The pancreatic β cell is a key site for mediating the effects of leptin on glucose homeostasis

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Summary

The hormone leptin plays a crucial role in maintenance of body weight and glucose homeostasis. This occurs through central and peripheral pathways, including regulation of insulin secretion by pancreatic β cells. To study this further in mice, we disrupted the signaling domain of the leptin receptor gene in β cells and hypothalamus. These mice develop obesity, fasting hyperinsulinemia, impaired glucose-stimulated insulin release, and glucose intolerance, similar to leptin receptor null mice. However, whereas complete loss of leptin function causes increased food intake, this tissue-specific attenuation of leptin signaling does not alter food intake or satiety responses to leptin. Moreover, unlike other obese models, these mice have reduced fasting blood glucose. These results indicate that leptin regulation of glucose homeostasis extends beyond insulin sensitivity to influence β cell function, independent of pathways controlling food intake. These data suggest that defects in this adipoinsular axis could contribute to diabetes associated with obesity.

Introduction

In many countries, the prevalence of diabetes is increasing at an alarming rate. The highest rates are observed among populations that have undergone changes in diet, physical activity, and life span that are associated with modernization and adoption of a "Western" lifestyle. The obesity that often develops with this lifestyle is clearly a risk factor for diabetes; ≈80% of subjects with diabetes are obese. The adipocyte-derived hormone leptin, which is present in the circulation at levels proportional to the mass of body fat stores (Maffei et al., 1995), may be a key factor linking diabetes and obesity. The effect of leptin as a satiety factor that can limit stores of lipid is well documented; however, leptin has other profound effects on metabolism (Ahima and Flier, 2000; Friedman and Halaas, 1998; Myers, 2004). Mice that are deficient in either leptin (*ob*/*ob* mice) or signaling via the leptin receptor (*db*/*db* mice) develop both obesity and diabetes. The severity of both conditions can be dramatically attenuated by leptin therapy (Pelleymounter et al., 1995) or restoration of leptin signaling (Kowalski et al., 2001).

Traditionally, the insulin resistance and hyperglycemia that develop in *ob*/*ob* and *db*/*db* mice have been attributed as a secondary consequence of obesity. However, several observations challenge this concept and suggest that the diabetes and obesity phenotypes can be segregated. Firstly, in rodents deficient in leptin or leptin signaling, hyperinsulinemia, and thus the disruption of normal glucose homeostasis, precedes changes in body mass (reviewed in Kieffer and Habener, 2000). Secondly, administration of leptin to *ob*/*ob* mice is able to reduce plasma glucose levels in hours, prior to changes in body weight (Kamohara et al., 1997; Kulkarni et al., 1997; Seufert et al., 1999a). Thirdly, even when administered for several days, low doses of leptin can virtually cure diabetes in *ob*/*ob* mice without altering body weight (Pelleymounter et al., 1995). Finally, pair feeding of *ob*/*ob* mice cannot correct plasma glucose levels to the same extent as leptin therapy (Schwartz et al., 1996). Therefore, leptin appears to have potent actions on glucose homeostasis that are independent of its effects on food intake and body weight.

The mechanisms by which leptin regulates glucose homeostasis independent of food intake are presently unclear. Studies with acute intracerebroventricular (ICV) leptin administration highlight a role for central actions of leptin in the hypothalamus in manipulating glucose metabolism independent of food intake and energy balance (Kamohara et al., 1997). Leptin may also regulate glucose homeostasis through direct actions on insulin-sensitive peripheral tissues such as liver, adipose, and muscle, where leptin receptors are found (Ghilardi et al., 1996). Indeed, leptin has been reported to directly modulate insulin effects in these tissues (Cohen et al., 1996; Muoio et al., 1997; Siegrist-Kaiser et al., 1997). Attenuated leptin receptor expression in adipose tissue yields hyperinsulinemia and glucose intolerance (Huan et al., 2003), supporting the notion that direct actions of leptin on adipose tissue are important for normal glucose homeostasis. However, genetic ablation of leptin receptors in the liver does not appear to influence plasma glucose or insulin levels (Cohen et al., 2001).

Several lines of evidence suggest that insulin-producing pancreatic β cells are a direct target of leptin action and thus a potential mechanism by which leptin can regulate glucose homeostasis. Pancreatic β cells express leptin receptors, including the long-form ObRb (Emilsson et al., 1997; Ghilardi et al., 1996; Kieffer et al., 1996; Seufert et al., 1999b), and in vitro studies indicate that leptin suppresses insulin synthesis and release (Emilsson et al., 1997; Kieffer et al., 1997; Kulkarni et al., 1997;

Ookuma et al., 1998; Seufert et al., 1999a, 1999b). Leptin appears to suppress insulin secretion by reducing glucose transport, cellular ATP levels and activating ATP-sensitive K^+ channels leading to cellular hyperpolarization (Harvey et al., 1997; Kieffer et al., 1997; Lam et al., 2004; Seufert et al., 1999b). As insulin is adipogenic and increases leptin levels (Kolaczynski et al., 1996; Saladin et al., 1995), feedback inhibition of leptin on insulin production as part of an "adipoinsular axis" could function as an endocrine brake to curtail further accumulation of fat (Kieffer and Habener, 2000).

In this study, we examined the effect of combined disruption of the leptin receptor gene in pancreatic β cells and hypothalamus. We find that this disruption leads to aberrant glucose homeostasis that is a consequence of irregular β cell function. Importantly, we find that the effects on glucose metabolism are independent of effects on food intake, thereby supporting the concept that leptin action on glucose homeostasis and food intake are independent.

Results

Lepr$^{flox/flox}$ *RIPcre tg*$^+$ mice have tissue-specific disruption of the leptin receptor gene

To examine the role of leptin in glucose homeostasis, we sought to generate mice with tissue-specific ablation of leptin receptor signaling. We made use of *Lepr*$^{flox/flox}$ mice, which have *loxP* sites flanking exon 17 of the leptin receptor gene (*Lepr*) (Balthasar et al., 2004; McMinn et al., 2004). Exon 17 of *Lepr* is present in the long, signaling form of the leptin receptor (ObRb) and encodes the JAK docking site. Upon Cre-mediated recombination at the *loxP* sites, exon 17 is excised (herein referred to as *Lepr*$^{\Delta 17}$), and a resulting frameshift mutation generates an altered 3′ terminus (McMinn et al., 2004) that no longer encodes tyr_{985} and tyr_{1138}, which are sites of JAK-mediated tyr phosphorylation. Mice homozygous for the *Lepr*$^{\Delta 17}$ allele are deficient in leptin-stimulated STAT phosphorylation (Coppari et al., 2005; Dhillon et al., 2006). The *Lepr*$^{flox/flox}$ mice were crossed with *RIPcre tg*$^+$ mice to produce *Lepr*^{flox} heterozygous offspring that were mated to each other to generate *Lepr*$^{flox/flox}$ and *Lepr*$^{flox/flox}$ *RIPcre tg*$^+$ mice. These mice were subsequently bred to generate animals for experiments. Both male and female *Lepr*$^{flox/flox}$ *RIPcre tg*$^+$ mice are fertile, unlike *db/db* mice. In all experiments, littermate *Lepr*$^{flox/flox}$ and *Lepr*$^{flox/flox}$ *RIPcre tg*$^+$ mice were compared to minimize differences in genetic background. *RIPcre tg*$^+$ mice have the *cre* transgene under the control of a 668 bp segment of the rat insulin II promoter (Postic et al., 1999). In addition to its well-characterized expression in pancreatic β cells (Postic et al., 1999), this promoter of the *RIPcre* transgene also leads to low-level Cre expression in neural derivatives, including the hypothalamus (Gannon et al., 2000).

To characterize the tissue-specific nature of Cre-mediated recombination in our *Lepr*$^{flox/flox}$ *RIPcre tg*$^+$ mice, we harvested genomic DNA from several tissues that have significant impact on glucose homeostasis and performed PCR using primers flanking the *loxP* sites of the *Lepr*^{flox} allele (Figure 1). PCR of genomic DNA from adipose, muscle, liver, islet, hypothalamus, and brain (minus the hypothalamus) tissue from *Lepr*$^{flox/flox}$ mice with and without the *RIPcre* transgene resulted in a product of ≈1370 bp being amplified. This product corresponded in size to the predicted size of the product from the *Lepr*^{flox} allele in the absence of Cre-mediated recombination. In *Lepr*$^{flox/flox}$ mice carrying the *RIPcre* transgene, an ≈ 950 bp product was also amplified in the DNA from islets and weakly amplified in DNA from hypothalamus tissue. This product corresponds in size to the expected product from the *Lepr*$^{\Delta 17}$allele. In the islets, this likely reflects a high degree of excision of the *Lepr*^{flox} allele, because islets are comprised mainly of β cells but also contain several noninsulin-producing cells in which Cre is not expressed (Postic et al., 1999). Thus the *Lepr*$^{flox/flox}$ *RIPcre tg*$^+$ mice have a substantial excision of the leptin receptor-signaling domain in the islets and partial excision in the hypothalamus.

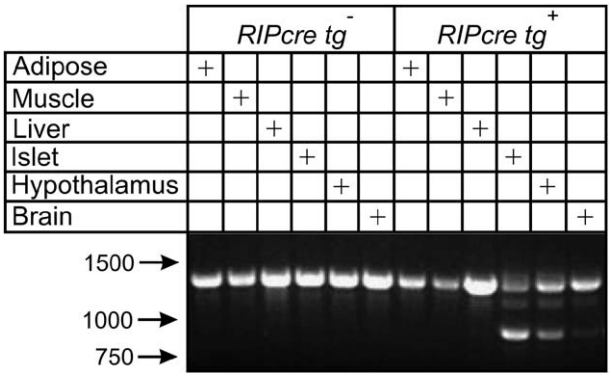

Figure 1. *RIPcre tg*$^+$ results in DNA excision at the *Lepr*^{flox} allele in islets and hypothalamus

Genomic DNA from tissues of *Lepr*$^{flox/flox}$ mice was used as template for PCR of the *Lepr*^{flox} allele. The predicted product sizes are 1369 bp for *Lepr*^{flox} and 952 bp for *Lepr*$^{\Delta 17}$. Arrows to the left mark the migration of molecular weight markers in bp.

Lepr$^{flox/flox}$ *RIPcre tg*$^+$ mice are obese with reduced fasting blood glucose

We tracked the body weight and fasting blood glucose of *Lepr*$^{flox/flox}$ *RIPcre tg*$^+$ mice and *Lepr*$^{flox/flox}$ littermate controls from 3–12 weeks of age. In both males and females, *Lepr*$^{flox/flox}$ *RIPcre tg*$^+$ mice had a statistically significant increase in body weight (Figures 2A and 2C) relative to controls. By 6 weeks of age, tissue-specific attenuation of leptin receptor signaling resulted in a gain of ≈13% in males ($p = 0.0122$) and 18% in females ($p = 0.0002$). By 12 weeks old, the female mice with the *RIPcre* transgene were still ≈18% heavier than those without the *RIPcre* transgene, while in males, the difference had grown to ≈33%. The *Lepr*$^{flox/flox}$ *RIPcre tg*$^+$ mice tended to be slightly longer in body length, however this difference was not statistically significant (91.0 ± 1.3 mm versus 88.6 ± 1.0 mm ($p = 0.08$) for males and 86.2 ± 1.1 mm versus 84.7 ± 2.2 mm ($p = 0.27$) for females for *Lepr*$^{flox/flox}$ *RIPcre tg*$^+$ and *Lepr*$^{flox/flox}$, respectively).

In addition to an increase in body weight, tissue-specific attenuation of leptin receptor signaling had an impact on fasting blood glucose levels (Figures 2B and 2D). At 3 weeks of age, *Lepr*$^{flox/flox}$ *RIPcre tg*$^+$ and *Lepr*$^{flox/flox}$ mice had similar fasting blood glucose levels. However, beyond this point up to 12 weeks old, the *Lepr*$^{flox/flox}$ *RIPcre tg*$^+$ mice typically had lower fasting blood glucose. When analyzed by ANOVA pairwise comparison, the difference in the average fasting blood glucose from 3–12 weeks of age was statistically significant for males ($p = 0.006$) and approaching significance in females ($p = 0.089$). The extent of the difference in fasting blood glucose was greater in the males than the females, with an average difference of

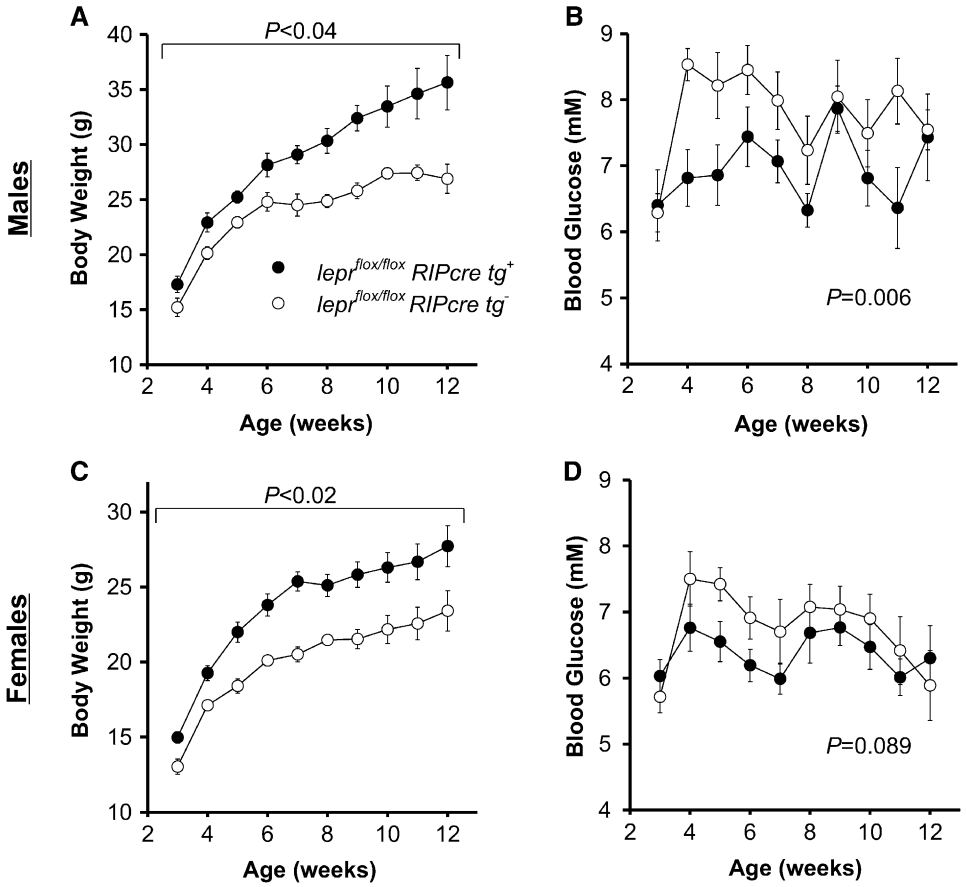

Figure 2. *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice have increased body weight and fasting hypoglycemia

Lepr$^{flox/flox}$ *RIPcre tg*$^{+}$ (closed symbols) and *Lepr*$^{flox/flox}$ littermates (open symbols) were fasted for 4 hr, and body weight and blood glucose were measured. Data are expressed as the average ± SEM, n ≥ 7. Panels (**A**) and (**C**) at each time point p < 0.04 for males and p < 0.02 for females. Panels (**B**) and (**D**), p values were determined by one-way ANOVA pairwise multiple comparison procedure with Holm-Sidak posthoc testing (Sigma Stat, SYSTAT software Inc.) over the 3–12 week period.

10.5% ± 2.5% in the males and 5.2% ± 2.1% in the females over 3–12 weeks of age.

The decreased fasting blood glucose in the *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice correlated with increased plasma insulin levels (Figure 3A). In 6-week-old male *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice, fasting insulin levels were 5.2-fold higher than *Lepr*$^{flox/flox}$ (p = 0.002). In females the situation was similar however the increase in insulin in the *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice was slightly less than the males at a 4.2-fold increase over the *Lepr*$^{flox/flox}$ mice (p = 0.004). In addition to the elevated plasma insulin levels, there was also a striking increase in leptin levels in both genders (Figure 3B). This increase in circulating leptin is consistent with increased adipose mass (Maffei et al., 1995). There was also a greater than 2-fold increase in plasma triglyceride levels in the *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice relative to *Lepr*$^{flox/flox}$ littermates in both genders (Figure 3C). In contrast, the level of plasma cholesterol was not altered in the *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice of either gender (Figure 3D).

The increased body weight and leptin levels of the *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice suggested that there might be increased adipose mass in these mice. To investigate this, we performed magnetic resonance measurements to obtain a ratio of lean/lipid mass (Figure 3E). The *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice had a significant 2-fold reduction in their lean/lipid composition compared to controls (p < 0.001). Surprisingly, this difference in body composition existed even in mice selected for similar body weight (24.5 ± 3.5 g versus 25.3 ± 1.7 g for *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ and *Lepr*$^{flox/flox}$). Magnetic resonance imaging revealed that there was a massive increase in both visceral and subcutaneous lipid in *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice (Figure 3F). This increased adiposity was particularly striking considering the similar body weight of the animals imaged (26.4 g versus 25.9 g for the *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ and *Lepr*$^{flox/flox}$ mice, respectively). Nonadipose tissue also had an increase in lipid accumulation. Hepatic triglyceride levels were 2.1 ± 0.3 and 7.7 ± 2.8 μg triglyceride/mg of liver in the *Lepr*$^{flox/flox}$ and *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice, respectively (n = 4; p = 0.045).

Energy expenditure but not food intake is altered in *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice

Since *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice may have attenuated leptin receptor signaling in the hypothalamus, we sought to investigate if leptin-sensitive markers of neuroendocrine function are altered in *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice. The circulating levels of corticosterone were increased in *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice, while ACTH, testosterone, and thyroxine were not significantly different relative to controls (Table S1 in the Supplemental Data available with this article online). Given the normal ACTH levels, the elevated corticosterone could result from direct effects of the hyperleptinemia in *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice on the adrenal cortex.

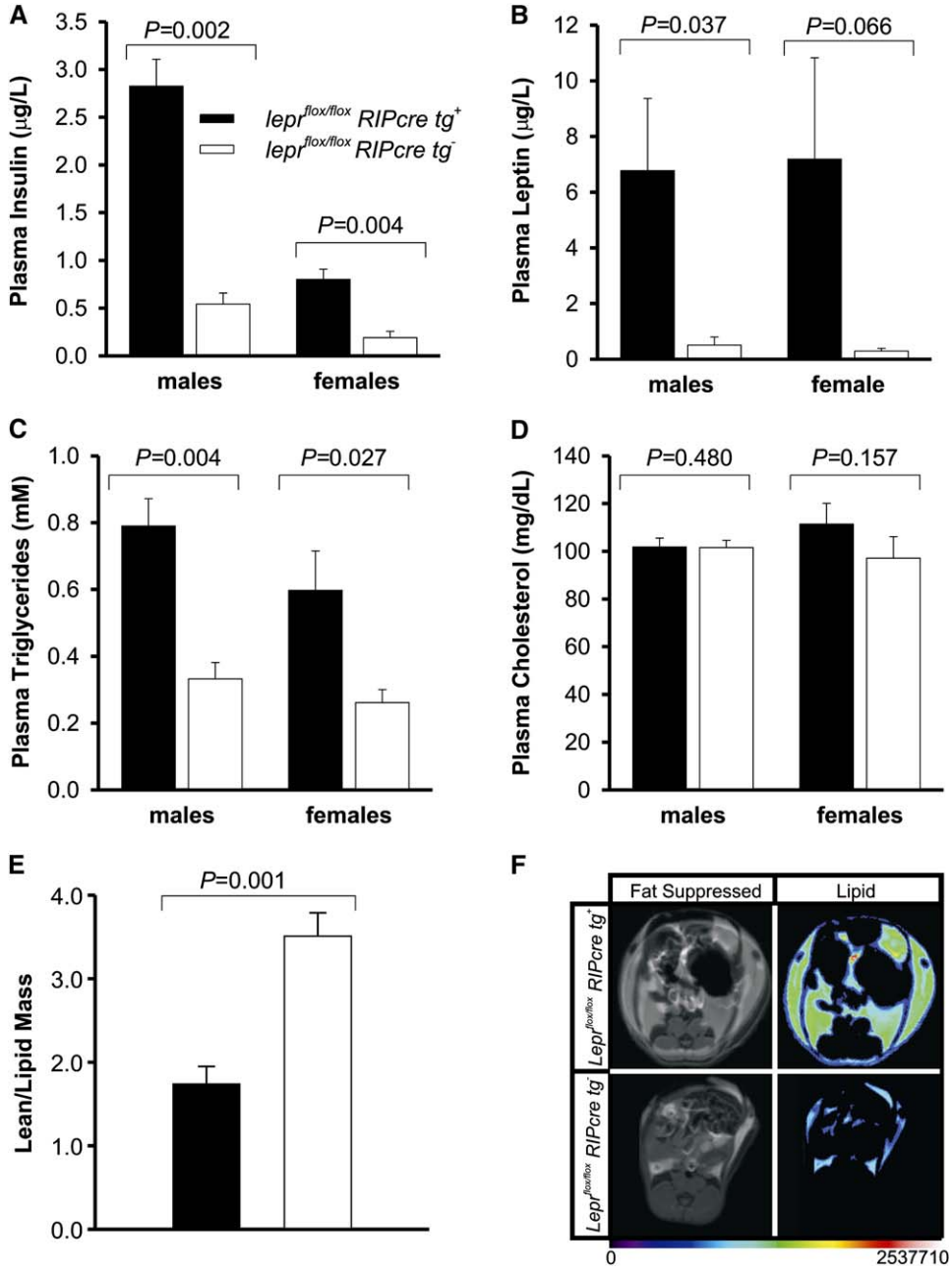

Figure 3. *Leprflox*$^{flox/flox}$ *RIPcre tg*$^{+}$ have elevated plasma insulin and leptin levels and have a decreased lean/lipid body composition

A–D) Plasma was collected from 4 hr fasted 6-week-old *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice (filled bars) and *Lepr*$^{flox/flox}$ littermate controls (open bars), n = 3.
E) Total body lean/lipid ratio in 5-week-old *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice (filled bars) and *Lepr*$^{flox/flox}$ littermate controls (open bars), n = 4.
F) MRI of 6-week-old mice.
In panels (**A**)–(**E**), data are expressed as the average ± SEM.

Since leptin is a potent regulator of food intake (Pelleymounter et al., 1995) and the hypothalamus appears to play a major role in this effect, we investigated if the increased body weight of these mice relative to their non-*RIPcre* transgenic littermates was a result of increased food intake. We monitored food intake in 5-week-old *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice and *Lepr*$^{flox/flox}$ controls over 5 days (Figure 4A). Food intake was comparable between the groups in both males and females, and differences were not statistically significant in either gender, a finding that was also made with 9-week-old mice (data not shown). By comparison, food intake in 5-week-old *db*/*db* mice was significantly greater relative to the *Lepr*$^{flox/flox}$ mice either with or without the *RIPcre* transgene ($p < 0.003$ in males and $p < 0.004$ in females). Thus, food intake does not appear to be altered in the *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice, although it remains possible that differences are present at ages that were not investigated.

The fact that the *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice do not have increased food intake suggests that the leptin signaling pathways in the hypothalamus controlling feeding remain functional. To further test this notion, we treated *db*/*db*, *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$,

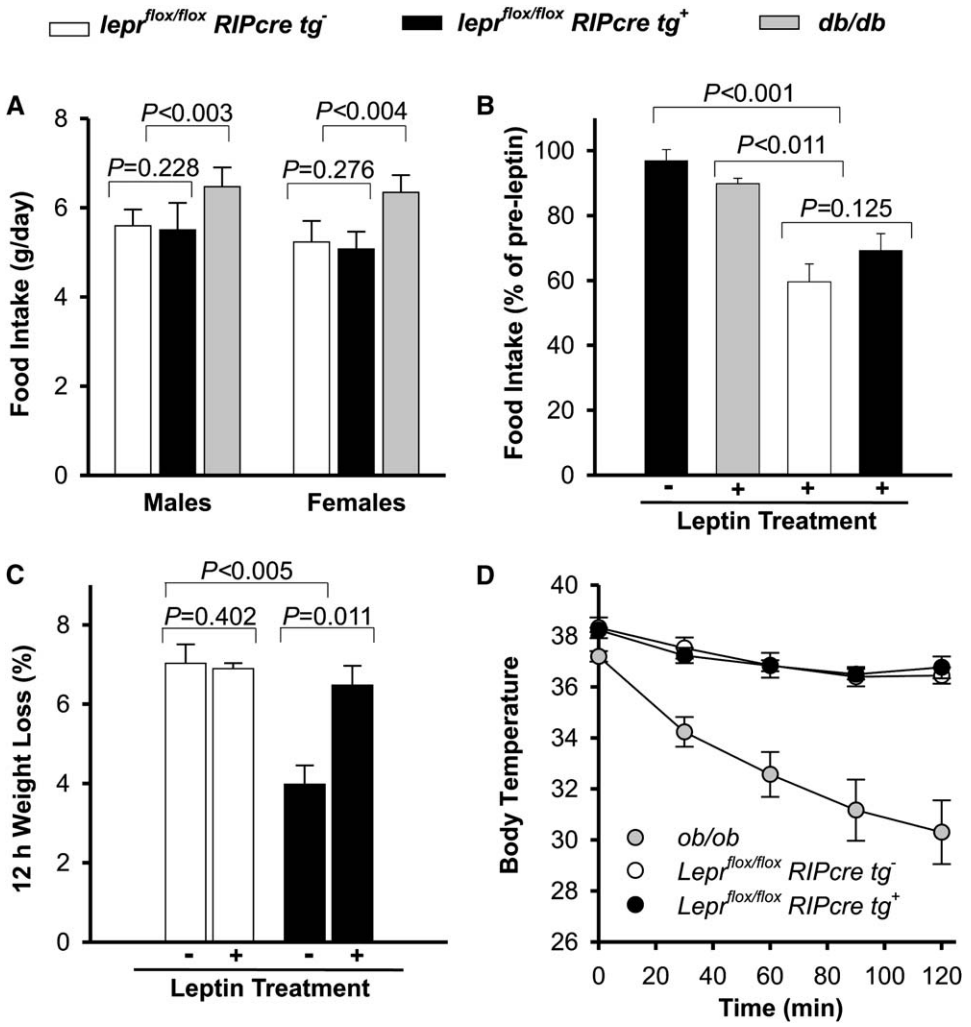

Figure 4. *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice do not have altered food intake but have decreased energy expenditure

A) Food intake in 5-week-old mice, *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ (filled bars), *Lepr*$^{flox/flox}$ (open bars), *db/db* (gray bars). Males n $\geq$ 4, females n $\geq$ 3.

B) Food intake in males following 2 days of leptin treatment (twice daily at 10 μg/g body weight) or vehicle (PBS), n$\geq$3.

C) Energy expenditure in 10-week-old male *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ (filled bars) or *Lepr*$^{flox/flox}$ (open bars) that were infused with either saline or leptin (2 μg/g body weight per 24 hr), n = 3 for each group. The starting body weights were 27.0 ± 0.9, 27.5 ± 1.2, 32.7 ± 2.5, and 32.2 ± 1.1g for *Lepr*$^{flox/flox}$ (saline and leptin) and *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ (saline and leptin), respectively.

D) Cold tolerance of male 12-week-old *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ (filled symbols), *Lepr*$^{flox/flox}$ (open symbols), each n = 4 and 29-week-old *ob/ob* (gray symbols) n = 3.

For all panels, data are expressed as the average ± SEM.

and *Lepr*$^{flox/flox}$ mice with leptin and monitored the effect on food intake (Figure 4B). Leptin treatment resulted in a substantial decrease in food intake in both the *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ and *Lepr*$^{flox/flox}$ mice. Although the magnitude of leptin's anorexic effect was slightly larger in the non-*RIPcre* transgenic mice, this difference was not statistically significant (p = 0.125). As expected, leptin treatment did not evoke a significant decrease in food intake in the *db/db* mice. Collectively these results signify that leptin action with respect to feeding is not altered in the *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice.

Differences in body weight in the absence of altered food intake could arise as a consequence of decreased energy expenditure. To explore the possibility that *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice might have reduced energy expenditure, we measured the change in body weight following a 12 hr fast (Figure 4C). At 10 weeks of age, the magnitude of weight loss over the 12 hr fasting period in *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice was only 57% that of the *Lepr*$^{flox/flox}$ controls ($p < 0.005$). Similar results were obtained in both genders of 6-week-old mice ($p < 0.005$, data not shown). The decreased weight loss of the *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice reveals that these mice have decreased energy expenditure. Interestingly, leptin treatment was able to normalize energy expenditure in the *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice to the level of the *Lepr*$^{flox/flox}$ mice. The absence of increased energy expenditure in the controls given exogenous leptin is consistent with previous studies (Hwa et al., 1997). Thus, despite a reduced energy expenditure, the data clearly reveal that leptin-sensitive central pathways controlling energy expenditure in the *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice are still intact and functional. Furthermore, unlike *ob/ob* mice, the *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice retained normal cold tolerance (Figure 4D). Since thermoregulation appears to be a central leptin-sensitive process (Kowalski

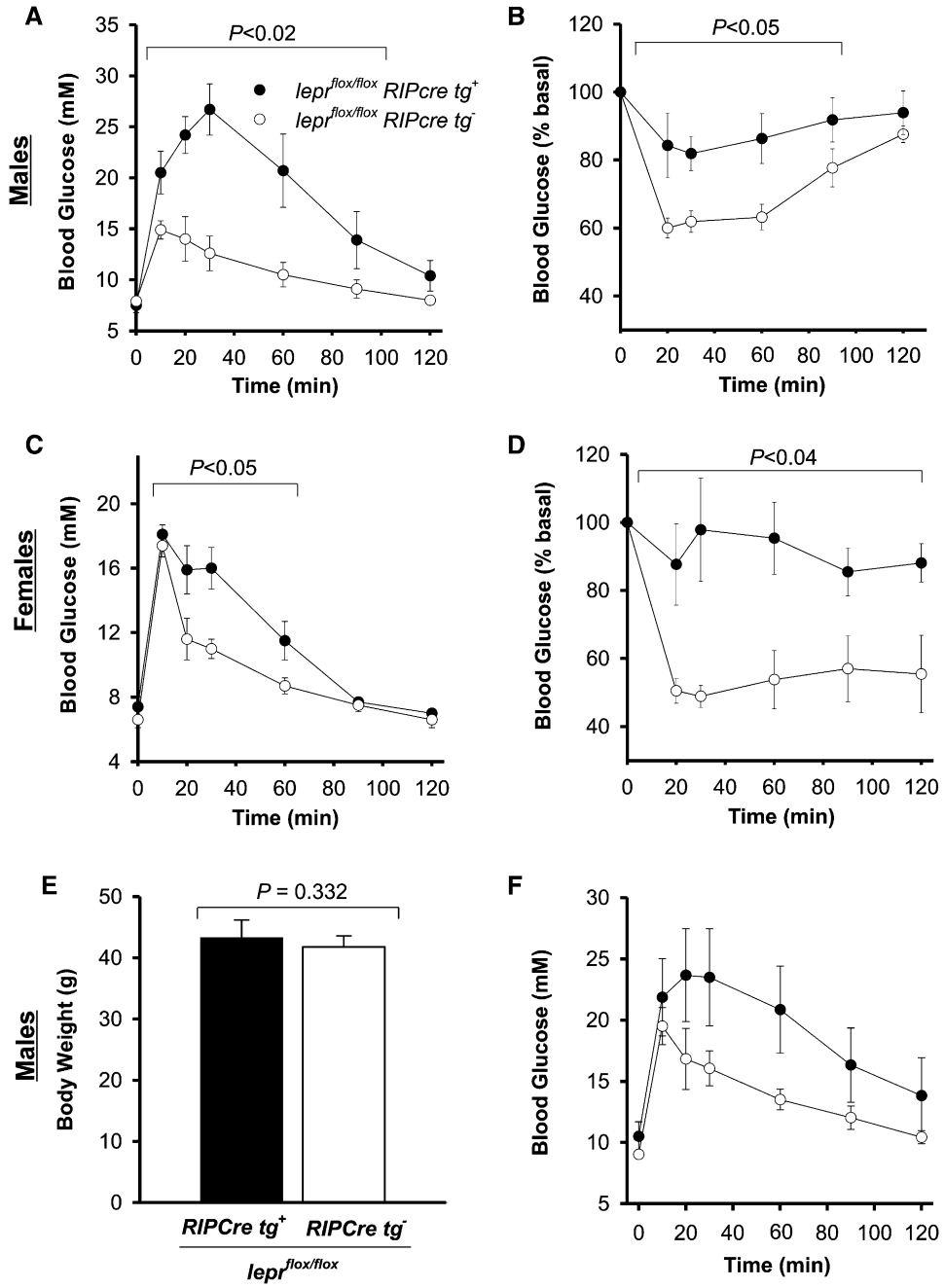

Figure 5. *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice have impaired glucose tolerance and insulin resistance

A–D) OGTT and ITT in 6-week-old *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ (closed symbols) and *Lepr*$^{flox/flox}$ littermates (open symbols). Panels (**A**) and (**B**), n = 4 *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ and n = 4 *Lepr*$^{flox/flox}$. Panel (**C**), n = 5 *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ and n = 7 for *Lepr*$^{flox/flox}$. Panel (**D**), n = 10 *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$, and n = 8 *Lepr*$^{flox/flox}$.

E–F) OGTT in weight-matched male *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ (closed bars/symbols) and *Lepr*$^{flox/flox}$ littermates (open bars/symbols) at 12–14 weeks old, n = 5 *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ and n = 4 for *Lepr*$^{flox/flox}$.

For all panels, data are expressed as the average ± SEM.

et al., 2001), this finding provides further support that hypothalamic leptin signaling is intact in the *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice.

Lepr$^{flox/flox}$ *RIPcre tg*$^{+}$ mice have disrupted glucose homeostasis

To evaluate if the tissue-specific attenuation of leptin receptor signaling impacted how the mice handle a glucose challenge, we performed oral glucose tolerance tests (OGTTs) on 6-week-old *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ and *Lepr*$^{flox/flox}$ controls (Figures 5A and 5C). In both genders, the *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice showed a prominent impairment in their ability to clear and normalize blood glucose relative to the *Lepr*$^{flox/flox}$ mice. This difference was statistically significant in both genders but was particularly striking in the males, with all time points measured from 10–90 min following gavage being significantly different ($p < 0.02$). The increase in blood glucose of the *Lepr*$^{flox/flox}$ *RIPcre*

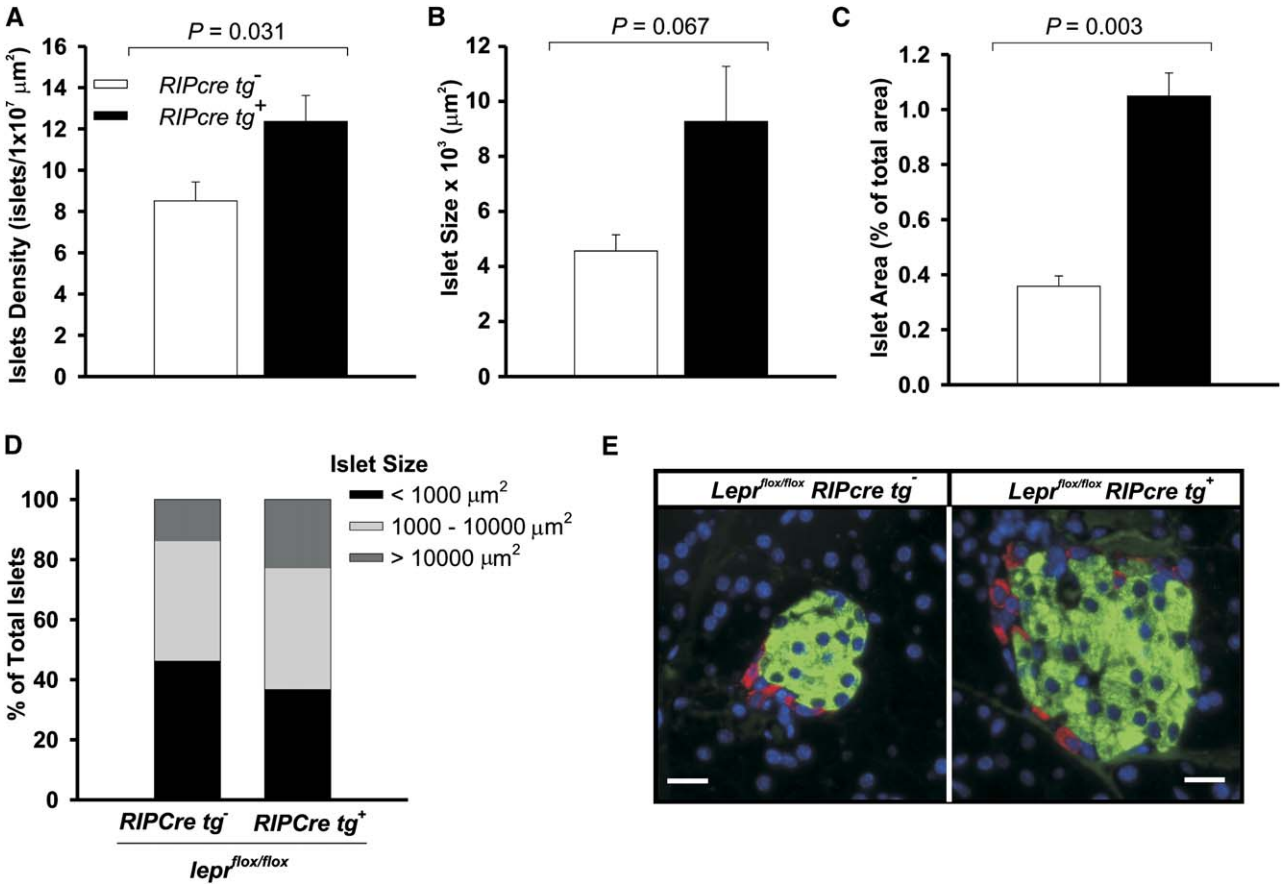

Figure 6. *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice have increased islet density and size

Pancreata from 6-week-old female *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ (filled bars) and *Lepr*$^{flox/flox}$ littermates (open bars) were sectioned and stained for insulin and glucagon.

A) Islet density expressed as the average number of islets per 1 × 10^7 μm^2 of pancreas area.

B) Islet size expressed as the average 2-dimensional islet size.

C) Total insulin-positive area expressed as the percent of total pancreas area.

D) Size distribution of islets expressed as the percentage of total islets.

E) Images of representative islets from *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ and *Lepr*$^{flox/flox}$ mice, green is anti-insulin, red is anti-glucagon, and blue is DAPI. The scale bar represents 25 μm.

For panels (**A**)–(**D**), data are expressed as the average ± SEM, n = 3 for *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ and n = 3 for *Lepr*$^{flox/flox}$.

tg^{+} mice relative to the controls over the 2 hr (determined by area under the curve) was 1.7-fold (p = 0.018) for males and 1.2-fold (p = 0.042) for the females. To determine if glucose intolerance in the *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice is simply a consequence of increased body mass, we performed OGTTs in weight-matched *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ and *Lepr*$^{flox/flox}$ mice (Figures 5E and 5F). Even in weight-matched animals, the *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice were less glucose tolerant than the *Lepr*$^{flox/flox}$ littermates (2281 ± 337 versus 1651 ± 87 by area under curve analysis for *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ and *Lepr*$^{flox/flox}$ mice, respectively, p = 0.075). This indicates that the glucose intolerance of *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice is at least partially independent of altered body weight.

Recently it has been reported that the *RIPcre* transgenic line we employed in this study may be glucose intolerant (Lee et al., 2006). To investigate this possibility in our mice, we measured OGT in *Lepr*$^{flox/wt}$ *RIPcre tg*$^{+}$ and littermate *Lepr*$^{flox/wt}$ *RIPcre tg*$^{-}$ mice (Figures S1A and S1B). In both genders, we found that glucose tolerance was not disrupted in the *Lepr*$^{flox/wt}$ mice by the presence of the *RIPcre* transgene. Thus the glucose intolerance we observe in the *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice is due to the Cre-mediated loss of leptin signaling and not the transgene itself.

Since the tissue-specific attenuation of leptin receptor signaling resulted in impaired glucose tolerance, we sought to determine if these mice displayed insulin resistance relative to controls. Following a 4 hr fast, insulin tolerance tests were performed on mice 6-8 weeks of age (Figures 5B and 5D). As expected, both male and female *Lepr*$^{flox/flox}$ controls responded to insulin with decreases in blood glucose; however the *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice had a blunted response to the exogenous insulin. This difference was statistically significant in both genders, yet the female *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice appeared to be more insulin resistant than their male counterparts.

Attenuated leptin receptor signaling results in an increase in islet area

Since the *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice were hyperinsulinemic, we examined if this corresponded to an increase in islet mass (Figure 6). In the pancreas of 6-week-old *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice, there was a statistically significant 46% increase in the density of islets (p = 0.031). In addition to there being more islets,

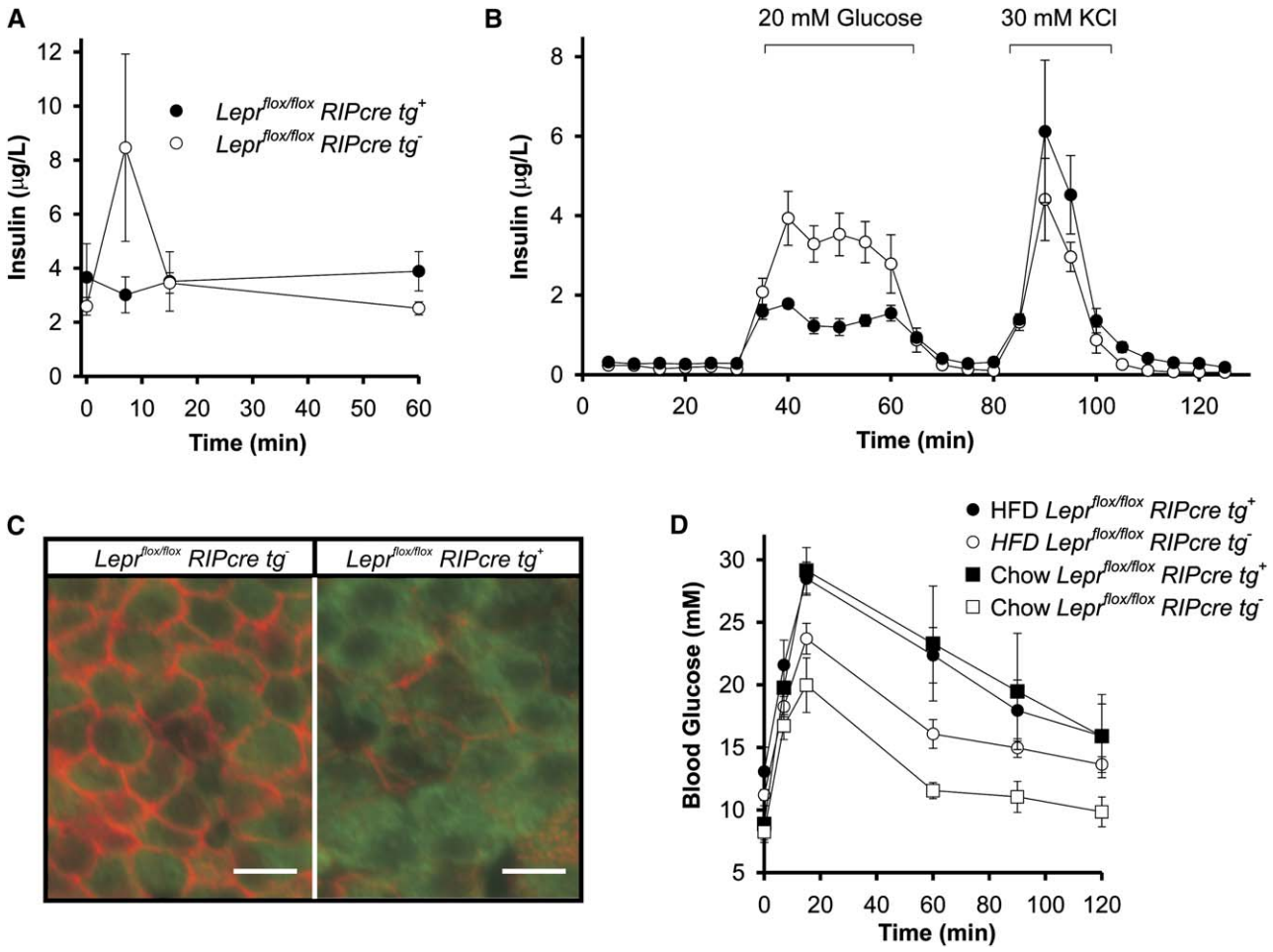

Figure 7. Glucose-stimulated insulin secretion is impaired in *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice

A) Plasma insulin levels in 14- to 15-week-old weight-matched male *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ (closed symbols, n = 4) and *Lepr*$^{flox/flox}$ littermates (open symbols, n = 3) following a glucose gavage.

B) Insulin secretion from perifused islets of 6-week-old male *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice (closed symbols, n = 3) and *Lepr*$^{flox/flox}$ littermates (open symbols, n = 3). Basal glucose levels are 3 mM.

C) Immunofluorescent images of representative islets from *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ and *Lepr*$^{flox/flox}$ mice, where green is anti-insulin, red is anti-GLUT2, and the scale bar represents a distance of 15 μm.

D) OGTT of male *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ (closed symbols) and *Lepr*$^{flox/flox}$ littermates (open symbols) fed either a high-fat diet (circles, n ≥ 8 for both genotypes) starting at 5 weeks old for 10 weeks or a chow diet (squares, n = 4 for both genotypes) for an equivalent time.

For panels (**A**), (**B**), and (**D**), data are expressed as the average ± SEM.

the average size of the islets was increased by ≈2-fold in the *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice, but this did not quite reach statistical significance. Collectively, the increase in islet number and islet size led to a substantial 2.9-fold increase in the islet area ($p = 0.003$) in mice with tissue-specific attenuation of leptin receptor signaling. The lack of leptin receptor signaling did not appear to alter the overall architecture of the islets as assessed by immunofluorescence (Figure 6E). In addition, somatostatin immunoreactivity was not altered by attenuation of leptin receptor signaling (data not shown).

Lepr$^{flox/flox}$ *RIPcre tg*$^{+}$ mice have defective glucose-stimulated insulin secretion

To investigate if the loss of glycemic control in the *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice is a consequence of insufficient plasma insulin in response to a glucose challenge, we measured plasma insulin levels in mice following a glucose gavage (Figure 7A). As expected, in control *Lepr*$^{flox/flox}$ mice there was a 2.3-fold increase in plasma insulin shortly after the gavage ($p = 0.098$). However, in *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice we were unable to detect an increase in plasma insulin in response to the glucose. To determine if this may be a consequence of defective glucose-stimulated insulin secretion from pancreatic β cells, we compared insulin release from perifused islets from *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ and *Lepr*$^{flox/flox}$ mice (Figure 7B). At basal glucose levels, there was low-level insulin secretion, with *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ islets secreting ≈1.4 fold more insulin ($p < 0.001$ by ANOVA pairwise comparison) than islets from *Lepr*$^{flox/flox}$ mice. However, in response to high glucose, relative to controls, there was 47% less insulin secretion (by area under curve analysis, $p = 0.019$) from the *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ islets. Despite the decreased glucose-stimulated insulin secretion, islets from *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice were equally responsive in insulin secretion to depolarization with 30 mM KCl as islets from *Lepr*$^{flox/flox}$ mice, thereby indicating that the impaired glucose-stimulated insulin secretion is not due to a general defect in insulin release.

A potential mechanism behind the disrupted glucose-stimulated insulin secretion in the *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ could be an impaired ability to properly sense glucose. To investigate this we examined GLUT2 expression in pancreatic β cells of *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ and *Lepr*$^{flox/flox}$ mice (Figure 7C). As expected, in *Lepr*$^{flox/flox}$ mice, there was abundant peripheral staining for GLUT2 in the β cells, however, the GLUT2 immunoreactivity was dramatically reduced in the *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice (Figure 7C).

Decreased GLUT2 expression and impaired glucose-stimulated insulin secretion can be associated with increased plasma lipid (Lee et al., 1994) and in fact may arise as a consequence of excessive lipid (Reimer and Ahren, 2002; Zhou and Grill, 1994). We sought to investigate how a high-fat feeding regime would impact the glucose tolerance in *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice. Following 10 weeks on a high-fat diet (HFD), glucose tolerance was measured in *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ and *Lepr*$^{flox/flox}$ mice (Figure 7D). As anticipated, relative to a chow diet, *Lepr*$^{flox/flox}$ mice fed a HFD were significantly less glucose tolerant (1596 ± 131 versus 2059 ± 103 by area under curve analysis for chow and HFD, respectively, $p = 0.014$). In contrast, relative to a chow diet, feeding *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice a HFD had no impact on glucose tolerance (2648 ± 404 versus 2578 ± 246 by area under curve analysis for chow and HFD respectively, $p = 0.440$).

Discussion

Using a Cre-LoxP recombination approach, we have generated mice in which the signaling domain of the leptin receptor gene is disrupted substantially in the pancreatic β cells and partially in the hypothalamus. This tissue-specific leptin receptor disruption results in mice with increased body weight, decreased lean/lipid mass, decreased energy expenditure, insulin resistance, increased islet area, impaired islet function, and glucose intolerance. Notably, the abnormalities in glucose homeostasis in these mice cannot be entirely accounted for by excessive food intake and obesity. Instead, the disrupted glucose homeostasis (in both the fed and fasting state) is a result of β cell malfunction.

A key distinction of the *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice from other models of deficient leptin receptor signaling is the reduced fasting blood glucose level. Mice in which leptin receptor signaling is impaired in POMC neurons (Balthasar et al., 2004), SF1 neurons (unpublished data) or synapsin I neurons (Cohen et al., 2001) within the brain have a similar degree of obesity as the *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice, but do not have reduced blood glucose levels. Moreover, absent leptin action throughout the body (*ob*/*ob* and *db*/*db*) results in obesity, insulin resistance, hyperinsulinemia, and hyperglycemia. In striking contrast to this, the *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice display chronic reduced fasting blood glucose. This provides evidence that leptin has effects on glucose homeostasis that are independent of the regulation of body weight.

The fasting hypoglycemia following attenuation of leptin receptor signaling in *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice likely arises as a consequence of elevated basal insulin output from the β cells. This is consistent with the differences we observe between genders, as the extent of the decreased blood glucose level in the *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice is greater in males than females, as is the hyperinsulinemia. Clearly the elevated basal plasma insulin level in the *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice is inappropriately high, as it results in excessively reduced glycemia relative to the nontransgenic littermate controls. While obesity-induced insulin resistance may contribute to the hyperinsulinemia, it is unlikely the primary cause in the *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice as such a mechanism would not be expected to overcompensate to the degree that reduced glycemia is maintained for several months. Moreover, previous documentation of the acute increase in blood glucose concomitant with decreased insulin levels within minutes of leptin injection into mice (Kulkarni et al., 1997) indicates that leptin regulation of insulin release is not secondary to improvements in insulin sensitivity. These findings support the notion that leptin normally functions to suppress insulin secretion, and in its absence, hyperinsulinemia ensues (Kieffer and Habener, 2000; Seufert, 2004).

A fundamental issue in the field regarding leptin action on glucose homeostasis is the relevant contribution of leptin at central versus peripheral sites. With the *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice, we cannot unambiguously attribute the disrupted glucose homeostasis specifically to central or peripheral pathways, but our results provide some insights. The fasting hypoglycemia that we observe arises as a consequence of increased insulin output from β cells. Consistent with this being a β cell autonomous response, we observed such increases in insulin secretion in the basal state in isolated islets. It is conceivable that a hypothalamic leptin signaling pathway may alter insulin secretion from β cells, persisting even after islets are removed from central control. However, other central leptin signaling pathways controlling food intake and energy expenditure are not severed in the *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice as they still responded acutely to exogenous leptin. Furthermore, although leptin regulated central pathways controlling insulin secretion are plausible, ICV administration of leptin does not appear to acutely alter plasma insulin levels (Kamohara et al., 1997), while peripheral administration does (Kulkarni et al., 1997; Seufert et al., 1999a). The hypothesis that loss of direct leptin signaling at β cells leads to basal hyperinsulinemia and fasting hypoglycemia is consistent with numerous studies showing leptin can inhibit insulin secretion from isolated islets (Emilsson et al., 1997; Kieffer et al., 1997; Kulkarni et al., 1997; Ookuma et al., 1998; Seufert et al., 1999a, 1999b). Thus, the fasting hyperinsulinemia and reduced fasting blood glucose in *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice appear to be more consistent with peripheral rather than central actions of leptin.

Despite a near 3-fold increase in islet area, *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice display striking hyperglycemia following a glucose load. Glucose intolerance in the presence of increased β cell mass is also observed in rodents with mutations in the leptin receptor gene (*db*/*db* mice and ZDF rats). Although the glucose intolerance is partially attributable to insulin resistance in *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$, it is not likely the primary reason. Female *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice handled the glucose load better than the male *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice, despite having greater resistance to insulin. Following a glucose challenge, we were unable to detect a surge in plasma insulin in *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice leading us to speculate that glucose-stimulated insulin secretion was impaired. Perifusion studies with islets isolated from *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice confirmed that these mice had attenuated glucose-stimulated insulin secretion relative to controls. Therefore, decreased glucose-dependent insulin secretion from β cells is probably the primary mechanism contributing to the glucose intolerance of the *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice.

Interestingly, while insulin secretion from islets isolated from *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice was impaired in response to

glucose, it was normal following depolarization with KCl. Thus the defect that results from chronically absent leptin signaling does not restrict the readily releasable pool of insulin. A potential mechanism of impaired glucose-stimulated insulin secretion is reduced GLUT2 expression in β cells as a consequence of excessive lipid, an observation made in *db*/*db* mice and ZDF rats. Leptin has been proposed to protect nonadipose tissue from excessive triglyceride accumulation and lipotoxicity (Unger, 2002). Moreover, excessive lipid accumulation in β cells results in decreased GLUT2 and impairment of glucose-stimulated insulin secretion (Lee et al., 1994; Wang et al., 1998). Thus, it is possible that the loss of leptin receptor signaling in β cells leads to excessive triglyceride accumulation, which secondarily impairs glucose-stimulated insulin secretion. Such a scenario is compatible with our finding that additional exposure to lipid via maintenance on a HFD could not further impair glucose homeostasis in the *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice.

In addition to the elevated plasma lipid levels, the *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice have a striking increase in lipid mass and a decrease in lean mass. Moreover, the increased lipid to lean mass of the *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ can occur in the absence of differences in total body weight. Thus the altered body composition is not strictly additional fat mass, as is observed in mice with attenuated leptin signaling in POMC and SF1-positive neurons (Dhillon et al., 2006). Such a change in body composition in the absence of increased body mass does not seem likely to arise as a consequence of the altered energy expenditure. Rather the altered composition suggests that these mice either have a propensity for adipogenesis or a preferential utilization of carbohydrates over lipids. Such changes are consistent with the actions of insulin, and thus may arise as a consequence of fasting hyperinsulinemia in the *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice. While hyperleptinemia may be expected to decrease ectopic lipid accumulation in tissues with functional leptin receptors such as liver (Unger, 2002), this appears to be overcome by the actions of insulin and elevated plasma triglycerides in this model.

In summary, our studies indicate that leptin has important actions on glucose homeostasis that are independent of the pathways by which leptin regulates food intake and body weight. Leptin appears to be important in restraining insulin secretion such that attenuated leptin action can cause hyperinsulinemia. As suggested by Coleman during early characterization of the *ob*/*ob* and *db*/*db* phenotypes (Coleman, 1978), it is quite conceivable that excessive insulin release could alone account for weight gain, insulin resistance, and fasting hypoglycemia. Thus leptin resistance leading to hyperinsulinemia may provide another mechanism by which obesity can contribute to diabetes. Furthermore, this hypothesis also provides a potential mechanism by which leptin resistance could lead to diabetes independent of the development of obesity. Indeed, Raskin and colleagues suggested that dysregulation of the adipoinsular axis could explain the obesity-independent hyperinsulinemia they observed in nondiabetic first-degree relatives of individuals with type 2 diabetes (Ishikawa et al., 1998). These hypotheses warrant further investigation.

Experimental procedures

Mice

C57BL/6-TgN(RIPcre)25Mgn (referred to as *RIPcre tg*$^{+}$) were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were housed with a 12 hr light/12 hr dark cycle and had ad libitum access to food (chow diet: 5015 Lab Diet, St Louis, MO, or high-fat diet when specified: #D12330, Research Diets, New Brunswick, NJ) and water. All procedures with animals were approved by the University of British Columbia Animal Care Committee and carried out in accordance with the Canadian Council on Animal Care guidelines. C57Bl6/J *RIPcre tg*$^{+}$ mice were mated with *Lepr*$^{flox/flox}$ (129 X FVB N6) to generate *Lepr*$^{flox/wt}$ either with or without the *RIPcre* transgene. These offspring (≈50% C57Bl6/J, 50% FVB, and less than 1% 129) were bred, with one mouse of each pair harboring the *RIPcre tg*$^{+}$. The offspring that were *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ and *Lepr*$^{flox/flox}$ *RIPcre tg*$^{-}$ (referred to as *Lepr*$^{flox/flox}$) were mated to produce *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice and *Lepr*$^{flox/flox}$ littermate controls for experiments. For all experiments, littermate *Lepr*$^{flox/flox}$ controls were compared with *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ mice in order to minimize differences in genetic background, which can affect the phenotype of leptin deficiency (Haluzik et al., 2004).

Plasma analyte analysis

Body weight and blood glucose were measured following a 4 hr fast. Blood glucose was measured with a One Touch Ultra Glucometer (Life Scan Inc., Burnaby, Canada) from the saphenous vein and leptin (mouse leptin ELISA, LINCO Research, St. Charles, MO), insulin (Ultrasensitive Mouse Insulin ELISA, Mercodia, Uppsala, Sweden), triglyceride (Serum Triglyceride kit, Sigma-Aldrich, St Louis, MO) and cholesterol (Cholesterol E kit, Wako Chemical, Richmond, VA) from plasma.

Analysis of ACTH, corticosterone, testosterone and total thyroxine

Blood samples were collected from 13- to 15-week-old fed *Lepr*$^{flox/flox}$ *RIPcre tg*$^{+}$ and *Lepr*$^{flox/flox}$ mice between 10 AM and 11 AM, either by the saphenous vein for the preparation of plasma or by cardiac puncture under isoflurane anesthesia for the preparation of serum. Levels of corticosterone (EIA kit from Assay Designs, Ann Arbor, MI), total thyroxine (RIA kit from Diagnostic Products Corporation, Los Angeles, CA), and testosterone (EIA kit from MP Biomedicals, Orangeburg, NY) were measured in serum. Levels of ACTH (RIA kit from Diagnostic Systems Laboratories, Inc., Webster, TX) were measured in plasma.

Islet isolation

Islets were isolated using the method described by Salvalaggio et al. (2002).

PCR analysis

Genomic DNA was prepared with DNeasy kits (Qiagen, Mississauga, Canada). Brain tissue was prepared as a homogenate of the brain minus the hypothalamus. For PCR reactions, 3 μl of genomic DNA was added to a 25 μl reaction containing 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 1 U/μl Platinum Taq DNA polymerase (all from Invitrogen, Burlington, Canada), and 1 μM of each forward and reverse primer (Integrated DNA Technologies Inc., Coralville, IA). For *Lepr*^{flox} PCR reactions the forward primer was ATG CTA TCG ACA AGC AGC AGA ATG ACG and the reverse primer CAG GCT TGA GAA CAT GAA CAC AAC AAC. PCR for the presence of the *RIPcre* transgene was performed with forward primer AAT GGG ACA AAC AGC AAA GTC C and reverse primer AGA TAA TCG CGA ACA TCT TCA GGT.

In vivo glucose-stimulated insulin secretion, oral glucose tolerance, and insulin tolerance tests

Mice were fasted for 4 hr and then either given an oral gavage of 1.5 mg/g body weight glucose as a 25% solution or an intraperitoneal injection of 0.4 U/kg human synthetic insulin (Novo Nordisk Canada Inc, Mississauga, Canada).

Islet morphology

Mice were given a 600 μl intraperitoneal injection of 2.5% avertin (Sigma-Aldrich, St Louis, MO), and pancreata were removed. Pancreata were fixed in 4% paraformaldehyde overnight at 4°C, rinsed in 70% ethanol, embedded in paraffin, and 3 μm sections were prepared. Sections were stained with guinea pig anti-insulin (LINCO Research, St. Charles, MO) and mouse anti-glucagon (Sigma-Aldrich, St Louis, MO) overnight at 4°C and then with goat anti-guinea pig Alexa Fluor 488 and donkey anti-mouse Alexa Fluor® 594 (Molecular Probes, Eugene, OR). For morphometric analysis, three randomly chosen fields of 0.26 cm^2 were measured for pancreas area as well as insulin-positive area using Openlab Software (Improvision, Lexington, MA).

For each pancreas, three sections separated by 30 μM were analyzed with the investigator blind to the genotype. For GLUT2 staining, pancreata (prepared as described above) were stained with rabbit anti-GLUT2 (Chemicon International Inc, Temecula, CA) overnight at 4°C and then with donkey anti-rabbit Alexa Fluor 594.

Islet perifusion analysis

Islets were cultured overnight, and then 100 islets from each mouse were loaded into separate chambers of an Acusyst-S perifusion apparatus (Endotronics Cell Culture Instrumentation, Minneapolis, MN) with Cytodex microcarrier beads (Sigma-Aldrich, St Louis, MO). Islets were equilibrated in chambers with pH 7.2 KRBB containing 0.5% BSA and 3 mM glucose for 45 min at a flow rate of 0.36 ml/min. At the indicated times, the concentration of glucose and KCl were altered.

Food intake studies

Mice were housed individually in cages with wire grid inserts on the cage floor. Mice were acclimatized to the cages for 2 days after which food intake (the difference in weight of food in the hopper and that remaining in hopper and on the floor 24 hr later) was measured for 5 days and averaged.

Assessment of energy expenditure

At the end of the dark cycle, osmotic pumps (2001D, Durect Corporation, Cupertino, CA) delivering either saline or 2 μg leptin/g body weight were implanted subcutaneously in mice. Mice were weighed and food was withdrawn for 12 hr, and then mice were weighed again.

Cold tolerance

Microchips (Bio Medic Data Systems) were implanted subcutaneously into mice that were housed individually in cages without bedding. Mice were placed in a 4°C environment, and body temperature was measured every 30 min for 2 hr using a Pocket Scanner (Bio Medic Data Systems).

Magnetic resonance imaging

Mice were euthanized and imaged in a Bruker Biospec 70/30 7 Tesla MRI Scanner (Bruker Biospin, Ettlingen, Germany). Images were acquired in the abdominal region in 1 mm axial slices with MSME T1-weighted pulse sequence, with and without fat suppression. Pseudocolored lipid images were obtained by subtracting images collected with fat suppression from images acquired without fat suppression.

Measurement of lean to lipid mass

Measurements were performed with a Bruker Biospec 70/30 7 Tesla MRI scanner (Bruker Biospin, Ettlingen, Germany). NMR signal from the body was acquired with a quadrature volume RF coil tuned to 300 MHz. The "free" water component corresponding to body fluids, e.g., urine and CSF, was typically less than 5% of the total signal. The ratio of lean/fat tissue is expressed as weight/weight was calculated from the NMR data as described in (Kunnecke et al., 2004).

Measurement of tissue triglycerides

Triglyceride was measured by a modified protocol (Briaud et al., 2001). Liver was homogenized with an ultra turrax in 3 ml of chloroform:methanol (2:1), and extracted twice with water. 1 ml of the organic layer was dried by $N_{2(g)}$ and 20 μl of Thesit (Sigma-Aldrich) was added and mixed under $N_{2(g)}$. Water (100 μl) was added and incubated at 37°C for 30 min with intermittent vortexing. Aliquots (2 and 20 μl) were assayed using the Serum Triglyceride Determination kit (Sigma-Aldrich) modified for a 96-well plate, calibrated with a trioleate (Sigma-Aldrich) standard curve.

Supplemental data

Supplemental data include one figure and one table and can be found with this article online at http://www.cellmetabolism.org/cgi/content/full/4/4/291/DC1/.

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