loaded directly onto an 8\(\%\) SDS-PAGE gel and blotted with an anti-leptin receptor polyclonal antibody as described previously (18). 1-\(\mu\)l plasma samples from AdCMV-OB-Re-treated rats or mice were run on SDS-PAGE directly to detect the levels of the soluble leptin receptor and leptin by Western blotting.

**Leptin Assay**—Circulating levels of leptin in plasma of rats and mice overexpressing the soluble leptin receptor were detected by Western blotting. Leptin levels in animals that did not receive AdCMV-OB-Re virus were measured by a rat leptin RIA kit (Linco Research, St. Louis, MO).

**Antibodies**—Polyclonal antibodies against leptin and the soluble leptin receptor were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) or as described previously (18, 22).

**RESULTS**

**Soluble Leptin Receptor Is Elevated in Leptin Nonresponsive ZDF Rats**—Leptin mRNA levels in the adipose tissue of genetically obese \(db/db\) mice and mice with hypothalamic lesions are increased by about 20-fold compared with lean controls. The level of circulating leptin is also elevated in plasma in these mice (23). In \(db/db\) mice, mutation of the leptin receptor results in a truncation of the long form leptin receptor and loss of leptin signaling (6, 7). Similar increases of both leptin mRNA and protein also occur in ZDF rats, which have a single amino acid mutation within the extracellular domain of the leptin receptor (Gln-269 \(\rightarrow\) Pro) (24). Because leptin signaling is absent in \(db/db\) mice or ZDF rats, elevated leptin levels in these mutant animals raises the possibility of a feedback control mechanism regulating leptin expression.

We asked if the expression of the soluble form of leptin receptor is changed when leptin signaling is absent, such as in \(db/db\) or ZDF rats. Previously, we have established an assay to measure the level of circulating leptin receptor with leptin-Sepharose beads, which is a useful method for concentrating available receptor protein for detection (18). The protein level of other forms of the leptin receptor is too low to be detected directly. Plasma from Zucker lean or ZDF rats was prepared and incubated with leptin-Sepharose resin to determine the circulating soluble leptin receptor level. The soluble leptin receptor present in plasma was estimated by its ability to bind an excess amount of added immobilized leptin that was coupled to Sepharose beads. Bound receptor protein is then separated by SDS-PAGE gel and detected with a polyclonal antibody. This method, however, cannot be used to concentrate and measure membrane-bound receptors, because leptin binding is very sensitive to the presence of detergents (data not shown).

To compare the relative abundance of the soluble leptin receptor in Zucker lean and ZDF rats, a fixed amount of plasma from lean Zucker rats and varying amounts of plasma from ZDF rats were used to incubate with leptin-Sepharose beads. The amount of receptor present in each sample did not saturate the binding capacity of the leptin resin used, because controls with a high level of recombinant receptor protein recovered from leptin-Sepharose resin or loaded directly to SDS-PAGE give rise to receptor signals with indistinguishable intensity (data not shown). We also measured the amount of leptin present in each sample using leptin radioimmunoassay (RIA). Fig. 1A shows the amount of leptin present in each plasma sample in Zucker lean and ZDF rats. There is about a 10-fold elevation of leptin in plasma from ZDF rats compared with lean rats. Fig. 1B is a Western blot of bound soluble leptin receptor from each plasma sample after incubation with the leptin-Sepharose beads. Samples containing 2-5 \(\mu\)l of plasma from ZDF rats generated signal density that is about equal to that from 100 \(\mu\)l of plasma from Zucker lean rats, indicating that the plasma concentration of soluble leptin receptor in ZDF rats is elevated by at least 20-fold. Although the plasma levels of the soluble leptin receptor from rats of the same genotype vary, a similar -fold increase of the soluble leptin receptor was found in ZDF rats compared with Zucker lean rats. This result demonstrates that, in the absence of leptin signaling, expression of both leptin and its soluble receptor is elevated in plasma.

**Overexpression of the Soluble Leptin Receptor Leads to a Parallel Rise of Circulating Leptin without Increasing Leptin Expression**—Because OB-Re level is elevated in plasma of ZDF rats, we asked if it plays a role in modulating the level of total circulating leptin. We chose to test this hypothesis by overexpressing the soluble leptin receptor in both Zucker lean and ZDF rats. High level in vivo expression of the soluble leptin receptor was achieved by administration of a recombinant adenovirus containing its cDNA (AdCMV-OB-Re). When transfected into 293T cells, virally expressed receptor protein migrated at the same position on SDS-PAGE as the soluble leptin receptor purified from plasma, suggesting that there is correct post-translational modification (data not shown). Before adenovirus-mediated overexpression of OB-Re, there were comparable amounts of leptin present in plasma samples in each
genotype as assayed with RIA; the levels of the soluble leptin receptor were also comparable in Zucker lean or ZDF rats using the leptin pull-down assay (data not shown). Two days after adenovirus injection, the leptin pull-down assay was repeated. Robust expression of the soluble leptin receptor was detected in both lean and ZDF rats that received AdCMV-OB-Re virus compared with those that received AdCMV-β-Gal virus (Fig. 2).

In parallel, we measured the levels of circulating leptin in these OB-Re-overexpressing rats with RIA. Surprisingly, the leptin level was over the upper limit of detection in both Zucker lean and ZDF rats overexpressing the soluble leptin receptor (data not shown). These results suggest that expression of the soluble leptin receptor leads to a concomitant increase of circulating leptin. The robust expression of OB-Re in vivo via adenovirus and a similar rise of circulating leptin prompted us to test if it is possible to detect both proteins from plasma directly. In normal rats, the amount of circulating leptin is too low to be detectable by direct blotting of plasma proteins. When samples from OB-Re-overexpressing rats were run on an 8% or 16% SDS-PAGE and detected with antibodies specific for soluble leptin receptor or leptin, strong signals were obtained for both proteins in lanes containing samples from rats that received AdCMV-OB-Re virus (Fig. 3). The signal for leptin or its soluble receptor was absent in rats that received AdCMV-β-Gal virus or were sham-operated, suggesting that the elevation of circulating leptin is the result of overexpression of its soluble receptor.

Adenoviruses that are injected via the jugular vein or tail vein preferentially infect the liver. To verify that the soluble leptin receptor we detected in plasma indeed comes from adenovirus-mediated overexpression of OB-Re but not from induction of endogenous soluble leptin receptor, we performed Northern blot analysis. Rats were sacrificed 2 weeks after treatment with AdCMV-OB-Re or AdCMV-β-Gal. Total RNA was prepared from liver and other tissues, and virally mediated expression of OB-Re was detected with a cDNA probe for OB-R. Fig. 4A shows that the livers of rats that received AdCMV-OB-Re contain a high level OB-Re mRNA, which is not detected in the livers of rats that received AdCMV-β-Gal. AdCMV-OB-Re-treated rats also have a high level of circulating OB-Re protein, demonstrating that it is produced from the adenoviruses administered (Fig. 4B).

To estimate the -fold increase of the soluble leptin receptor
and leptin after adenovirus treatment, we performed serial dilution analysis of both proteins in plasma. Plasma samples from AdCMV-OB-Re virus-treated ZDF rats were diluted and compared with undiluted sample before adenovirus injection. Antibody against leptin or its soluble receptor was used to detect the amount of each protein present in all samples. Fig. 5 shows that, with compared with the undiluted plasma sample obtained prior to virus treatment, a rise of about 25-fold for leptin and about 100-fold for its soluble receptor was achieved.

**Soluble Leptin Receptor Does Not Affect Leptin Expression—**

Hyperleptinemia induced by its soluble receptor may be explained by the following three possibilities: increased leptin synthesis, increased leptin stability by binding to its overexpressed soluble receptor or other proteins, or a combination of both. We first tested whether the soluble leptin receptor affects leptin expression at the transcriptional level by Northern blot analysis. In both **ob/ob** and **db/db** mice, expression of leptin is increased by up to 20-fold at the mRNA level (24). To test whether the soluble leptin receptor plays a role in leptin expression in animals with normal leptin function, we overexpressed the soluble leptin receptor in wild type mice for 3 days and compared the leptin mRNA levels in mice that received AdCMV-OB-Re virus or AdCMV-β-Gal control virus. This time point was chosen, because adenovirus-mediated overexpression reaches a peak level 2–3 days after virus injection.

Total RNA was prepared from the white adipose tissue of mice treated with AdCMV-OB-Re or AdCMV-β-Gal and blotted with a cDNA probe specific for leptin. As shown in Fig. 6, mice that received OB-Re virus expressed various amounts of the soluble leptin receptor. In all cases, the levels of circulating leptin were also elevated. However, the leptin mRNA is not increased in adipose tissue in response to OB-Re overexpression. We also examined other tissues to test whether they express leptin after adenovirus-mediated OB-Re overexpression. No leptin signal was detected from all other tissues examined (data not shown).

**Fig. 4. Liver-specific expression of adenoviruses encoding the soluble leptin receptor.** Rats that received adenoviruses encoding the soluble leptin receptor (Re) or β-galactosidase (β-Gal) were sacrificed, and total RNA was prepared from liver and other tissues. A, OB-Re mRNA was detected in the liver of rats that received OB-Re virus but not control virus (β-Gal). B, the expressed protein was also readily detected by Western blotting of plasma protein with an antibody recognizing the leptin receptor.

**Fig. 5. Titration of the -fold increase of leptin and its soluble receptor.** Plasma (1 μl) from ZDF rats with overexpression of the soluble leptin receptor was undiluted, or diluted 1:5, 1:10, 1:25, 1:50, 1:100, and 1:200 in PBS. Total protein in diluted samples was adjusted by adding plasma from lean rats that did not overexpress the soluble leptin receptor. These samples were loaded side by side with plasma from two control ZDF rats that received AdCMV-β-Gal virus (−). Receptor and leptin signals were compared after Western blotting to estimate the -fold increase of both leptin and its soluble receptor after overexpression of OB-Re. The signal in all lanes above the 49-kDa marker arose from IgG in plasma that reacted with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody.

**Fig. 6. Overexpression of the soluble leptin receptor does not lead to increase of leptin expression.** A, adenoviruses encoding OB-Re or β-galactosidase (β-Gal) were injected into the tail vein of 10-week-old C57B/6J mice. Receptor expression and the level of circulating leptin were simultaneously monitored by Western blotting of plasma samples with receptor- or leptin-specific antibodies. B, mice were sacrificed, and epididymal fat was removed from each mouse. Total RNA was prepared and loaded onto a 1.2% agarose gel blot and blotted with a CDNA probe specific for leptin. The amount of RNA loaded in each lane is shown at the bottom. Leptin mRNA was not increased in mice that received adenoviruses encoding the soluble leptin receptor.

**The Soluble Leptin Receptor Protects Leptin from Degradation in ob/ob Mice—** To further understand the mechanism by which leptin levels are increased by OB-Re overexpression, **ob/ob** mice were given leptin continuously via a subcutaneous miniature osmotic pump. This method has been used successfully to achieve delivery of leptin at controlled rates (25). **ob/ob** mice have no endogenous leptin due to a mutation in its coding region (1). Consequently, all circulating leptin will be derived from material released from the pump. If the soluble leptin receptor increases the stability of leptin, we would predict that it would cause an elevation in circulating leptin. As shown in Fig. 7, the soluble leptin receptor reached a very high level in mice that received AdCMV-OB-Re, but not in those that received AdCMV-β-Gal. As predicted, circulating leptin is also elevated by manyfold in mice that received AdCMV-OB-Re. The increased leptin level in OB-Re-overexpressing mice can be completely explained by its delayed clearance.

**Overexpressed Soluble Leptin Receptor Enhances Leptin’s Ef-**
Receptor (Re) or β-galactosidase (β-Gal). Plasma samples were prepared at 2-day intervals to monitor the circulating concentration of leptin and its soluble receptor. Leptin signal was entirely from material released from the pump, because ob/ob mice have no endogenous leptin. Mice with OB-Re overexpression have a higher circulating level of leptin (left four lanes). The band above leptin signal in all lanes represents nonspecific cross-reactions of the antibodies used.

**DISCUSSION**

In a previous study, we demonstrated that the soluble leptin receptor circulates in plasma and is capable of binding to leptin (18). Other groups also found that soluble leptin receptor level is elevated by up to 40-fold at late stages of mouse pregnancy (19). Similarly, elevated leptin levels in fa/+ pups compared with lean controls (fa+/+ and +/+ ) may also be the combined effect of increased leptin expression and delayed clearance (28). The mechanism governing the expression of soluble leptin receptor remains to be determined.

The main site of expression of the soluble leptin receptor in vivo is not known. Previously, we failed to detect a signal for OB-Re when Northern blot analysis was performed (15). Available data have demonstrated that OB-Re is expressed by the placenta in mice. Its expression starts at day 14 of pregnancy, peaking just before parturition to about 40-fold the level found in nonpregnant mice (19, 29). In rats and humans, the pregnancy-associated rise of circulating leptin and its soluble receptor is relatively modest, achieving only a 2-fold increase versus a more than 40-fold increase in mice (30). Alternatively, soluble leptin receptor may also be produced by proteolytic cleavage of membrane-associated receptor.

Why is leptin expression in the adipose tissue of db/db mice or ZDF rats increased? One possibility is the feedback inhibition of leptin via its receptor in wild type animals. It is known that OB-R is expressed on the surface of adipocytes (6, 10). At a certain threshold concentration, leptin may directly signal the adipocyte to stop its own production utilizing the receptor on the surface of these adipocytes. Alternatively, the central nervous system may also respond to leptin signaling by releasing neurotransmitters or neuropeptides to control the synthesis and release of leptin into circulation. A loss of these pathways due to leptin signaling deficiency in db/db mice or ZDF rats may contribute to the increased production of leptin.

Our findings provided a plausible explanation of several unexplained observations regarding the level of circulating leptin. For example, there is a lack of correlation between the leptin mRNA level and protein level in some obese subjects. The leptin mRNA level is lower in obese subjects than expected based on differences of circulating leptin (31). This could be explained by the presence of increased soluble leptin receptor in these individuals. A support for this hypothesis came from a recent clinical study of patients with the same mutations of the leptin receptor that truncate OB-R 5’ of the transmembrane region. The truncated product resembles the soluble leptin receptor. As a result, circulating levels of leptin in these patients are very high (32), analogous to the elevation of leptin by its soluble receptor that we reported here. Similarly, the circulating level of leptin in C57BL/6J db/db mice was unchanged after 15 days of food restriction, whereas its level became undetectable in lean mice 6 days after food restriction (24). The persistence of circulating leptin in food-restricted db/db mice may also be regulated by its soluble leptin receptor. Alternatively, other leptin binding protein in serum may also play a role (33, 34).

We did not observe a major change of food intake or body weight in Zucker lean rats overexpressing the soluble leptin receptor. Although OB-Re overexpression raises circulating leptin by manyfold, it is obviously inactive when bound to its soluble receptor. The slightly enhanced leptin effect on food intake and body weight in ob/ob mice may be due to a higher level of free leptin in the presence of OB-Re overexpression. When a large pool of leptin-receptor complex is present in circulation, bound leptin is constantly released, resulting in a net increase of free leptin in plasma. Conversely, when new circulating leptin synthesis and release of leptin into circulation. A loss of these pathways due to leptin signaling deficiency in db/db mice or ZDF rats may contribute to the increased production of leptin.

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leptin release is low, such as during food restriction, free leptin may not decrease as rapidly if a pool of leptin-receptor complex preexists in plasma. Studies are underway to determine whether bound leptin affects leptin signal transduction through its long form receptor in vitro.

The new mechanism in regulation of circulating leptin concentration reported here has added an additional level of complexity to the modulation of leptin’s biological activity and availability. In the absence of leptin signaling, increased output of its soluble receptor results in an elevation of circulating leptin that is not from leptin overexpression. The leptin that is bound by its soluble receptor appeared to be inactive but may be made available for release into circulation and activate leptin responses. It remains to be determined what is the main site of expression of the soluble leptin receptor and how its own stability is regulated. A fuller understanding of these issues should shed more light on the mechanisms of action used by leptin in the control of so many distinct yet important physiological processes, ranging from appetite and body weight to fertility, immune function, and bone formation.

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