Disruption of leptin receptor expression in the pancreas directly affects β cell growth and function in mice

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Obesity is characterized by hyperinsulinemia, hyperleptinemia, and an increase in islet volume. While the mechanisms that hasten the onset of diabetes in obese individuals are not known, it is possible that the adipose-derived hormone leptin plays a role. In addition to its central actions, leptin exerts biological effects by acting in peripheral tissues including the endocrine pancreas. To explore the impact of disrupting leptin signaling in the pancreas on β cell growth and/or function, we created pancreas-specific leptin receptor (ObR) KOs using mice expressing Cre recombinase under the control of the pancreatic and duodenal homeobox 1 (Pdx1) promoter. The KOs exhibited improved glucose tolerance due to enhanced early-phase insulin secretion, and a greater β cell mass secondary to increased β cell size and enhanced expression and phosphorylation of p70S6K. Similar effects on p70S6K were observed in MIN6 β cells with knockdown of the ObR gene, suggesting crosstalk between leptin and insulin signaling pathways. Surprisingly, challenging the KOs with a high-fat diet led to attenuated acute insulin secretory response to glucose, poor compensatory islet growth, and glucose intolerance. Together, these data provide direct genetic evidence, from a unique mouse model lacking ObRs only in the pancreas, for a critical role for leptin signaling in islet biology and suggest that altered leptin action in islets is one factor that contributes to obesity-associated diabetes.

Introduction

The factors that promote β cell failure and increase the incidence of diabetes in obese individuals are not fully understood. The presence of hyperglycemia and hyperphagia in obese individuals, despite the presence of high levels of circulating insulin and leptin, suggests these individuals are resistant to the actions of both hormones (1). The hypothalamic actions of leptin are relatively well characterized; however, the expression of the long form of the leptin receptor (ObRb) in peripheral tissues, including the endocrine pancreas, indicates that leptin can also exert peripheral actions independent of its effects in the hypothalamus (2). For example, in vitro studies have reported inhibitory effects of leptin on insulin gene expression and insulin secretion in β cell lines and isolated murine and human islets (2–4). Furthermore, leptin treatment of ob/ob mice reversed hyperinsulinemia (5). Although the db/db mouse, which carries a mutation in the ObR gene (6), manifests hyperinsulinemia and hyperplastic islets (7), it is unclear whether the alterations in islet growth and function in db/db mice are due to a lack of direct leptin action in β cells or secondary to the effects of insulin resistance in peripheral tissues.

To directly assess the role of leptin signaling in the pancreas, we used the Cre-loxP technique to create a mouse model that is deficient in ObRs only in the pancreas. We report that the expression of Cre using the Pdx1 (pancreatic and duodenal homeobox 1) promoter results in efficient recombination of a loxP-containing ObR gene in the pancreas. Mice lacking ObRs in the pancreas (pancreas-ObR-KO) and fed normal chow manifested improved glucose tolerance, enhanced first-phase insulin response to glucose, and islet hyperplasia.

We also report upregulation in the islets of expression and phosphorylation of proteins in the insulin/IGF-I signaling pathway that are known to be important for the maintenance of islet growth and function (8–19). Qualitatively similar signaling data were observed in MIN6 β cells with a knockdown of ObR using siRNA. Challenging the mice with a high-fat diet (HFD), however, led to significantly greater glucose intolerance in the KOs compared with controls. Together, these data provide genetic evidence for cross-talk between leptin and insulin signaling pathways in the regulation of islet biology, with implications for β cell dysfunction in obesity.

Results

Creation of the pancreas-specific ObR-KO mice. We created a mouse with a pancreas-specific KO of ObR by breeding mice carrying obr in which exon 1 was flanked with loxP sites (obrlox/lox; ObRlox) (20) with mice expressing cre driven by the Pdx1 promoter (21).

Both control (ObRlox) and KO mice were born at the expected Mendelian frequency, survived to adulthood, and were fertile. We performed RT-PCR analysis to evaluate efficiency of recombination of the ObRlox allele in the pancreas triggered by Cre expression. Expression of the gene coding for ObR was virtually absent in
the pancreas and islets but unaffected in extrapancreatic tissues in the KOs, indicating pancreas-specific recombination (Figure 1, A and B). The mRNA for ObRb assessed by in situ hybridization was expressed equally in the hypothalamus in both ObRlox and KO mice and confirmed the specificity of recombination (Figure 1C). In all cases, KO mice were compared with ObRlox controls.

Similar body weights, food intake, and fat mass in ObRlox control and KO mice. We next evaluated the physiological actions of leptin in the hypothalamus. Body weight, food intake, serum leptin concentrations, and perigonadal fat pad weights of 4-month-old male and female KO mice were similar to those of age- and sex-matched ObRlox controls (Figure 1, D–G). These findings confirm that ObRs in hypothalamus were functional in KO mice and that KO of ObRs in the pancreas did not modify body weight, food intake, fat mass, or circulating leptin levels in 4-month-old mutant animals.

Enhanced early-phase insulin secretion and improved glucose tolerance in KO mice. Leptin signaling suppresses insulin secretion (2–4). To evaluate the effects of pancreas-specific ObR deletion on β cell secretory function in vivo, we measured acute insulin release in response to glucose stimulation. In male and female ObRlox controls, a significant increase in insulin secretion was observed 2 minutes following i.p. glucose injection, consistent with an acute-phase insulin secretory response (Figure 2A). Interestingly, in KO mice, the acute-phase insulin secretory response to glucose was enhanced by 2-fold in males and 3-fold in females compared with controls (Figure 2A). Consequently, upon i.p. glucose challenge, KO mice showed a significantly lower glucose excursion, consistent with improved glucose tolerance (Figure 2B). Fasting circulating insulin levels in KO mice were approximately 2-fold higher than those in controls; the difference was statistically significant.

Figure 1
Evidence for deletion of ObR and physiological effects of pancreas-specific disruption of the ObR in pancreas-ObR-KO mice. (A) Representative data of expression of ObR gene in islets and non-islet pancreatic tissue in 6-month-old ObRlox and KO mice, as assessed by RT-PCR. Amylase was used as a marker for non-islet pancreas samples and β-actin as an internal control. n = 3. (B) Representative data of expression of ObR gene assessed by RT-PCR in whole-brain extract, hypothalamus, liver, lung, kidney, white adipose tissue (WAT), spleen, small intestine, heart, and skeletal muscle in 6-month-old ObRlox (lox) and KO mice. n = 3. (C) In situ hybridization for ObRb showing that it is localized in hypothalamus of both ObRlox and KO mice. Body weight (D), 24-hour ad libitum food intake (E), fasting serum leptin concentration (F), and perigonadal fat pad weights (G) in 4-month-old male and female ObRlox and KO mice. n = 4–6; all P = NS. Data are shown as mean ± SEM. Scale bars: 500 μm.
in females, and levels tended to be higher in males (P = 0.07), indicating increased basal insulin secretion (Figure 2C). Higher circulating levels of insulin were also observed in the random-fed state in KO mice compared with controls (male: 4.7 ± 0.3 versus 2.3 ± 0.4 ng/ml, P < 0.05, n = 5; female: 3.6 ± 0.4 versus 1.9 ± 0.3 ng/ml, P < 0.05, n = 4–5). Blood glucose levels in KO mice did not differ from those in ObRlox mice in the fasting state (Figure 1D) and tended to be lower, although the difference was not statistically significant in random-fed states (male: KO, 102 ± 14 versus ObRlox, 110 ± 18 mg/dl, n = 5; female: KO, 114 ± 16 versus ObRlox, 131 ± 20 mg/dl, n = 5). Whole-body insulin sensitivity evaluated by i.p. insulin tolerance tests (ITTs; 1 U Humulin/kg body weight) showed no differences between the groups (Figure 2E). Fasting plasma glucagon levels did not show significant differences between groups (male: ObRlox, 66 ± 12 versus KO, 52 ± 14 pg/ml; female: ObRlox, 58 ± 14 versus KO 64 ± 16 pg/ml, n = 5; P = NS).  

To evaluate insulin secretion in vitro, we cultured freshly isolated islets for 48 hours in glucose at physiological concentrations and then subjected single, size-matched islets from ObRlox or KO mice to step-up glucose stimulation. We observed significantly enhanced (P < 0.05) Ca^{2+} flux and insulin secretion in response to an increase in glucose concentration from 3 to 8 mM in the KOs (Figure 2, F and G). Further, consistent with previous reports (2, 3), leptin treatment suppressed Ca^{2+} flux and insulin secretion in control islets, while no effects were evident in the KO islets. These results suggest that absence of leptin signaling...
allows enhanced insulin secretion from cultured islets, consistent with the observations in vivo.

Islet hyperplasia and increased expression of insulin signaling proteins in KO islets. In addition to growth factors, leptin signaling has also been implicated in β cell growth in vitro (22). To evaluate whether disruption of ObRs in the pancreas affects islet morphology and β cell growth in vivo, we performed immunohistochemical analyses of pancreas sections using a cocktail of antibodies to non-β cell hormones (23, 24). We observed a 2-fold increase in β cell mass in KO mice that was secondary to an increase in β cell size (Figure 3, A–C) but not due to enhanced mitosis (control, 0.27% ± 0.2% versus KO, 0.37% ± 0.2% BrdU+ β cells [total 500 β cells counted], n = 4; P = NS). We did not observe differences in expression of cleaved caspase-3, suggesting that the increase in β cell mass observed in KO mice is unlikely due to altered β cell apoptosis (data not shown).

To explore the pathways that mediate an increase in islet hyperplasia, we considered previous reports that proteins in the insulin/IGF-I pathways regulate β cell size and survival (8–19). Western blotting of islet lysates showed significantly enhanced phosphorylation of PKB/Akt at Ser473 (p-Akt), p70 S6 kinase at Thr389 (p-p70S6K), and FoxO1 at Ser256 (p-FoxO1), and α-tubulin as a loading control. The relative expression of p-Akt, p-p70S6K, and p-FoxO1 normalized to each total protein is shown in the graph. *P < 0.05 versus ObRlox; mean ± SEM, n = 4. (E) Immunofluorescence for Glut-2 and insulin in pancreas sections of ObRlox and KO mice. Scale bars: 50 μm. Western blots of total islet lysates for Glut-2 (E, lower panel), p-PTEN, and PTEN (F), normalized to α-tubulin. The relative expression of p-PTEN normalized to α-tubulin is shown in the graph. *P < 0.05 versus ObRlox; n = 4. (G) Expression of SOCS-3 and preproinsulin in ObRlox and KO islets assessed by quantitative real-time PCR. *P < 0.05 versus ObRlox; n = 4–6. Data were obtained from pancreas or islet samples from 4- to 6-month-old mice and are shown as mean ± SEM.

Figure 3
Increase in islet size and β cell mass and enhanced expression of insulin signaling proteins in islets of pancreas-ObR-KO mice. (A) H&E staining or immunofluorescence staining for insulin and glucagon in pancreas sections of ObRlox and KO mice. Scale bars: 100 μm. (B) Cell mass estimated by morphometric analysis. *P < 0.05 versus ObRlox; n = 4. (C) Immunofluorescence staining for insulin and β-catenin in pancreas sections to determine β cell size. Relative β cell size (mean from n ≥ 200 cells counted) is shown in the graph. *P < 0.05 versus ObRlox; mean ± SEM, n = 3. Scale bars: 10 μm. (D) Western blots of total islet lysates for p-Akt (Ser473), Akt, p-p70S6K (Thr389), p70S6K, p-FoxO1 (Ser256), FoxO1, and α-tubulin as a loading control. The relative expression of p-Akt, p-p70S6K, and p-FoxO1 normalized to each total protein is shown in the graph. *P < 0.05 versus ObRlox; n = 4–6. (E) Immunofluorescence for Glut-2 and insulin in pancreas sections of ObRlox and KO mice. Scale bars: 50 μm. Western blots of total islet lysates for Glut-2 (E, lower panel), p-PTEN, and PTEN (F), normalized to α-tubulin. The relative expression of p-PTEN normalized to α-tubulin is shown in the graph. *P < 0.05 versus ObRlox; n = 4. (G) Expression of SOCS-3 and preproinsulin in ObRlox and KO islets assessed by quantitative real-time PCR. *P < 0.05 versus ObRlox; n = 4–6. Data were obtained from pancreas or islet samples from 4- to 6-month-old mice and are shown as mean ± SEM.
To further explore the possible mechanisms underlying the increased insulin-secretory function with enhanced insulin receptor signaling pathway in KO mouse islets, we evaluated the expression of glucose transporter–2 (Glut-2) in β cells. Immunofluorescence analyses showed similar expression patterns of Glut-2 in both groups (Figure 3E, upper panel). Further, Western blotting of islet lysates showed the tendency of Glut-2 to decrease in KO s, but the difference was not statistically significant (Figure 3E, lower panel), suggesting that alterations in Glut-2 expression are unlikely to contribute substantially to the higher insulin secretion in the KOs.

Since phosphatase and tensin homolog (PTEN), a major negative regulator of the PI3K/Akt signaling pathway, was recently reported to be regulated by leptin in hypothalamic cell lines and pancreatic β cells (25), we evaluated phosphatase activity and protein content in KO islets. By Western blotting, we observed a mild but significant upregulation of PTEN phosphorylation (p-PTEN) in the KOs compared with controls (Figure 3F), while no differences were observed in total PTEN protein expression (KO, 122% ± 14% of control; n = 4; P = NS). These data are compatible with enhanced PI3K/Akt signaling in the KOs, in part due to the attenuation of PTEN, a negative regulator of insulin signaling.

SOCS-3 also acts as a negative regulator of insulin signaling and has been reported to link cytokine signaling with insulin resistance (26). In β cells, SOCS-3 has been reported to be induced by leptin and inhibit proinsulin gene expression (27). Consistent with these observations, we observed a significant decrease in the expression of SOCS-3 by quantitative real-time PCR in KO islets that was associated with a mild but significant increase in proinsulin gene expression (Figure 3G). These results confirm the link between SOCS-3 and insulin gene expression and provide additional evidence for a regulatory role for leptin/insulin signaling in β cell secretory function.

Enhanced insulin signaling in MIN6 β cells with Obr gene knockdown.
To further evaluate the potential interactions between insulin and leptin signaling pathways in vitro, we utilized MIN6 β cells with Obr gene knockdown using siRNA. Transfection of siRNA oligonucleotides for mouse Obr (siObr) resulted in an approximately 80% reduction in Obr gene expression in MIN6 β cells and was confirmed by quantitative real-time PCR (Figure 4A). In control MIN6 β cells transfected with scrambled RNA, lepin treatment promoted tyrosine phosphorylation of Stat3 (p-Stat3) and Jak2 (p-Jak2), while virtually no effect was observed in siObr-knockdown cells (Figure 4B), indicating that lepin-stimulated Jak2/Stat3 signaling is attenuated in cells with Obr knockdown.

Next, to assess alteration in proteins in the insulin-induced signal- ing pathways in MIN6 β cells with Obr knockdown, we treated MIN6 β cells with insulin after transfection of siObr or scrambled RNA. MIN6 β cells with Obr knockdown showed an increase in protein expression of p70S6K and FoxO1, in the basal state, and elevated insulin-induced phosphorylation of PKB/Akt (Ser473), p70S6K (Thr389), and FoxO1 (Ser256) (Figure 4C). We also observed mildly elevated PTEN phosphorylation in MIN6 β cells with Obr knockdown in both basal and lepin-treated states (Figure 4D). These findings in MIN6 β cells are in agreement with signaling data in islets from pancreas-Obr-KO mice (Figure 3, D and F) and suggest that the phenotypes in KOs are due to a primary defect in β cells and unlikely due to altered lepin action in non-β cells, neural, or other circulating factors. Indeed, no significant differences were detected in circulating glucagon or somatostatin (data not shown) levels between groups.

Impaired glucose tolerance in pancreas-Obr-KO mice induced by HFD feeding. In addition to its role in islet function and survival, lepin modulates lipid metabolism in islets (28, 29). To explore whether the enhanced insulin secretion observed in the KOs on a normal chow diet protects the mutants in the face of caloric excess, we fed control and KO mice with an HFD or normal chow for 12 weeks. After high-fat feeding, as expected, mice in both groups were significantly heavier and exhibited insulin resistance compared with their littermates on normal chow (Figure 5A) and were equally resistant (Figure 5B). After high-fat feeding, as expected, mice in both groups were significantly heavier and exhibited insulin resistance compared with their littermates on normal chow diet, confirming the effects of diet-induced obesity (Figure 5, A and B). Further, the HFD-fed mice in the KO and control groups responded by gaining similar body weights (Figure 5A) and were equally insulin resistant (Figure 5B). Surprisingly, however, the KO mice on HFD exhibited impaired glucose tolerance that was, in part, due to blunted acute insulin response to glucose compared with the controls (Figure 5, C–E). Furthermore, while control mice on HFD developed islet...
hyperplasia to compensate for insulin resistance compared with mice on normal chow, the KO mice showed a significantly reduced islet mass (61.8% of control) after high-fat feeding (Figure 3A and Figure 5F). Leptin has been reported to regulate lipid metabolism in β cells and to protect islets from the effects of lipid overload (28, 30). Lipid accumulation in the islets, in turn, is known to impair islet secretory function and viability (28). Thus, it is possible that switching the diet from normal chow to high-fat in mice lacking leptin signaling in islets leads to lipid accumulation in the islets and consequent poor insulin secretory response and attenuated islet growth, leading to altered glucose homeostasis. Further molecular studies are necessary to dissect the pathways involved in leptin modulation of lipid metabolism and in the cross-talk with growth factor pathways regulating the β cell proliferation response in these mutants in the context of an HFD.

**Discussion**

To directly assess the role of ObR signaling in the growth and function of pancreatic β cells, we created a genetic model using a mouse expressing Cre-recombinase driven by the Pdx1 promoter for 2 reasons. First, the absence of Cre expression in the brain of the PdxCre mouse eliminates the possibility of recombination in the hypothalamus, which is rich in ObR-positive neurons (21). Second, this approach allowed us to examine the effect of recombination of the ObR gene around E8.5, when the Pdx1 promoter is switched on earlier than expression of the rat insulin promoter (21). Our studies provide genetic evidence for a direct role for leptin in the endocrine pancreas, in contrast to a recent report that used a mouse expressing Cre recombinase on the rat insulin promoter that is expressed later during development (~E9.5) and is also expressed in some neurons in the hypothalamus (31). Considering the dominant role of leptin in the hypothalamus, it is likely that the islet phenotype in mice generated with the rat insulin promoter–driven Cre is masked by alterations in hypothalamic function affecting insulin sensitivity and fat mass.

Previous studies have reported that leptin signaling interacts with proteins in the insulin-signaling pathway to exert its biological actions in several tissues. For example, leptin activates the IRS/PI3K pathway in hypothalamic neurons to regulate food intake and body weight (1) and to stimulate glucose transport and glycogen synthesis in muscle cells (32). In hepatocytes, leptin activates IRS-associated PI3K and PDE3B to promote inhibition of cAMP elevation by glucagon (33, 34). In β cell lines and isolated islets, leptin inhibits insulin secretion through PI3K-dependent PDE3B

**Figure 5**

Diet-induced obesity leads to impaired glucose tolerance in pancreas-ObR-KO mice. Body weight (A), ITT results (B), glucose tolerance test (GTT) results (C), and area under the curve for the GTT results (D) in 4-month-old male ObRlox and KO mice on regular chow (Chow) or HFD for 12 weeks. *P < 0.05, HFD (n = 10) versus Chow (n = 6); †P < 0.05, KO mice on HFD versus ObRlox mice on HFD, n = 5; ‡P < 0.05, ‡P < 0.01, KO mice on HFD versus KO mice on Chow, n = 5 (KO) or 3 (Chow). (E) Percent change in plasma insulin levels after i.p. glucose injection (3 g/kg body weight) in ObRlox and KO mice on HFD for 12 weeks. $P = 0.07; n = 4. (F) Left: H&E staining in representative pancreas sections of ObRlox and KO mice on HFD for 12 weeks. Scale bars: 100 μm. Right: Mean islet area from at least 10 islets from 5 individual mice for each genotype. **P < 0.01 versus ObRlox. Data are shown as mean ± SEM.
The reduced SOCS-3 and mildly increased preproinsulin gene expression in the islets of KO mice are compatible with the reports that SOCS-3 is a leptin-induced negative regulator of leptin signaling (1) and an in vitro study showing inhibition of preproinsulin gene expression by leptin induction of SOCS-3 (27). Thus, the reduced expression of SOCS-3 in the KO islets is consistent with enhanced phosphorylation of PI3K/Akt signaling in islets lacking ObR expression (26). The reduced SOCS-3 expression might contribute, in part, to enhanced insulin secretion and islet hyperplasia by upregulating insulin gene expression and insulin signaling in islets lacking leptin signaling. Finally, the elevated PTEN phosphorylation in KO islets and MIN6 β cells with ObR knockdown could indirectly enhance signaling via the PI3K/Akt pathway in the β cells, since phosphorylation of PTEN leads to inactivation of its phosphatase activity. One interpretation of these studies is that absence of leptin signaling allows enhanced action of insulin on β cells in an autocrine/paracrine manner to promote secretory function and/or growth.

To explore whether the effect of enhanced acute-phase secretion and improved glucose tolerance observed in the KO mice on normal chow persists and protects KOs against the effects of diet-induced obesity, we fed the KO mice with an HFD. Surprisingly, we observed glucose intolerance that was significantly worse in the KOs compared to that in the controls. Indeed, these findings bring into context the role of leptin in regulating lipid metabolism and islet function/growth (28, 29). For example, expression of wild-type ObRs in the islets of Zucker diabetic fatty rats attenuates accumulation of lipid content and rescues diabetogenic phenotypes such as impaired insulin secretion and β cell apoptosis (28). Thus, the attenuated acute-phase secretory response and an inadequate compensatory islet growth response in the KOs on the HFD may be due to secondary effects of lipid overload in the islets as a consequence of absent leptin signaling and is one possible mechanism underlying the impaired glucose tolerance in the pancreas-ObR-KO mice (summarized in Figure 6). Alternatively, insulin resistance at the level of the β cell itself in the KOs may directly contribute, in part, to a failure of the β cell to compensate for HFD-induced insulin resistance. In any case, the specific interactions among leptin signaling, lipid metabolism, and growth factor proteins contributing to insulin resistance in β cells in the context of obesity warrants further investigation.

A distinct difference between the pancreas-Ob-R-KO model reported in this article, created using Pdx-Cre, and that reported by Covey et al, using RIP-Cre (31), is the recombination in the hypothalamus in the latter. Although the mice reported by Covey et al. did not manifest enhanced food intake, the increased body weight, fat mass, serum leptin levels, and plasma and liver triglyceride content, even when the mice were fed normal chow, together can directly contribute to glucose intolerance and poor insulin sensitivity in these mice (20, 38). In contrast, the pancreas-ObR-KOs on normal chow did not manifest alterations in insulin sensitivity but exhibited improved glucose tolerance due to enhanced insulin secretion that is consistent with a lack of tonic inhibitory action of leptin on β cell secretion (2, 3). Furthermore, we also provide evidence for crosstalk between leptin and insulin signaling pathways that contributes to alterations in β cell growth and function. Thus, the phenotype in pancreas-Ob-R-KO mice reflects the direct effects of absent leptin signaling in the islets, independent of the hypothalamus, and provides a straightforward model to study the role of leptin signaling in the development of obesity-associated diabetes. It will be especially instructive to explore whether disruption of both leptin and insulin signaling in the β cells, akin to leptin and insulin resistance associated with diet-induced obesity, leads to a greater susceptibility to development of diabetes.

In summary, we have created a unique genetic model to directly demonstrate the consequences of lack of functional ObRs in islet β cells. It is tempting to speculate that leptin resistance in pancreatic
β cells is one factor that contributes to the hyperinsulinemia, β cell failure, and consequent glucose intolerance in the obese state.

Methods

Animals and genotyping. ObRlox mice were created by homologous recombination using the ObR gene with loxP sites flanking exon 1, as previously described (20), and were maintained on a C57BL/6 background. Transgenic mice expressing Cre recombinase under the control of the mouse Pax6 promoter (Pdx-Cre mice) (21) were maintained on a C57BL/6 background and bred with Obflox mice. All animals were housed in specific pathogen–free facilities and maintained on a 12-hour light/12-hour dark cycle and fed standard rodent chow at the Foster Animal Laboratory, Brandeis University, Waltham, Massachusetts, USA. We used both male and female mice for all experiments. All protocols for animal use were approved by the Institutional Animal Care and Use Committee of the Joslin Diabetes Center and Brandeis University and were in accordance with NIH guidelines.

For the high-fat feeding study, 6-week-old ObRlox control and pancreas-ObRKO male mice were fed either an HF diet or regular chow for 12 weeks as described previously (11). Body weights were followed weekly, and glucose tolerance tests (GTTs) and ITTs were performed during the last week of feeding as described below.

Genotyping was performed on DNA isolated from the tails of 3- to 4-week-old mice by PCR. The primers for the oblox gene were 5′-GTCACCTAGGT-TAATGTATCT-3′ (forward) and 5′-TCTAGCCCTCCAGACTGAC-3′ (reverse) and for the Cre transgene were 5′-TGCCAGACCAATGTGACGC-3′ (forward) and 5′-CCAGTTACGGATATAGTTCATG-3′ (reverse).

Physiological measurements. Food intake of the mice was measured as described previously (39). We determined blood glucose values from whole venous blood using an automated glucose monitor (Glucometer Elite, Bayer) and measured serum insulin and leptin levels by ELISA, using mouse insulin or leptin standards (Crystal Chem Inc.). GTTs and acute insulin secretion tests were performed on animals that had been fasted overnight for 16 hours (8, 9). For GTTs, glucose levels were measured from blood collected from the tail immediately before and 15, 30, 60, and 120 minutes after i.p. glucose injection. For acute insulin secretion tests, blood samples were collected before (time 0) and 2 and 5 minutes after i.p. glucose injection (3 g/kg body weight). ITTs were performed as previously described (9). Blood glucose levels were determined immediately before and 15, 30, and 60 minutes after injection of insulin at random-fed state (1 U/kg body weight) (Humulin R; Lilly).

RT-PCR and quantitative real-time PCR. Islets were isolated from mice as described previously (3). Total RNA from islets, non-islet tissue, and specific tissues was extracted by Turbo RNA Isolation kit (Invitrogen) and treated with DNase I (Ambion). The RNA was then reverse transcribed and amplified by semiquantitative PCR by using primer sets as described previously (11). Overall, 30 cycles were performed. The primers for mouse insulin (Linco) and Texas red–conjugated donkey anti–guinea pig IgG (Jackson ImmunoResearch Laboratories Inc.) were used in this study to amplify the target sequences of interest. The primers were designed to ensure sequence specificity and to match the cDNA sequences of the respective genes. The amplified products were resolved by electrophoresis on a 1% agarose gel and visualized by ethidium bromide staining.

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