Leptin Deficiency in Rats Results in Hyperinsulinemia and Impaired Glucose Homeostasis

Anna M. D’souza, Ali Asadi, James D. Johnson, Scott D. Covey, and Timothy J. Kieffer

Department of Cellular and Physiological Sciences (A.M.D., A.A., J.D.J., T.J.K.), Department of Biochemistry and Molecular Biology (S.D.C.), and Department of Surgery (J.D.J., T.J.K.), University of British Columbia, Vancouver British Columbia, Canada V5Z 4E3

Leptin, an adipocyte-derived hormone, has well-established anorexigenic effects but is also able to regulate glucose homeostasis independent of body weight. Until recently, the ob/ob mouse was the only animal model of global leptin deficiency. Here we report the effects of leptin deficiency on glucose homeostasis in male and female leptin knockout (KO) rats. Leptin KO rats developed obesity by 6 to 7 weeks of age, and lipid mass was increased by more than 2-fold compared with that of wild-type (WT) littermates at 18 weeks of age. Hyperinsulinemia and insulin resistance were evident in both males and females and were sustained with aging. Male KO rats experienced transient mild fasting hyperglycemia between 14 and 25 weeks of age, but thereafter fasting glucose levels were comparable to those of WT littermates up to 36 weeks of age. Fasting glucose levels of female KO rats were similar to those of WT littermates. Male KO rats exhibited a 3-fold increase in the proportion of $\beta$-cell area relative to total pancreas at 36 weeks of age. Islets from 12-week-old KO rats secreted more insulin when stimulated than islets from WT littermates. Leptin replacement via miniosmotic pump (100 $\mu$g/d) reduced food intake, attenuated weight gain, normalized glucose tolerance, and improved glucose-stimulated insulin secretion and insulin sensitivity. Together, these data demonstrate that the absence of leptin in rats recapitulates some of the phenotype previously observed in ob/ob mice including development of hyperinsulinemia, obesity, and insulin resistance. (Endocrinology 155: 1268–1279, 2014)

Leptin is a 167-amino acid hormone expressed predominantly in adipocytes. Circulating leptin levels are proportionate to total adipose mass and are acutely regulated by changes in energy status as a result of fasting or feeding, independent of body weight (1). Leptin signaling is an important mediator of food intake, adiposity, and energy expenditure (2). In ob/ob mice, a homozygous mutation in the gene encoding leptin (ob) results in the absence of leptin (3). These leptin-deficient mice are obese and hyperphagic and have an impaired thermoregulatory capacity marked by reduced basal body temperature compared with that of wild-type (WT) littermates (4). These metabolic perturbations are accompanied by a redistribution of fat storage to nonadipose tissue including liver, pancreas, and muscle, which can disrupt insulin signaling (5, 6).

Leptin deficiency in ob/ob mice results in hyperglycemia and hyperinsulinemia (7). This is also evident in db/db mice and Zucker diabetic fatty (ZDF) rats, both of which lack functional leptin receptors as a result of a spontaneous mutation in the gene encoding the leptin receptor (8, 9). Islets from ob/ob mice, db/db mice, and ZDF rats have increased $\beta$-cell mass compared with WT littermates and also have impaired glucose-stimulated insulin secretion (10–13). These aberrations in glucose metabolism were initially thought to be a consequence of obesity; however, leptin therapy normalized fasting glucose and reduced plasma insulin levels by 80% without significant reductions in body weight in leptin-deficient ob/ob mice (14, 15). Similarly, pair feeding ob/ob mice to match food in-
take of WT littermates did not effectively improve glucose metabolism, whereas leptin therapy was able to normalize glucose tolerance, suggesting that the effects of leptin on glucose homeostasis are independent of food intake (16). Furthermore, glucose-stimulated insulin secretion in ob/ob islets treated with leptin was increased by 15% at 5.5 mM glucose and 85% at 16.7 mM glucose (17). The beneficial effects of leptin therapy on glucose homeostasis are also evident in animal models without leptin deficiency or genetic defects in leptin signaling. Leptin treatment in the UCD-T2DM rat, a model of polygenic type 2 diabetes resulted in increased insulin sensitivity and reduced fasting glucose without any changes in food intake (18). Together, these results reveal that leptin is able to alter glucose homeostasis independent of energy balance.

The phenotype resulting from leptin deficiency differs between ob/ob mice and humans. In leptin-deficient humans, severe obesity occurs, but fasting glucose levels are typically in the normal range (19, 20). In contrast, adult ob/ob mice are obese and have fasting glucose levels of >12 mM (7, 14, 21). Thus, although the ob/ob mouse reflects some aspects of impaired leptin function observed in humans, it does not accurately portray all aspects of this condition. Rats have an extensive history of use in medical research because they are more physiologically similar to humans than mice. Genetic modifications were previously exclusive to mice; however, through the use of zinc finger nuclease technology, a leptin knockout (KO) rat has been generated via a 151-bp deletion of exon 1. The initial characterization of these leptin-deficient rats revealed obesity, hyperphagia, and elevated insulin levels (22); however, other aspects of glucose metabolism were not reported. Here we demonstrate that unlike ob/ob mice and more similar to humans, leptin-deficient rats do not develop fasting glucose levels >12 mM. Thus, the leptin KO rat may represent a more clinically meaningful animal model of impaired leptin function than the ob/ob mouse.

Materials and Methods

Animals

Leptin deficiency results in infertility (23); therefore, male and female rats that were heterozygous for the mutated leptin gene (Sprague-Dawley background strain) were obtained from Sigma SAGE Laboratories and were mated to produce male and female WT, heterozygous, or KO rats. Offspring were housed on a 12-hour light/12-hour dark cycle and had ad libitum access to a standard chow diet (5015; LabDiet) and water. Animals were genotyped using genomic DNA amplified by PCR. The sequence of the forward primer was AAGAAGAAGAGACCCCCAGCGGAGGAAA and that of the reverse primer was CTATTGTTTTCATGTCAGCAGCATGACAAC. The predicted sizes for the WT and KO lep alleles were 471 and 320 bp, respectively. All animal experiments were approved by the University of British Columbia Animal Care Committee and performed in accordance with the Canadian Council on Animal Care guidelines.

Cold tolerance and body composition analysis

Seventeen-week-old male KO rats and WT littermates were implanted with sterile temperature transponders (IPTT-300; Bio Medic Data Systems, Seaford, DE) as described previously (24). Body temperature was measured every 15 minutes for a total of 90 minutes using a hand-held scanner (DAS-5007; Bio Medic Data Systems). Lean to lipid mass was measured in anesthetized 18-week-old male rats using a BioSpec 70/30 7-T magnetic resonance imaging (MRI) scanner (Bruker Biospin). A nuclear magnetic resonance (NMR) signal from the body was acquired using a quadrature volume radiofrequency coil tuned to 300 MHz. The lean and fat masses were calculated from the NMR data as described previously (25). Images of fat distribution in 18-week-old male and female rats were captured from the thoracic and abdominal regions and acquired in 1-mm longitudinal sections using MRI.

Biochemical and hormonal assays

Body weight and blood glucose were measured after a 4-hour fast. Blood glucose was monitored via a One Touch Ultra Glucometer (LifeScan) from the saphenous vein. Plasma glucagon was measured using an RIA (Millipore). Plasma insulin, C-peptide, and adiponectin were measured using ELISAs from ALPCO. Glucagon-like peptide-1 (GLP-1) was measured using the total GLP-1 (v2) kit from Meso Scale Discovery. GH was measured from plasma samples using an ELISA from Millipore. Hepatic and gastrocnemius muscle triglycerides were measured by a protocol modified from that of Braud et al (26) as described previously (21). Triglycerides from plasma and extracted from hepatic and gastrocnemius muscle were measured using a serum triglyceride determination kit (Sigma-Aldrich).

Glucose and insulin tolerance tests

Glucose tolerance tests (GTTs) were performed after an overnight (16-hour) fast. Glucose (1.5 g/kg body weight) was administered by oral gavage. Blood was collected from the saphenous vein in heparin-coated tubes for analysis of plasma insulin levels. To assess insulin sensitivity, rats were fasted for 4 hours followed by an ip injection of 0.5 U/kg body weight of insulin (Novolin, Novo Nordisk) and measurement of blood glucose levels.

Western blot analysis

Liver samples were collected at 36 weeks of age. Western blotting were performed as described previously (27), using antibodies against phosphorylated Akt (4060), total Akt (9272), phosphorylated ERK1/2 (4370), and total ERK1/2 (9102) with β-tubulin (2146) used as a loading control. All primary antibodies were from Cell Signaling Technology with the exception of β-actin (Novus Biologicals).

Islet isolation and perifusion

Islets were isolated from 12-week-old male and female KO rats and WT littermates by collagenase injection into the pancreatic ducts (28) with subsequent mesh filtration as described by Salvalaggio et al (29). Pancreata were distended by injecting 15 mL of collagenase (0.19 mg/mL Liberase TL grade; Roche Diagnostics) in Hanks’ solution and then were digested in collage-
nase solution for 25 minutes in a 37°C water bath. The resulting tissue was washed 3 times in Hank’s solution (with CaCl₂), resuspended in Ham’s F10 medium (Sigma-Aldrich), and poured onto a 100-µm nylon mesh cell strainer (BD Biosciences). Captured islets were then hand-picked and cultured overnight, and 100 islets from each rat were loaded into temperature- and CO₂-controlled chambers of an AcuSyst-S Perifusion apparatus (Endotronics). The perifusion protocol consisted of a 1-hour baseline period during which chambers were perfused with 3 mM glucose, followed by perifusion with 3 mM glucose (30 minutes), 20 mM glucose (30 minutes), 3 mM glucose (20 minutes), 3 mM glucose plus 30 mM KCl (30 minutes), 3 mM glucose (20 minutes), and 15 mM arginine (30 minutes). All perifusion solutions were made in HEPES-buffered Krebs-Ringer bicarbonate buffer containing 0.5% BSA and delivered at 2 mL/min. Fractions were collected every 5 minutes, and insulin was assayed using a rat insulin RIA (Millipore), pooling data collected from isolated male and female islets.

Pancreas morphology and immunohistochemistry

At 36 weeks of age, rats were euthanized with CO₂, and pancreata were fixed in 4% paraformaldehyde, rinsed in 70% ethanol, embedded in paraffin, and sectioned at 5-µm thickness (Wax-it Histology Services). Three pancreas sections per male rat, separated by at least 200 µm, were immunostained for insulin (1:1000, I8510; Sigma-Aldrich), glucagon (1:1000, G2654; Sigma-Aldrich), and glucose transporter-2 (GLUT-2) (1:300, 07–1402; Millipore). Secondary incubation was performed at room temperature for 1 hour using Alexa Fluor–conjugated antibodies (1:1000; Life Technologies). Sections were imaged with an ImageXpress Micro microscope and associated software (Molecular Devices). Relative β- or α-cell areas consisted of the total insulin-positive or glucagon-positive area expressed relative to the whole pancreas.

Leptin therapy via miniosmotic pumps

Male KO rats (16 weeks old) received 100 µg/d rat recombinant leptin (Peprotech) delivered via miniosmotic pump (28-day infusion, 2ML4; ALZET). Osmotic pumps were implanted s.c to enable controlled, continuous peptide delivery (30); WT littermates received miniosmotic pumps infusing water, which was the vehicle used to dissolve leptin.

Statistical analysis

Data analysis was performed using Prism 6.0 (GraphPad Software Inc). Results are expressed as means ± SEM. Statistical analyses were performed by Student t tests or ANOVA with Bonferroni post hoc comparisons where appropriate. Statistical significance was defined as P < .05.

Results

Leptin-deficient rats have increased adiposity and poor thermoregulation

Leptin has well-established effects on energy balance, so we characterized the effects of leptin deficiency in rats by measuring metabolic parameters including body weight and adiposity. Deletion of 151 bp within exon 1 of the lep gene resulted in male KO rats that were significantly heavier than littersmates by 7 weeks of age and were 50% heavier by 12 weeks of age (P < .01) (Figure 1A). Similarly, body mass was significantly higher in female KO rats by 6 weeks of age and was 60% greater than that of WT littersmates by 12 weeks of age (P < .01) (Figure 1B). MRI confirmed that the increase in body mass was attributable to increased visceral and subcutaneous adiposity in both male and female KO rats (Figure 1C). Total fat mass, as determined by NMR analysis, was 2-fold higher in 18-week-old KO males and 2.8-fold higher in KO females than in their respective WT littersmates (P < .05) (Figure 1D). Lean mass did not differ between KO rats and WT littersmates (P > .05). Levels of adiponectin, an adipose tissue-derived hormone that is inversely related to obesity and insulin resistance (31, 32), were significantly higher in male and female KO rats at 9 weeks of age than in WT littersmates, whereas only female KO rats maintained higher adiponectin levels than WT littersmates at 34 weeks of age (P < .05) (Figure 1E).

Ectopic lipid accumulation has been observed previously in ob/ob mice (33) and ZDF rats, which lack functional leptin receptors (34). To determine whether ectopic lipid accumulation was present in leptin-deficient rats, we quantified and compared muscle and hepatic lipid content of 36-week-old KO rats and WT littersmates. Muscle triglyceride content in KO male and female rats was 4.6- and 4.2-fold greater than that in their respective WT littersmates. Similarly, hepatic triglyceride content in KO male and female rats was 1.8- and 3.3-fold higher than that in their respective WT littersmates (P < .05) (Table 1 and Supplemental Figure 1A published on The Endocrine Society’s Journals Online web site at http://end.endojournals.org).

Obesity can be a consequence of both increased energy intake and reduced energy expenditure. Previous studies have suggested that leptin regulates energy expenditure in part through brown adipose tissue thermogenesis (35); indeed, leptin deficiency in mice has been shown to diminish their capacity for thermoregulation (36). At ambient temperature, body temperature of male and female KO rats and WT littersmates did not differ (Figure 1F). When KO rats were subjected to a cold (4°C) environment for 90 minutes, body temperature was reduced by ~1°C after 15 minutes and remained this way for the duration of cold exposure, whereas the body temperature of WT littersmates did not change (P < .05) (Figure 1F).

Leptin-deficient rats have impaired fasting glucose regulation

To investigate whether leptin deficiency in rats affects glucose levels, fasting blood glucose and plasma insulin were measured in KO rats at multiple ages. Fasting glucose...
levels were initially comparable to those of WT rats, but by 8 weeks of age fasting glucose levels were typically 1.5-fold higher in male KO rats than in WT littermates and were significantly elevated between 14 and 25 weeks of age ($P < .05$) (Figure 2A). In contrast, female KO rats maintained fasting glucose levels similar to those of WT littermates, except at 12 weeks of age (Figure 2B). These findings are in line with previous reports indicating that female rats tend to be protected against increases in fasting glucose levels compared with males, an effect that may be mediated by estrogen (37, 38).

Previously, Vaira et al (22) reported that KO rats have elevated fasting plasma insulin levels compared with those of WT littermates at 4 weeks of age. Here we report sustained fasting hyperinsulinemia in male and female KO rats up to 36 weeks of age. At 6 weeks of age, plasma insulin levels were 22-fold higher in male KO rats than in WT littermates ($12.58 \pm 1.82$ ng/mL vs $0.57 \pm 0.12$ ng/mL, $P < .05$) (Figure 2C), whereas plasma insulin levels of female KO rats were 54-fold higher than those of WT littermates ($37.38 \pm 3.84$ ng/mL vs $0.70 \pm 0.02$ ng/mL) (Figure 2D).

In addition to insulin, glucagon and GH also contribute to regulation of glucose homeostasis. Fasted plasma glucagon levels were elevated in KO males ($215.20 \pm 16.44$ ng/mL vs $136.39 \pm 8.92$ ng/mL, $P < .01$) and KO females ($190.10 \pm 13.82$ ng/mL vs $114.70 \pm 8.73$ ng/mL, $P < .01$) compared with those of their respective littermate controls (Figure 2, E and F). A trend for reduced GH levels in male and female KO rats compared with those of their respective WT littermates between the ages of 10 and 14 weeks was observed ($P = .05$ and not significant, respectively) (Figure 2F). Despite a similar trend for concomitant hyperinsulinemia, hyperglucagonemia, and reduced GH lev-

**Table 1. Liver and Muscle Lipid Content**

<table>
<thead>
<tr>
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<th>WT</th>
<th>KO</th>
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<td>Liver triglycerides, nmol/mg tissue</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>6.60 ± 1.49</td>
<td>12.03 ± 1.53a</td>
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<tr>
<td>Female</td>
<td>5.83 ± 0.66</td>
<td>19.07 ± 0.44b</td>
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<tr>
<td>Liver cholesterol, nmol/mg tissue</td>
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<tr>
<td>Male</td>
<td>5.30 ± 0.59</td>
<td>5.58 ± 0.32</td>
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<tr>
<td>Female</td>
<td>4.73 ± 0.47</td>
<td>6.57 ± 0.73</td>
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<tr>
<td>Muscle triglycerides, nmol/mg tissue</td>
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</tr>
<tr>
<td>Male</td>
<td>0.14 ± 0.02</td>
<td>0.65 ± 0.13a</td>
</tr>
<tr>
<td>Female</td>
<td>0.11 ± 0.02</td>
<td>0.46 ± 0.12a</td>
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Data are means ± SEM.

a $P < .05$ by Student $t$ test.

b $P < .001$ by Student $t$ test.
els, female KO rats do not develop a transient elevation in fasting glucose levels, whereas male KO rats do.

Glucose tolerance and insulin sensitivity are disrupted in leptin-deficient rats

To further characterize the effects of leptin deficiency on glucose handling, glucose tolerance and insulin sensitivity were assessed using an oral GTT. After an overnight fast, blood glucose levels were comparable between 7-week-old male and female KO and WT rats. However, by 20 minutes postgavage, blood glucose levels were higher in both male (Figure 3A) and female (Figure 3B) KO rats compared with those in WT controls. Area under the curve analysis of the glucose response revealed glucose intolerance in male KO rats (1274.50 ± 1100.61 vs 978.00 ± 24.39, \(P < .05\)) by one-way ANOVA (A–D) and the Student t test (E and F).

To assess glucose tolerance in older rats, we performed GTTs on 34-week-old rats (Figure 3, E–H). Glucose tolerance and impaired glucose-stimulated insulin secretion (GSIS) occur in KO rats. A and B, An oral GTT was performed on 7-week-old male (A) and female (B) rats after an overnight fast (1.5 g/kg glucose). C and D, Plasma was collected at each time point to measure GSIS in male (C) and female (D) rats. A GTT was repeated at 34 weeks of age in aging male (E) and female (F) rats, with corresponding measurement of plasma insulin levels (G, males; H, females). Data are expressed as means ± SEM (n = 4–7). *, \(P < .05\) by two-way ANOVA.

Figure 2. Leptin-deficient rats are hyperinsulinemic, and males experience transient hyperglycemia. A–D, Fasting blood glucose levels (A and B) and plasma insulin (C and D) were measured in male and female rats. E and F, Fasted glucagon levels (E) and GH levels (F) were measured in male and female rats at 26 weeks of age and 10 to 14 weeks of age, respectively. Data are expressed as means ± SEM (n = 3–9). *, \(P < .05\) by one-way ANOVA (A–D) and the Student t test (E and F).

Figure 3. Glucose intolerance and impaired glucose-stimulated insulin secretion (GSIS) occur in KO rats. A and B, An oral GTT was performed on 7-week-old male (A) and female (B) rats after an overnight fast (1.5 g/kg glucose). C and D, Plasma was collected at each time point to measure GSIS in male (C) and female (D) rats. A GTT was repeated at 34 weeks of age in aging male (E) and female (F) rats, with corresponding measurement of plasma insulin levels (G, males; H, females). Data are expressed as means ± SEM (n = 4–7). *, \(P < .05\) by two-way ANOVA.
erance of WT rats at 34 weeks of age was comparable to results at 7 weeks of age, whereas glucose tolerance deteriorated with age in KO rats (Figure 3, E and F). Glycemic excursion was 1.4-fold greater in 34-week-old male KO rats \((1390.50 \pm 111.36 \text{ vs } 895.63 \pm 38.67, \text{ } P < .05)\), and 1.5-fold greater in 34-week-old female KO rats \((1364.50 \pm 102.02 \text{ vs } 917.88 \pm 19.54 \text{ } P < .05)\) compared with that in WT littermates of the same age. Blood glucose levels peaked at 60 minutes in KO rats at 34 weeks of age in comparison to 30 minutes at 7 weeks of age. Although KO rats had pronounced hyperinsulinemia relative to that in WT rats, older female KO rats failed to show increased insulin levels until 60 minutes after glucose administration and increases in insulin levels were evident in male KO rats only after 120 minutes \((P < .05 \text{ by two-way ANOVA})\). Fasting insulin levels are regulated by insulin output from the pancreas as well as clearance by the liver and kidney. In a leptin-deficient state, ob/ob mice have reduced insulin clearance \((39)\). To assess insulin clearance, plasma C-peptide levels were measured during the glucose challenge at 34 weeks of age and compared with insulin levels. A reduced C-peptide to insulin ratio indicates reduced clearance. Despite having 10- to 15-fold higher C-peptide levels \((P < .05)\) (Supplemental Figure 2A), KO rats had a lower C-peptide to insulin ratio than WT littermates, indicating reduced insulin clearance \((P < .05)\) (Supplemental Figure 2B). These findings suggest that deficits in insulin clearance in addition to increased basal secretion may contribute to hyperinsulinemia in this model.

Because hyperinsulinemia is often associated with obesity and insulin resistance \((40, 41)\), we next tested whether insulin resistance might contribute to the diminished glucose tolerance observed in leptin KO rats. Sixty minutes after ip injection of insulin, glucose levels in 11-week-old male and female WT rats were reduced by 52% and 48%, respectively, compared with baseline (Figure 4, A and B). In contrast, blood glucose levels were unchanged in KO rats after insulin injection, indicating significant insulin resistance. The integrated change in blood glucose levels (represented as area over the curve [AOC]) remained positive in WT males and females \((270.17 \pm 76.32 \text{ and } 363.03 \pm 101.44 \text{ mM, respectively})\) but was negative for KO males \((-18.61 \pm 79.51 \text{ mM})\) and females \((-7.33 \pm 111.68 \text{ mM})\). At 34 weeks of age, both male and female KO rats remained insulin resistant compared with WT rats \((\text{AOC } = 168.33 \pm 66.21 \text{ in male WT rats vs } 451.15 \pm 17.42 \text{ in KO rats and } \text{AOC } = 513.03 \pm 21.46 \text{ in female WT rats vs } -44.64 \pm 85.68 \text{ in KO rats})\) (Figure 4, C and D, respectively). Hepatic phosphorylation of Akt (S473) was reduced by 1.7-fold \((P = .05)\) (Figure 4E), whereas phosphorylation of Erk1 (T202/Y204) and Erk2 (T185/Y187) was reduced by 2.4-fold relative to that in WT animals \((P < .05)\) (Figure 4F). Thus, KO rats had significant impairments in hepatic insulin sensitivity concomitant with hyperinsulinemia, which was sustained with aging.

To investigate the effects of leptin deficiency on postprandial glucose metabolism, 13-week-old rats were subjected to an overnight fast followed by 1 hour of free feeding. Data are expressed as means ± SEM (n = 4–6). *, \(P < .05\) KO vs WT by the Student \(t\) test. ITT, insulin tolerance test.

Figure 4. Leptin deficiency results in insulin resistance. A–D, Insulin sensitivity was measured after ip injection of insulin \((0.5 \text{ U/kg body weight})\) in male (A) and female (B) rats at 11 weeks of age and again at 34 weeks of age (C, male; D, female). E and F, Phosphorylation \((P)\) of Akt \((\text{E}; \text{ Ser473})\) and Erk1/2 \((\text{F}; \text{ Thr202/Tyr204 of Erk1 and Thr185/Tyr187 of Erk2})\) was assessed in liver lysates of 36-week-old male and female animals. E and F, Blood glucose \((\text{E})\) and plasma insulin levels \((\text{F})\)
1 hour of free feeding, blood glucose levels of male KO rats were 1.7-fold higher than those of WT littermates (10.32 ± 0.93 mM vs 6.06 ± 0.20 mM, \( P < .05 \)) (Figure 4E). Similarly, female KO rats exhibited a 1.5-fold increase in blood glucose levels compared with those in WT rats after 1 hour of free feeding (12.00 ± 1.30 mM vs 7.92 ± 0.69 mM, \( P < .05 \)). Interestingly, these elevations in postprandial blood glucose levels occurred in KO rats despite hyperinsulinemia in the fasted and fed state (12.68 ± 1.71–74.75 ± 3.25 ng/mL in males and 29.64 ± 6.54–160.72 ± 46.60 ng/mL in females, \( P < .05 \), WT vs KO, by two-way ANOVA). Feeding also increased insulin levels in WT rats (0.20 ± 0.07–0.55 ± 0.14 ng/mL in males and 0.20 ± 0.03–0.59 ± 0.13 ng/mL in females), but to a lesser extent.

Leptin deficiency results in increased relative \( \beta \)-cell area and insulin secretion

A defining feature of \( ob/ob \) mice is their large pancreatic islets consisting predominantly of \( \beta \)-cells (13). To determine whether this is also the case in KO rats, we stained pancreas sections taken from 3 different regions for insulin and glucagon. Islets from 36-week-old male KO rats appear larger (Supplemental Figure 1B), and \( \beta \)-cell area relative to total pancreas area, as determined by immunostaining, was 3-fold higher than that of WT rats (1.74 ± 0.46% vs 0.54 ± 0.05% in KO rats, \( P < .05 \)) (Figure 5B). No differences in relative \( \alpha \)-cell areas were observed between WT and KO rats (0.014 ± 0.002% vs 0.016 ± 0.003%, \( P > .05 \)) (Figure 5C). Despite having greater \( \beta \)-cell area, insulin levels did not significantly increase in KO rats until 120 minutes after the glucose gavage (Figure 3, C and D, G and H), indicating a potential deficit in stimulus secretion coupling. GLUT-2 is believed to be a key component of \( \beta \)-cell glucose sensing in rodents (42). Whereas GLUT-2 staining was clearly evident in WT rats, GLUT-2 immunoreactivity within KO islets appeared to be reduced (Figure 5A). Although there was a delay in glucose-stimulated insulin secretion, reduced GLUT-2 does not seem to result in reduced insulin output in KO islets.

We next determined whether changes in islet morphology were associated with alterations to islet function ex vivo by subjecting islets isolated from 12-week-old rats to perifusion. Basal (3 mM glucose) insulin secretion was mildly higher from KO islets than from WT islets (6.10 ± 0.61 ng/mL vs 3.07 ± 0.43 ng/mL, \( P < .05 \)) (Figure 5D). In response to 20 mM glucose, islets from KO rats secreted, on average, 2.5-fold more insulin than WT islets (16.4 ± 5.03 ng/mL vs 5.66 ± 0.93 ng/mL, \( P = .058 \)).

Insulin secretion (averaged over the duration of exposure to stimuli) was higher than that in WT islets in response to 30 mM KCl (31.8 ± 9.31 ng/mL vs 8.61 ± 0.47 ng/mL, \( P < .05 \)) and 15 mM arginine (45.7 ± 14.3 ng/mL vs 19.3 ± 3.58 ng/mL, \( P = .12 \)). These data are consistent with results obtained in vivo in which KO rats had a greater increase in plasma insulin than WT rats after 1 hour of free feeding (Figure 4H).

Leptin therapy reverses the obese, glucose-intolerant phenotype

Next we sought to determine whether a reversal in phenotype could be induced by introducing recombinant leptin to 15-week-old leptin-deficient male rats via subcutaneous miniosmotic pump. Before leptin therapy, plasma leptin values were undetectable (lower limit of detection = 0.15 ng/mL) in KO rats, whereas fasting WT levels were...
2.72 ± 0.41 ng/mL. Seven days after leptin therapy was started, plasma leptin levels in leptin-treated KO rats were 5-fold higher than those in WT rats with vehicle (water) treatment (circulating leptin = 27.5 ± 2.59 ng/mL in KO rats vs 5.11 ± 0.61 ng/mL in WT rats, P < .001) (Figure 6A). Before leptin therapy, WT and KO rats had similar rates of weight gain. However, KO rats experienced an attenuation of weight gain after leptin pump implantation, whereas vehicle-treated WT rats continued to gain weight (P < .05) (Figure 6B). The attenuated weight gain observed in leptin-treated KO rats was partly attributed to decreased food intake. Before leptin therapy, leptin-deficient rats were consuming 1.6-fold more food than WT littermates (43.3 g/d vs 26.7 g/d; P < .001). Vehicle-treated WT littermates continued to consume between 25 and 35 g of food per day; however, 7 days after initiation of leptin therapy, KO rats were consuming an average of 13.33 ± 0.93 g of food per day, a 70% decrease from preleptin values (P < .05) (Figure 6C).

It is well established that leptin can suppress insulin release via the bidirectional adipoinsular axis (43). We therefore sought to determine whether leptin therapy was able to reduce plasma insulin levels in KO rats. Before leptin treatment, KO rats had circulating insulin levels averaging 12.74 ± 3.05 ng/mL (Figure 6D). After 2 weeks of leptin therapy, KO rats remained hyperinsulinemic relative to WT rats, but insulin levels were reduced by 62% (4.83 ng/mL ± 1.32 ng/mL, P < .05) (Figure 6D). In addition to reductions in body weight and plasma insulin, leptin therapy also resulted in reduced circulating plasma triglyceride levels. After 3 weeks of leptin therapy, plasma triglyceride levels were reduced from preleptin values of 3.19 ± 0.47 mg/mL to 1.68 ± 0.46 mg/mL (P < .05) (Figure 6E).

Reduced circulating insulin levels were also accompanied by improved glucose tolerance and insulin sensitivity. Before leptin therapy, KO rats were glucose intolerant compared with WT controls (area under the curve = 637.5 ± 144.2 vs 252.5 ± 61.8, P < .05) (Figure 7A) and exhibited basal hyperinsulinemia (5.06 ± 0.66 ng/mL vs 0.327 ± 0.03 ng/mL) and impaired glucose-stimulated insulin secretion (Figure 7B). After 3 weeks of leptin therapy, glucose tolerance was comparable to that of WT littermates (P > .05) (Figure 7A) and basal insulin levels in

Figure 6. Leptin therapy reduces weight gain, food intake, and fasting insulin levels in KO rats. Male KO rats (15 weeks old) were treated with continuous leptin infusion via miniosmotic pump (100 µg/d); WT littermate controls received pumps infusing vehicle (water). A–E, Plasma leptin levels (A), body weight (B), food intake (C), fasted plasma insulin levels (D), and plasma triglyceride levels (E) were measured before and after pump implantation in WT and KO rats. F, Two weeks after pump implantation, plasma GLP-1 levels were measured in WT and KO rats after an overnight fast and 1 hour of free feeding. Data are expressed as means ± SEM (n = 4–6 per group). *, P < .05 WT vs KO by two-way ANOVA; ***, P < .001 WT vs KO on day 14 by the Student t test.
KO rats were reduced by 57% (from 5.06 ng/mL to 2.20 ng/mL; \( P < 0.05 \)) (Figure 7B). Furthermore, the kinetics of glucose-stimulated insulin secretion in KO rats more closely resembled that of WT rats during the GTT. Before initiation of leptin therapy, ip injection of insulin (0.75 U/kg) induced a mild reduction in blood glucose levels (20% compared to baseline) in 14-week-old male KO rats by 90 minutes after injection compared with a 54% reduction in WT (\( \bullet \)) rats (Figure 7C). After 8 days of leptin therapy, insulin sensitivity was restored in KO rats and the reduction in blood glucose in the 45 minutes after insulin injection was comparable between KO rats and WT littermates (Figure 7C). Leptin has been demonstrated previously to stimulate release of the gut peptide GLP-1 (44), a hormone that has many glucose-lowering actions (45). However, leptin-treated KO rats did not have significantly elevated GLP-1 levels (Figure 6F), suggesting that this may not be the mechanism mediating improvements to glucose homeostasis in this model.

**Discussion**

The \( \text{o}b/\text{ob} \) mouse has been the favored model for studying the effects of leptin deficiency on energy balance and glucose homeostasis. Vaira et al (22) reported that leptin KO rats, much like their murine equivalents, have impaired energy balance, glucose intolerance and elevated insulin levels at 4 weeks of age (22). In the current study, we expanded on these findings to more thoroughly characterize energy balance and glucose handling in both male and female rats at different ages.

Comparable to \( \text{o}b/\text{ob} \) mice (6), leptin KO rats double their body weight between 4 and 8 weeks of age and have increased ectopic triglyceride accumulation in liver and muscle. These findings are similar to recent observations with leptin-deficient F344/NS1c rats generated by \( N\)-ethyl-\( N\)-nitrosourea mutagenesis (46), as well as previous reports of elevated hepatic triglycerides observed in adult ZDF rats (47) and \( \text{o}b/\text{ob} \) mice (48). Consistent with previous reports of hyperglucagonemia and hyperinsulinemia in \( \text{o}b/\text{ob} \) mice (6, 13), glucagon levels were 1.6-fold higher in KO rats than in WT rats at 26 weeks of age, and insulin levels were at times \( \approx 50 \)-fold higher in KO rats than in WT rats. Analysis of C-peptide levels demonstrated that the dramatic hyperinsulinemia in KO rats may be partly attributed to reduced insulin clearance. Insulin resistance and decreased hepatic insulin signaling in KO rats suggested that there might be reduced sensitivity of the \( \alpha \)-cell to paracrine insulin signaling (49). This, combined with the absence of leptin signaling, which is known to suppress glucagon secretion in \( \alpha \)-cells (50), could contribute to the elevated glucagon levels observed. With further aging or additional \( \beta \)-cell insults such as consuming a high-fat diet, \( \beta \)-cell failure might occur, resulting in diabetes, as has been observed in ZDF rats (51).

Despite the similarities outlined above, some phenotypic inconsistencies were observed between the leptin KO rat and \( \text{o}b/\text{ob} \) mouse. The transiently elevated fasting glucose levels in male KO rats have also been observed in \( \text{o}b/\text{ob} \) mice (13); however, the degree of hyperglycemia was substantially lower than the fasting blood glucose levels reported in adult \( \text{o}b/\text{ob} \) mice (14, 31, 52). In addition, adiponectin was increased in male and female KO rats than in WT littermates at 9 weeks of age, whereas it...
was reduced in ob/ob C57BL/6J mice (31). Unlike ob/ob mice (53), the body temperature of KO rats was comparable to that of WT rats at ambient temperature. Within 1 hour of cold exposure, ob/ob mice experience a rapid and steep decline in body temperature (53). In contrast, body temperature of KO rats was only mildly reduced within the first 15 minutes of cold stress and remained constant for the duration of cold exposure. Although this finding suggests differences in thermoregulatory capacity between leptin-deficient species, longer durations of cold exposure may be required to elucidate the effects on thermoregulatory capacity due to differences in surface area between rats and mice.

In light of previous reports showing that leptin improves metabolic phenotype and glucose handling, we evaluated whether recombinant leptin administration could reverse the obese, glucose-intolerant phenotype in KO rats. Similar to previous reports in mice (2, 14, 16, 36), administration of leptin to KO rats dramatically reduced weight gain compared with preleptin values. In addition, leptin therapy reduced circulating insulin levels in KO rats by 2.6-fold within 7 days. This was probably due to a combination of leptin acting directly on β-cells to inhibit insulin secretion (54) and indirectly via the sympathetic nervous system (55). Leptin treatment in KO rats also resulted in a ~50% reduction in plasma triglyceride levels. This may have resulted from leptin modulating hepatic lipoprotein production and metabolism (56, 57). Leptin therapy also resulted in improved insulin sensitivity and glucose tolerance, which is consistent with previous studies in both mice and rats that underwent leptin therapy (58, 59). Although food intake initially decreased in leptin-treated KO rats, there was a gradual recovery in food intake over the duration of leptin therapy. Analysis of plasma leptin levels after pump implantation demonstrated a reduction in circulating levels of immunoreactive leptin in KO rats after 24 days. We speculate this could be a result of altered pump function in the KO rats or altered leptin clearance.

Taken together, our findings demonstrate that the function of leptin in glucose homeostasis is largely conserved between rats, mice, and humans. Compared with murine models, rats more closely mimic responsiveness to environmental influences such as diet and stress (60). The mild perturbations to fasting glucose levels that we observed in leptin KO rats are more consistent with the normal fasting glucose levels of leptin-deficient humans (19, 20). However, most obese humans are not leptin deficient but rather have high plasma leptin levels associated with a state of leptin resistance (61). The leptin KO rat may be used to understand the metabolic consequences of impaired leptin function that occurs in both leptin-deficient and leptin-resistant states. The larger size of rats than mice provides additional benefits of greater blood volume and increased sequential blood sampling, allowing for easier measurements of physiological parameters. Although the glucose handling of other rat models of impaired leptin action (eg, ZDF rat and Koletzky rat) has been well established, the absence of functional leptin receptors makes it challenging to assess the effects of leptin therapy on glucose homeostasis. Here we clearly show that leptin replacement in KO rats rapidly and dramatically improved glucose homeostasis and energy metabolism.

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Address all correspondence and requests for reprints to: Timothy J. Kieffer, 2350 Health Sciences Mall, University of British Columbia, Vancouver, BC, Canada V6T 1Z3. E-mail: tim.kieffer@ubc.ca.

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