High Circulating Leptin Receptors with Normal Leptin Sensitivity in Liver-specific Insulin Receptor Knock-out (LIRKO) Mice

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Shmuel E. Cohen[‡1], Efi Kokkotou[‡§1,2], Sudha B. Biddinger[‡], Tatsuya Kondo[‡], Rolf Gebhardt[¶], Juergen Kratzsch[‖], Christos S. Mantzoros[§], and C. Ronald Kahn[‡3]

From the [‡]Joslin Diabetes Center, [§]Beth Israel-Deaconess Medical Center and Harvard Medical School, Boston, Massachusetts 02215, the [¶]Institute of Biochemistry, University of Leipzig, D-04103 Leipzig, Germany, and the [‖]Institute of Laboratory Medicine, Clinical Chemistry, and Molecular Diagnostics, University Hospital, D-04103 Leipzig, Germany

Liver-specific insulin receptor knock-out (LIRKO) mice display hyperinsulinemia, abnormal glucose metabolism, and progressive liver dysfunction. In addition, circulating leptin levels appear to be increased more than 10-fold. However, food intake, body weight, and adipose mass are not significantly altered in LIRKO mice compared with wild-type littermates. Using a ligand immunofunctional assay, we found that the apparent increase in circulating leptin in LIRKO mice is because of an 80-fold increased serum level of soluble leptin receptor. Gene expression analysis by microarray and real time PCR reveals the liver as the source of soluble leptin receptor in LIRKO mice, with an increase in expression of the short (Ob-Ra), long (Ob-Rb), and soluble (Ob-Re) forms of the leptin receptor. Direct control of leptin receptor expression by insulin could also be demonstrated in isolated hepatocytes from normal mice. Despite the markedly increased levels of leptin receptor in their circulation, LIRKO mice exhibit normal or even enhanced leptin sensitivity, as assessed by their physiological and molecular responses to exogenous leptin administration and their lower base-line hypothalamic levels of SOCS3 mRNA. Thus, insulin signaling in the liver plays an important role in control of leptin receptor expression and shedding. In the LIRKO mouse, this is lost, leading to markedly increased leptin receptors into the circulation. These high levels of circulating leptin receptor bind leptin and likely alter its clearance, but do not inhibit leptin action and may actually potentiate leptin action. In this manner, insulin signaling in liver plays an important role in leptin homeostasis and fine modulation of leptin action.

Leptin is a major sensor of body fat stores. Normally, as fat mass increases, circulating leptin levels rise and act at the hypothalamus to decrease appetite and increase energy expenditure thus limiting further weight gain (1). Although some rare forms of obesity are caused by leptin or leptin receptor mutations (2, 3), in most humans and rodents with obesity there is leptin resistance, and thus leptin is unable to normally suppress overeating or enhance energy expenditure (4). In obese states, leptin resistance is accompanied by hyperinsulinemia and insulin resistance.

Cross-talk exists between leptin and insulin-regulated pathways at multiple levels. In the brain, both insulin and leptin act to inhibit appetite (1). In pancreatic islets, leptin has a direct inhibitory effect on insulin secretion (5). Additionally, various mechanisms of cross-talk between leptin and insulin signaling in the liver have been described recently (6). At the cellular level, Cohen *et al.* (7) found that leptin attenuated insulin-induced tyrosine phosphorylation of the insulin receptor substrate (IRS)[4]-1 in human hepatoma cells. On the other hand, in rodent hepatoma cells, our laboratory found that insulin-induced association of phosphatidylinositol 3-kinase with IRS-1 was increased after leptin preincubation, whereas association of the p85α regulatory subunit of phosphatidylinositol 3-kinase with IRS-2 was diminished significantly (8), suggesting differential effects of leptin on individual insulin pathways.

We have previously developed a mouse model of hepatic insulin resistance using the Cre-Lox system to inactivate the insulin receptor gene specifically in liver (LIRKO) (9). These mice develop secondary hyperinsulinemia from a combination of increased β cell mass and decreased insulin clearance by the liver. They also display focal dysplasia and hyperplastic nodules in their livers and a 50% reduction in albumin levels, as well as a similar reduction in serum triglycerides and free fatty acids (9, 10), which could be explained, at least in part, by the inability of insulin to promote triglyceride synthesis in liver and by reduced lipolysis in adipose tissue. However, despite their pronounced hyperinsulinemia and insulin resistance, LIRKO mice are not

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[3] To whom correspondence should be addressed: Joslin Diabetes Center, One Joslin Place, Boston, MA 02215. Tel.: 617-732-2635; Fax: 617-732-2593; E-mail: c.ronald.kahn@joslin.harvard.edu.

[4] The abbreviations used are: IRS, insulin receptor substrate; LIRKO, liver insulin receptor knockout; Ob-R, leptin receptor; POMC, proopiomelanocortin; AgRP, agouti-related protein; NPY, neuropeptide Y; SOCS-3, suppressor of cytokine signaling-3; ELISA, enzyme-linked immunosorbent assay; RT, reverse transcription; PBS, phosphate-buffered saline; IRE, insulin-response element; TBP, TATA-binding protein.

obese, thus providing a unique model to study the effect of insulin on leptin homeostasis.

Leptin production, mainly from the adipose tissue, leptin bioavailability, and leptin sensitivity of its target cells represent the main regulators of the actions of leptin under physiological conditions, the last two being mediated by leptin receptors. Leptin receptors (Ob-R) occur in several forms as a result of alternative splicing (11). The long form of the receptor (Ob-Rb) serves in leptin signaling, and its functional inhibition by molecules such as SOCS3 represents a major source of leptin resistance during obesity (12, 13). In addition, there are at least four forms of leptin receptor with transmembrane and short intracellular domains (Ob-Ra, Ob-Rc, Ob-Rd, and Ob-Rf). All isoforms share the ligand-binding domain, but only the long form has the full cytoplasmic domain containing all signal transduction motifs (14). Both the long and the short forms of receptor can also be shed into the circulation by the action of surface proteases (15). Furthermore, alternative splicing creates a secretory (soluble) form of receptor (Ob-Re, also known as sOb-R), which lacks a transmembrane domain, and is directly released into the circulation, whereas by binding leptin, it controls the amount of free leptin and the rate of leptin clearance (16).

In this study using the LIRKO mouse, we demonstrate an important role of insulin signaling in liver in control of hepatic expression of the various isoforms of the leptin receptor gene, leading to increasing secretion and shedding of leptin receptors. This produces high apparent circulating levels of leptin, but it does not inhibit leptin action and may actually sensitize to these effects. Thus, the liver may modulate leptin homeostasis and function in insulin-resistant states.

EXPERIMENTAL PROCEDURES

Mice—LIRKO mice were generated using the Cre/loxP system for site-specific excisional DNA recombination (17) by crossing mice carrying a floxed insulin receptor [$IR^{lox/lox}$] and albumin-Cre transgenic mice heterozygous for the floxed allele $IR^{lox/+}$ as described previously (9). The MIRKO (17) and FIRKO (18) mice were generated in a similar fashion using tissue-specific Cre-transgenics. Other animals described in this study were obtained from The Jackson Laboratories. All protocols for animal care and use were approved by the Animal Care Committee of the Joslin Diabetes Center and were in accordance with National Institutes of Health guidelines. The body composition of 16-week-old LIRKO and control [$IR^{lox/lox}$] mice was measured by Dual-Energy X-ray Absorbiometry (DEXA) using a Lunar PIXImus densitometer (GE Healthcare).

Leptin Treatment—Recombinant mouse leptin was obtained from Dr. A. F. Parlow, Scientific Director, National Hormone and Peptide Program, Harbor-UCLA Medical Center. For the prolonged leptin treatment experiment, 2-month-old male LIRKO and their lox littermates mice ($n = 4–6$/group) were treated with leptin (4 μg/g body weight) or PBS intraperitoneally at 0700 and 1900 daily for 4 days, and sacrificed 14 h after their last leptin injection at 0900 in a randomly fed state. A similar cohort of mice ($n = 4$/group) was used likewise for the short term experiments, with the difference that mice were sacrificed 30 min after their first injection.

Biochemical Measurements—Serum insulin was measured by ELISA (Crystal Chem Inc.). Total serum leptin was measured by ELISA (R & D Systems). sOb-R levels were determined by a ligand immunofunctional assay, as described previously (19). Briefly, the wells of a microtiter plate were coated with the anti-sOb-R IgG and blocked against nonspecific binding with 1% bovine serum albumin in PBS. Twenty microliters of murine rsOb-R (R & D Systems) standards ranging between 10 pM and 1000 nM or serum samples were added to the wells and incubated with an excess of biotinylated leptin in assay buffer (1% goat γ-globulin, 0.1% bovine serum albumin, and 0.05% Tween 20 in PBS) overnight at 4 °C. The complex consisting of sOb-R and biotinylated leptin was detected by europium-labeled streptavidin (PerkinElmer Life Sciences) and the Victor system (PerkinElmer Life Sciences). The resulting signal is based on both leptin binding and immunological recognition of the sOb-R. The assay detected sOb-R in a range between 20.7 pM and 769 nM, and spiking of 3.1 μM leptin yielded a mean recovery of 90.1%. Intra-assay as well as inter-assay coefficients of variation were below 10.7% ($n = 12$) and 12.4% ($n = 10$), respectively. Results of dilution and sOb-R spiking experiments demonstrated a recovery of 103.0 ± 10.7% ($n = 4$) and 94.2 ± 11.9% ($n = 3$), respectively, which is within the expected range for immunoassays. Free leptin index was calculated by dividing the total serum leptin (nanomolar) by the circulating sOb-R (nanomolar) concentration. Western blot analysis of sOb-R in sera of control and LIRKO mice was performed as described previously (20). Five microliters of serum was fractionated under reducing conditions by 6% SDS-PAGE, transferred to nitrocellulose membrane, and detected by the polyclonal antiserum against sOb-R (R & D Systems).

Gene Expression Analysis—Tissues were immediately collected from animals, snap-frozen in liquid nitrogen, and stored at −80 °C. Total RNA was isolated using RNeasy columns (Qiagen), and cDNA was synthesized using the Superscript™ Choice System (Invitrogen) or the Advantage RT for PCR kit (BD Biosciences) and $(dT)_{18}$ for priming. Biotinylated cRNA was prepared using the Bioarray High Yield RNA transcript labeling kit (Enzo Biochem). Murine 74Av2 chips (Affymetrix) were hybridized in triplicate with RNA pooled from 3 to 4 animals in the Affymetrix Core Lab at the Joslin Diabetes Center. The Affymetrix microarray analysis was facilitated by the Affymetrix 5.0 software, and the filters of significance applied have been described elsewhere (21). Ob-Ra, Ob-Rb, or Ob-Re cDNA was amplified in duplicate in a 40-μl real time PCR (SYBRGreen, PE Biosystems), in an ABI Prism 7700 sequence detection system (PE Biosystems). The primer sets were specific for each receptor isoform, and sense and antisense primers were aligned to different exons to avoid genomic DNA amplification. Primer3 software facilitated primer design. The following primer sets were used and presented from 5′ to 3′: Ob-Ra and Ob-Rb (common) sense, aatgacgcagggctgtatgt; Ob-Ra antisense (specific), atggactgttgggaagttgg; Ob-Rb antisense (specific), tcaggctccagaagaagagg; Ob-Re sense, tgaagatgatggaatgaagtgg; Ob-Re antisense, agttggcatccttatttgaggt; leptin sense, caagcagtgcctatccaga; leptin antisense, aagcccaggaatgaagtcca; SOCS3 sense, gggagccccttttgtagactt; SOCS3 antisense, ctggtactcgcttttggagc; TATA-binding protein

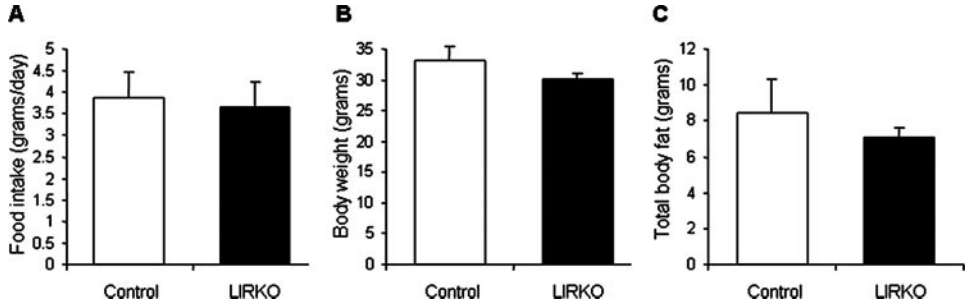

FIGURE 1. **LIRKO mice are not obese.** *A,* daily food intake; *B,* body weight; and *C,* fat mass in 16-week-old male LIRKO and control mice.

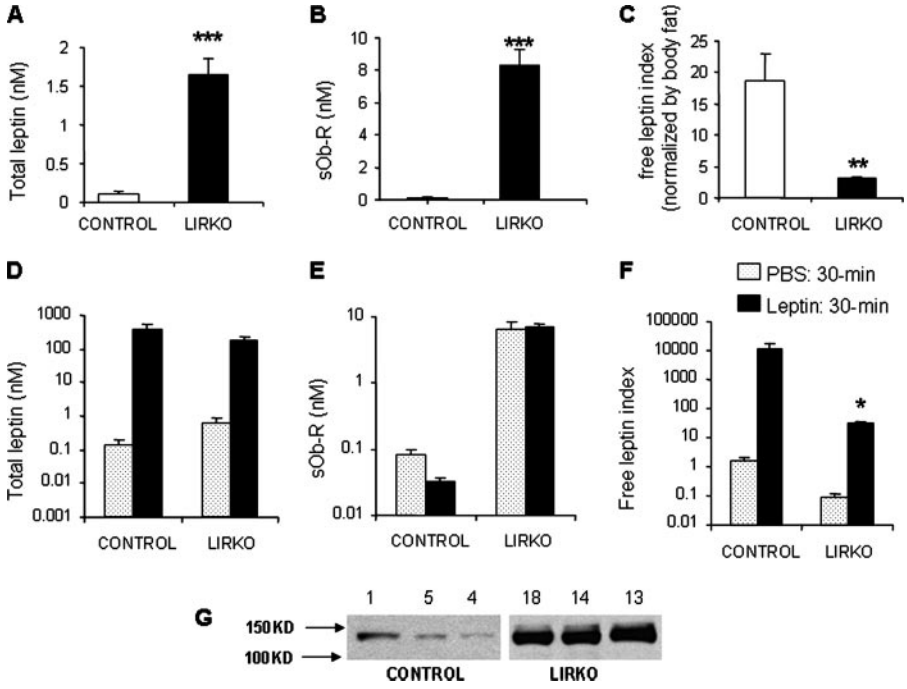

FIGURE 2. **Apparent hyperleptinemia and increased circulating levels of soluble leptin receptor in LIRKO mice.** LIRKO mice are represented by *dark bars* and their controls by *open bars* (n = 15–17/group). *A,* serum total leptin was measured using an ELISA. *B,* sOb-R was measured by a ligand immunofunctional assay; *C,* free leptin index was calculated from the above and normalized by grams of total body fat. LIRKO mice had ~10-fold increase (***, $p < 0.001$) in serum total leptin and an ~60-fold increase in circulating sOb-R (***, $p < 0.001$), resulting in an ~6-fold decrease in their free serum leptin (**, $p < 0.01$). *D–F* show the same parameters as the above, in PBS (*gray bars*) or leptin-treated (*dark bars*) control or LIRKO mice. Serum was collected 30 min after the leptin injection. Leptin treatment resulted in an ~3000-fold increase in total serum leptin levels in control mice and ~300-fold in LIRKO mice (*D*) but had no effect on the sOb-R levels (*E*). Still, free leptin after leptin injection (*F*) was significantly lower in LIRKO mice compared with control mice (*, $p < 0.05$). *G,* Western blot for sOb-R in sera of control (mouse *1, 5,* and *4*) and LIRKO mice (mouse *18, 14,* and *13*). Serum proteins were fractionated in an SDS-polyacrylamide gel, transferred to a solid support, and incubated with an antibody against sOb-R.

(TBP) sense, acccttcaccaatgactcctatg; TBP antisense, tgactgcagcaaatcgcttgg. Amplification of NPY, POMC, and AgRP has been described previously (22). Results are expressed as arbitrary mRNA units normalized by TBP expression.

Isolation and Treatment of Primary Hepatocytes—Hepatocytes from livers of male C57BL/6 mice were prepared by collagenase perfusion as described previously for rat hepatocytes (23). Viability of cells, assessed by trypan blue exclusion, was >90%. Hepatocytes were cultured on collagen-coated 6-well Petri dishes (Costar) in 1 ml of Williams medium E supplemented with 10% bovine calf serum at a density of 125,000 cells/cm^2, as described previously (24). After an initial period of 4 h, medium and unattached cells were removed, and the cells transferred to serum-free Williams medium E and incubated for 20 h with or without hormones (0.1 μM insulin, 160 ng/ml leptin). Thereafter, cells were harvested, and total RNA was prepared as described above. Each experimental condition was run three times.

Statistical Analysis—Statistical analysis of the data was performed with SigmaPlot 2000 for Windows Version 6.00 (SPSS) and StatView (Abacus Concepts) using a two-tailed unpaired t test and analysis of variance factorial with Bonferroni/Dunn correction for multiple comparisons. A probability value $p <$ 0.05 was considered significant. Results are expressed as mean ± S.E. unless otherwise indicated.

RESULTS

LIRKO Mice Are Characterized by Hyperleptinemia in the Absence of Obesity—As noted previously, LIRKO mice are of normal body weight (30.2 ± 0.9 *versus* 33.2 ± 2.3 g) and have similar levels of total body fat as assessed by DEXA (7.1 ± 0.5 *versus* 8.4 ± 1.9 g) and food intake (3.64 ± 0.6 *versus* 3.86 ± 0.6 g/day) as their 16-week-old male controls (Fig. 1). Despite this, total serum leptin levels as determined by ELISA were more than 10-fold elevated in LIRKO mice compared with controls (1.6 ± 0.2 *versus* 0.15 ± 0.03 nM, respectively; $p <$ 0.001) (Fig. 2*A*), and this was true in both males and females and at ages 1 to 4 months (data not shown).

The presence of high circulating leptin levels with normal weight in LIRKO mice suggested the possibility of some factor(s) that might modify the action of leptin, and one consideration in this regard would be increased levels of circulating leptin receptor. Indeed, measurement of circulating sOb-R by ligand immunofunctional assay revealed a >80-fold increase in the serum of LIRKO mice as compared with controls (8.3 ± 0.9 *versus* 0.1 ± 0.01 nM; $p <$ 0.001) (Fig. 2*B*). As a result, calculated free serum leptin index, normalized by body fat, was 6-fold lower in LIRKO mice than in controls (3.0 ± 0.3 *versus* 18.8 ± 4.2, respectively; $p <$ 0.01) (Fig. 2*C*). Indeed, even after acute IP leptin injection, although levels of total circulating leptin markedly increased in both LIRKO and control mice (187 ± 37 *versus* 373 ± 175 nM; respectively) (Fig. 2*D*) and sOb-R levels remained unaffected by leptin treatment (6.7 ± 1.0 nM *versus*

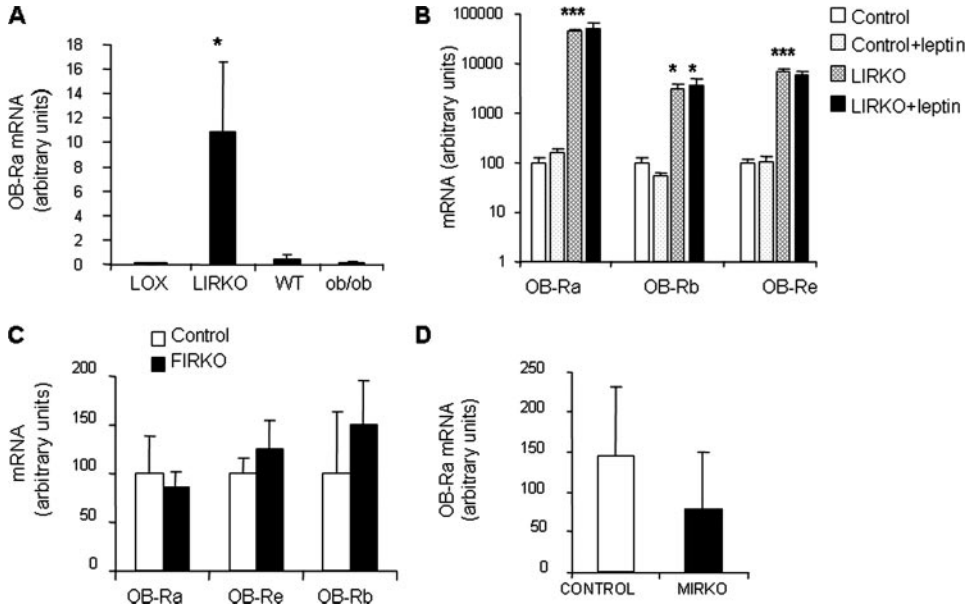

FIGURE 3. **Leptin receptor mRNA expression is dramatically up-regulated in the liver of LIRKO mice.** *A,* Affymetrix microarray analysis of liver samples shows that LIRKO mice express higher (*, $p < 0.05$) levels of Ob-Ra than their controls (wild type (*WT*) and lox/lox) or other insulin-resistant (ob/ob) mouse models. Data are expressed as the mean of three microarray chips (representing 3–4 mice) ± S.D. *B,* real time PCR analysis of various leptin receptor isoforms in liver of control and LIRKO mice, treated with either PBS (*gray bars*) or leptin (*dark bars*) for 4 days. LIRKO mice had significantly (**, $p < 0.01$) up-regulated levels of all the leptin receptor isoforms that did not changed by leptin treatment. *C,* white adipose tissue expression of the three leptin receptor isoforms was not up-regulated in FIRKO mice (*dark bars*) compared with their controls (*open bars*), as evaluated by quantitative real time PCR. *D,* Ob-Ra mRNA expression is not up-regulated in the muscle of MIRCO mice, as evaluated by Affymetrix microarray analysis.

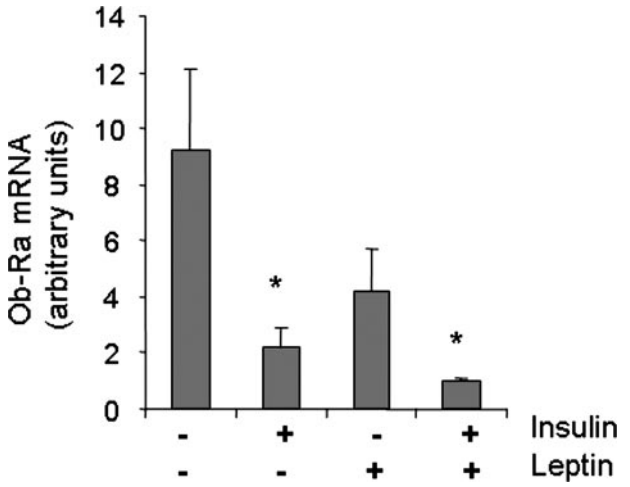

FIGURE 4. **Insulin treatment inhibits Ob-Ra mRNA expression *in vitro*.** Hepatocytes were isolated from C57BL/6J mice and placed in culture. Cells were treated with 0.1 μM of insulin, 160 ng/ml leptin, or combination for 20 h, and Ob-Ra mRNA expression was evaluated by real time RT-PCR. *, $p < 0.05$.

0.04 ± 0 nM) (Fig. 2*E*), the high level of circulating receptor resulted in markedly lower free leptin levels in LIRKO mice than in control mice (30.4 ± 8.5 *versus* 12,297 ± 6,213 respectively, $p < 0.05$) (Fig. 2*F*), confirming the vast excess of sOb-R in this mouse model of hepatic insulin resistance (Fig. 2, *B* and *E*).

LIRKO Mice Have Increased Liver Production of Leptin Receptors—The source of the increased circulating sOb-R in LIRKO mice was the liver. Thus, on Affymetrix microarray analysis, Ob-Ra mRNA expression was >400-fold higher in the LIRKO liver than in controls ($p < 0.05$) (Fig. 3*A*). The up-regulation of leptin receptor in liver of LIRKO mice was confirmed by real time RT-PCR using isoform-specific primers for Ob-Ra, Ob-Rb, and Ob-Re. Indeed, there was a marked up-regulation of all three leptin receptor isoforms in the liver of LIRKO mice (Ob-Ra 467.6 ± 31.9-fold, $p < 0.001$; Ob-Rb 31.5 ± 6.3-fold, $p < 0.01$; Ob-Re 69.1 ± 11.9-fold, $p < 0.001$) (Fig. 3*B*). The increase in leptin receptor expression appeared to be a unique feature of the liver of the LIRKO mouse, because there was no alteration in leptin receptor expression in epididymal fat, skeletal muscle, and kidney of LIRKO mice (data not shown) or in epididymal fat of fat-specific insulin receptor knock-out (FIRKO) mice (18) (Fig. 3*C*) or skeletal muscle of muscle-specific insulin receptor knock-out mice (MIRKO) (25) (Fig. 3*D*), as assessed by real time PCR (LIRKO and FIRKO) or Affymetrix microarray analysis (MIRKO).

Insulin Treatment Down-regulates Ob-Ra Expression in Isolated Hepatocytes—The increased Ob-Ra expression in the liver of the LIRKO mice could be due to a direct effect of tissue-specific loss of insulin signaling or secondary to other changes in the liver previously described in these mice (9). To gain some insight to the mechanism, we treated normal isolated mouse hepatocytes with insulin and leptin, as a positive control (26). We found that 20 h of insulin treatment resulted in ~75% down-regulation of Ob-Ra mRNA expression (2.1 ± 0.7 arbitrary units) compared with control (9.2 ± 2.9 arbitrary units, $p < 0.05$) (Fig. 4).

Leptin Sensitivity Is Not Compromised in LIRKO Mice—Although there is some debate, it has been suggested that high levels of circulating leptin receptor or high levels of circulating leptin might lead to leptin resistance. To determine whether the LIRKO mice were leptin-resistant, we injected 2-month old male LIRKO and control mice twice a day intraperitoneally with 4 μg/g body weight of recombinant mouse leptin for 4 days. Following leptin treatment, body weight of both LIRKO and control mice decreased significantly over the 4 days, with the fall being slightly, but not significantly, greater on day 1 in the LIRKO mice *versus* the controls (Fig. 5*A*). Assessment of food intake revealed that LIRKO mice actually had a more dramatic and rapid response to exogenous leptin than controls. Thus, in LIRKO mice there was a 50% reduction in food intake on day 1 of treatment that persisted throughout the 4 days of leptin injections, whereas leptin-treated control mice decreased food intake slowly over 4 days to eventually approach the same reduction on day 4 that the LIRKO mice achieved on day 1 (Fig. 5*B*). Moreover, serum insulin levels, which were

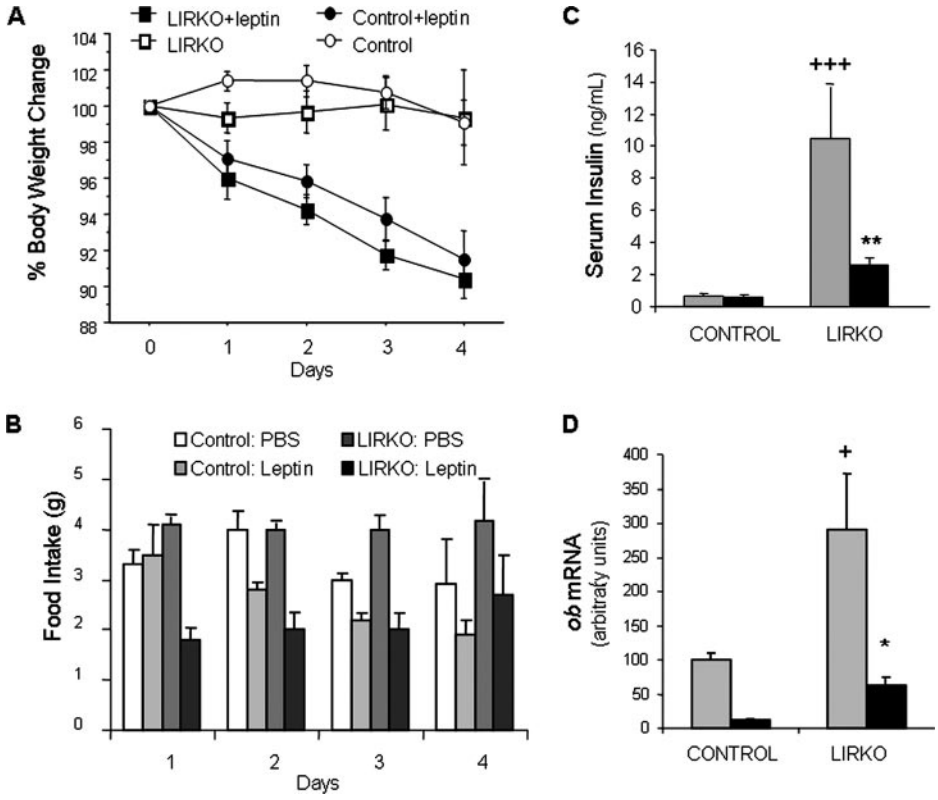

FIGURE 5. **LIRKO mice respond appropriately to exogenous leptin.** Control (*circles*) or LIRKO mice (*squares*) (n = 4–6/group) were treated with either leptin (4 μg/g body weight) (*filled symbols*) or PBS (*open symbols*) twice a day for 4 days, and their weight was monitored daily. *A,* in the graph presented, the percentage change in body weight because of leptin treatment was similar among LIRKO and control mice. *B,* daily food intake was monitored in parallel in the same mice. LIRKO mice reached their maximum reduction in food intake within the 1st day of leptin treatment and the control mice on the 3rd day. *C,* LIRKO mice were found to be severely hyperinsulinemic (+++, $p < 0.001$) when compared with control mice. Leptin treatment (*dark bars*) compared with PBS treatment (*gray bars*) resulted in a significant drop in the serum insulin levels only in LIRKO mice (**, $p < 0.01$). *D,* white adipose tissue *ob* gene expression, as evaluated by real time RT-PCR, is higher in LIRKO mice compared with control mice (+, $p < 0.05$), but it decreased more (*, $p < 0.05$) in absolute value with leptin treatment in LIRKO mice than in control mice (n = 4–6/group). *Gray bars* = mice treated with PBS; *black bars* = mice treated with leptin.

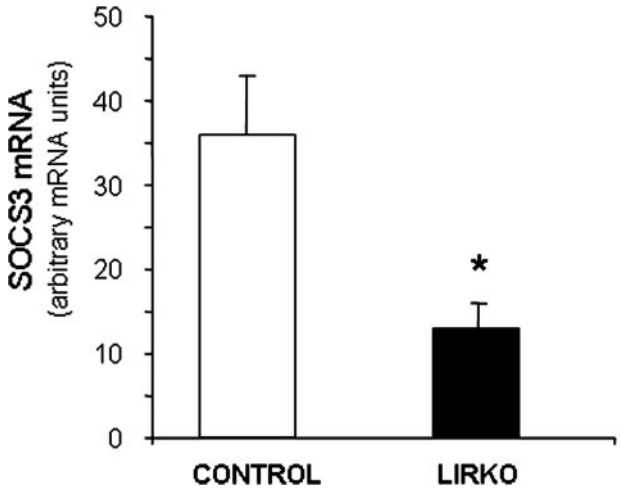

FIGURE 6. **Hypothalamic SOCS3 expression in LIRKO mice.** Base-line SOCS3 mRNA expression in the hypothalamus, as evaluated by real time RT-PCR, was found to be lower in LIRKO mice (*dark bars*) compared with wild-type mice (*open bars*) (*, $p < 0.05$), indicating increased hypothalamic leptin sensitivity in the LIRKO mice.

initially 18-fold increased ($p < 0.001$) in the LIRKO mice, were decreased by 75% with leptin treatment ($p < 0.01$), although there was no effect in control mice (Fig. 5*C*). Likewise, the ~2.5-fold elevated leptin gene expression in WAT of LIRKO mice compared with control mice ($p < 0.05$) was also suppressed following leptin treatment more in LIRKO mice than controls ($p < 0.05$) (Fig. 5*D*). This is consistent with a previous study from our group in which we administered continuous leptin infusion via an osmotic pump (24 μg/day) in 12-week-old male LIRKO and control mice for 4 days, which also resulted in similar weight loss, reduction in food intake, and drop in serum insulin levels in the two genotypes (10).

Hypothalamic Leptin Sensitivity in LIRKO Mice—SOCS3 mRNA expression in the hypothalamus has been regarded as another potential indicator and mechanism of central leptin resistance (27). Interestingly, SOCS3 levels were ~50% lower in LIRKO mice than in controls ($p < 0.05$) (Fig. 6). We also measured mRNA expression of several neuropeptides known to be targets of leptin in the hypothalamus both in the basal state and after 4 days of leptin (or PBS) treatment. This study revealed that both genotypes had similar neuropeptide levels at base line and both responded with appropriate down-regulation of the orexigenic peptides NPY (Fig. 7*A*) and AgRP (Fig. 7*B*) and appropriate up-regulation of the anorexigenic peptide precursor POMC (Fig. 7*C*). Taken together, these data indicate an increase, rather than a decrease, in leptin sensitivity in the LIRKO mice and are compatible with the suppression of food intake in response to exogenous leptin administration presented in Fig. 5*B*.

DISCUSSION

Leptin resistance is a common component of obesity in humans and rodents (28), but the role of circulating leptin receptor as a regulator of leptin action has just started to emerge. The current study of a mouse model with high levels of soluble leptin receptor resulting from genetic ablation of insulin signaling in the liver (9) has allowed us to demonstrate that even in the presence of markedly elevated levels of circulating leptin receptor both from shed and secreted isoforms, sensitivity to both endogenous and exogenous leptin appears to be completely normal. This conclusion is based on reduction of food intake and significant weight loss in LIRKO mice treated with leptin, to a degree similar to that observed in similarly treated wild-type mice (this study and see Ref. 10). In addition, several physiological targets of leptin action (29), such as *ob* gene expression, insulin, and other appetite-regulating neu-

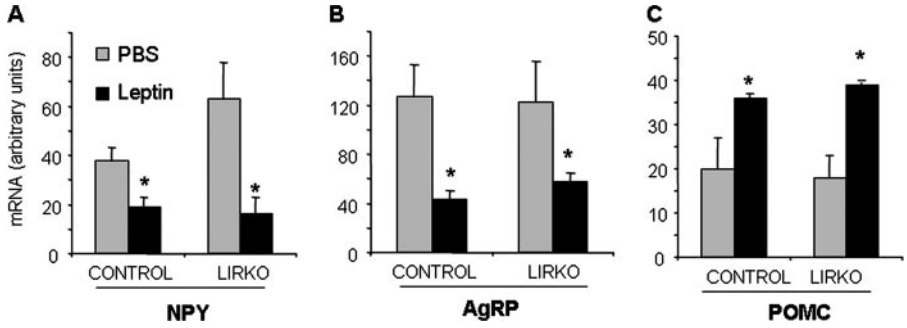

FIGURE 7. **Hypothalamic appetite regulating neuropeptide responses in LIRKO mice are not compromised.** After 4 days of leptin (*dark bars*) or PBS (*gray bars*) treatment, hypothalamic expression of NPY, AgRP, and POMC was evaluated in LIRKO and control mice by real time RT-PCR. Compared with PBS treatment, leptin treatment resulted in a significant down-regulation of NPY (*A*) and AgRP (*B*) and up-regulation of POMC (*C*) (*, $p < 0.05$) to a similar degree in both genotypes.

ropeptides in the hypothalamus also respond appropriately to exogenous leptin administration in LIRKO mice. Moreover, hypothalamic SOCS3 expression, a well accepted marker of central leptin resistance (13, 27), is also lower in LIRKO mice. Thus, high levels of circulating leptin receptors (and the associated hyperleptinemia) do not cause leptin resistance; rather it appears that free circulating leptin may be the better determinant of leptin sensitivity. Such a statement is further corroborated by *in vitro* experiments showing that increased sOb-R does not directly interfere with leptin signaling at the cellular level but can regulate the availability of free leptin and its clearance (16, 30). Thus, when cells bearing Ob-Rb are incubated with leptin in the presence of high levels of sOb-R, leptin signaling can be inhibited, but this can be easily reversed by increasing the concentration of free leptin, despite the high levels of sOb-R in the media. Interestingly, and in further support of increased leptin sensitivity associated with high sOb-R levels, overexpression of sOb-R in *ob/ob* mice actually enhances, rather than suppresses, the effects of leptin treatment (31). In analogy to the LIRKO mice, humans that carry a mutant allele of the leptin receptor gene, which results in enhanced shedding of the extracellular leptin binding domain of the receptor, exhibit very high levels of total leptin but normal levels of free leptin and are not obese (32, 33). Typically, in lean subjects the majority of circulating leptin is in its bound form, whereas during obesity serum levels of soluble leptin receptor decrease resulting in more free leptin available to signal in its target cells; conversely, weight loss has been associated with an increase in circulating leptin receptors in both humans and rodents (34–37).

This study of the LIRKO mouse also revealed important aspects of the role of liver in leptin physiology. First, our study demonstrates that the liver may be a site of significant sOb-R production, at least in some pathological states. Indeed, serum leptin levels are higher in patients with liver cirrhosis, as are the levels of leptin receptor mRNA expression in liver, although the levels of circulating sOb-R in these studies have not been directly assessed (38). Second, in liver, leptin receptor expression is clearly under the control of insulin, as evidenced by its markedly elevated levels in the LIRKO mouse, as well as by the significant insulin-mediated down-regulation of Ob-Ra in isolated hepatocytes as presented here (Fig. 4). In fact, in states of low insulin levels, as in men subjected to a 72-h fast (39), in women with anorexia nervosa (40, 41), and in children with type 1 diabetes (42), serum levels of sOb-R are increased.

Our finding of leptin receptor mRNA up-regulation in the liver as a result of the lack of insulin signaling in this organ has been recently recapitulated in mice lacking IRS signaling in liver (43). Although the transcriptional regulators of leptin receptor expression and of specific splice products are largely unknown, a preliminary sequence analysis of the 5′-untranslated region of the leptin receptor gene using the consensus insulin-response elements (IRE) core sequence (44–46) T(G/A)TTT(T/G)(G/T) revealed multiple putative IREs, including one located within the first 2 kb. In addition to leptin receptor, hepatic expression of many genes that regulate metabolism or growth is under the control of insulin signaling (45, 47). In the LIRKO mouse in particular, the inability of insulin to signal through functional IREs in the promoter of peroxisome proliferator-activated receptor-γ coactivator (PGC)-1, phosphoenolpyruvate carboxykinase, and tyrosine aminotransferase, glucose-6-phosphatase results in significant liver up-regulation of these genes, which might account, at least in part, for their hyperglycemia, hyperlipidemia, and insulin resistance (9, 48).

In aggregate, the current and previous studies clearly indicate that in states of increased sOB-R levels associated with increased free leptin, there is improved, rather than decreased, leptin sensitivity. Moreover, the study of the LIRKO mouse revealed the importance of the liver in the regulation of leptin bioavailability.

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High Circulating Leptin Receptors with Normal Leptin Sensitivity in Liver-specific Insulin Receptor Knock-out (LIRKO) Mice

Shmuel E. Cohen, Efi Kokkotou, Sudha B. Biddinger, Tatsuya Kondo, Rolf Gebhardt, Juergen Kratzsch, Christos S. Mantzoros and C. Ronald Kahn

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