Leptin resistance is a common feature of obesity and the metabolic syndrome. However, the regulated expression of the leptin receptor (Ob-R) has not been studied in detail. Expression profiling of liver mRNA in leptin-treated wild-type mice revealed a marked increase in leptin receptor mRNA levels, which had not previously been described. This was confirmed by isoform-specific real-time PCR, which showed a >25-fold increase in the mRNAs encoding the short forms (Ob-Ra, Ob-Re) and a >10-fold increase in the mRNA encoding the long (Ob-Rb) form of the leptin receptor in liver. In parallel, we also observed induction of plasma-soluble leptin receptor (SLR) protein by leptin administration, pair feeding, and short term food restriction. However, induction of SLR by leptin is abolished in mice with selective deletion of Ob-R from liver using Cre-Loxp technology. These data suggest that the liver is a major source of Ob-R mRNA expression under conditions of negative energy balance. Membrane-bound Ob-R is then shed into the circulation as SLR. Our study thus reveals an unexpected role of the liver in modulating total circulating leptin levels and possibly its biological activity.

The hormone leptin is the afferent signal in an endocrine program regulating energy balance, metabolism, and neuroendocrine function (1). Mice lacking leptin (ob/ob) or its receptor (db/db) are massively obese and hyperphagic (2–5). Leptin administration in wild-type and ob/ob mice causes a dose-dependent reduction in food intake and body weight (6). Leptin elicits metabolic effects that cannot be explained by its actions on food intake alone, characterized by selective depletion of peripheral tissue lipid and enhanced insulin sensitivity (7, 8). A small number of humans with morbid obesity and marked peripheral tissue lipid and enhanced insulin sensitivity (7, 8). A small number of humans with morbid obesity and marked lipid accumulation are associated with selective deletion of leptin and the Receptor (Ob-Re), which in rodents can be alternatively spliced into five different isoforms (Ob-Ra–e) (4). Ob-Rb is the only isoform that has been shown to mediate signal transduction in vivo and is expressed most abundantly in the brain (15). Physiological and genetic studies have shown that the effects of leptin are mediated via its action in the brain (16, 17). However, the leptin receptor is broadly expressed, and the role of leptin in peripheral tissues and of isoforms other than Ob-Rb is less well defined (18). One of the splice variants of the leptin receptor, Ob-Re, does not encode a transmembrane domain and is secreted. Although Ob-Re mRNA has been identified in rodents, the Ob-Re splice form has not been found in humans; however, soluble leptin receptor (SLR)1 circulates in human plasma (19, 20). In vitro and in vivo studies indicate that SLR may be generated by ectodomain shedding of membrane-spanning receptors mediated by a metalloprotease (21, 22). The relative contribution of Ob-Re mRNA-derived SLR to ectodomain shedding-derived SLR in plasma is not known, as the sizes of circulating SLR generated by either mechanism are indistinguishable by Western blotting analysis.

The generation of SLR protein is regulated under physiological conditions. In mice, expression of Ob-Re mRNA in the placenta is strongly induced at later stages of pregnancy, causing an up to 40-fold increase in SLR (23). In humans, levels of SLR are inversely related to adiposity (24). Previously, we and others reported that SLR is the major determinant of plasma leptin levels and acts to stabilize circulating leptin without increasing leptin transcription in adipose tissue (25, 26). We also found both in vitro and in vivo that similar to human SLR, murine SLR may be generated by ectodomain shedding of membrane spanning receptors (21). Subsequent in vitro studies demonstrated that, when leptin is bound to its soluble receptor, the leptin-SLR complex is incapable of activating Ob-Rb, although it does not inhibit the action of free leptin (27). These results suggest that SLR serves to sequester leptin from productive interactions with its signaling receptor.

In aggregate, these findings point to SLR as an important determinant of leptin action. However, the nutritional regulation of SLR levels remained incompletely studied, and its tissue site of production was unknown. In this study, we report the induction of Ob-R mRNA in the liver by leptin administration, with a parallel increase in plasma levels of SLR. We also show that SLR is induced by pair feeding and fasting. Induction of SLR was not observed in mice with selective deletion of Ob-R in the liver.
Liver Leptin Receptor Induction

Liver generated using the Cre-LoxP system. These data indicate that the liver is a major source of SLR expression in states of negative energy balance and demonstrate a novel role for the liver in modulating leptin action.

**MATERIALS AND METHODS**

**Reagents—**Recombinant leptin was from Sigma, from Dr. A. F. Parlow (National Hormone and Peptide Program, Torrance, CA), or Amgen, Inc. (Thousand Oaks, CA). Alzet 2002 mini-osmotic pumps were from Alza (Palo Alto, CA). Insulin and leptin radioimmunoassay kits were from Alpcyo Diagnostics (Windham, NH) and R&D Systems (Minneapolis, MN), respectively. Glucose Trinder reagent was from Sigma. Oligonucleotide microarrays were from Affymetrix (Santa Clara, CA). TRiso reagent was from Invitrogen. Reverse transcription reagents used for real-time PCR were from Roche Applied Science. Probes for real-time PCR were from Applied Biosystems (Foster City, CA) and primers were from Genosys (The Woodlands, TX). Anti-FLAG-horseradish peroxidase antibody was from Sigma. Hybrid-N+ membrane, Rapid-hyb buffer, ECL and ECL + Western blotting detection reagents and gel filtration columns were from Amersham Biosciences. Protease inhibitor mixture was from Roche Diagnostics. [α-32P]dCTP was from PerkinElmer Life Sciences. The random primer labeling kit was from Amersham Biosciences. X-ray film was from Denville Scientific Inc. (Metuchen, NJ).

**Animals—**Male C57Bl/6 and ob/ob mice at 10 weeks of age were purchased from The Jackson Laboratories (Bar Harbor, ME). C57Bl/6 and ob/ob mice were fed a standard rodent chow. The mice were maintained between 21–23 °C on a 12-h light, 12-h dark cycle. Mice were housed and cared for by the staff at The Rockefeller University and were individually housed and cared for by the staff at The Rockefeller University Resource Centers. For leptin treatment, the mice were individually housed and maintained between 21–23 °C on a 12-h light, 12-h dark cycle. Mice were treated with PBS or the same dose of leptin for up to 10 days. Pair-fed mice were treated with PBS but only allowed to eat as much as leptin-treated mice voluntarily consumed. For food deprivation experiment, another cohort of mice were treated with PBS or the same dose of leptin for up to 10 days. Pair-fed mice were treated with PBS but only allowed to eat as much as leptin-treated mice voluntarily consumed. For food deprivation experiments, animals had their food removed and were sacrificed 24 h later.

**Northern Blotting Analysis of Ob-R RNA Levels in Liver—**Total RNA was extracted from the livers of mice treated with or without leptin. To prepare a probe that would detect all forms of Ob-R, PCR was performed on a plasmid encoding full-length Ob-Rt DNA, pCDA3.1 (from Dr. D. Reitman, The Rockefeller University, NY). PCR was performed using primers corresponding to part of the ectodomain of Ob-R (21). To detect Ob-Re mRNA, three copies of Ob-Re-specific cDNA sequence were ligated in tandem in a head-to-tail fashion and subcloned into pBluescript SK(+), named pBS33. Each copy of the Ob-Re-specific sequence was amplified using the following primers, each containing an EcoRI site for subcloning: forward primer, 5'-CGGAATTCCGATGTTGATCTGAG-3'; reverse primer, 5’-CGGAGATCCGAGCCGAGGCTTTTC-3’; probe labeled with FAM dye. As a control for the input amount, each sample was assayed in duplicate and amplified with the ABI Prism 7700® sequence detection system (PerkinElmer Life Sciences). Ob-R probes were labeled with VIC dye for cyclophilin. The sequences of the primers and probes were as follows: Ob-R, 5'-AAAGCTATAGGAATGGTAATTC-3'; Ob-R, 5'-AGGAGTTCGCTTCACTTCA-3'; and Ob-R reverse primer, 5’-TATGCAGGTTAAGTGCGAGCTATC-3’. For isofrom-specific quantification, all assays were done using the same probe 5’-AAATTTTACGCTTGGCACCAGAAACATT-3’ and the same forward primer 5’-GGTTGGGAGGAGGCGATTCCA-3’. Reverse primers were as follows: for Ob-Ra, 5’-ATTGGTTACAGGCTTGGCACCAGAAACATT-3’; and Ob-Rb, 5’-TTGCTGAACTGAAATGGCTTTGCGC-3’. Recombinant adenoviruses encoding FLAG-Ob-Rb or β-galactosidase were injected into the jugular vein of the mice. Each mouse received 2 × 1011 total virus particles in 0.2 ml of PBS under anesthesia by mouse/rat mixture (500 mg of ketamine, 10 mg of xylazine, 10 mg of acepromazine in 20 ml of sterile H2O). Three days after virus injection, 50 μl of plasma sample from each mouse were collected from the tail vein. Mice were sacrificed by cervical dislocation, and livers were dissected immediately, washed with PBS, and snap-frozen in liquid nitrogen. Total protein was extracted by homogenization in lysis buffer (50 mM HEPES, pH 7.5, 1% Nonidet P-40, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1× protease inhibitor mixture) on ice. Liver lysate was centrifuged at 20,000 × g at 4 °C for 10 min, and the supernatant was harvested and stored for use.

To determine the levels of SLR in plasma or Ob-Rb in liver, 20 μl of plasma or 10 μl of total liver lysate from virus-infected mice was diluted with PBS to 1 ml and incubated with leptin-Sepharose resin at 4 °C overnight. Leptin beads were processed as above.

**RESULTS**

**Expression Profiling of Livers of Leptin-treated Wild-type Mice—**Oligonucleotide microarrays were examined to evaluate the molecular effects of leptin treatment on the livers of wild-type mice. We previously used expression profiling to show that leptin induces robust changes in the expression profile of ob/ob mice, and in the process identified stearoyl-CoA desaturase-1 (SCD-1) as a key mediator of the metabolic effects of leptin (7, 29). Wild-type mice were treated with leptin or saline via implanted osmotic pumps for up to 8 days. As an additional control to evaluate the effects of a shift from negative to positive energy balance, another cohort of mice were treated with leptin for 8 days, after which treatment was withdrawn and the animals were allowed to feed freely (leptin withdrawal).
weight and food intake of these mice is shown in Fig. 1, A and B. Food intake reached its nadir at day 3, which has previously been shown to be temporally associated with the complete depletion of adipose tissue mass (7). Thereafter, food intake rebounded and weight loss stabilized. Upon withdrawal of leptin, mice were markedly hyperphagic and returned to baseline body weight within 4 days.

Plasma levels of leptin, insulin, and glucose were measured in each animal. Leptin levels were significantly increased at days 6 and 8 of treatment, with levels $\approx$10-fold that of untreated controls (Fig. 1C). Upon withdrawal of leptin, at day 9, leptin levels were virtually undetectable, as these mice had been treated with leptin until their adipose stores were completely depleted and thus lacked both exogenous and endogenous leptin. This hypoleptinemia stimulated the marked hyperphagia observed in the leptin withdrawal group, leading to a rapid return to basal body weight and normalization of plasma leptin levels. Plasma insulin levels were significantly reduced at days 6 and 8 of leptin treatment, but upon leptin withdrawal, increased significantly, as these mice rapidly shifted from a state of negative to positive energy balance (Fig. 1D). Glucose levels were also reduced with leptin treatment, but returned to baseline levels upon leptin withdrawal (Fig. 1E).

Leptin-treated or saline-treated control mice were sacrificed at 6 and 8 days of treatment and at 1, 2, 3, and 4 days after leptin withdrawal. Liver RNA was isolated and cRNA was synthesized and hybridized to oligonucleotide microarrays. RNA levels in each sample were normalized to those from livers of untreated wild-type mice. We were interested in identifying genes that were either markedly induced or repressed upon chronic leptin treatment and subsequently normalized upon leptin withdrawal (30). We hypothesized that these genes would be important mediators of the effects of leptin on energy homeostasis.

Induction of Leptin Receptor (Ob-R) mRNA by Leptin Treatment—One gene found to be strongly induced by chronic leptin treatment and normalized upon leptin withdrawal was that encoding Ob-R, the leptin receptor. Ob-R encodes five splice variants, denoted Ob-Ra–e. Ob-Rb, the long form, has been shown to mediate signal transduction and is expressed at its highest levels in hypothalamic nuclei, important in regulating food intake and energy homeostasis. Ob-Re, which encodes SLR, binds leptin and circulates in the plasma. SLR can also be generated by ectodomain shedding of membrane-bound forms of Ob-R. Although the leptin receptor is broadly expressed, a physiological role in the liver has not previously been identified.

Real-time PCR (Taqman) was performed to validate the microarray data. Relative to controls, at 6 and 8 days of leptin treatment, Ob-R RNA was induced $\approx$15- and 70-fold, respectively (Fig. 2A). Upon leptin withdrawal and a switch to positive energy balance, Ob-R RNA levels rapidly returned to control levels. The data in Fig. 2A were generated using primers and probes that amplify all Ob-R splice variants. Real-time PCR using primers and probes specific to individual splice variants showed that Ob-Ra, Ob-Rb, and Ob-Re all followed a qualitatively similar trend, with robust induction at days 6 and 8 of leptin treatment and return to basal levels with leptin withdrawal (Fig. 2B).

Induction of SLR by Chronic Leptin Treatment—The marked induction of the RNA encoding multiple Ob-R isoforms suggested that chronic leptin treatment might be associated with increased plasma-soluble leptin receptor protein SLR by ectodomain shedding of membrane-bound isoforms. To test this prediction, a separate time course experiment was performed in which wild-type mice were treated with the same dose of
leptin for either 1, 2, 3, 4, 5, or 10 days. As additional controls, groups of free-fed saline-treated mice and pair-fed saline-treated mice were also studied. Pair-fed mice were only allowed to eat as much food as leptin-treated mice voluntarily consumed. No significant difference in body weight was noted between leptin-treated and pair-fed mice (data not shown). As in Fig. 1B, food intake reached its lowest level at day 3 of leptin administration, and thereafter, weight loss leveled off and food intake rebounded.

Plasma was isolated at each time point, and SLR levels were measured using a leptin-Sepharose pull-down assay (see “Materials and Methods”). SLR levels were slightly induced, relative to saline-treated controls, as early as day 1 of leptin treatment (Fig. 3A). However, SLR was significantly up-regulated at day 3, the time point at which food intake was at its nadir and animals were in a state of maximal negative energy balance. SLR levels remained up-regulated at the remaining time points, with a slight decrease at day 10. SLR was also induced by pair feeding, although not nearly as dramatically as by leptin treatment. This suggests that although leptin may be the most potent stimulus for inducing SLR, other conditions that induce a state of negative energy balance, such as pair feeding, may also be associated with SLR up-regulation.

Short Term Fasting Elevates Plasma-soluble Leptin Receptor Levels—Leptin treatment of mice causes a reduction in food intake, which creates a negative energy balance. To determine the effects of nutrient reduction or deprivation, we measured SLR levels in fasted mice. As shown in Fig. 3B, short term acute food deprivation of wild-type mice also resulted in increased soluble leptin receptor levels. In aggregate, these results demonstrated that both leptin administration and food restriction cause a rise in plasma soluble leptin receptor in wild-type mice. These findings provide examples of additional mechanisms regulating SLR induction in vivo. However, the up-regulation of SLR with pair feeding or fasting was much less potent than that with leptin.

Increased SLR Levels Are Not Due to Increased Ob-Re RNA Levels—The above data indicate that leptin administration induced liver Ob-R RNA, with a concomitant increase in plasma SLR levels. We hypothesized that this phenomenon was the result of cleavage of the membrane-bound receptor. To test this theory, we evaluated the alternative hypothesis that increased SLR levels were derived from an up-regulation of Ob-Re RNA. An Ob-Re isoform-specific real-time PCR assay was not possible, because the unique portion of the Ob-Re message is too short to generate specific primers and probes. Therefore, Northern blotting was done using a pan-Ob-R probe and an Ob-Re-specific probe generated from concatamers of the unique Ob-Re sequence. Blotting with the pan-Ob-R probe revealed a robust increase in RNA levels at days 6 and 8 of leptin treatment (Fig. 4A). Upon withdrawal of leptin (Fig. 4A, lanes 5–8), Ob-R RNA levels rapidly diminished to undetectable levels, as shown in Fig. 2A. As a comparison, placenta RNA, which is the richest known source of Ob-Re RNA, was run in the last lane. Pan-Ob-R RNA levels from this tissue were even greater than those from leptin-treated livers, but the band migrated at a lower molecular weight, indicating that the induction of Ob-R RNA in liver was accounted for by induction of membrane-bound isoforms. To confirm this prediction, Fig. 4A, lower panel, shows the same samples blotted with an Ob-Re-specific probe. Only placenta showed a detectable signal, suggesting that the increased Ob-R RNA levels in leptin-treated liver and associated up-regulation of plasma SLR were because of increased RNA levels and cleavage of membrane-bound Ob-R isoforms.

Production of SLR Protein by the Liver—Although our data established liver Ob-R RNA induction as a physiological source of increased plasma SLR levels in leptin-treated animals, we wished to determine the extent to which the liver was responsible for plasma SLR production in states of negative energy balance. Previously reported microarray analysis of white adipose tissue and skeletal muscle RNA from leptin-treated mice did not detect any induction in Ob-R RNA levels. To examine
this question more directly, we treated Ob-R\textsuperscript{AlbKO} mice with leptin. These mice, generated by Cre-loxP technology, have a hepatocyte-specific depletion of Ob-R and thus have a significant reduction in liver Ob-R RNA levels. A time course experiment was performed in which Ob-R\textsuperscript{AlbKO} or littermate controls were treated with leptin or saline for 10 days. Both groups of mice showed equivalent reductions in body weight and food intake (data not shown) with similar kinetics to the time course in Fig. 1. Saline-treated Ob-R\textsuperscript{AlbKO} and littermate controls had similar levels of plasma SLR (Fig. 4B). Although leptin treatment of the controls led to a robust increase in plasma SLR levels (Fig. 4B, lane 2), as shown in Fig. 3, leptin-treated Ob-R\textsuperscript{AlbKO} only showed a moderate increase in plasma SLR levels (Fig. 4B, lanes 6–8). This indicates that the liver is the predominant source of increased plasma SLR in leptin-treated animals.

Shedding of Membrane-bound Ob-R Is Independent of Leptin Action—To determine whether ectodomain shedding of Ob-R requires leptin action, ob/ob mice were fasted for 24 h (as for wild-type mice) or infused with adenoviruses encoding Ob-Rb or a control protein, \(\beta\)-galactosidase. Similar to that in wild-type mice (Fig. 3B), levels of plasma-soluble leptin receptor became elevated after a 24-h fast (Fig. 5A).

Liver and plasma samples were prepared 3 days after virus infection to detect adeno virally expressed full-length Ob-Rb as well as shed SLR. Ob-Rb was tagged at the N terminus with a FLAG tag so that shedding from virally generated Ob-Rb could be easily detected with a FLAG antibody. Blotting of total liver lysates confirmed that Ob-Rb was expressed in the livers of Ad-Ob-Rb-infused mice (Fig. 5, B and C, upper panels). As expected, Ad-Ob-Rb expression in wild-type mice resulted in the appearance of two circulating forms of Ob-R (Fig. 5, B and C, lanes 3 and 4, lower panels), as we reported previously (21). In the ob/ob mouse that is deficient for leptin, the same species of circulating Ob-R fragments were also observed (Fig. 5, B and C, lane 5, lower panels), which were not detected in Ad-\(\beta\)-Gal-treated mice (Fig. 5, B and C, lanes 1 and 2, lower panels). Because no leptin was given to ob/ob mice either during fasting or adenovirus expression, these data demonstrate that shedding is independent of leptin action.

**DISCUSSION**

In this report, we demonstrated that soluble leptin receptor is induced by both leptin treatment and food deprivation. Microarray analysis of liver from leptin-treated mice showed an unexpected and robust induction in Ob-R mRNA levels, accounted for by increased levels of RNA encoding Ob-Ra–c, the membrane-bound forms of the receptor (4). This was associated with increased levels of plasma SLR. Although SLR is preferentially induced by leptin administration, pair feeding and short term food restriction in both wild-type and ob/ob mice also increased levels of SLR. The increased levels of plasma SLR appear to be the result of cleavage and shedding of membrane-bound Ob-R, as leptin treatment and food restriction had no effect on levels of Ob-Re RNA, which encodes a soluble form of the receptor. We further showed that the leptin-mediated increase in plasma SLR was predominantly, or perhaps entirely, derived from the liver. Ob-R\textsuperscript{AlbKO} mice, which have a
Cre-mediated deletion of Ob-R in hepatocytes, only showed a modest induction in plasma SLR following leptin treatment. This small level increase may be derived from residual Ob-R, as the efficiency of Ob-R deletion varies between mice but never reaches 100% efficiency. Finally, to understand the trigger for membrane shedding, we showed that cleavage of membrane-bound Ob-R is independent of leptin action.

Although induction of liver Ob-R RNA and plasma SLR was first identified using a microarray screen for leptin-induced genes in wild-type liver, we further showed that pair feeding and starvation may also induce SLR. This suggests that a state of negative energy balance, rather than leptin administration per se, is the stimulus for SLR up-regulation. Induction of SLR by a 72-h fast has also been reported in humans (19). SLR induction in leptin-treated mice was temporally associated with the time of most rapid weight loss. Some signal during induction in leptin-treated mice was temporally associated by a 72-h fast has also been reported in humans (19). SLR bound Ob-R is independent of leptin action.

The efficiency of Ob-R deletion varies between mice but never modest induction in plasma SLR following leptin treatment. Cre-mediated deletion of Ob-R in hepatocytes, only showed a 10% decrease in Ob-R RNA levels in humans have been shown to be inversely related to the occurrence of obesity. As SLR binding stabilizes leptin and increases its half-life, it may act as a buffer serving to maintain free plasma leptin levels in humans. Some signal during induction in rodents and humans. SLR has been shown to be highly induced during pregnancy, secondary to increased Ob-Re production by the placenta (23). This may represent one means to limit leptin action and increase energy storage during pregnancy, with clear adaptive value. On the other hand, SLR levels in humans have been shown to be inversely related to the measures of adiposity (24). Obese humans may have inappropriately low levels of SLR and consequently be exposed to chronically elevated free leptin. This could be an important factor in the pathogenesis of leptin resistance.

As SLR binding stabilizes leptin and increases its half-life, it is also plausible that SLR facilitates leptin action. In fact, SLR may act as a buffer serving to maintain free plasma leptin levels within a specified range. When plasma leptin levels are low, SLR may potentiate leptin action, and when plasma leptin levels are high, SLR may inhibit leptin action. Such a role has previously been demonstrated for insulin-like growth factor and the family of insulin-like growth factor binding proteins (31). Further studies are currently underway to better define the role of SLR induction in rodents and humans.

The above data clearly establish a role for liver Ob-R in generating plasma SLR. However, the possibility exists that ablation of liver Ob-R would also block leptin signaling in the liver (32), which could affect the release of a hormone/cytokine that regulates Ob-R cleavage or Ob-Re mRNA levels in another tissue. The biochemical events leading to cleavage of the membrane-bound receptor and the regulation of SLR-leptin binding and dissociation remain to be elucidated. Although we have shown that the induction of liver Ob-R RNA is closely associated with increased plasma SLR levels, the increased Ob-R, particularly that of the signaling isofrom Ob-Rb, may be associated with a signaling role within the liver.

These data represent the first demonstration of a role for liver Ob-R in modulating the biological effects of leptin. The induction of Ob-R and plasma SLR levels appears to represent an additional level of control over the physiological actions of leptin. SLR may play an important role in energy homeostasis, and alterations in SLR levels may be relevant to the pathogenesis of leptin resistance and obesity. Further work may point to SLR as a therapeutic target for the treatment of obesity and metabolic disorders.

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