Strain-Dependent Stimulation of Growth in Leptin-Treated Obese \( \text{db/db} \) Mice

ABRAM M. MADIEHE, SADIE HEBERT, TIFFANY D. MITCHELL, AND RUTH B. S. HARRIS

Department of Foods and Nutrition, University of Georgia (A.M.M., T.D.M., R.B.S.H.), Athens, Georgia 30602; and Pennington Biomedical Research Center (S.H., T.D.M., R.B.S.H.), Baton Rouge, Louisiana 70808

Leptin increases the proliferation of various cell types in vitro, and we reported that background strain influences the metabolic responses to leptin in \( \text{db/db} \) mice, which express short-form, but not long-form, leptin receptors. Here, we examined the effects of leptin on growth of young \( \text{C57BL/6J} \), \( \text{C57BL/6J} \), and \( \text{C57BL/3J} \) \( \text{db/db} \) mice. Intraperitoneal infusions of 20 \( \mu \)g leptin/d for 26 d increased the food intake of \( \text{C57BL/6J} \) mice by 15% \((P < 0.01)\), but had no effect in \( \text{C57BL/3J} \) \( \text{db/db} \) mice. Leptin-infused \( \text{C57BL/6J} \) \( \text{db/db} \) mice gained more weight (-20%; \( P < 0.04 \)) than PBS-infused controls. The increased weight was sustained after leptin infusion ended. Leptin had no effect on weight gain or food intake of \( \text{C57BL/3J} \) \( \text{db/db} \) mice, which only express the soluble leptin receptor. A single leptin injection increased MAPK phosphorylation in liver by 40% \((P < 0.001)\) and that in muscle tissues by 20% \((P < 0.001)\) in \( \text{C57BL/6J} \) mice, but did not change phosphorylation in \( \text{C57BL/3J} \) \( \text{db/db} \) mice. These results suggest that leptin increases the weight gain of \( \text{C57BL/6J} \) \( \text{db/db} \) mice by activating the MAPK pathway through a mechanism that is dependent on short-form leptin receptors. This response may be masked by activation of the long-form receptor in wild-type animals that lose body fat during leptin treatment. (Endocrinology 143: 3875–3883, 2002)

Leptin, A PRODUCT of the \( \text{ob} \) gene, is released predominantly by white adipose tissue and plays a significant role in the regulation of food intake and the control of body weight. These effects of leptin are mediated by central receptors (1, 2), and it has been proposed that leptin acts as a feedback signal to the brain, adjusting energy intake and thermogenesis to keep the size of body energy stores constant (3). Originally it was assumed that leptin would function as a negative feedback signal in the regulation of energy balance, inhibiting food intake in response to positive energy balance (4). There is now a significant amount of evidence to suggest that a low concentration of leptin is a signal of energy deficit and limits the activity of energy-expensive functions, such as reproduction (5). In addition to its role in regulating energy balance, it is now clear that leptin has many other metabolic and reproductive effects (6).

Leptin exerts its effects through the leptin receptor (Ob-R), which has at least five splice variants (Ob-Ra, -Rb, -Rc, -Rd, and -Re) (3). This receptor bears strong sequence homology to the class I cytokine receptor family that signals via Janus-activated kinase (JAK) and signal transducers and activators of transcription pathways (7, 8). Mutations in the Ob-R gene occur in \( \text{db/db} \) mice (3, 9) and \( \text{fa/fa} \) rats (10, 11), producing severe obesity. The long form of the receptor, Ob-Rb, is highly expressed in the hypothalamus and is the predominant signaling isoform (3, 12). This receptor is essential for the inhibition of food intake by leptin (1, 2), and its activity is dependent upon the activation of phosphoinositide 3-kinase (13). ObRe is a circulating receptor (14) that appears to act as a binding protein. The short-form leptin receptors (Ob-Ra, Ob-Rc, and Ob-Rd) are ubiquitously expressed, and, because they lack the cytoplasmic domains necessary for signaling of signal transducer and activators of transcription, they were predicted to be signaling inactive (15). However, these receptors contain the conserved box 1 motif present in cytokine receptors (16), which is required for the association of JAK1 and JAK2 with cytokine receptor proteins (17). Leptin has been shown to activate phosphoinositide 3-kinase in cultured hepatocytes, and it is assumed that this is mediated by short-form leptin receptors because the cells express very low levels of ObRb (18). In addition, transfection studies have shown that both Ob-Rb and Ob-Re induce leptin-dependent activation of the MAPK pathways (14, 19). These protein kinases play a central role in regulating the activity of many nuclear transcription factors involved in inflammatory, immune, and proliferative responses (20) and are activated by various mitogenic hormones and growth factors.

Mutational analyses of the Ob-R showed that genetically obese and diabetic \( \text{db/db} \) mice have a spontaneous frameshift mutation in the Ob-R gene causing a splicing defect, which upon translation results in the production of a mutant protein with a short cytoplasmic domain (3). Obese \( \text{fa/fa} \) rats have a point mutation in the Ob-R gene that leads to decreased binding affinity for leptin (11, 14). The \( \text{db/db} \) mice are unresponsive to leptin treatment with regard to decreases in food intake and body weight, which indicated that they had a defect in the receptor signaling system. The commonly investigated \( \text{db} \) mutant is the \( \text{C57BL/Ks} \) \( \text{db/db} \) mouse (commercially available from The Jackson Laboratory, Bar Harbor, ME). Expression of the \( \text{db} \) mutation on the \( \text{C57BL/6} \) \( \text{BL/6j} \) background results in a phenotype that shows less severe diabetes than the \( \text{BL/Ks} \) mice (21, 22). Although the mutations in \( \text{BL/Ks} \) and \( \text{BL/6j} \) \( \text{db/db} \) mice result in the production of only the short and circulating isoforms of the leptin receptor (3, 23), another point mutation in the \( \text{db} \) gene causes the formation of a premature stop codon that pro-
duces animals that express only the circulating isoform, ObRe (24). This mutation is expressed in the BL/6 background, C57BL/6 Lepr<sup>ob</sup> (10), and is named C57BL/6J (BL/6J) db/db. None of these db/db mice expresses the long-form leptin receptor; therefore, they are expected to be totally unresponsive to leptin, assuming that Ob-Rb is the signaling-competent isoform. We recently reported, however, that administration of exogenous leptin to db/db mice causes metabolic changes that are dependent on the background strain (21), and that leptin improved the diabetic status of 12-wk-old male BL/6J db/db mice, but not that of BL/Ks db/db mice. The aim of this study was to examine the effects of leptin on the growth of young db/db mice raised on different background strains. The studies described here provide evidence that leptin infusion increases the rate of weight gain of 5-wk-old BL/6J db/db mice and that the response to chronic leptin infusion is minimized by the BL/Ks background genome. The results also demonstrate that the effects of leptin are dependent on the membrane-bound receptors, because no effect was observed in BL/6J db/db mice that express only ObRe. In addition, acute administration of leptin activates MAPK in peripheral tissues of BL/6J db/db mice, which may potentially account for their increased weight gain during leptin infusion.

Materials and Methods

**Experiment 1: leptin infusion in young male BL/6J db/db mice**

Previous experiments with db/db mice demonstrated that ip leptin infusions partially reversed the hyperglycemia of 12-wk-old male BL/6J, but not BL/Ks, db/db mice. The objective of this study was to determine whether young BL/6J db/db mice, in which obesity and diabetes were less severe, were more responsive to leptin than the older mice. The mice used in this experiment were obtained from a breeding colony maintained at the Pennington Center and were housed individually in cages with grid floors with access to water and chow (Purina 5001,Ralston Purina Co., St. Louis, MO) in a room maintained at 78–80 F with lights on 12 h/d from 0600 h. All procedures were approved by the Pennington Biomedical Research Center’s investigational animal care and use committee. Sixteen 4-wk-old BL/6J db/db mice were weight-matched into two groups of eight animals each. Body weights and food intakes were recorded daily for 1 wk. Each mouse was anesthetized using isoflurane and fitted with an Alzet miniosmotic pump (model 2004, DirecCorp., Cupertino, CA) placed ip. One group of db/db mice was infused with PBS, and the other group received 20 μg leptin/d in PBS (0.25 μl/h of 3.33 mg/ml recombinant murine leptin; R&D Systems, Inc., Minneapolis, MN) for 26 d. After 2 d of infusion, the mice were fasted for 5 h, and blood samples were collected by tail bleeding midday for measurement of serum concentrations of glucose (Kit 510, Sigma, St. Louis, MO), corticosterone (corticosterone RIA kit; ICN Biomedicals, Inc., Costa Mesa, CA), and free fatty acids (FFA; NEFA C kit, WAKO Chemicals USA, Inc., Richmond, VA).

On d 7 of infusion, mice were fasted for 5 h before the start of an oral glucose tolerance test (OGTT). Mice were gavaged with 50 mg glucose, and blood samples were collected by tail bleeding at 0, 10, 30, and 60 min for determination of glucose and serum insulin (rat insulin RIA kit, Linco Research, Inc., St. Charles, MO) concentrations. The remaining samples were combined for measurement of serum leptin (mouse leptin RIA kit, Linco Research, Inc.). On d 12 of infusion, mice were fasted for 5 h before measurement of insulin sensitivity. A small blood sample was collected from the tail for measurement of glucose (Accu-Chek Instant, Roche, Mannheim, Germany) and insulin. Mice then received ip. injections of 0.75 U/kg insulin (Iletin R, Eli Lilly & Co., Inc., Indianapolis, IN). Insulin was measured 15 min after the injection, and glucose was measured 15, 30, 45, and 60 min after the injection. Mice were killed by decapitation between 1100–1300 h after a 4-h fast on d 26 of infusion. Trunk blood was collected for measurements of serum glucose, insulin, FFA, corticosterone, and leptin. The liver, pancreas, spleen, and four fat pads were weighed and returned to the carcass for body composition analysis (25).

**Experiment 2: leptin infusion in young male BL/6J/Ks mice**

Results from the previous experiment showed an unexpected increase in the growth of BL/6J db/db mice infused with leptin. The objective of this experiment was to determine whether BL/Ks db/db mice were similarly responsive to leptin, as we previously reported that background strain affects the responsiveness of db/db mice to leptin (21). Twelve 4-wk-old BL/Ks db/db mice were purchased from The Jackson Laboratory. They were housed as described above. Seven BL/Ks db/db mice were fitted with Alzet pumps that delivered leptin (20 μg/d for 26 d), and five BL/Ks db/db mice were fitted with pumps that delivered PBS. The experimental design was exactly the same as that in experiment 1, except that insulin sensitivity was not measured in the BL/Ks db/db mice because there was a surge in serum glucose within 30 sec of the insulin injection, presumably due to stress-induced gluconeogenesis, that masked any effect of insulin on glucose clearance.

**Experiment 3: leptin infusion in young female BL/6J db/db mice**

The preceding two experiments demonstrated that male BL/6J, but not BL/Ks, db/db mice grew faster when they were infused with leptin. In a previous study we reported differential effects of leptin on glucose metabolism in male and female BL/6J db/db mice (21). Therefore, the objectives of this experiment were to determine whether female BL/6J db/db mice grew faster if they were infused with leptin, and whether increased weight gain in leptin-infused mice was reversed once the infusion ended. Twelve 4-wk-old female BL/6J db/db mice were housed as described in experiment 1, and body weights were recorded daily. Each mouse was fitted with an Alzet pump that delivered either 20 μg leptin/d (model 1002; 0.25 μl/h of 3.33 mg/ml leptin in PBS) or PBS for 14 d. Mice were maintained for an additional 10 d after the infusion ended. Fasting blood glucose was measured before infusion and at 3-d intervals during and after the infusion period.

**Experiment 4: leptin infusion in young male and female BL/3J mice**

The experiments described above demonstrated that leptin infusion promoted weight gain in both male and female BL/6J db/db mice, which are deficient in Ob-Rb but express all of the short-form leptin receptors. The objective of this experiment was to determine whether female BL/3J db/db mice grew faster if they were infused with leptin, and whether increased weight gain in leptin-infused mice was reversed once the infusion ended.

Male and female BL/3J db/db mice were housed as described for experiment 1. After 1 wk of baseline measurements of food intake and body weight, nine male and seven female mice were fitted with pumps that delivered 20 μg leptin/d for 14 d (see experiment 3). Seven male and six female control mice were infused with PBS. The mice were killed after 13 d of infusion, and organs were weighed and returned to the carcass to determine carcass fat content. Serum concentrations of FFA, leptin, and glucose were measured.

**Experiment 5: leptin dose response in db/db BL/6J mice**

To confirm the leptin responsiveness of male and female db/db BL/6J mice a small dose-response study was carried out. Male and female db/db mice (n = 5/group) were infused for 7 d with 0, 5, or 10 μg/d leptin from Alzet pumps (model 1007D; 0.5 μl/h of 0.42 mg/ml leptin or 0.83 mg/ml leptin in PBS). One group of male db/db mice was also infused with 20 μg leptin/d (0.5 μl/h of 1.66 mg/ml leptin) to confirm the results from experiment 1. Daily food intakes and body weights were recorded, and an OGTT was performed on d 5 of infusion. Mice were killed on d 7 of
infusion for measurements of serum hormone levels and body composition.

**Experiment 6: leptin effect on MAPK**

The experiments described above demonstrate that leptin infusion promotes weight gain in BL/6J db/db mice, but not in BL/ks or BL/3J db/db mice. In vitro proliferation studies have shown that leptin induces proliferation in various cell types by activating the MAPK pathway (14, 27, 28). The objective of this experiment was to examine whether the increased rate of weight gain in response to leptin infusion was associated with increased activation of MAPK that could potentially be mediated by the short-form leptin receptors. Leptin was given as a single injection based on observations by Kim et al. (23) that injection of 1 mg/kg leptin activates MAPK in peripheral tissues of rats.

Experimental procedures were approved by University of Georgia investigational animal care and use committee. Twelve 8-wk-old female BL/6J db/db and eight female BL/3J db/db mice were individually housed at 72–74°F, with lights on 12 h/d from 0600 h, and had free access to water and nonpurified chow diet (Purina 3001, Ralston Purina Co.) until the beginning of the experiment. After 3 d of handling and baseline measurements of body weight, mice were weight-matched and divided into PBS- and leptin-treated groups. Mice were fasted for 4 h before injections to ensure a postabsorptive state. Mice were injected ip with either PBS or 30 μg leptin (1–mg/kg) and were decapitated exactly 1 h later. Fat pads (inguinal, parametrial, retroperitoneal, and perirenal) were dissected out and weighed. Parametrial fat pads, gastrocnemius muscle, and a piece of liver from each mouse were snap-frozen in liquid nitrogen and stored at −80°C. Blood was collected for measurements of serum concentrations of leptin and insulin.

Small pieces of liver, muscle, and parametral fat were homogenized in buffer [20 mm Tris-HCl (pH 7.5), 5 mm EDTA, 0.15 mm NaCl, and 10% glycerol] containing protease (1 μg/ml leupeptin, 1 μg/ml aprotinin, and 2 μm phenylmethylsulfonylfluoride) and phosphatase inhibitors (10 mm NaF, 2 mm sodium vanadate, and 1 mm molybdate). After homogenization, Triton X-100 and Nonidet P-40 were added to final concentrations of 1%, and homogenates were incubated on ice for 15 min with shaking. A crude cytosolic fraction was prepared by centrifuging the homogenate at 4°C for 10 min at 3000g. The supernatant was subjected to SDS-PAGE in a buffer with 10% polyacrylamide gel in 25 mm Tris, 192 mm glycine; and 0.1% SDS, pH 8.3. Proteins were transferred to a polyvinylidene difluoride membrane (Immobilon P, Millipore Corp., Bedford, MA) in a buffer with 25 mm Tris, 192 mm glycine, and 20% methanol. Phosphorylated p44/42 MAPK was detected by Western blot using anti-p44/42 phospho-MAPK and total MAPK antibodies (Upstate Biotechnology, Inc., Lake Placid, NY). The blots were developed using the Renaissance chemiluminescence kit (Perkin-Elmer Life Sciences, Inc., Boston, MA) and were exposed to BioMax-MR x-ray films (Eastman Kodak Co., Rochester, NY). X-Ray films were scanned using a flatbed scanner, and the image was digitized using the UN-SCAN-IT gel version 5.1 software (Silk Scientific, Orem, UT).

**Statistical analysis**

Statistically significant differences in single time point measurements between leptin-infused and PBS-infused animals of a specific genotype were determined by unpaired t test (Statistica, StatSoft, Tulsa, OK). Differences in body weight, food intake, and serum glucose and insulin during the OGTT and insulin sensitivity test were determined by repeated measures ANOVA using day or time as the repeated measure and leptin as an independent variable. Post hoc comparisons of treatment means at different time points were tested by t test, and significance levels were unadjusted for multiple comparisons (SAS for Windows, release 6.12, SAS Institute, Inc., Cary, NC).

**Results**

**Experiment 1: leptin infusion in young male BL/6J mice**

Intraperitoneal infusion of 20 μg/d caused a nonsignificant increase in serum leptin concentrations in db/db mice (Table 1). Male BL/6J db/db mice infused with 20 μg leptin/d gained significantly more weight than control BL/6J db/db mice infused with PBS (Table 1; treatment, P < 0.10; day, P < 0.001; day × treatment, P < 0.0001). Body weight recorded before the pump was inserted on d 0 was used as a covariate in the analysis, because it had a significant (P < 0.03) effect on subsequent weight gain. Daily comparisons of body weight showed that the difference between PBS- and leptin-infused mice was significant (P < 0.04) from d 15 to the end of the experiment (Fig. 1). The food intake of leptin-infused BL/6J db/db mice was also significantly greater than that of PBS-infused mice from d 5–19 of infusion, excluding d 7, 11, 12, and 18 (Fig. 2; treatment, P < 0.01; day, P < 0.0001; day × treatment, P < 0.0001).

Two days of leptin infusion significantly increased serum corticosterone concentrations in BL/6J db/db mice, but at the

**Table 1. Serum measures for db/db BL/6J and BL/Ks male mice in experiments 1 and 2**

|                      | PBS     | Leptin  | PBS     | Leptin  |
|----------------------|---------|---------|---------|---------|
| Serum levels on d 2   |          |         |         |         |
| Glucose (mmol/liter)  | 10 ± 1† | 10 ± 1‡ | 16 ± 1§ | 16 ± 2‡ |
| FFA (μEq/liter)       | 1314 ± 121† | 1345 ± 144‡ | 876 ± 40§ | 911 ± 43‡ |
| Corticosterone (ng/ml)| 124 ± 25† | 180 ± 38‡ | 189 ± 55§ | 198 ± 34‡ |
| Leptin, d 7 (ng/ml)   |          |         |         |         |
| Serum levels at end of study |         |         |         |         |
| Glucose (mmol/liter)  | 28 ± 5  | 26 ± 4  | 40 ± 7  | 34 ± 4  |
| Insulin (ng/ml)       | 8.4 ± 2.0 | 7.2 ± 1.0 | 5.8 ± 2.0 | 6.1 ± 1.1 |
| FFA (μEq/liter)       | 1498 ± 134† | 1605 ± 147‡ | 1104 ± 87§ | 946 ± 59§ |
| Corticosterone (ng/ml)| 199 ± 69† | 65 ± 12§ | 262 ± 25§ | 136 ± 17‡ |

Carcass composition

|                      | Start weight (g) | Weight gain (g/26 d) | Fat (g) | Protein (g) |
|----------------------|-----------------|----------------------|---------|-------------|
|                      | 31.6 ± 0.3      | 12.3 ± 0.4†          | 17.9 ± 0.9 | 6.2 ± 0.4   |
|                      | 10.2 ± 0.6      | 17.9 ± 0.4          | 18.1 ± 0.7 | 6.2 ± 0.2   |
|                      | 5.7 ± 0.2       | 6.2 ± 0.2           | 6.2 ± 0.2 | 6.2 ± 0.2   |

Data are the mean ± SEM for groups of eight mice. db/db mice were infused with 20 μg leptin/d for 26 d. Serum collected at the end of the study was after 26 d of infusion.

Significant differences (P < 0.05) between groups are indicated by the different letter symbols.

Significant difference (P < 0.02) between leptin-infused and PBS-infused BL/6J mice.
end of the experiment corticosterone was significantly lower in leptin-treated mice than in controls (Table 1). Leptin had no effect on glucose or insulin concentrations during the OGGT (data not shown). There were no significant differences in body composition of PBS- and leptin-infused BL/6J db/db mice, although there were small increases in both fat and lean tissue of the leptin-infused mice (Table 1).

Experiment 2: leptin infusion in male BL/6J db/db mice

Leptin infusion caused a nonsignificant increase in serum leptin concentrations in BL/6J db/db mice similar to that in BL/6J db/db mice (Table 1), but the body weight of BL/6J db/db mice was not affected by leptin treatment (Fig. 1). The food intake of PBS-infused BL/6J db/db mice declined toward the end of the infusion period, resulting in a significant difference in leptin intake in treated and control mice on d 20–25 of the experiment (Fig. 2; treatment, $P = 0.0001$; day, $P < 0.0001$; day × treatment, $P < 0.0009$). Leptin had no effect on serum metabolites measured after 2 d of infusion (Table 1) or on glucose and insulin levels in BL/6J db/db during the OGGT (data not shown). The BL/6J db/db mice were significantly more hyperglycemic than the BL/6J db/db mice (fasting glucose: BL/6J controls, 9.5 ± 1.8 mmol/liter; BL/6J db/db mice, 16.0 ± 3.1 mmol/liter; $P < 0.04$) and did not release insulin in response to a glucose challenge. Leptin had no effect on the body composition of BL/6J db/db mice. Serum corticosterone was significantly lower in leptin-treated BL/6J db/db mice than in their controls at the end of the experiment (Table 1).

Experiment 3: leptin infusion in female BL/6J db/db mice

Female BL/6J db/db mice infused with leptin for 2 wk gained more weight than their controls. This difference did not reach statistical significance (Fig. 3; leptin, $P < 0.07$; day, $P < 0.001$; interaction, $P = 0.05$), but the increased weight gain was maintained even after leptin infusion stopped. Leptin had no effect on fasting glucose concentrations in female BL/6J db/db mice at any time during the experiment (data not shown), and there were no significant differences in body composition of leptin-treated and control BL/6J db/db mice (data not shown).
Experiment 4: leptin infusion in BL/3J db/db mice

There was no effect of leptin infusion on body weight (Fig. 4) or food intake (data not shown) in either male or female BL/3J db/db mice. The leptin infusions also did not change fasting glucose or insulin concentrations in these mice (Table 2), and there was no effect of leptin on carcass fat content (Table 2).

Experiment 5: leptin dose response in BL/6J mice

Male BL/6J db/db mice infused with 20 μg leptin/d for 7 d gained more weight than their PBS-infused controls (Fig. 5; leptin, \( P = \text{NS} \); day, \( P < 0.0001 \); day \( \times \) leptin, \( P < 0.04 \)), but there was no significant effect on food intake or serum insulin and glucose concentrations during the OGTT (data not shown). Lower doses of leptin tended to increase weight gain in male mice, but the difference was not significant (Fig. 5). The 10 μg leptin/d infusion did not change serum glucose concentrations measured during the OGTT, but reduced the amount of insulin released in response to the glucose challenge in female BL/6J db/db mice (area under the curve above baseline: control, 60 ± 26 ng/ml/min; 5 μg leptin, 141 ± 52 ng/ml/min; 10 μg leptin, 17 ± 15 ng/ml/min; gender, \( P < 0.001 \); leptin, \( P < 0.02 \); time, \( P < 0.0001 \); gender \( \times \) time, \( P < 0.0002 \); gender \( \times \) time \( \times \) leptin, \( P < 0.09 \)).

Experiment 6: leptin effect on MAPK

A single leptin injection significantly increased serum leptin levels compared with a saline injection, without having any effect on fat pad or liver weight (Table 3). This leptin treatment significantly (\( P < 0.001 \)) increased phosphorylation of liver and muscle MAPK in BL/6J db/db mice, but did not activate MAPK in white adipose tissue (Fig. 6). Leptin injection had no effect on the phosphorylation of MAPK in the liver, muscle, or fat pad of BL/3J db/db mice (Fig. 7).

Discussion

The results of this study show that leptin increases the rate of weight gain of obese diabetic BL/6J db/db mice, but not that of BL/Ks db/db mice, and that this response is dependent on the presence of membrane-bound, short-form leptin receptors, because no effect was observed in BL/3J db/db mice that lack all membrane-bound leptin receptors. In addition, leptin administration induced MAPK activation in the liver and muscle, but not in the adipose tissue of BL/6J db/db mice that expressed short-form leptin receptors. Kim et al. (23) found that an iv injection of leptin stimulated phosphorylation of MAPK in fat, muscle, and liver of rats. The body composition of leptin-infused mice in experiment 1 described here suggested that the increased weight gain was due to an increase in overall growth, including body fat. The failure of a leptin injection to stimulate phosphorylation in adipose tissue from BL/6J db/db mice may have been secondary to the obesity of the mice, because it has been reported that adipose tissue expression of the cytokine signaling inhibitor, SOCS3 (suppressor of cytokine signaling), is

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**TABLE 2.** Serum measures and carcass fat content in BL/3J db/db mice in experiment 4

|          | Male PBS | Male Leptin | Female PBS | Female Leptin |
|----------|----------|-------------|------------|---------------|
| Glucose (nmol/liter) | 24 ± 2<sup>a</sup> | 30 ± 4<sup>b</sup> | 31 ± 3<sup>b</sup> | 24 ± 1<sup>a</sup> |
| FFA (μEq/liter) | 1051 ± 174<sup>a</sup> | 868 ± 64<sup>b</sup> | 1034 ± 84<sup>b</sup> | 850 ± 69<sup>a</sup> |
| Leptin (ng/ml) | 53 ± 5<sup>a</sup> | 70 ± 6<sup>b</sup> | 79 ± 5<sup>a</sup> | 60 ± 5<sup>a</sup> |
| Carcass fat (%) | 63 ± 2<sup>a</sup> | 66 ± 3<sup>b</sup> | 70 ± 2<sup>b</sup> | 64 ± 2<sup>a</sup> |

Data are the mean ± SEM for groups of seven or eight mice. The BL/3J db/db mice were infused with 20 μg leptin/d or PBS for 2 wk before being killed for collection of blood and determination of carcass fat content. Both leptin and glucose were significantly higher \( (P < 0.05) \) in female than in male mice. <sup>a,b</sup> Significant differences between groups are indicated by the different letter symbols.

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**Fig. 4.** Daily body weights of male and female BL/3J db/db mice in experiment 4. Obese mice were infused with 20 μg leptin/d for 14 d. There was no effect of leptin on body weight in either sex.

**Fig. 5.** A, Daily body weights of male BL/6J db/db mice infused with PBS or 20 μg leptin/d for 7 d in experiment 5. The difference in body weight did not reach statistical significance \( (P < 0.07) \). B, Weight change in male BL/6J db/db mice infused with PBS, 5 μg leptin/d, or 10 μg leptin/d for 7 d.
increased in obese rats (29). Alternatively, there may be differences in leptin-induced activation of MAPK in peripheral tissues of leptin-infused and leptin-injected mice. There was no effect of leptin on weight gain or MAPK activity in BL/6J\textsuperscript{db/db} mice that express only the circulating leptin receptor. This study, therefore, provides evidence that leptin, in the absence of a functional Ob-Rb that decreases food intake and body weight, induces activation of MAPK through the short-form leptin receptors. This activation of MAPK may be a potential mediator of leptin effects on weight gain in young BL/6J\textsuperscript{db/db} mice (30).

Experiments from other laboratories have indicated that central or peripheral leptin treatment produces no response in older \textit{db/db} mice (1, 31). These studies focused on the effects of leptin on food intake and body weight regulation, and used \textit{db/db} mice maintained on the BL/Ks background, a strain that has been shown to be more diabetogenic than the BL/6J background strain (22). Measurements made in these experiments confirmed that fasting glucose is elevated in BL/Ks \textit{db/db} mice compared with BL/6J \textit{db/db} mice, but that FFA levels are lower in BL/Ks mice. We recently reported that leptin partially reversed hyperglycemia in male BL/6J \textit{db/db} mice, but not in BL/Ks \textit{db/db} mice (21), which suggests that the metabolic effects of leptin in \textit{db/db} mice are dependent on the background strain. We were unable to measure insulin sensitivity in the BL/Ks mice, because the insulin injection produced immediate gluconeogenesis, which may be considered an indicator of stress. At the end of the study corticosterone was significantly reduced in leptin-treated \textit{db/db} mice from both strains, but to a lesser degree in BL/Ks.

### TABLE 3. Fat pad weight and serum leptin in BL/6J and BL/3J \textit{db/db} mice used in experiment 6

|                   | BL/6J       | BL/3J       |
|-------------------|-------------|-------------|
| Fat pad weight (mg) | 9498 ± 762  | 8611 ± 724  |
| Leptin (ng/ml)     | 154 ± 22\textsuperscript{a} | 1101 ± 66\textsuperscript{b} |
| Fat pad weights/body weight | 0.22 ± 0.02 | 0.23 ± 0.004 |

Data are the mean ± SEM for groups of four or six mice. The mice were injected with 30 µg leptin or PBS and decapitated after 1 h for collection of blood and determination of fat pad weights. Fat pad weight is the sum of inguinal, parametrial, retroperitoneal, and perirenal fat pads.

\textsuperscript{a,b} Leptin injection significantly increased serum leptin levels compared to saline (\(P < 0.0001\)), as indicated by \textit{superscripts} within strains.
than in BL/6 db/db mice. This suggests that the activity of the hypothalamic-pituitary-adrenal axis in BL/Ks mice is also up-regulated compared with that in BL/6 db/db mice.

Leptin has been shown to induce proliferation in various tissues, including adrenal, epithelial, endothelial cells, T cells, and monocytes (2, 14, 32–34), and to promote angiogenesis (34, 35) through activation of MAPK (2). This study provides evidence that leptin activates MAPK in vivo, and that this activation occurs even in the absence of long-form leptin receptors. Therefore, this activation of MAPK may potentially be involved in the increased growth of young, leptin-infused BL/6 db/db mice. Our results are consistent with in vivo studies in which high levels of leptin induced colonic epithelial cell proliferation in BL/6 db/db mice, which was suggested to provide a possible explanation for the observed associations between obesity and the increased incidences of colon cancer (36). In addition, an increase in lean body mass was observed in C57BL/6 db/db mice that were parabiosed to ob/ob mice (37). In the parabiosis experiment the ob/ob mice lost significant amounts of body fat, presumably in response to the leptin delivered in the blood supply from the db/db partner. Simultaneously the db/db partners of the ob/ob mice experienced a 30% increase in body protein content. It was concluded that leptin stimulated the release of a growth factor in ob/ob mice, which then crossed the parabiotic union to stimulate protein accumulation in the db/db partner. If this were true, then the increased weight gain of C57BL/6 mice in experiment 1 described here was not attributable to a direct effect of leptin, but, rather, to leptin-induced stimulation of another growth factor that was active in the mice.

A number of reports have indicated that db/db mice are unresponsive to the effects of leptin injection on food intake (16, 31, 38). In this study a continuous peripheral infusion of leptin significantly increased the food intake of BL/6 db/db mice, which may have been a primary cause of the increased weight gain of the mice or could have been secondary to the energy requirement of an increased growth rate. Infusion from Alzet pumps does not mimic the oscillatory nature or the diurnal rhythm of endogenous leptin release, but it does produce chronic, physiologically relevant changes in circulating leptin concentrations that are equivalent to those present in obesity, rather than the large intermittent elevations of leptin that are produced by daily injections. Circulating leptin levels are extremely high in db/db mice, and leptin is transported into the brain via saturable transport systems (39, 40). Thus, it is unlikely that the small change in leptin caused by the peripheral infusion could have changed central concentrations of leptin, supporting the idea that the response of db/db mice to leptin was mediated in the periphery.

Although Ob-Rb is considered to be the signaling-competent receptor due to presence of domains required for postreceptor protein-protein interactions, the short-form Ob-R has also been shown to perform signaling in vitro. When cells expressing Ob-Ra were treated with leptin, the expression of several immediate early genes, such as c-fos, c-jun, and junB, were induced (17, 41). The induction of these genes after leptin treatment was shown to be mediated by MAPK (17, 19). This activation of MAPK in vitro suggested that the effects of leptin on peripheral tissues could be mediated by short-form leptin receptors, which are expressed in much greater numbers than ObRb in peripheral tissues. The question remained, however, whether leptin was a growth factor in vivo. The experiments described here provide evidence that leptin infusion increases the weight gain of BL/6 db/db mice, and that leptin activates MAPK in insulin-responsive peripheral tissues. Recently, leptin was shown to activate MAPK in wild-type mice, but not in BL/Ks db/db mice (23), suggesting that activation of MAPK requires the long-form leptin receptor. These results are consistent with our observations that leptin does not increase the weight gain of db/db mice on the BL/Ks background. Therefore, the increased growth observed in BL/6 db/db mice is attributable to both the presence of short-form leptin receptors and the increased sensitivity to leptin of this strain of db/db mice compared with BL/Ks db/db mice (21).

Both in vivo and in vitro studies have demonstrated that leptin induces insulin resistance in adipose tissue (27); therefore, the increased adiposity of BL/Ks lean mice compared with BL/6J mice seen in this study may be associated with their insensitivity to leptin. Furthermore, BL/3J db/db mice that lack the membrane-bound receptors are heavier than BL/6J db/db mice, whereas BL/Ks db/db mice have equivalent adiposity to BL/3J db/db mice (data not shown). Thus, it is possible that genetic modifiers present in BL/Ks but absent in BL/6J mice inactivate short-form leptin receptors and produce similar levels of adiposity as in BL/3J db/db mice. It should be noted that the we have previously reported differences in the response of lean heterozygous (db/+ ) BL/6J and BL/Ks mice, which suggests that some features of the leptin receptor signaling system may be inactivated by the BL/Ks genetic background (21). Although our data point out major differences between these two strains, they do not identify the potential modifying factors responsible for these differences. It is possible that the growth response to leptin in BL/6J db/db mice results from the interaction of alleles at the diabetes locus with unknown genetic factors in the BL/6J or BL/Ks genomes. Therefore, our studies highlight the importance of genetic control in studies designed to determine the causes of obesity and diabetes, as previously emphasized by Hummel and colleagues (22). In humans, genetic polymorphisms detected at leptin and leptin receptor loci have been shown to modulate the relationships between different components of the metabolic syndrome. Leptin receptor, β3-adrenergic receptor, and glucocorticoid receptor gene polymorphisms have been associated with an augmented clustering of metabolic abnormalities in response to overfeeding, and genetic factors also seem to modify the responsiveness of metabolic syndrome features to endurance training (42). Furthermore, a strong interaction between genetic background and macronutrient content of the diet on body composition has been described in mice. AKR/J mice have a greater percentage of carcass fat and are more responsive to the effects of dietary fat on body composition than SWR/J mice (43). Therefore, an improved understanding of the effects of genetic background on leptin sensitivity may help in the prevention of obesity and diabetes.

In conclusion, the studies described here characterized the effects of leptin on the growth of young db/db mice raised on
different genetic background strains. The results provide evidence that chronic leptin infusion increases the growth rate of BL/6j db/db, but not BL/Ks db/db, mice. These differential effects in mice of different background strains suggest that in earlier experiments with BL/Ks db/db mice (16, 31, 38) the effects of leptin on body weight and growth were not detected because of the insensitivity of the BL/Ks strain to leptin. The method of leptin administration may also play a role in determining the response in db/db mice. In experiments described here we administered leptin as a continuous peripheral infusion, whereas others gave daily injections of leptin, and we previously found that metabolic responses to leptin can be detected in leptin-infused BL/6j db/db mice, but not in mice receiving daily injections of leptin (21). The experiments described here also provide evidence that short-term leptin receptors, in addition to being transport receptors, perform signaling in vivo by activating the MAPK pathway and may mediate increased weight gain in leptin-treated BL/6j db/db mice. Obese humans and animals have high circulating levels of leptin, but are resistant to leptin’s effects on body weight regulation. It is, therefore, conceivable that the leptin-mediated increase in growth and activation of MAPK observed in this study could be involved in various disease states associated with obesity. Furthermore, our observations place more emphasis on the importance of the background genome on the sensitivity to leptin, which is becoming relevant based on various genetic polymorphisms that may modulate gene–gene interactions and the relationships between crucial components in the development of obesity and diabetes in humans. An improved understanding of the effects of genetic background on the responsiveness to leptin may help in the development of strategies for the prevention of obesity and diabetes.

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Address all correspondence and requests for reprints to: Dr. Abram Madiehe, Department of Foods and Nutrition, Dawson Hall, Athens, Georgia 30602. E-mail: madihe@arches.uga.edu.

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