Partial leptin deficiency favors diet-induced obesity and related metabolic disorders in mice

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Begriche K, Lettérón P, Abbey-Toby A, Vadrot N, Robin M-A, Bado A, Pessayre D, Fromenty B. Partial leptin deficiency favors diet-induced obesity and related metabolic disorders in mice. Am J Physiol Endocrinol Metab 294: E939–E951, 2008.—Partial leptin deficiency is not uncommon in the general population. We hypothesized that leptin insufficiency could favor obesity, nonalcoholic steatohepatitis (NASH), and other metabolic abnormalities, particularly under high calorie intake. Thus, mice partially deficient in leptin (ob/+ and their wild-type (+/+) littersmates were fed for 4 mo with a standard-calorie (SC) or a high-calorie (HC) diet. Some ob/+ mice fed the HC diet were also treated weekly with leptin. Our results showed that, when fed the SC diet, ob/+ mice did not present significant metabolic abnormalities except for elevated levels of plasma adiponectin. Under high-fat feeding, increased body fat mass, hepatic steatosis, higher plasma total cholesterol, and glucose intolerance were observed in +/+ mice, and these abnormalities were further enhanced in ob/+ mice. Furthermore, some metabolic disturbances, such as blunted plasma levels of leptin and adiponectin, reduced UCP1 expression in brown adipose tissue, increased plasma liver enzymes, β-hydroxybutyrate and triglycerides, and slight insulin resistance, were observed only in ob/+ mice fed the HC diet. Whereas de novo fatty acid synthesis in liver was decreased in +/+ mice fed the HC diet, it was disinhibited in ob/+ mice along with the restoration of the expression of several lipogenic genes. Enhanced expression of several genes involved in fatty acid oxidation was also observed only in ob/+ animals. Leptin supplementation alleviated most of the metabolic abnormalities observed in ob/+ fed the HC diet. Hence, leptin insufficiency could increase the risk of obesity, NASH, glucose intolerance, and hyperlipidemia in a context of calorie overconsumption.

METHODS

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kcal/kg), 35% simple carbohydrates (1,400 kcal/kg, mainly sucrose) and 18% protein (700 kcal/kg). Food consumption was measured with both diets. To measure food consumption with the HC diet (which presents a pastry consistency), it was manually transformed into pellets that were frozen until their utilization. Food consumption was then assessed every day for 6 consecutive wk.

In another experiment, leptin was administered in ob/ob mice fed the HC diet. In these investigations, three groups of mice were studied for 4 mo, namely +/+ mice fed the HC diet and ob/+ mice fed the same diet and treated or not with leptin. Recombinant murine leptin [50 μg dissolved in 250 μl of phosphate-buffered saline (PBS)] purchased from R&D (Lille, France) was injected intraperitoneally once/wk to leptin-supplemented mice, whereas the same volume of PBS was administered to the other animals. In this experiment, all mice were killed 18 h after the last injection of leptin. Preliminary investigations in six young ob/+ mice fed the SC diet showed that plasma leptin concentrations assessed 1 and 18 h after a single injection were higher (1.225 ± 106 and 4.83 ± 0.54, respectively) compared with basal levels (3.31 ± 0.48 ng/ml). The mean daily food intake in mice treated with leptin was unchanged during the first 6 wk of supplementation, although the food consumption assessed the day after each injection was significantly reduced by 20%. Thus, leptin injections induced cycles of transient hypophagia followed by recovery of food intake. All experiments were performed in agreement with national guidelines for the proper use of animals in biomedical research. Moreover, our investigations were performed in a laboratory accredited by the French Direction des Services Vétérinaires (accreditation n°B 75-18-02) and with the approval of the French National Medical Research Institute.

**Body composition and blood and tissue sampling.** Fat mass and lean mass (the latter representing water and proteins) were determined by dual-energy X-ray absorptiometry (DEXA), using a Piximus apparatus (Lunar, Madison, WI) as described previously (20). Fed mice were first studied on the day before the onset of the investigations and then at the end of the 4-mo experiments. This allowed us to determine changes from baseline in each animal.

Unless otherwise indicated, blood was drawn from the retroorbital sinus with heparinized capillary Pasteur pipettes. Blood was collected either in the postabsorptive state (referred to as the fed state) or after an overnight period of fast (referred to as the fasted state). In some experiments, blood was collected in +/+ and ob/+ mice before the 4-mo investigation. After sampling, blood was immediately put on ice and subsequently centrifuged in a refrigerated table-top centrifuge. Plasma was then assessed every day for 6 consecutive wk.

Inflammatory infiltrates (black arrows) are made of lymphocytes, whereas the blue arrows show hepatocytes dying through necrosis.

**Liver histology and in situ detection of apoptosis.** To evaluate necroinflammation and fibrosis, liver fragments from fed animals were fixed in 10% neutral formalin and embedded in paraffin. Next, 5-μm sections were cut and then stained with specific dyes. Examination of the sections was performed by an experienced pathologist (A. Abbey-Toby) without knowledge of the treatment. Necroinflammation was estimated after hematoxylin-eosin staining on 10 different fields at ×200 magnification and semiquantified as 0 (no necroinflammation), 1 (mild necroinflammation), and 2 (moderate necroinflammation), depending of the number and the size of the inflammatory infiltrates (Fig. 1). Portal and perisinusoidal fibrosis were evaluated thanks to Masson’s trichrome and picro-Sirius red staining, respectively. For the detection of neutral lipids, liver cryosections were stained with Oil Red O. Steatosis, evaluated as the percentage of hepatocytes containing vacuoles of fat, was assessed on 10 different fields at ×200 magnification (Fig. 1). In situ detection of apoptosis was performed with the terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling (TUNEL) assay using the TACS TdT kit (R&D Systems, Abdingdon, UK) as described previously (33).

**Plasma studies and assessment of leptin in eWAT.** Plasma triglycerides, glucose, ALT, LDH, total cholesterol, iron, ferritin, β-hydroxybutyrate, plasma nonesterified fatty acids, and total antioxidant status (expressed as Trolox equivalents) were measured on an automatic analyzer (Olympus AU400). Triglycerides, glucose, alanine aminotransferase (ALT), lactate dehydrogenase (LDH), total cholesterol, iron, and ferritin were measured with commercial kits (OSR6133, OSR6121, OSR6107, OSR6126, OSR6116, OSR6186, and OSR61138, respectively) from Olympus Diagnostic (Rungis, France), whereas β-hydroxybutyrate, nonesterified fatty acids, and total antioxidant status were measured with commercial kits (RB1007, FA115, and NX2332, respectively) from RANDOX Diagnostic.
(Montpellier, France). Insulin, leptin, and adiponectin were measured using double-antibody RIA kits (RI-13K, ML-82K, and MADP-60HK, respectively) purchased from Linco Research (St. Charles, MO). Glucagon-like peptide-1 (GLP-1) was assessed with the GLP-1-(7-36) active ELISA kit purchased from Linco Research. TNFα was determined as the KRC 3012 ELISA kit from Biosource International (Camarillo, CA).

To measure leptin in adipose tissue, an eWAT fragment was homogenized in Krebs-Ringer buffer (100 mg/ml) with a protease inhibitor cocktail (1 μM/ml Sigma-Aldrich, Saint-Quentin Fallavier, France) as described previously (20). After centrifugation (10 min at 10,000 g), the infranatant was used to measure leptin with a mouse leptin ELISA kit (Crystal Chem, Downers Grove, IL).

Lipids and triglycerides, de novo fatty acid synthesis, and microsomal triglyceride transfer protein activity in the liver. Hepatic total lipids and triglycerides were measured in fed mice as described previously (33). De novo fatty acid synthesis in liver was assessed in fed mice by using the method previously described by Stansbie et al. (40). Briefly, 150 μCi of °H2O was injected intraperitoneally into mice in the fasted absorptive state. Two hours later, liver was quickly removed to extract fatty acids (40). After counting the radioactivity, mice in the postabsorptive state. Two hours later, liver was quickly removed to extract fatty acids (40). After counting the radioactivity, the rate of de novo fatty acid synthesis was calculated as micromoles of °H2O incorporated into fatty acids per hour and per gram of liver. To determine the [3H]H2O-specific activity, blood was also drawn before liver removal and centrifuged to determine the disintegrations per minute by counting 10 μl of plasma. The specific activity of tritiated water was then determined for each mouse by dividing the disintegrations per minute measured in plasma by the micromoles of °H2O.

Glucone and insulin tolerance tests. Intraperitoneal glucose tolerance test (IPGTT) was performed in mice after a 12-h overnight fast. At 10:00 AM, 0.6 g/kg body wt was injected intraperitoneally into mice and blood was collected by tail bleeding at 0, 15, 30, 45, 60, 90, and 120 min for measurement of blood glucose by using One-touch Accu-Check Glucometer (Roche, Paris, France). In one IPGTT experiment, a small volume of blood was also drawn from the retroorbital sinus at 0, 30, 60, 90, and 120 min for subsequent determination of leptin in adipose tissue, an eWAT fragment was homogenized in Krebs-Ringer buffer (100 mg/ml) with a protease inhibitor cocktail (1 μM/ml Sigma-Aldrich, Saint-Quentin Fallavier, France) as described previously (20). After centrifugation (10 min at 10,000 g), the infranatant was used to measure leptin with a mouse leptin ELISA kit (Crystal Chem, Downers Grove, IL).

DNA isolation and real-time quantitative PCR analysis. Total hepatic RNA was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction. Total RNA from iBAT and eWAT was extracted using the Lupid RNaseasy kit (Qiagen, Courtabouef, France). RNA integrity was assessed with the RNA 6000 Nano LabChip kit (Agilent, Waldbronn, Germany). Real-time quantitative PCR was subsequently performed on selected genes expressed in liver, iBAT, and eWAT (Table 1). To this end, reverse transcription was performed with 2 μg of total RNA in a reaction buffer composed of 20 mM Tris·HCl (pH 8.3), 375 mM KCl, 15 mM MgCl2, 10 mM dithiothreitol, 0.5 mM of each deoxynucleoside triphosphate, 250 ng of random primers, 2 U RNase inhibitor, and 10 U Moloney murine leukaemia virus reverse transcriptase. The reaction was carried out at 37°C for 50 min, and the mixture was then heated at 70°C for 15 min. Real-time quantitative PCR was subsequently performed on an aliquot (5 μl) of the reverse transcription reaction with 0.25 μM of each primer (Table 1) and 10 μl of Master SYBR Green mix (Sigma-Aldrich) in a Chromo IV light cycler apparatus (Bio-Rad, Marnes-La-Coquette, France). The PCR conditions were one cycle at 94°C for 3 min followed by 40 cycles at 94°C for 30 s and 60°C for 1 min. Amplification of specific transcripts was confirmed by melting curve profiles generated at the end of each run. Moreover, PCR specificity was further ascertained with an agarose gel electrophoresis by checking the length of the PCR products. Expression of the mouse ribosomal protein S6 was used as reference, and the 2−ΔΔCt method was employed to express the relative expression of each selected gene.

Western blot analysis. Frozen liver fragments (ca. 100 mg) were homogenized in a PBS solution containing 0.1% Triton and protease inhibitors. Homogenates were then centrifuged at 4,500 g at 4°C to remove tissue debris. Protein content was measured in the supernatants by using the Lowry assay. To assess the hepatic expression of fatty acid synthase (FAS), total and phosphorylated acetyl-CoA carboxylase (ACC and phospho-ACC, respectively), manganese superoxide dismutase (MnSOD), and cytochrome P450 2E1 (CYP2E1) proteins (ca. 50 μg) underwent SDS-polyacrylamide electrophoresis (8% polyacrylamide for FAS, phospho-ACC, and ACC and 12% for MnSOD and CYP2E1) transfer to nitrocellulose membrane (Hybond ECL; Amersham Biosciences) and immunoblotting with rabbit polyclonal antibodies against FAS (Santa Cruz Biotechnology, Santa Cruz, CA), ACC and phospho-ACC (Upstate, Lake Placid, NY), MnSOD (Stressgen, Ann Arbor, MI), and CYP2E1 (Oxford Biomedical Research, Oxford, MI). Blots were incubated with appropriate secondary antibodies, and protein bands were revealed by enhanced chemiluminescence (Amersham Pharmacia, Orsay, France). To normalize protein loadings, blots were stripped and incubated with monoclonal mouse antibodies against β-actin (Sigma-Aldrich). Protein bands were quantified using a Helwett Packard Scanjet 4570c scanning unit and ImageMaster1D software (Pharmacia Biotech).

Glutathione levels and aconitase activity in liver. Reduced glutathione (GSH) levels were determined by a method adapted from Griffith, as previously described (33). To assess hepatic aconitase activity, frozen liver fragments (ca. 20 mg) were homogenized in 500 μl of buffer containing 50 mM Tris·HCl, pH 7.4, 0.2 mM sodium citrate, and 0.05 mM MgCl2. Homogenates were then centrifuged at 800 g at 4°C for 10 min, and supernatants were then sonicated for 20 s. Aconitase activity was subsequently assessed on 200-μg proteins in the presence of 1 mM sodium citrate, 1 mM NADP+, and 2 U isocitrate dehydrogenase. Samples were preincubated at 37°C for 5 min, and aconitase activity (expressed as nmol of generated NADPH·min−1·mg protein−1) was then assessed from the increased absorption measured at 340 nm for 5 min.

Statistical analyses. Data are presented as means ± SE. When four groups were compared, two-way analysis of variance (ANOVA) with the factors of genotype (+/+ or ob+/+) and diet (SC or HC) was performed to assess statistical significances. When the ANOVA indicated a significant interaction between factors, individual means were compared with least significant difference (LSD) post hoc test. When three groups were compared, statistical analysis was performed by one-way ANOVA followed by an LSD post hoc test. Because the different parameters investigated were considered to be relatively dependent on each other, corrections for multiple comparisons were not performed. In experiments with only two sets of data, the Student’s t-test was used.

RESULTS

Food consumption, caloric intake, body fat mass, and lean mass. The daily food consumption was 4.22 ± 0.06 and 4.07 ± 0.05 g/animal, respectively, in +/+ and ob/+ mice fed the SC diet and 2.43 ± 0.05 and 2.25 ± 0.06 g/animal, respectively, in +/+ and ob/+ mice fed the HC diet. Consequently, the daily caloric intake was 11.9 ± 0.2 and 11.5 ± 0.1 kcal/animal, respectively, in +/+ and ob/+ mice fed the SC diet and 12.9 ± 0.3 and 12.0 ± 0.3 kcal/animal, respectively, in +/+ and ob/+ mice fed the HC diet (P < 0.05 for the diet factor, 2-way...
ANOVA). Thus, although food consumption was reduced in +/+ and ob/+ mice fed the HC diet compared with the SC diet, the daily caloric intake was slightly, but significantly, augmented in HC animals, with no difference between +/+ and ob/+ mice. Initial body fat mass was significantly increased in ob/+ mice (Fig. 2), as described previously (5). After 4 mo, body adiposity was augmented significantly in mice fed the HC diet and particularly in ob/+ mice (Fig. 2). Consequently, the gain of body fat mass was the highest in ob/+ mice fed the HC diet, whereas body lean mass was significantly reduced in this group of mice (Fig. 2). Body weight was significantly augmented in mice fed the HC diet. However, the gain of body weight in ob/+ mice fed the HC diet was not significantly higher than in +/+ mice fed the same diet since the gain of lean mass was less in ob/+ mice (Fig. 2).

Leptin in plasma and eWAT. Initial plasma levels of leptin in ob/+ mice were similar to those in +/+ mice (Fig. 3), most likely due to increased body adiposity (Fig. 2), as previously suggested (5). Indeed, relative values of plasma leptin (that is, expressed per gram of fat mass) were significantly lower in ob/+ mice (Fig. 3). After 4 mo, plasma leptin was significantly augmented in mice fed the SC diet, with a trend toward lower values in ob/+ mice. However, relative values of plasma leptin were significantly decreased in ob/+ mice compared with +/+ mice (Fig. 3). In mice fed the HC diet, leptin content in eWAT was also significantly reduced in ob/+ mice (Fig. 3). However, these data cannot be generalized for other fat depots since leptin expression presents depot-specific differences (48).

**Table 1. Sequences of primers used for real-time quantitative PCR**

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Accession No.</th>
<th>Primer Sequences</th>
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<td>F: 5’-CAATTCCTCTSCAGACAGTCTGA-3’</td>
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<td>FAS</td>
<td>NM_007988</td>
<td>R: 5’-CCCAAGAAGATCCGAGTCTGA-3’</td>
</tr>
<tr>
<td>L-CPT I</td>
<td>NM_013495</td>
<td>F: 5’-ACCCACCCAGAAGGCGTTC-3’</td>
</tr>
<tr>
<td>HK II</td>
<td>NM_013820</td>
<td>R: 5’-AAGGAGATGGCGAGGATAG-3’</td>
</tr>
<tr>
<td>L-PK</td>
<td>NM_013631</td>
<td>F: 5’-TTGGCTCTATCCGTCAGGCC-3’</td>
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<tr>
<td>MCAD</td>
<td>U07159</td>
<td>R: 5’-ACCAACCTACGAGAACCAG-3’</td>
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<td>M-CPT I</td>
<td>NM_009948</td>
<td>F: 5’-ACATTCCTATGCCATATGCTC-3’</td>
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<tr>
<td>PPARα</td>
<td>NM_01144</td>
<td>R: 5’-CTTAAGCGATGACCACTCCT-3’</td>
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<td>PPARγ</td>
<td>U10374</td>
<td>F: 5’-ACTGATGCAATGGACAGATTAG-3’</td>
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<tr>
<td>PGC-1α</td>
<td>NM_008904</td>
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<td>PEPCK</td>
<td>NM_011044</td>
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<td>S6</td>
<td>NM_009096</td>
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<td>UCP1</td>
<td>U63419</td>
<td>F: 5’-GTTGAACACACCGCAGCC-3’</td>
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<td>UCP2</td>
<td>AF111999</td>
<td>R: 5’-AAGCGATGTAAGGGACAC-3’</td>
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ACC1, acetyl-CoA carboxylase-1; F, forward; R, reverse; FAS, fatty acid synthase; HK, hexokinase II; L-CPT I, liver carnitine palmitoyltransferase I; M-CPT I, muscle carnitine palmitoyltransferase I; PPARα and -γ, peroxisome proliferator-activated receptor-α and -γ; PGC-1α, PPARγ coactivator-1α; PEPCK, phosphoenolpyruvate carboxykinase; S6, ribosomal protein S6; SCD1, stearoyl-CoA desaturase-1; SREBP-1c, sterol response element-binding protein-1c; UCP1 and -2, uncoupling protein-1 and -2.

**Plasma insulin and adiponectin, IPGTT, and IPITT.** Initial plasma levels of insulin were similar between +/+ and ob/+ mice (data not shown). After 4 mo, insulin was significantly augmented in mice fed the HC diet (Table 2). Initial plasma levels of adiponectin tended to be higher (P = 0.08) in ob/+ mice (+59%), and after 4 mo of SC diet adiponectin was significantly higher in these mice compared with +/+ mice (Table 2). However, under HC feeding plasma adiponectin was similar between ob/+ and +/+ animals (Table 2).

Initial IPGTT in ob/+ mice showed a slight but significant glucose intolerance that remained similar after 4 mo of SC diet (Fig. 4). In contrast, the loss of glucose tolerance was greatly enhanced under HC feeding (Fig. 4). Furthermore, the highest insulin levels measured during the IPGTT were observed in ob/+ mice fed the HC diet (Fig. 4).
Consequently, IPITT was carried out to assess whole body insulin sensitivity. Initial IPITT showed no major difference between \(+/+\) and \(ob/+\) mice (data not shown). After 4 mo, there was a slight loss of insulin sensitivity under HC feeding, although insulin insensitivity was not significantly higher in \(ob/+\) mice when area under the curve was taken into account (data not shown). However, basal glycemia and glycemia 15 min after insulin injection were significantly augmented by 27 and 39%, respectively, in \(ob/+\) mice compared with \(+/+\) mice (\(n=10\) mice in each group).

Other plasma parameters. Initial plasma levels of glucose, triglycerides, and total cholesterol were similar between fed \(ob/+\) and \(+/+\) mice (data not shown). After 4 mo, plasma triglycerides, ferritin, and GLP-1 in the fed state were significantly increased in \(ob/+\) mice fed the HC diet (Table 2). Total cholesterol was significantly augmented in mice fed the HC diet, with the highest levels observed in \(ob/+\) mice (Table 2). In the fasted state, plasma triglycerides and total cholesterol were significantly increased in mice fed the HC diet (Table 2). Moreover, total cholesterol was significantly higher in \(ob/+\) mice compared with \(+/+\) mice (Table 2). Last, plasma \(\beta\)-hydroxybutyrate was significantly augmented in \(ob/+\) mice under HC feeding (Table 2).

**Plasma ALT and LDH, liver histology, and TUNEL.** Initial plasma levels of ALT tended to be increased by 33% (\(P = 0.07\)) in \(ob/+\) mice. After 4 mo, ALT and LDH levels were moderately but significantly augmented in \(ob/+\) mice fed the HC diet (Table 2). Next, liver histology was evaluated as described in methods (Fig. 1). After 4 mo, necroinflammation...
was observed only in mice fed the HC diet, with a higher severity in ob/+ mice. Indeed, the mean score of necroinflammation was 0.3 and 1.0 in +/+ and ob/+ mice, respectively (n = 10 mice/group). The inflammatory infiltrates were made predominantly of lymphocytes (Fig. 1, C and D), with a few macrophages in portal areas (not shown). Steatosis was clearly more abundant with the HC diet and more marked in ob/+ mice compared with +/+ mice. Indeed, the mean percentage of hepatocytes with steatosis was 3.8 and 8.8% in +/+ and ob/+ mice, respectively, fed the standard diet (n = 4 mice/group), whereas it was 32.5 and 46.0% in +/+ and ob/+ mice, respectively, fed the HC diet (n = 10 mice/group). When steatosis was significant, fatty hepatocytes were distributed uniformly throughout the lobule (Fig. 1B). Portal and perisinusoidal fibrosis were nearly absent whatever the group of mice (data not shown). Last, the TUNEL assay revealed only rare apoptotic nuclei, including in ob/+ mice fed the HC diet (data not shown).

Body and plasma parameters after 2 mo. After 2 mo, body weight tended to be increased by ca. 4% in mice fed the HC diet, but the difference was not significant (P = 0.10 for the diet factor, 2-way ANOVA). At this time, blood was withdrawn in the fed state in 12 mice/group. Plasma glucose and ALT were unchanged among the different groups of mice (data not shown). Total cholesterol in plasma was 2.34 ± 0.08 and 2.69 ± 0.08 mM, respectively, in +/+ and ob/+ mice fed the SC diet, and 4.09 ± 0.16 and 4.48 ± 0.18 mM, respectively, in +/+ and ob/+ mice fed the HC diet (P < 0.05 for the genotype and diet factors, 2-way ANOVA). Surprisingly, plasma triglycerides were significantly reduced in mice fed the HC diet. Indeed, triglycerides were 1.22 ± 0.11 and 1.39 ± 0.11 mM, respectively, in +/+ and ob/+ mice fed the SC diet.
and 0.95 ± 0.06 and 0.98 ± 0.08 mM, respectively, in +/+ and ob/+ mice fed the HC diet (P < 0.05 for the diet factor, 2-way ANOVA). Thus, the metabolic abnormalities in ob/+ mice fed the HC diet were restricted to hypercholerolemia after 2 mo, although no interaction between genotype and diet was observed at this time. Interestingly, hypercholerolemia in ob/+ mice fed the HC diet seemed to precede body fatness.

Hepatic lipids and triglycerides and de novo fatty acid synthesis. After 4 mo, hepatic lipids tended (P = 0.17) to be higher by 25% in ob/+ mice fed the HC diet compared with +/+ mice fed the same diet (data not shown). Hepatic triglycerides were significantly higher under HC feeding, with the highest accumulation in ob/+ mice (Fig. 5). De novo fatty acid synthesis in liver was significantly reduced in +/+ mice fed the HC diet (Fig. 5), possibly due to the inhibitory action of exogenous fat on hepatic de novo lipogenesis (3, 37). How-

Table 2. Plasma parameters in +/+ and ob/+ mice after 4 mo of SC or HC diet

<table>
<thead>
<tr>
<th></th>
<th>SC Diet</th>
<th>HC Diet</th>
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<tbody>
<tr>
<td></td>
<td>+/+ Mice</td>
<td>ob/+ Mice</td>
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<tr>
<td>Insulin, ng/ml</td>
<td>1.23±0.15</td>
<td>1.28±0.23</td>
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<tr>
<td>Adiponectin, pg/ml</td>
<td>26.5±12.5</td>
<td>72.5±19.5</td>
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<tr>
<td>Glucose, mM</td>
<td>10.8±0.4</td>
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<tr>
<td>Triglycerides, mM</td>
<td>1.01±0.06</td>
<td>1.08±0.06</td>
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<tr>
<td>Total cholesterol, mM</td>
<td>2.37±0.08</td>
<td>2.78±0.11</td>
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<tr>
<td>Antioxidant status, mM</td>
<td>0.94±0.03</td>
<td>0.96±0.18</td>
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<tr>
<td>TNFα, pg/ml</td>
<td>8.9±1.6</td>
<td>10.2±1.5</td>
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<td>Iron, μmol/l</td>
<td>23.3±1.0</td>
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<td>Ferritin, ng/ml</td>
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<td>ALT, UI/l</td>
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<td>LDH, UI/l</td>
<td>621±56</td>
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<td>β-Hydroxybutyrate, mM</td>
<td>0.81±0.07</td>
<td>0.88±0.11</td>
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</table>

Results are means ± SE for 18 mice/group, except for insulin, adiponectin, TNFα, and glucagon-like peptide-1 (GLP-1) (n = 7–12 mice/group). +/+, Wild-type mice; ob/+ , mice partially deficient in leptin; SC, standard caloric; HC, high caloric; G, genotype; D, diet; G × D, interaction between genotype and diet; ALT, alanine aminotransferase; LDH, lactate dehydrogenase; NEFA, nonesterified fatty acids. Blood was drawn in the fed or the fasted state as specified in the table. Letters indicate an effect of G, the type of D, or G × D (P < 0.05, 2-way ANOVA). In case of interaction, symbols indicate statistical significance between groups [P < 0.05, least significant difference (LSD) post hoc test]. *Different from +/+ mice; †different from mice fed SC diet.

The mRNA expression of lipogenic genes was, in general, significantly higher in ob/+ mice fed the SC diet and lower in +/+ mice fed the HC diet (Table 3). However, this expression increased in ob/+ mice under HC feeding (Table 3). Expression of the glycolytic enzymes glucokinase and liver-type pyruvate kinase was significantly augmented in ob/+ mice (Table 3). Concerning gluconeogenesis, phosphoenolpyruvate carboxykinase expression was decreased in mice fed the HC diet, whatever the genotype (Table 3). Moreover, the mRNA levels of glucose-6-phosphatase and peroxisome proliferator-activated receptor (PPAR)-γ coactivator-1α (PGC-1α) were also reduced in +/+ and ob/+ mice under HC feeding (Table 3), thus suggesting that hepatic gluconeogenesis was not increased in these mice. Interestingly, the expression of genes involved in fatty acid oxidation (FAO) was decreased in ob/+ mice fed the SC diet but significantly augmented under HC feeding (Table 3). Finally, uncoupling protein (UCP)2 expression was increased with the HC diet in +/+ mice, but this upregulation was significantly reduced by 30% in ob/+ mice (Table 3).

Expression of the FAS protein was significantly increased in ob/+ mice fed the HC diet (Fig. 6). Phospho-ACC (the phosphorylated and inactivated form of ACC) was reduced by the HC diet, but with this diet total ACC was decreased and augmented in +/+ and ob/+ mice, respectively (Fig. 6). Consequently, the phospho-ACC/ACC ratio was strongly reduced in ob/+ mice under HC feeding (Fig. 6), thus suggesting that ACC was mostly in its active form in these mice.

Assessment of oxidative stress in liver. Hepatic GSH levels, aconitase activity, and expression of MnSOD, CYP2E1, and heat shock protein 70 were measured to evaluate oxidative stress. However, only hepatic CYP2E1 expression was significantly increased (by 48%) in ob/+ mice fed the HC diet compared with +/+ mice fed the SC diet (data not shown). We also assessed the mRNA expression of several genes that can be induced in stress conditions, namely heat shock protein 70, glutathione S-transferase A4, glucose-related protein 78, and TNF receptor 1. However, no change was detected, particularly in ob/+ mice fed the HC diet (data not shown).

Investigations on brown and white adipose tissue. After 4 mo, the weight of IBAT was 128 ± 11 and 126 ± 21 mg in +/+ and ob/+ mice, respectively, under standard feeding and 187 ± 21 and 177 ± 20 mg in +/+ and ob/+ mice, respectively, fed the HC diet (n = 6–9 mice/group, P < 0.05 for the diet factor, 2-way ANOVA). Next, we assessed mRNA levels of genes involved in mitochondrial biogenesis (PGC-1α), oxidative metabolism [PPARα, muscle carnitine palmitoyltransferase I (M-CPT I)], and oxidative phosphorylation uncoupling (UCP1). Expression of M-CPT I, PPARα, and PGC-1α was...
ca. 25% in Hexokinase II expression was also significantly decreased by 

moderately but significantly upregulated in ob/+ mice fed the SC diet (Table 3). HC feeding significantly augmented UCP1 and PGC-1α expression in +/+ mice, but this increase was blunted in ob/+ mice under SC feeding (Table 3). PPARγ expression was similarly downregulated in +/+ and ob/+ mice fed the HC diet. In contrast, the increased expression of M-CPT I observed under SC feeding in +/+ mice was further upregulated in ob/+ mice (Table 3).

Genes involved in adipogenesis and lipogenesis [sterol regulatory element-binding protein-1c (SREBP-1c), PPARγ, FAS] and FAO (M-CPT I) were also studied in eWAT. Expression of SREBP-1c, FAS, and M-CPT1 was moderately but significantly decreased in ob/+ mice under SC feeding (Table 3). SREBP-1c and PPARγ expression were similarly downregulated in +/+ and ob/+ mice fed the HC diet (Table 3). Hexokinase II expression was also significantly decreased by ca. 25% in +/+ and ob/+ mice fed the HC diet (data not shown). FAS expression was reduced under HC feeding in +/+ mice and was further downregulated in ob/+ mice (Table 3). M-CPT I expression was also decreased under HC feeding in +/+ mice, but it was almost restored in ob/+ mice (Table 3).

Effects of leptin supplementation. Leptin supplementation fully prevented the gain of body adiposity in ob/+ mice fed the SC diet, and this was associated with a partial restoration of plasma leptin levels (Fig. 7). Leptin also prevented the accumulation of liver lipids and triglycerides in ob/+ mice under HC feeding and significantly reduced hepatic de novo lipogenesis (Fig. 7). Leptin supplementation also ameliorated plasma total cholesterol, liver enzymes, and GLP-1 (Table 4) as well as blood glucose and plasma insulin during the IPGTT (data not shown). However, leptin did not prevent the loss of lean mass in ob/+ mice fed the HC diet (data not shown). In liver, leptin treatment fully or partially prevented the increased expression of PPARγ, SREBP-1c, ACC1, stearoyl co-A desaturase 1, liver-type pyruvate kinase, and liver carnitine palmitoyltransferase I (L-CPT I) induced in ob/+ mice under HC feeding, whereas it did not change glucokinase, PPARα, and medium-chain acyl-CoA dehydrogenase expression (Table 5). In iBAT, leptin prevented the upregulation of M-CPT I observed in ob/+ mice fed the HC diet, but it further decreased UCP1 expression and significantly upregulated PPARα (Table 5). PGC-1α expression (Table 5) and the weight of iBAT (data not shown) were unchanged by leptin. In eWAT, leptin significantly decreased the expression of SREBP-1c, PPARγ, and FAS (Table 5). Interestingly, leptin normalized M-CPT I expression that was upregulated in ob/+ mice under HC feeding.

DISCUSSION

This study shows that, under HC feeding, partial leptin deficiency in ob/+ mice greatly favors body fatness (Fig. 2), moderate steatohepatitis (see RESULTS), postabsorptive hypertriglyceridemia (Table 2), glucose intolerance (Fig. 4), and mild insulin resistance (see RESULTS). Furthermore, leptin supplementation prevented almost all of these metabolic disturbances (Fig. 7 and Tables 4 and 5). Since food overconsumption is prevalent in many countries, our data suggest that
identification of individuals with low levels of leptin could prove to be useful for the prevention of obesity and related metabolic disorders.

Partial leptin deficiency could be common in humans, since it is estimated that 5–10% of obese subjects are low-leptin secretors (11, 24). Since obesity itself upregulates leptin expression in WAT, the prevalence of partial leptin deficiency could be much greater in the general population, although data regarding this major issue are still lacking. Interestingly, a common promoter polymorphism (−2548G/A) in the leptin (ob) gene significantly influences leptin expression in WAT and its plasma concentrations, although there are some discrepancies regarding the impact of each ob variant on plasma leptin levels (16, 18, 24, 27). Nevertheless, inherited low levels of leptin can increase the risk of being overweight (or obese) in humans (8, 16, 32) and mice (under standard diet) (5). A short report also suggested that the −2548G/A polymorphism can significantly modulate the grade of steatosis and fibrosis in patients with nonalcoholic fatty liver disease (29). Our data clearly indicate that the deleterious consequences of leptin insufficiency are greatly favored by calorie overconsumption.

Indeed, a positive interaction between the ob/l+ genotype and the HC diet was found for body fat mass (Fig. 1), plasma triglycerides and total cholesterol in the fed state (Table 2), and the expression of different genes involved in fat metabolism in liver and adipose tissues (Table 3), whereas an additive (i.e., independent) effect was observed for glucose intolerance (Fig. 4), liver triglycerides (Fig. 5), plasma total cholesterol in the fasted state, and plasma ALT (Table 2). Interestingly, our data on body adiposity are partly reminiscent of some observations made in rats heterozygous (fa/+) for the leptin receptor mutation fa. Indeed, a positive interaction between genotype (fa/+) and diet (high fat) was found in young male adult rats for the epididymal fat pad weight (26), although subsequent investigations from the same group found only an additive effect for these factors (17). In these studies, however, there was no interaction between the genotype and diet factors (and no additive effect) regarding plasma cholesterol and triglycerides and liver triglycerides (17, 26). Hence, although partial deficiencies in leptin and leptin receptor could similarly predispose to obesity in the context of high-fat and/or high-calorie diets, their respective impacts on dyslipidemia and fatty liver may be divergent.

Increased body fat mass in ob/l+ mice fed the HC diet is probably due to an insufficient production of leptin by WAT (Fig. 3). Indeed, an adequate leptin production in response to calorie overconsumption is required to curb the expansion of body adiposity thanks to leptin-induced reduction of calorie intake and increased energy expenditure (11, 47). In mice, leptin-mediated increased UCP1 expression in BAT plays a key role in the appropriate stimulation of thermogenesis (47). However, this adaptive UCP1 upregulation was blunted in ob/l+ mice under HC feeding (Table 3).

Partial leptin deficiency also favored hepatic steatosis, especially with the HC diet (Fig. 5 and RESULTS). Under HC feeding, fatty liver in ob/l+ mice could result from the accumulation of both exogenous and de novo synthesized fat. Accumulation of exogenous fat is suggested by increased plasma triglycerides in the fed state (Table 2). Moreover, although hepatic de novo lipogenesis was decreased in +/+ mice under HC feeding, this adaptive downregulation was lost in ob/l+ mice (Fig. 5). A lack of suppression of hepatic de novo lipogenesis has been reported in Zucker obese and diabetic (fa/fa) rats fed a high-fat diet (3), suggesting that disinhibition of fatty acid synthesis could be due to leptin insufficiency. Importantly, the lack of suppression of hepatic de novo lipogenesis in ob/l+ mice fed the HC diet was accompanied by increased expression of several enzymes involved in glycolysis, fatty acid synthesis, or desaturation and triglyceride biosynthesis (Table 3 and Fig. 6).

Some adaptive responses in the liver can occur to limit triglyceride accretion, such as increased MTP activity and FAO (4, 22, 42). Whereas MTP activity was unchanged in ob/l+ mice under HC feeding (see RESULTS), we found increased expression of PPARα, L-CPT I, and MCAD (Table 3). Moreover, plasma β-hydroxybutyrate was augmented (Table 2), thus suggesting higher hepatic FAO. Interestingly, increased levels of plasma ketone bodies have been reported in patients with nonalcoholic steatohepatitis (NASH), and mitochondrial FAO is enhanced in liver of ob/l+ mice and in diet-induced obese rats (4, 35, 42). Augmented hepatic FAO in fatty liver despite active de novo lipogenesis may involve increased PPARα and CPT I expression associated with the decreased affinity of this mitochondrial FAO enzyme for its physiological inhibitor, malonyl-CoA (4). Besides limiting fatty liver, en-
showed in the graphs are means ± SE for 6 mice in each group. iBAT, interscapular brown adipose tissue; eWAT, epididymal white adipose tissue; G-6-Pase, glucose-6-phosphatase. After 4 mo, the mRNA expression of different enzymes and transcription factors involved in lipogenesis, glycolysis, gluconeogenesis, fatty acid oxidation, oxidative phosphorylation uncoupling, and mitochondrial biogenesis was assessed by quantitative PCR (qPCR) in liver, eWAT, and iBAT.

Table 3. mRNA expression in liver, iBAT, and eWAT of enzymes and transcription factors involved in glucose or lipid metabolism in +/+ and ob/+ mice after 4 mo of SC or HC diet

<table>
<thead>
<tr>
<th></th>
<th>SC Diet</th>
<th>ob/+ Mice</th>
<th>HC Diet</th>
<th>ob/+ Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Liver</strong></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>SREBP-1c</td>
<td>1.00±0.09</td>
<td>1.73±0.05*</td>
<td>1.22±0.09</td>
<td>2.50±0.07*</td>
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<tr>
<td>PPARγ</td>
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<td>0.72±0.04†</td>
<td>1.89±0.07†</td>
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<td>0.39±0.05†</td>
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<tr>
<td>ACC</td>
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<td>0.80±0.04†</td>
<td>1.69±0.04†</td>
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<tr>
<td>FAS</td>
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<td>mGAT1</td>
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<td>1.75±0.20</td>
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<tr>
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<td>1.81±0.24</td>
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<td>3.07±0.08†</td>
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<tr>
<td>PEPC</td>
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<td>0.83±0.09</td>
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<tr>
<td>G-6-Pase</td>
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<td>0.54±0.04†</td>
<td>0.61±0.04†</td>
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<tr>
<td>PGC-1α</td>
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<td>0.52±0.05†</td>
<td>0.77±0.06*</td>
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<tr>
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<td>0.94±0.04</td>
<td>1.64±0.05†</td>
</tr>
<tr>
<td>L-CPT I</td>
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<td>0.89±0.06</td>
<td>1.33±0.08†</td>
<td>1.73±0.05†</td>
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<tr>
<td>MCAD</td>
<td>1.00±0.10</td>
<td>0.81±0.06*</td>
<td>1.07±0.04</td>
<td>1.38±0.05†</td>
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<tr>
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<td>1.40±0.03*</td>
<td>3.74±0.13†</td>
<td>2.60±0.24†</td>
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<tr>
<td><strong>iBAT</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UCP1</td>
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<td>0.95±0.04</td>
<td>1.76±0.07†</td>
<td>1.50±0.03†</td>
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<td>M-CPT I</td>
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<td>PPARα</td>
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<td>1.38±0.04*</td>
<td>1.40±0.04†</td>
<td>1.52±0.05†</td>
</tr>
<tr>
<td>PGC-1α</td>
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<td>1.30±0.04*</td>
<td>1.61±0.07†</td>
<td>1.31±0.05†</td>
</tr>
<tr>
<td><strong>eWAT</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SREBP-1c</td>
<td>1.00±0.04</td>
<td>0.89±0.07*</td>
<td>0.58±0.04†</td>
<td>0.63±0.05†</td>
</tr>
<tr>
<td>PPARα</td>
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<td>1.09±0.04</td>
<td>0.55±0.04</td>
<td>0.64±0.05</td>
</tr>
<tr>
<td>FAS</td>
<td>1.00±0.05</td>
<td>0.83±0.07*</td>
<td>0.24±0.05†</td>
<td>0.16±0.05†</td>
</tr>
<tr>
<td>M-CPT I</td>
<td>1.00±0.04</td>
<td>0.75±0.05*</td>
<td>0.58±0.04†</td>
<td>0.89±0.06†</td>
</tr>
</tbody>
</table>

Results are means ± SE for 6 mice in each group. Letters above the graphs indicate statistical significance between groups (P < 0.05, 2-way ANOVA). In case of interaction, symbols indicate statistical significance between groups (P < 0.05, LSD post hoc test). *Different from +/+ mice; †different from mice fed SC diet.

hanced hepatic FAO and ketogenesis could also provide a significant source of energy in tissues such as skeletal muscle, which present limited glucose and lipid oxidizing capacities in the context of obesity and insulin resistance (14, 34, 41, 45). Interestingly, ketone bodies could also constitute a cue for the brain to curb food intake (36).

Higher plasma levels of GLP-1 could also represent an adaptive response in ob/+ mice fed the HC diet (Table 2).

Fig. 6. Protein expression of acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) in liver. At the end of the 4-mo experiments, liver expression of total ACC, the phosphorylated form of ACC (phospho-ACC), and FAS were assessed by Western blot analysis. Data showed in the graphs are means ± SE for 5–7 mice in each group. Letters above the graphs indicate an effect of G or G × D (P < 0.05, 2-way ANOVA). In case of interaction, signs above the bars indicate statistical significance between groups (P < 0.05, LSD post hoc test). *Different from +/+ mice; †different from mice fed SC diet.
Indeed, this intestine-derived incretin stimulates glucose-dependent insulin release, reduces appetite, and directly decreases the expression of lipogenic genes on hepatocytes (6, 19). Importantly, GLP-1 secretion and plasma levels can be reduced in obesity and type 2 diabetes (2, 19). However, the insulin-resistant state, hyperglycemia, and/or hyperglycagone mia per se, rather than obesity, could play a key role in the reduction of GLP-1 secretion (19, 31, 46). In our study, insulin resistance was mild (see RESULTS) and glycemia in the insulin-resistant state, hyperglycemia, and/or hyperglycagone mia per se, rather than obesity, could play a key role in the reduction of GLP-1 secretion (19, 31, 46). In our study, insulin resistance was mild (see RESULTS) and glycemia in the fasted and fed state was unchanged in ob/+ mice fed the HC diet (Table 2), and this could explain why plasma levels of GLP-1 were not reduced. Interestingly, leptin appears to favor GLP-1 secretion (2), but plasma GLP-1 was not augmented in +/+ mice fed the HC diet (Table 2), which presented the highest plasma concentrations of leptin (Fig. 3). Thus, further investigations would be needed to identify the factors that could have favored GLP-1 secretion in ob/+ mice fed the HC diet.

Higher plasma adiponectin in ob/+ mice under standard feeding (Table 2) suggests an adaptation in response to leptin insufficiency. Like leptin, adiponectin acts centrally to reduce insulin resistance. Like leptin, adiponectin acts centrally to reduce insulin resistance. Like leptin, adiponectin acts centrally to reduce insulin resistance.

Table 5. Effects of leptin supplementation on gene expression in liver and adipose tissues in ob/+ mice after 4 mo of HC diet

<table>
<thead>
<tr>
<th>Gene</th>
<th>+/+ Mice</th>
<th>ob/+ Mice</th>
<th>ob/+ Mice treated with leptin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td>PPARγ</td>
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<td>1.48±0.11*</td>
</tr>
<tr>
<td></td>
<td>SREBP-1c</td>
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<td>2.65±0.12*</td>
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<td></td>
<td>ACC1</td>
<td>1.00±0.07</td>
<td>1.88±0.08*</td>
</tr>
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<td></td>
<td>SCD1</td>
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<td>2.94±0.18*</td>
</tr>
<tr>
<td></td>
<td>GK</td>
<td>1.00±0.06</td>
<td>1.22±0.08*</td>
</tr>
<tr>
<td></td>
<td>L-PK</td>
<td>1.00±0.08</td>
<td>2.13±0.10*</td>
</tr>
<tr>
<td></td>
<td>PPARα</td>
<td>1.00±0.06</td>
<td>1.89±0.07*</td>
</tr>
<tr>
<td></td>
<td>L-CPT I</td>
<td>1.00±0.06</td>
<td>1.47±0.07*</td>
</tr>
<tr>
<td></td>
<td>MCAD</td>
<td>1.00±0.09</td>
<td>1.38±0.08*</td>
</tr>
<tr>
<td>iBAT</td>
<td>UCP1</td>
<td>1.00±0.07</td>
<td>0.86±0.08*</td>
</tr>
<tr>
<td></td>
<td>PGC-1α</td>
<td>1.00±0.08</td>
<td>0.82±0.06*</td>
</tr>
<tr>
<td></td>
<td>PPARα</td>
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</tr>
<tr>
<td></td>
<td>M-CPT I</td>
<td>1.00±0.05</td>
<td>1.19±0.06*</td>
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<td>eWAT</td>
<td>SREBP-1c</td>
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<tr>
<td></td>
<td>PPARγ</td>
<td>1.00±0.08</td>
<td>1.19±0.10</td>
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<td></td>
<td>FAS</td>
<td>1.00±0.07</td>
<td>0.66±0.06*</td>
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<td>HK II</td>
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<tr>
<td></td>
<td>M-CPT I</td>
<td>1.00±0.08</td>
<td>1.54±0.11*</td>
</tr>
</tbody>
</table>

Results are means ± SE for 5 mice. After 4 mo of leptin supplementation, the mRNA expression of different genes was assessed by qPCR in liver, iBAT, and eWAT in the different groups of mice. S6 expression was used to normalize mRNA levels of the different target genes. *Different from +/+ mice, P < 0.05; †different from ob/+ mice not treated with leptin, P < 0.05.
body weight (30), favors FAO in liver and muscle, and presents insulin-sensitizing effects (23). Nevertheless, hepatic phospho-ACC was not augmented in ob/+ mice fed the SC diet (Fig. 6), suggesting that higher adiponectin levels failed to activate AMP kinase in ob/+ liver. Importantly, HC feeding suppressed this increase in plasma adiponectin (Table 2), and this could have favored body fatness and other metabolic disorders in ob/+ mice. Free fatty acids, reactive oxygen species, and TNFα can reduce adiponectin expression in WAT (13, 38).

Further studies would be needed to determine the exact mechanism(s) whereby calorie overconsumption suppresses adiponectin expression in ob/+ mice.

Hepatocyte death was due primarily to necrosis in ob/+ mice fed the HC diet (Table 2 and RESULTS). Importantly, consumption. Should such recommendations fail, leptin supplementation to avoid energy overconsumption suppresses adiponectin expression in ob/+ mice.

Hepatocyte death was due primarily to necrosis in ob/+ mice fed the HC diet (see Fig. 7, Table 4, and RESULTS). Interestingly, improved glucose tolerance seemed to

be independent of adiponectin since its plasma levels were not elevated in leptin-deficient mice. Finally, fibrosis was absent in ob/+ mice fed the HC diet (see Fig. 7, Table 4, and RESULTS). However, leptin did not normalize the expression of several other genes, including UCP1 and PGC-1α in iBAT. Although not expected, lower UCP1 expression could be secondary to the reduction of body fatness, as reported previously (44).

Ob/+ mice fed an HC diet could be used as a helpful model to study body fatness, moderate NASH, and other metabolic disorders commonly observed in obese individuals. Beyond this experimental aspect, our data suggest that investigations would be needed to determine the exact prevalence of leptin insufficiency, particularly in wealthy countries. If early detection of low leptin secretors can be achieved, such individuals should benefit from dietary counseling to avoid energy overconsumption. Should such recommendations fail, leptin supplementation could be an option.

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