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. First published March 18, 2008; doi:10.1152/ajpendo.00379.2007.—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 mass, hepatic steatosis, increased 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

Animals, diets, and treatment with leptin. Five-week-old male C57BL/6J+/+ mice (wild type, henceforth referred to as +/+ mice) and C57BL/6-ob/ob+ mice (partially deficient in leptin, referred to as ob+/+ mice) were purchased from Janvier (Le-Genest-St-Isle, France). After 1 wk of acclimatization, +/+ and ob+/+ mice were split into two groups and fed ad libitum for 4 mo on either a standard chow (A04 biscuit; UAR, Villemoisson-sur-Orge, France) or a high-fat, high-sucrose diet (referred to as HC diet; purchased from SAFE, Auby, France). Thus, four different groups of mice were studied, namely +/+ and ob+/+ mice fed the standard-calorie (SC) diet and +/+ and ob+/+ mice fed the HC diet. The SC diet brings 2,820 kcal/kg of food and contains 3% fat (270 kcal/kg), 48% complex carbohydrates (1,910 kcal/kg, primarily starch), and 16% protein (640 kcal/kg). The HC diet brings 5,320 kcal/kg and includes 36% fat (primarily lard, 3,220 kcal/kg).
kcal/kg), 35% simple carbohydrates (1,400 kcal/kg, mainly saccharose) 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/+ 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 animal house accredited by the French Direction des Services Vétérinaires, respectively (2 different livers with ca. 5 and 80% of fatty hepatocytes dying through necrosis. 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 and stored at −20°C until analysis. All mice were killed by cervical dislocation. Liver, epididymal white adipose tissue (eWAT), and interscapular brown adipose tissues (iBAT) were quickly removed and frozen in liquid nitrogen and kept at −80°C until use. In some experiments, liver fragments were processed for histology and in situ detection of apoptosis.

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 on 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

Fig. 1. Evaluation of steatosis and necroinflammation in mice. Steatosis and necroinflammation were evaluated with Oil Red O and hematoxylin-eosin stainings, respectively. A and B are representative of 2 different livers with ca. 5 and 80% of fatty hepatocytes, respectively [A: ob/+ mouse fed the standard-calorie (SC) diet; B: ob/+ mouse fed the high-calorie (HC) diet]. C and D show necroinflammation with score 1 and 2, respectively (C: +/+ mouse fed the HC diet; D: ob/+ mouse fed the HC diet). Inflammatory infiltrates (black arrows) are made of lymphocytes, whereas the blue arrows show hepatocytes dying through necrosis.
of 3H incorporated into fatty acids per hour and per gram of liver. To
calculate the rate of de novo fatty acid synthesis was calculated as micromoles
removed to extract fatty acids (40). After counting the radioactivity,
mice in the postabsorptive state. Two hours later, liver was quickly
homogenized in Krebs-Ringer buffer (100 mg/ml) with a protease
inhibitor cocktail (1 μ/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 lepbin with a mouse
leptin ELISA kit (Crystal Chem, Downers Grove, IL).

Lipids and triglycerides, de novo fatty acid synthesis, and micro-
somal 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 3H2O was injected intraperitoneally into
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 3H incorporated into fatty acids per hour and per gram of liver. To
determine the [3H2O]-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 disinte-
grations per minute measured in plasma by the micromoles of water
(i.e., 517 nmol for 10 μl of plasma, assuming that plasma is 93% water)
as described previously (40). Microsomal triglyceride transfer protein (MTP) activity was determined in liver with a commercial kit
(Roar Biomedical, New York, NY) as described previously (25).

Glucose and insulin tolerance tests. Intraperitoneal glucose toler-
ance test (IPGTT) was performed in mice after a 12-h overnight fast.
At 10:00 AM, d-glucose (2 g/kg body wt) was injected intraperito-
neally 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 an
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
measurement of plasma insulin by using an insulin ELISA kit from
Crystal Chem. Intraperitoneal insulin tolerance test (IPTT) was
performed in mice after a 5-h fast. Human insulin (Actrapid) pur-
chased from Novo Nordisk (Chartres, France) was injected intraperi-
toneally (0.5 U/kg body wt), and blood was collected by tail bleeding
with the timing detailed above for IPGTT. Areas under the curve
during IPGTT and IPTT were then calculated by the linear trapezoi-
dal method.

RNA 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 ex-
tected using the Lipid RNeasy kit (Qiagen, Courtaboeuf, 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 leukemia
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
Cromvo IV light cycle 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 check-
ing the length of the PCR products. Expression of the mouse ribo-
somal 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 superna-
tants by using the Lowry assay. To assess the hepatic expression of
fatty acid synthase (FAS), total and phosphorylated acetyl-CoA car-
boxylase (ACC and phosphoryl ACC, respectively), manganese super-
oxide dismutase (MnSOD), and cytochrome P450 2E1 (CYP2E1)
proteins (ca. 50 μg) underwent SDS-polyacrylamide electrophoresis
(8% polyacrylamide for FAS, phosphoryl ACC, and ACC and 12% for
MnSOD and CYP2E1) transfer to nitrocellulose membrane (Hybond
ECL, Amersham Biosciences) and immunoblotting with rabbit poly-
clonal antibodies against FAS (Santa Cruz Biotechnology, Santa
Cruz, CA), ACC and phosphoryl ACC (Upstate, Lake Placid, NY),
MnSOD (Stressgen, Ann Arbor, MI), and CYP2E1 (Oxford Biomed-
ical Research, Oxford, MI). Blots were incubated with appropriate
secondary antibodies, and protein bands were revealed by enhanced
chemiluminescence (Amersham Pharmacia, Orsay, France). To nor-
malize protein loadings, blots were stripped and incubated with
monoclonal mouse antibodies against β-actin (Sigma-Aldrich). Pro-
tein bands were quantified using a Helwett Packard Scanjet 4570c
scanning unit and ImageMaster1D software (Pharmacia Biotech).

Glutathione levels and aconitase activity in liver. Reduced glu-
thione (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 indi-
cated a significant interaction between factors, individual means were
performed 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 differen-
t 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 Stu-
ent’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

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Diet was not significantly higher than in weight was significantly augmented in mice fed the HC diet. Body mass was significantly reduced in this group of mice (Fig. 2). Consequently, the gain of body fat mass was the highest in mice fed the HC diet, whereas body lean mass was significantly lower in mice (Fig. 3). After 4 mo, plasma leptin was significantly augmented in mice fed the HC 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 directly compared with other fat depots since leptin expression presents depot-specific differences.

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ACC1, acetyl-CoA carboxylase-1; F, forward; R, reverse; FAS, fatty acid synthase; GK, glucokinase; mGPAT1, glycerol phosphate acyltransferase mitochondrial isoform; HK II, hexokinase II; L-CPT I, liver carnitine palmitoyltransferase I; L-PK, liver-type pyruvate kinase; MCAD, medium-chain acyl-CoA dehydrogenase; 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.

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 HC 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 directly compared with other fat depots since leptin expression presents depot-specific differences (48).

**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 fed the HC diet. Indeed, basal glycemia was 9.2 ± 0.6 and 11.7 ± 0.7 mM in +/+ and ob/+ mice, respectively, whereas glycemia 15 min after insulin administration was 7.3 ± 0.5 and 10.2 ± 0.5 mM, respectively (n = 10 mice in each group).

Other plasma parameters. Initial plasma levels of glucose, 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 β-hydroxybutyrate was significantly increased 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

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**Fig. 2. Variations of body fat mass, lean mass, and body weight over the 4-mo period of investigations.** Body fat mass (A) and lean mass (B) were determined by dual-energy X-ray absorptiometry (DEXA). C: mice were also weighed on the occasion of DEXA measurements, and variations of body weight were also calculated. Results are means ± SE for 18-22 mice in each group. Letters above the graphs indicate an effect of the genotype (G), the type of diet (D), or an interaction between genotype and diet (G × D) (P < 0.05, 2-way ANOVA). In case of interaction, signs above the bars indicate statistical significance between groups (P < 0.05, least significant difference (LSD) post hoc test). *Different from +/+ mice; †different from mice fed SC diet.
was observed only in mice fed the HC diet, with a higher severity in ob/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/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 SC diet were restricted to hypercholesterolemia after 2 mo, although no interaction between genotype and diet was observed at this time. Interestingly, hypercholesterolemia 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 lipidogenesis (3, 37). However, de novo FA synthesis was disinhibited in ob/+ mice fed the HC diet (Fig. 5). Last, MTP activity was unchanged whatever the group of mice (data not shown).

mRNA and protein expression in liver. 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 30% in ob/+ mice, but this upregulation was significantly reduced by 30% in ob/+ mice (Table 3).

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 tissues. 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...
Fig. 4. Intraperitoneal glucose tolerance test (IPGTT). IPGTT was assessed first before the onset of the investigations (A) and then after 4 mo (B). For a matter of clarity, signs symbolizing statistical significance were omitted for the IPGTT curve. Areas under the curves (AUC) were also calculated for each group of mice. After 4 mo, insulin was also measured during the IPGTT at different times after glucose injection. For initial IPGTT, results are means ± SE for 10 and 12 mice in each group (*different from +/+ mice, P < 0.05). For IPGTT performed after 4 mo, levels of blood glucose are means ± SE for 10–12 mice in each group. Letters above the AUC graph indicate an effect of G and of D (P < 0.05, 2-way ANOVA). Levels of plasma insulin during the IPGTT are means ± SE for 5 mice in each group.

Effects of leptin supplementation. Leptin supplementation fully prevented the gain of body adiposity 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 (Table 3). PPARγ expression was similarly induced in +/+ and ob/+ mice fed the HC diet. In contrast, the increased expression of M-CPT I observed in ob/+ 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 upregulated 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).

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/+ 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/+ 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/+ 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/+ 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/+ 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/+ 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/+ 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/ob 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-
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>+/+ Mice</th>
<th>ob/+ Mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver</td>
<td></td>
<td></td>
<td></td>
<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†G, D, G × D</td>
</tr>
<tr>
<td>PPARγ</td>
<td>1.00±0.04</td>
<td>1.65±0.06*</td>
<td>0.72±0.04†</td>
<td>1.89±0.07†G, G × D</td>
</tr>
<tr>
<td>SCD1</td>
<td>1.00±0.06</td>
<td>1.34±0.06*</td>
<td>0.39±0.05†</td>
<td>1.47±0.05†G, D, G × D</td>
</tr>
<tr>
<td>ACC1</td>
<td>1.00±0.06</td>
<td>1.14±0.05*</td>
<td>0.80±0.04†</td>
<td>1.69±0.04†G, D, G × D</td>
</tr>
<tr>
<td>FAS</td>
<td>1.00±0.10</td>
<td>1.61±0.24</td>
<td>0.44±0.12</td>
<td>1.71±0.46G</td>
</tr>
<tr>
<td>mtG3AT1</td>
<td>1.00±0.06</td>
<td>2.17±0.20</td>
<td>0.82±0.05</td>
<td>1.75±0.20G, D</td>
</tr>
<tr>
<td>GK</td>
<td>1.00±0.05</td>
<td>1.53±0.13</td>
<td>1.16±0.06</td>
<td>1.81±0.24G</td>
</tr>
<tr>
<td>L-PK</td>
<td>1.00±0.06</td>
<td>1.97±0.06*</td>
<td>1.33±0.10</td>
<td>3.07±0.08†G, D, G × D</td>
</tr>
<tr>
<td>PEPCK</td>
<td>1.00±0.09</td>
<td>1.36±0.11</td>
<td>0.80±0.08</td>
<td>0.83±0.09G, D</td>
</tr>
<tr>
<td>G-6-Pase</td>
<td>1.00±0.06</td>
<td>0.74±0.05*</td>
<td>0.54±0.04†</td>
<td>0.61±0.04†G, D, G × D</td>
</tr>
<tr>
<td>PGC-1α</td>
<td>1.00±0.09</td>
<td>0.86±0.07</td>
<td>0.52±0.05†</td>
<td>0.77±0.06*D, G × D</td>
</tr>
<tr>
<td>PPARα</td>
<td>1.00±0.08</td>
<td>0.84±0.04*</td>
<td>0.94±0.04</td>
<td>1.64±0.05†G, D, G × D</td>
</tr>
<tr>
<td>L-CPT I</td>
<td>1.00±0.09</td>
<td>0.89±0.06</td>
<td>1.33±0.08†</td>
<td>1.73±0.05†G, D, G × D</td>
</tr>
<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†D, G × D</td>
</tr>
<tr>
<td>UCP2</td>
<td>1.00±0.04</td>
<td>1.40±0.03*</td>
<td>3.74±0.13†</td>
<td>2.60±0.24†G, D, G × D</td>
</tr>
<tr>
<td>iBAT</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UCP1</td>
<td>1.00±0.05</td>
<td>0.95±0.04</td>
<td>1.76±0.07†</td>
<td>1.50±0.03†G, D, G × D</td>
</tr>
<tr>
<td>M-CPT I</td>
<td>1.00±0.06</td>
<td>1.33±0.05</td>
<td>1.92±0.05</td>
<td>2.28±0.05G, D</td>
</tr>
<tr>
<td>PPARγ</td>
<td>1.00±0.06</td>
<td>1.38±0.04*</td>
<td>1.40±0.04†</td>
<td>1.52±0.05†G, D, G × D</td>
</tr>
<tr>
<td>PGC-1α</td>
<td>1.00±0.05</td>
<td>1.30±0.04*</td>
<td>1.61±0.07†</td>
<td>1.31±0.05*D, G × D</td>
</tr>
<tr>
<td>eWAT</td>
<td></td>
<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†D, G × D</td>
</tr>
<tr>
<td>PPARγ</td>
<td>1.00±0.05</td>
<td>1.09±0.04</td>
<td>0.55±0.04</td>
<td>0.64±0.05D</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†G, D, G × D</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†D, G × D</td>
</tr>
</tbody>
</table>

Results 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 in the different groups of mice. Expression of S6 was used to normalize mRNA levels of the different target genes. 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, 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 hyperglycagomina 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 ob/+ 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 feeding (10.2 ± 0.3 vs. 13.1 ± 0.1 on June 25, 2017 http://ajpendo.physiology.org/ Downloaded from

Table 4. Effects of leptin supplementation on plasma parameters in ob/+ mice after 4 mo of HC diet

<table>
<thead>
<tr>
<th>Parameter</th>
<th>(+/+ Mice)</th>
<th>(ob/+ Mice)</th>
<th>(ob/+ Mice treated with leptin)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose, mM</td>
<td>10.7 ± 1.1</td>
<td>10.2 ± 0.4</td>
<td>9.1 ± 0.4</td>
</tr>
<tr>
<td>Triglycerides, mM</td>
<td>0.94 ± 0.10</td>
<td>1.14 ± 0.09</td>
<td>1.02 ± 0.09</td>
</tr>
<tr>
<td>Total cholesterol, mM</td>
<td>3.45 ± 0.31</td>
<td>4.65 ± 0.27*</td>
<td>3.33 ± 0.37†</td>
</tr>
<tr>
<td>β-Hydroxybutyrate, mM</td>
<td>0.07 ± 0.01</td>
<td>0.09 ± 0.01</td>
<td>0.11 ± 0.01</td>
</tr>
<tr>
<td>ALT, UI/l</td>
<td>82 ± 18</td>
<td>116 ± 28</td>
<td>134 ± 25</td>
</tr>
<tr>
<td>LDH, UI/l</td>
<td>83.7 ± 127</td>
<td>173 ± 330*</td>
<td>951 ± 190</td>
</tr>
<tr>
<td>Insulin, ng/ml</td>
<td>2.74 ± 0.45</td>
<td>2.47 ± 0.43</td>
<td>2.71 ± 0.48</td>
</tr>
<tr>
<td>Adiponectin, μg/ml</td>
<td>14.7 ± 2.4</td>
<td>17.8 ± 1.7</td>
<td>16.5 ± 1.6</td>
</tr>
<tr>
<td>Iron, μmol/l</td>
<td>18.0 ± 0.7</td>
<td>21.1 ± 3.0</td>
<td>17.6 ± 0.7</td>
</tr>
<tr>
<td>Ferritin, ng/ml</td>
<td>80.6 ± 13.5</td>
<td>105.2 ± 14.1</td>
<td>106.0 ± 15.9</td>
</tr>
<tr>
<td>GLP-1, pmol/l</td>
<td>3.9 ± 0.1</td>
<td>5.7 ± 0.4*</td>
<td>4.0 ± 0.1†</td>
</tr>
</tbody>
</table>

Results are means ± SE for 7–10 mice/group. Blood was drawn in the fed state.*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, plasma TNFα was unchanged (Table 2), and there was no overt hepatic oxidative stress (see RESULTS). Hence, the excess of fat could have induced necrosis through the accumulation of long-chain fatty acids (or their acyl-CoA derivatives), which are detrimental on various cell components and metabolisms (12, 43). Finally, fibrosis was absent in ob/+ mice fed the HC diet (see RESULTS). Because leptin is a key mediator of fibrogenesis (4, 28), leptin insufficiency could have hampered the development of fibrosis in ob/+ mice.

Leptin supplementation prevented most of the disorders observed 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 augmented by leptin supplementation (Table 4). Treatment with leptin also normalized the upregulated expression of several genes in liver, iBAT, and eWAT (Table 5). 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.

ACKNOWLEDGMENTS

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