Restoration of Lepr in β cells of Lepr null mice does not prevent hyperinsulinemia and hyperglycemia

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ABSTRACT

Objective: The adipose-derived hormone leptin plays an important role in regulating body weight and glucose homeostasis. Leptin receptors are expressed in the central nervous system as well as peripheral tissues involved in regulating glucose homeostasis, including insulin-producing β cells of the pancreas. Previous studies assessing the role of leptin receptors in β cells used Cre-loxP to disrupt the leptin receptor gene (Lepr) in β cells, but variable results were obtained. Furthermore, recombination of Lepr was observed in the hypothalamus or exocrine pancreas, in addition to the β cells, and Lepr in non-β cells may have compensated for the loss of Lepr in β cells, thus making it difficult to assess the direct effects of Lepr in β cells. To determine the significance of Lepr exclusively in β cells, we chose to selectively restore Lepr in β cells of Lepr null mice (Lepr<sup>b<sup>b</sup>loxTB<sup>b</sup>loxTB).

Materials and methods: We used a mouse model in which endogenous expression of Lepr was disrupted by a loxP-flanked transcription blocker (Lepr<sup>b</sup>loxTB<sup>b</sup>loxTB), but was restored by Cre recombinase knocked into the Ins<sup>1</sup> gene, which is specifically expressed in β cells (Ins<sup>1</sup>Cre). We bred Lepr<sup>b</sup>loxTB<sup>b</sup>loxTB and Ins<sup>1</sup>Cre mice to generate Lepr<sup>b</sup>loxTB<sup>b</sup>loxTB and Lepr<sup>b</sup>loxTB<sup>b</sup>loxTB Ins<sup>1</sup>Cre mice, as well as Lepr<sup>wt/</sup><sup>wt</sup> and Lepr<sup>b</sup>loxTB<sup>b</sup>loxTB Ins<sup>1</sup>Cre littermates. Male and female mice were weighed weekly between 6 and 11 weeks of age and fasting blood glucose was measured during this time. Oral glucose was administered to mice aged 7—12 weeks to assess glucose tolerance and insulin secretion. Relative β and α cell area and islet size were also assessed by immunostaining and analysis of pancreas sections of 12—14 week old mice.

Results: Male and female Lepr<sup>b</sup>loxTB<sup>b</sup>loxTB mice, lacking whole-body expression of Lepr, had a phenotype similar to db/db mice characterized by obesity, hyperinsulinemia, glucose intolerance, and impaired glucose stimulated insulin secretion. Despite restoring Lepr in β cells of Lepr<sup>b</sup>loxTB<sup>b</sup>loxTB mice, fasting insulin levels, blood glucose levels and body weight were comparable between Lepr<sup>b</sup>loxTB<sup>b</sup>loxTB Ins<sup>1</sup>Cre mice and Lepr<sup>b</sup>loxTB<sup>b</sup>loxTB Ins<sup>1</sup>Cre mice were similar to that observed in Lepr<sup>b</sup>loxTB<sup>b</sup>loxTB Ins<sup>1</sup>Cre mice. Analysis of pancreatic insulin positive area revealed that restoration of Lepr in β cells of Lepr<sup>b</sup>loxTB<sup>b</sup>loxTB mice did not prevent hyperplasia of insulin positive cells nor did it rescue Glut-2 expression.

Conclusion: Collectively, these data suggest that direct action of leptin on β cells is insufficient to restore normal insulin secretion and glucose tolerance in mice without leptin receptor signaling elsewhere.

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Keywords Leptin receptor; Hyperinsulinemia; β cell

1. INTRODUCTION

Obesity is often associated with elevated circulating insulin levels (hyperinsulinemia), glucose intolerance, and reduced insulin sensitivity [1,2]. The molecular mechanisms underlying the increased insulin secretion and islet hyperplasia that are often concomitant with obesity remain unclear, but the adipose-derived hormone leptin may be a potential link between adipose tissue and β cells. Leptin has an established role in regulating food intake, adipose metabolism, and glucose homeostasis through signaling in the central nervous system (CNS) [3,4]. In addition, leptin receptors are distributed in several peripheral tissues involved in glucose regulation including the liver, adipose, and pancreas [5,6]. Within these tissues, the long isoform of the leptin receptor (Leprb) is believed to mediate the glucoregulatory actions of leptin [7].

In vitro studies have demonstrated that Lepr is expressed in murine and human pancreatic β cells, as well as in β cell lines [8—11]. Previous studies have attempted to assess whether leptin has a direct action on β cell function, but the results are contradictory. While some studies have found that incubation of human or rodent islets with leptin did not alter the expression or secretion of insulin [12—14], other studies demonstrated reduced insulin expression and secretion from...
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islets treated with leptin [8.12.15–18]. The inhibitory actions of leptin on β cell function are supported by in vivo characterization of glucose homeostasis and circulating insulin levels in mice lacking leptin or its receptor. In leptin deficient mice (ob/ob) and mice with mutated Lepr (db/db), hyperinsulinemia develops prior to increases in body weight, and islet hyperplasia is evident in these mice [19–21]. When ob/ob mice are treated with leptin, there is a reduction of insulin gene expression and secretion and a reduction of circulating insulin levels within hours [22]. Moreover, doses of leptin insufficient to reduce body weight are sufficient to reverse hyperinsulinemia, indicating that the insulin-lowering effects of leptin can occur independent of weight loss [23].

To elucidate the mechanism by which leptin can regulate insulin secretion and expression, previous studies have employed Cre-loxP technology. Disruption of Lepr in β cells was achieved by Covey et al. using a rat insulin promoter driving Cre expression (RIPCre) [24]. LeprloxP/lox RIPCre mice were obese, hyperinsulinemic, glucose intolerant, and experienced islet hyperplasia relative to their LeprloxP/lox littermates. Interestingly, a similar approach by Morioka et al. and their colleagues to disrupt Lepr using a Pdx1 (pancreatic duodenal homeobox 1) promoter to drive Cre expression resulted in mice with mildly elevated fasting insulin levels but the absence of obesity [25]. Furthermore, Morioka et al. reported improved glucose tolerance and elevated fasting insulin levels in mice lacking Lepr [23].

Lepr, and its receptor Leprb, are expressed in many tissues, including the pancreas, liver, brain, and muscle. Leptin receptors are also expressed in other peripheral tissues and the central nervous system (CNS) to make up for the life-long loss of direct action of leptin in β cells. To determine the actions of leptin in β cells, we exploited a murine model in which a transcriptional blockade flanked by two loxP sites is placed between exon 16 and exon 17 of Lepr (LeprloxTB/loxTB), resulting in impaired leptin signaling [29]. To assess the direct actions of leptin in β cells, we crossed these mice with Ins1Cre mice to selectively delete Lepr in pancreatic β cells [28]. Unlike previous studies, which reported elevated plasma insulin levels following knockout of Lepr [24,25], knockout of Lepr in β cells using Ins1Cre did not induce elevated plasma insulin levels. Moreover, knockout of Lepr using Ins1Cre did not result in glucose intolerance or obesity. While the studies described above used very similar approaches to assess the role of Lepr in β cells, differences in genetic background and tissue specificity of Cre lines may have contributed to the different phenotypes observed upon deletion of Lepr. Furthermore, these knockout studies are limited by the potential compensation by Lepr expressed either in other peripheral tissues or the central nervous system (CNS) to make up for the life-long loss of direct action of leptin in β cells. To determine the actions of leptin in β cells, we exploited a murine model in which a transcriptional blockade flanked by two loxP sites is placed between exon 16 and exon 17 of Lepr (LeprloxTB/loxTB), resulting in impaired leptin signaling [29]. To assess the direct actions of leptin in β cells, we crossed these mice with Ins1Cre mice to selectively restore Lepr in pancreatic β cells, while functional leptin receptors were absent in other tissues. This provided a unique model with which to assess whether the direct actions of leptin in β cells are sufficient to prevent hyperinsulinemia.

2. METHODS

2.1. Experimental animals

LeprloxTB/loxTB mice (JAX no. 018989, 50% FVB, 50% C57BL/6J, <1% 129) and Ins1Cre mice (JAX no. 026801, C57BL6/J) [29] were obtained from Jackson Laboratory (Bar Harbor, ME, USA). LeprloxTB/loxTB mice were then bred with Ins1Cre mice for two generations to yield LeprloxTB/loxTB, LeprloxTB/loxTB, LeprloxTB/loxTB, Ins1Cre, LeprloxTB/loxTB, and LeprloxTB/loxTB mice on a B6.FVB hybrid background. Offspring were born at the expected Mendelian ratio. To assess the specificity of Ins1Cre mediated recombination, mt/mg reporter mice (JAX no. 007676, B6.129, Jackson Laboratory), which possess a membrane-bound Tomato fluorescent protein (mT) flanked by two unidirectional loxP sites, followed by the open reading frame for membrane bound enhanced green fluorescent protein (EGFP, mG) driven by the chicken β actin promoter, were crossed with Ins1Cre mice to generate homozygous mt/mg mice with or without 1 allele for Ins1Cre. Mice were housed on a 12 h light/12 h dark cycle and had ad libitum access to food (chow diet 2918, Harlan Laboratories, Madison WI, USA) and water. At 13 weeks of age, animals were fasted for 4 h prior to euthanasia. Pancreata were collected and weighed prior to fixation. All procedures with animals were approved by the University of British Columbia Animal Care Committee and carried out in accordance with the Canadian Council of Animal Care guidelines.

2.2. Metabolic measurements

Body weight and blood glucose were measured in mice fasted for 4 h. Blood was collected from the saphenous vein to measure blood glucose using a One Touch Ultra Gluco meter (Life Scan, Burnaby, Canada). Insulin was measured in plasma (Mous eUltrasonic insulin ELISA, Alpco Diagnostics, Salem, NH, USA) collected at 7 and 11 weeks of age. At 9 weeks of age, LeprloxTB/loxTB Ins1Cre mice and littermate control mice underwent a 6h fast prior to oral gavage of dextrose (1.5 g/kg, 40% solution) followed by measurement of blood glucose and collection of plasma at time = 0, 15, 30, and 60 min to assess glucose tolerance and glucose stimulated insulin secretion.

2.3. Islet isolation

Islets were isolated from 11 to 13 week old male and female LeprloxTB/loxTB Ins1Cre and LeprloxTB/loxTB littermate mice as previously described [30]. Briefly, mice were euthanized by CO2, and 2 ml of Hank’s balanced salt solution (HBSS) containing 1000 units/ml of type XI collagenase (Sigma—Aldrich, St. Louis, MO) was immediately injected into the pancreatic duct. Pancreata were digested at 37 °C for 11 min and then washed with ice cold HBSS containing 1 mM CaCl2. Islets were then hand picked into RPMI 1640 (Sigma Aldrich) containing 0.5% BSA, 100 units/ml penicillin and 100 µg/ml streptomycin. Islets were then flash frozen in liquid nitrogen and stored at −80 °C.

2.4. Gene deletion

Immediately following euthanasia, mice were decapitated and their brains were isolated. Hypothalamus was then dissected on ice and flash frozen. To assess gene deletion, genomic DNA (gDNA) was extracted from flash frozen hypothalamic tissue and islets as previously described [31]. Deletion of the transcriptional blockade upstream of exon 17 in the Lepr gene was assessed using Taqman qPCR probes (IDT) (for primer sequences see Table 1; for location of primers in gene see Figure 1A). The degree of recombination was determined using the Pfaffi method in which the deltaCT value of the flanked region of Lepr (target gene) was divided by the deltaCT value of a region upstream of the loxP sites (reference gene).

2.5. Islet morphology and immunohistochemistry

Pancreata were collected from 12 to 14 week old mice, fixed overnight in 4% PFA, rinsed in 70% ethanol, embedded in paraffin, and processed for sectioning by Wax-it Histology Services (Vancouver, Canada). Three sections separated by 200 µm per mouse were immunostained for insulin (rabbit anti-insulin antibody, Cat# C2793, 1:1000, Cell Signaling) and glucagon (mouse anti-glucagon antibody, Cat# A2001, Cell Signaling) using the avidin-biotin-peroxidase complex method.
Exon 18 extracted from islets (B) and hypothalamus (C). Mice expressing recombination was assessed by measuring the ratio of qPCR product obtained using primers within and outside of the TB region (primers 1 and 2, respectively in A) of gDNA collected from pancreata of 6-week old mice (H). Plasma insulin levels were measured in 8-week old mice following a gavage of 40% glucose (1.5 g/kg body weight) to assess glucose tolerance and glucose stimulated insulin secretion (I, J). Values are presented as individual data points in B, C, E, and J with a line indicating the median and the shaded area spanning the range of statistical significance (n = 3–5 per group).

Table 1 Sequences of primers used for quantitative PCR. Primer names as depicted in Figure 1A. Primer set 1 was used to amplify sequence within the transcriptional blockade region of Lepr while Primer set 2 was used to amplify exon 17 within Lepr.

| Primer name | Sequence |
|-------------|----------|
| 1 (forward) | 5’ GTG AGA TCA TGA GAC CCT AAA 3’ |
| 1 (reverse) | 5’ GGA ACT CAA GAC CAT CTA TCA 3’ |
| 1 (HEX probe) | 5’ TTC TGA ATT GGT GTC CCT GGA GCC 3’ |
| 2 (forward) | 5’ CCT TTC CAG ATA ATG CCT GAT AGA 3’ |
| 2 (reverse) | 5’ GAG CCA CAC TTA GCT CCA ATA 3’ |
| 2 (FAM probe) | 5’ TAG GGC GGA TGA ACC AGC AAA TGT 3’ |

Figure 1: Lepr was restored in mice with a transcriptional blockade (TB) upstream of exon 17 in Lepr by recombination of loxP sites flanking the TB using Ins1Cre (A). Percent recombination was assessed by measuring the ratio of qPCR product obtained using primers within and outside of the TB region (primers 1 and 2, respectively in A) of gDNA extracted from islets (B) and hypothalamus (C). Mice expressing mT/mG were used to determine the extent of recombination in islets with Ins1Cre. Representative images were collected from pancreata of mT/mG Ins1Cre and mT/mG mouse co-stained for insulin (red) and EGFP (green) and DAPI (gray) (D; n = 3–4). Expression of GFP indicates Cre-mediated recombination. Scale bar represents 100 μm. Fasted insulin levels were measured at 5 weeks of age (E), along with fasting glucose levels (F) and body weight (G) between 5 and 2017 The Authors. Published by Elsevier GmbH. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).
considered statistically significant. All values are expressed either as individual data points with median or as mean ± SEM where indicated. Unless otherwise specified, a one-way ANOVA (or non-parametric Kruskal–Wallis test), two-way ANOVA, or Student’s t-test were performed, with a Tukey post-hoc test.

3. RESULTS

3.1. Validation of Lepr restoration in β cells

To assess recombination of the LeprloxTB/loxTB site in the presence of Ins1Cre, islets were isolated from 11 to 13 week old LeprloxTB/loxTB and LeprloxTB/loxTB Ins1Cre mice, and gDNA was extracted for analysis by qPCR. A ~75% rate of recombination of Lepr was observed in islets of LeprloxTB/loxTB Ins1Cre mice, while the recombination of Lepr in islets of LeprloxTB/loxTB mice was less than 10% (Figure 1B). Recombination of Lepr in islets was compared to recombination of Lepr in the hypothalamus to assess the tissue specificity of Ins1Cre. The percent of recombination in the hypothalamus was minimal and was comparable between LeprloxTB/loxTB Ins1Cre and LeprloxTB/loxTB mice (Figure 1C).

Next, we crossed Ins1Cre mice with mT/mG reporter mice to determine whether Ins1Cre produced recombination in β cells. These double fluorescent reporter mice express membrane-targeted dimer Tomato prior to Cre-mediated excision and membrane-targeted enhanced green fluorescent protein (EGFP) after excision [33]. Immunostaining of EGFP in fixed pancreas from mT/mG Ins1Cre and mT/mG control mice revealed EGFP expression co-localized in β cells of mT/mG Ins1Cre mice (Figure 1D), indicating that Cre-induced recombination occurred in β cells. To determine whether replacing 1 insulin allele with Cre influenced glucose homeostasis and metabolism, we also assessed body weight, fasting glucose, fasting leptin levels, and insulin sensitivity in male mT/mG Ins1Cre mice between the ages of 4−12 weeks. Fasting insulin, blood glucose, and body weight levels were similar between mT/mG Ins1Cre mice and mT/mG mice (Figure 1E−G). Similarly, no differences in insulin sensitivity, glucose tolerance, or glucose stimulated insulin secretion were observed (Figure 1H−J). Therefore, our characterization of the Ins1Cre line confirms and extends previously published data [34] and demonstrates that knock-in of Cre into one allele of Ins1 does not appear to significantly affect glucose homeostasis.

3.2. Lepr in β cells is not sufficient to regulate islet morphology or β cell area

It has been previously reported that whole body mutation of Lepr [21] or knockout of Lepr in the pancreas of Chow-fed mice [25] results in increased β cell area. To determine if restoration of Lepr in β cells is sufficient to prevent the increase in β cell area, we immunostained pancreas sections collected from 12 to 14 week old LeprloxTB/loxTB Ins1Cre, LeprloxTB/loxTB and LeprloxTB/loxTB mice with insulin, glucagon, and DAPI (Figure 2A). Quantification revealed that LeprloxTB/loxTB insulin positive area was ~10 fold higher compared to LeprloxTB/loxTB Ins1Cre mice with insulin, glucagon, and DAPI (Figure 2A). Restoration of Lepr in LeprloxTB/loxTB Ins1Cre mice resulted in a trend for reduced insulin positive area compared to LeprloxTB/loxTB mice (p = 0.06). In contrast, no differences in α cell area, as determined by glucagon positive immunostaining, were observed between experimental groups (Figure 2D). Analysis of islet size revealed a significantly lower proportion of small islets (<500 μm²) in LeprloxTB/loxTB and LeprloxTB/loxTB Ins1Cre mice compared to LeprloxTB/loxTB mice, while LeprloxTB/loxTB and LeprloxTB/loxTB Ins1Cre mice had a greater number of large (>10,000 μm²) islets than LeprloxTB/loxTB littermates. To determine if restoration of Lepr in β cells is able to prevent the loss of Glut-2 expression in LeprloxTB/loxTB mice, pancreas sections were co-immunostained for Glut-2, insulin, and DAPI (Figure 2B). Glut-2 immunoreactivity was clearly present in LeprloxTB/loxTB Ins1Cre mice, but generally lacking in LeprloxTB/loxTB and LeprloxTB/loxTB Ins1Cre mice. Together, these findings suggest that direct action of leptin in β cells may not be sufficient to modify islet size or rescue Glut-2 expression in mice lacking leptin receptors elsewhere.

3.3. Restoration of Lepr in β cells has no significant impact on body weight, insulin levels, or fasting glucose levels

Along with increased insulin levels, obesity has been reported in LeprloxTB/loxTB RIPCre mice [24]. To examine the effects of restoration of Lepr in β cells, we measured body weight weekly between 6 and 11 weeks of age and collected plasma at 13 weeks of age to measure leptin levels. Body weight of male and female LeprloxTB/loxTB Ins1Cre mice was comparable to LeprloxTB/loxTB Ins1Cre mice. Male LeprloxTB/loxTB Ins1Cre mice weighed 50% more than LeprloxTB/loxTB littermate mice, while female LeprloxTB/loxTB Ins1Cre mice weighed 80% more than their respective control littermates (Figure 3A, B). Similar to prior studies with LeprloxTB/loxTB mice [29], we observed hyperleptinemia in LeprloxTB/loxTB male and female mice, with leptin levels that were ~150 times higher compared to LeprloxTB/loxTB Ins1Cre littermates (LeprloxTB/loxTB = 252.9 ± 26.7 ng/ml, LeprloxTB/loxTB Ins1Cre = 1.65 ± 0.20 ng/ml). Despite restoring Lepr in β cells, fasting leptin levels of LeprloxTB/loxTB Ins1Cre mice (279.8 ± 11.67 ng/ml) were comparable to those of LeprloxTB/loxTB mice. To assess the effects of β cell-specific signaling of leptin on insulin secretion and glucose homeostasis, we monitored blood glucose weekly between 6 and 11 weeks of age and collected plasma from male and female mice at 7 and 11 weeks of age to measure insulin levels. Hyperinsulinemia was evident in LeprloxTB/loxTB Ins1Cre mice and plasma insulin levels were comparable to that of LeprloxTB/loxTB mice (Figure 3C, D). In contrast, plasma insulin levels of LeprloxTB/loxTB and LeprloxTB/loxTB Ins1Cre mice were ~20 fold lower than LeprloxTB/loxTB Ins1Cre mice. Similar to fasting insulin levels, fasting glucose levels were elevated in LeprloxTB/loxTB Ins1Cre and LeprloxTB/loxTB Ins1Cre mice compared to LeprloxTB/loxTB and LeprloxTB/loxTB Ins1Cre mice (Figure 3E, F). However, by 10−11 weeks of age, female LeprloxTB/loxTB Ins1Cre mice exhibited significantly higher glucose levels than their LeprloxTB/loxTB littermates. Together these findings suggest that selective restoration of Lepr in β cells does not prevent obesity and hyperinsulinemia in mice lacking leptin receptors elsewhere. Moreover, restoration of Lepr in β cells does not improve fasting glucose levels of male or female LeprloxTB/loxTB Ins1Cre mice relative to their respective littermate LeprloxTB/loxTB mice.

3.4. LeprloxTB/loxTB Ins1Cre mice are glucose intolerant and have an exaggerated insulin response to glucose

We next assessed the effects of restoring Lepr in β cells on insulin secretion in both male and female LeprloxTB/loxTB Ins1Cre mice. Following a 6 h fast, LeprloxTB/loxTB mice were euglycemic, while LeprloxTB/loxTB and LeprloxTB/loxTB Ins1Cre mice were hyperglycemic (blood glucose >11 mM; Figure 4A, D). Fifteen minutes after glucose gavage, blood glucose levels of male and female LeprloxTB/loxTB and LeprloxTB/loxTB Ins1Cre mice peaked between 15 and 18 mM. In contrast, glucose levels of LeprloxTB/loxTB and LeprloxTB/loxTB Ins1Cre mice peaked above the limit of detection (33.3 mM, represented as a dashed line on the graph), indicating they were severely glucose intolerant. Analysis of area under the curve (AUC) revealed that LeprloxTB/loxTB and LeprloxTB/loxTB Ins1Cre mice were significantly glucose intolerant compared to control littermate mice (Figure 4B, E). In response to glucose gavage, male and female LeprloxTB/loxTB and LeprloxTB/loxTB Ins1Cre mice had fasting plasma insulin levels between 0.1 and 0.7 ng/ml, and insulin levels peaked at 0.3—
1.1 ng/ml at the 15-minute time point (Figure 4C, F). In contrast, Lepr<sup>loxTB/loxTB</sup> and Lepr<sup>loxTB/loxTB</sup> Ins1Cre mice had fasting insulin levels that were ~10-fold higher than Lepr<sup>wt/wt</sup> mice and peak glucose stimulated insulin secretion levels that were between 15 and 30-fold higher than Lepr<sup>wt/wt</sup> mice. Fasting as well as glucose stimulated plasma insulin levels were comparable between Lepr<sup>loxTB/loxTB</sup> and Lepr<sup>loxTB/loxTB</sup> Ins1Cre mice. Despite similar glucose levels in response to oral glucose gavage, basal plasma insulin levels and glucose stimulated insulin secretion was different between male and female mice. Female Lepr<sup>loxTB/loxTB</sup> and Lepr<sup>loxTB/loxTB</sup> Ins1Cre mice had lower basal insulin levels compared to males (Figures 3C, D and 4C, F), and insulin secretion in response to glucose was much higher in female Lepr<sup>loxTB/loxTB</sup> as compared to male mice of the same genotype (Figure 4C, F).

4. DISCUSSION

To examine the effects of restoring Lepr in β cells on insulin secretion and morphology, we crossed mice expressing Cre knocked into the Ins1 gene with Lepr<sup>loxTB/loxTB</sup> mice, which have a transcriptional blockade inserted between exon 16 and exon 17 of Lepr, resulting in impaired leptin signaling. Disruption of the leptin receptor signaling domain in Lepr<sup>loxTB/loxTB</sup> mice results in a phenotype similar to db/db mice, characterized by obesity, hyperinsulinemia, hyperglycemia, insulin resistance, and islet hyperplasia [35,36]. Cre-mediated removal of the transcriptional blockade and restoration of Lepr can be achieved in Lepr<sup>loxTB/loxTB</sup> mice in a cell-type selective manner, enabling the assessment of the physiological impact of Lepr in specific tissues.

Figure 2: Representative images were taken from pancreas sections of Lepr<sup>wt/wt</sup>, Lepr<sup>loxTB/loxTB</sup>, and Lepr<sup>loxTB/loxTB</sup> Ins1Cre mice immunostained for insulin (INS; red), glucagon (GCG; green), and DAPI (gray) (A). Presence of Glut-2 in β cells was determined by co-staining pancreas sections for insulin (red) Glut-2 (green) and DAPI (white) in Lepr<sup>wt/wt</sup>, Lepr<sup>loxTB/loxTB</sup>, and Lepr<sup>loxTB/loxTB</sup> Ins1Cre mice (B). Scale bar represents 100 μm. Relative β and α cell area were calculated by measuring total insulin and glucagon positive areas, respectively, relative to total pancreas area (C, D). Islet size was approximated by measuring insulin positive area per islet. Islet size was divided into 6 categories and frequency of islets in each category was determined relative to total number of islets measured per animal (E). Values are presented as individual data points with a line representing the median and the shaded area indicating the range of values. *P < 0.05 compared to Lepr<sup>wt/wt</sup> Ins1Cre. A non-parametric one-way ANOVA with Kruskal–Wallis post hoc analysis was used to determine statistical significance (n = 4–5 per group).
Restoration of Lepr in β cells of Lepr<sup>loxTB/loxTB</sup> Ins1Cre mice did not alter the development of hyperinsulinemia, hyperglycemia, glucose intolerance, and obesity, suggesting that leptin signaling in β cells does not suppress insulin secretion. Moreover, islet hyperplasia was still present in Lepr<sup>loxTB/loxTB</sup> Ins1Cre mice, similarly to what is reported in db/db mice [35]. However, as we did not follow the mice for an extended period of time, our studies cannot rule out the possibility that the restoration of Lepr in β cells may have prevented age related β cell failure that eventually results in overt severe diabetes, like in db/db mice [37,38].

A distinct difference between the present study and previous studies in which Lepr was selectively deleted [24,25,28] is the inability of compensation to occur from non-β cells in Lepr<sup>loxTB/loxTB</sup> Ins1Cre mice. In prior studies that examined the role of leptin signaling in β cells by deletion of Lepr, it is unclear whether Lepr acting in other tissues influenced pancreas morphology and β cell function. The ability of non-β cell leptin receptor action to influence β cell function is evident in mice with restored Lepr in brains of db/db mice. Over-expression of Leprb driven by synapsin 1 and neuron specific enolase promoters in db/db mice resulted in normalized islet morphology and function.

Figure 3: Body weight was measured weekly between 6 and 11 weeks of age in male (A) and female (B) Lepr<sup>wt/wt</sup>, Lepr<sup>wt/wt</sup> Ins1Cre, Lepr<sup>loxTB/loxTB</sup>, and Lepr<sup>loxTB/loxTB</sup> Ins1Cre, mice. Fasting plasma insulin levels at 7 and 11 weeks of age in male (C) and female mice (D). Values are presented as individual data points with a line indicating the median and the shaded region spanning the range of values. Fasting blood glucose levels were monitored in male (E) and female (F) mice. Values in A, B, E, & F are presented as mean ± SEM. *P < 0.05 comparing Lepr<sup>loxTB/loxTB</sup> Ins1Cre and Lepr<sup>wt/wt</sup> using a two-way repeated measures ANOVA with Tukey post hoc analysis (n = 4–13 females, n = 3–5 males).
Previous studies have reported partial correction of body weight [39]. These authors concluded that Lepr in the CNS is able to rescue the diabetic and obese phenotype of db/db mice. To prevent Lepr in non-β cells from influencing β cell function, we selectively re-expressed Lepr in β cells of mice lacking Lepr elsewhere. Our results suggest that Lepr in β cells is not sufficient to prevent hyperinsulinemia and glucose intolerance from occurring. It is likely that Lepr in the CNS and other islet cell populations are involved in inhibiting insulin secretion and preventing hyperplasia of β cells.

Previous studies have reported Lepr expression in β cells of human and rodent islets and cell lines [9,40–43]; however, in another study, non-detectable levels of Lepr were reported in β cells from dispersed islets using RT-qPCR [28]. More recently, studies using single-cell RNA-seq to determine the transcriptome profiles of human and murine islets revealed that the expression of Lepr was higher in δ cells than β cells [44,45]. Though insulin secretion and expression was reduced in isolated murine and human islets treated with leptin [8,10,12], this effect may have been a result of leptin acting on δ cells. Collectively, this suggests that despite restoration of Lepr in LeprloxTB/loxTBIns1Cre mice, the relatively low levels of Lepr in β cells may not be sufficient to provoke improvements in β cell function in the absence of leptin receptor signaling in other pancreatic islet populations. The presence of hyperinsulinemia and islet hyperplasia despite restoration of Lepr in β cells of LeprloxTB/loxTB mice may be due to hyperleptinemia that was present in LeprloxTB/loxTBIns1Cre mice. Plasma leptin levels were ~150-fold higher in LeprloxTB/loxTBIns1Cre mice compared to control mice. Hyperleptinemia has been previously observed in LeprloxTB/loxTB [29], and in mice with disruption of Lepr in peripheral tissues [46]. This increase of plasma leptin levels was attributed to the disruption of Lepr in white adipose tissue (WAT), which led to inactivation of a negative feedback loop regulating leptin synthesis in WAT [46]. Indeed the presence of hyperleptinemia can cause leptin resistance [47]. Thus, despite restoration of Lepr in β cells, the chronically elevated leptin levels in LeprloxTB/loxTBIns1Cre mice may have resulted in a state of leptin resistance in β cells of LeprloxTB/loxTBIns1Cre mice.

5. CONCLUSION

In summary, selective restoration of Lepr in pancreatic β cells of Lepr knockout mice does not prevent the development of hyperinsulinemia and β cell expansion. These findings suggest that leptin receptor signaling directly in β cells does not regulate β cell function. We propose that actions of leptin on β cells are secondary to action of leptin signaling in other tissues. Whether this involves a combination of leptin signaling in other islet cell populations and in the CNS warrants further study.

ACKNOWLEDGMENTS

We thank Shannon O’Dwyer for technical assistance. This work was supported by the Canadian Institutes of Health Research. A.M.D. was supported by the National Sciences and Engineering Research Council of Canada.

CONFLICT OF INTEREST

None declared.

REFERENCES

[1] Odileye, O.E., de Courten, M., Pettitt, D.J., Ravussin, E., 1997. Fasting hyperinsulinemia is a predictor of increased body weight gain and obesity in Pima Indian children. Diabetes 46:1341–1345.

[2] Bray, G.A., 1979. Human obesity and some of its experimental counterparts. Annales de la Nutrition et de l’Alimentation 33:17–25.
Brief Communication

[3] Schwartz, M.W., Woods, S.C., Porte Jr., D., Seeley, R.J., Baskin, D.G., 2000. Central nervous system control of food intake. Nature 404:661–671.

[4] Elmquist, J.K., Elias, C.F., Saper, C.B., 1999. From lesions to leptin: hypothalamic control of food intake and body weight. Neuron 22:221–232.

[5] Cohen, P., Yang, G., Yu, X., Soukas, A.A., Wolfish, C.S., Friedman, J.M., et al., 2005. Induction of leptin receptor expression in the liver by leptin and food deprivation. Journal of Biological Chemistry 280:10024–10039.

[6] Fei, H., Okano, H.J., Li, C., Lee, G.H., Zhao, C., Darnell, R., et al., 1997. Anatomic localization of alternatively spliced leptin receptors (Ob-R) in mouse brain and other tissues. Proceedings of the National Academy of Sciences of the United States of America 94:7001–7005.

[7] Ghielardi, N., Ziegler, S., Wiestner, A., Stoffel, R., Heim, M.H., Skoda, R.C., 1996. Defective STAT signaling by the leptin receptor in diabetic mice. Proceedings of the National Academy of Sciences of the United States of America 93:6231–6235.

[8] Kulkarni, R.N., Wang, Z.L., Wang, R.M., Hurley, J.D., Smith, D.M., Ghatei, M.A., et al., 1997. Leptin rapidly suppresses insulin release from insulinoma cells, rat and human islets and, in vivo, in mice. Journal of Clinical Investigation 100:2729–2736.

[9] Pei, H., Okano, H.J., Li, C., Lee, G.H., Zhao, C., Darnell, R., et al., 1997. Leptin receptors expressed on pancreatic beta-cells. Biochemical and Biophysical Research Communications 224:522–527.

[10] Emilsson, V., Liu, Y.L., Cawthorne, M.A., Morton, N.M., Davenport, M., 1997. Expression of the functional leptin receptor mRNA in pancreatic islets and direct inhibitory action of leptin on insulin secretion. Diabetes 46:313–316.

[11] Islam, M.S., Morton, N.M., Harsson, A., Emilsson, V., 1997. Rat insulinoma-derived pancreatic beta-cells express a functional leptin receptor that mediates a proliferative response. Biochemical and Biophysical Research Communications 238:851–855.

[12] Poitout, V., Rouault, C., Guerre-Millo, M., Briaud, I., Reach, G., 1998. Inhibition of insulin secretion by leptin in normal rodent islets of Langerhans. Endocrinology 139:822–826.

[13] Leclercq-Meyer, V., Malaisse, W.J., 1998. Failure of human and mouse leptin to affect insulin, glucagon and somatostatin secretion by the perfused rat pancreas at physiological glucose concentration. Molecular and Cellular Endocrinology 141:111–118.

[14] Leclercq-Meyer, V., Considine, R.V., Sener, A., Malaisse, W.J., 1996. Do leptin receptors play a functional role in the endocrine pancreas? Biochemical and Biophysical Research Communications 229:794–798.

[15] pallett, A.L., Morton, N.M., Cawthorne, M.A., Emilsson, V., 1997. Leptin inhibits insulin secretion and reduces insulin mRNA levels in rat isolated pancreatic islets. Biochemical and Biophysical Research Communications 238:267–270.

[16] Ookuma, M., Ookuma, K., York, D.A., 1998. Effects of leptin on insulin secretion from isolated rat pancreatic islets. Diabetes 47:219–223.

[17] Ishida, K., Murakami, T., Mizuno, A., Iida, M., Kuvajima, M., Shima, K., 1997. Leptin suppresses basal insulin secretion from rat pancreatic islets. Regulatory Peptides 70:179–182.

[18] Zhao, A.Z., Bornfeldt, K.E., Beavo, J.A., 1998. Leptin inhibits insulin secretion by activation of phosphodiesterase 3B. Journal of Clinical Investigation 102:869–873.

[19] Coleman, D.L., 1978. Obese and diabetes: two mutant genes causing diabetes-obesity syndromes in mice. Diabetologia 14:141–148.

[20] Coleman, D.L., Hummel, K.P., 1973. The influence of genetic background on the expression of the obese (Ob) gene in the mouse. Diabetologia 9:287–293.

[21] Coleman, D.L., Hummel, K.P., 1974. Hyperinsulinemia in pre-weaning diabetes (db) mice. Diabetologia 10(Suppl):607–610.

[22] Seufert, J., Kieffer, T.J., Habener, J.F., 1999. Leptin inhibits insulin gene transcription and reverses hyperinsulinemia in leptin-deficient ob/ob mice. Proceedings of the National Academy of Sciences of the United States of America 96:674–679.

[23] Pellemounter, M.A., Cullen, M.J., Baker, M.B., Hecht, R., Winters, D., Boone, T., et al., 1995. Effects of the obese gene product on body weight regulation in ob/ob mice. Science 269:540–543.

[24] Covey, S.D., Wideman, R.D., McDonald, C., Unniappan, S., Huyhn, F., Asadi, A., et al., 2006. The pancreatic beta cell is a key site for mediating the effects of leptin on glucose homeostasis. Cell Metabolism 4:291–302.

[25] Monioka, T., Astilma, E., Hu, J., Dishinger, J.F., Kurpad, A.J., Elias, C.F., et al., 2007. Disruption of leptin receptor expression in the pancreas directly affects beta cell growth and function in mice. Journal of Clinical Investigation 117:2860–2868.

[26] Honig, G., Liu, A., Berger, M., German, M.S., Tecott, L.H., 2010. Precise pattern of recombination in serotonergic and hypothalamic neurons in a Pdx1-Cre transgenic mouse line. Journal of Biomedical Science 17:82.

[27] Wicksteed, B., Bussova, M., Yan, W., Opland, D.M., Plank, J.L., Reintir, R.B., et al., 2010. Conditional gene targeting in mouse pancreatic sCels: analysis of ectopic Cre transgene expression in the brain. Diabetes 59:3090–3098.

[28] Soelding, H., Hodson, D.J., Adriabassens, A., Gribble, F.M., Reimann, F., Trapp, S., et al., 2015. Limited impact on glucose homeostasis of leptin receptor deletion from insulin- or proglucagon-expressing cells. Molecular Metabolism 4:619–630.

[29] Berglund, E.D., Vania, C.R., Donato Jr., J., Kim, M.H., Chuang, J.C., Lee, C.E., et al., 2012. Direct leptin action on POMC neurons regulates glucose homeostasis and hepatic insulin sensitivity in mice. Journal of Clinical Investigation 122:1000–1009.

[30] Salvaglio, P.R., Deng, S., Ariyan, C.E., Miller, I., Zawalich, W.S., Basadonna, G.P., et al., 2002. Iset filtration: a simple and rapid purification procedure that avoids ficoll and improves islet mass and function. Transplantation 74:877–879.

[31] Aljarni, S.M., Martinez, I., 1997. Universal and rapid salt-extraction of high quality genomic DNA for PCR-based techniques. Nucleic Acids Research 25:4692–4693.

[32] D’Souza, A.M., Asadi, A., Johnson, J.D., Covey, S.D., Kieffer, T.J., 2014. Leptin deficiency in rats results in hyperinsulinemia and impaired glucose homeostasis. Endocrinology 155:1268–1279.

[33] Muzumdar, M.D., Tasic, B., Miyamichi, K., Li, L., Luo, L., 2007. A global double-fluorescent Cre reporter mouse. Genesis 45:593–605.

[34] Thores, B., Tarussio, D., Maestro, M.A., Rovira, M., Heikela, E., Ferrer, J., 2015. Ins1(Cre) knock-in mice for beta cell-specific gene recombination. Diabetologia 58:558–565.

[35] Gapp, D.A., Leiter, E.H., Coleman, D.L., Schweizer, R.W., 1983. Temporal changes in pancreatic islet composition in C57BL/6J-db/db (diabetes) mice. Diabetologia 25:439–443.

[36] Hummel, K.P., Dickie, M.M., Coleman, D.L., 1966. Diabetes, a new mutation in the mouse. Science 153:1127–1128.

[37] Berglund, O., Frankel, B.J., Hellman, B., 1978. Development of the insulin secretory defect in genetically diabetic (db/db) mouse. Acta Endocrinologica 87:543–551.

[38] Kjorholm, C., Akerfeldt, M.C., Boin, T.J., Laybutt, D.R., 2005. Chronic hyperglycemia, independent of plasma lipid levels, is sufficient for the loss of beta-cell differentiation and secretory function in the db/db mouse model of diabetes. Diabetes 54:2755–2763.

[39] De Luca, C., Kowalski, T.J., Zhang, Y., Elmquist, J.K., Lee, C., Kilimann, M.W., et al., 2005. Complete rescue of obesity, diabetes, and infertility in db/db mice by neuron-specific LEPRI-B transgenes. Journal of Clinical Investigation 115:3484–3493.

[40] Tanizawa, Y., Okuya, S., Ishihara, H., Asano, T., Yada, T., Oka, Y., 1997. Direct stimulation of basal insulin secretion by physiological concentrations of leptin in pancreatic beta cells. Endocrinology 138:4513–4516.
[41] Fehmann, H.C., Peiser, C., Bode, H.P., Stamm, M., Staats, P., Hedettoft, C., et al., 1997. Leptin: a potent inhibitor of insulin secretion. Peptides 18:1267—1273.

[42] Kieffer, T.J., Heller, R.S., Leech, C.A., Holz, G.G., Habener, J.F., 1997. Leptin suppression of insulin secretion by the activation of ATP-sensitive K+ channels in pancreatic beta-cells. Diabetes 46:1087—1093.

[43] Seufert, J., Kieffer, T.J., Leech, C.A., Holz, G.G., Moritz, W., Ricordi, C., et al., 1999. Leptin suppression of insulin secretion and gene expression in human pancreatic islets: implications for the development of adipogenic diabetes mellitus. Journal of Clinical Endocrinology and Metabolism 84:670—676.

[44] Benner, C., van der Meulen, T., Caceres, E., Tigyi, K., Donaldson, C.J., Huising, M.O., 2014. The transcriptional landscape of mouse beta cells compared to human beta cells reveals notable species differences in long non-coding RNA and protein-coding gene expression. BMC Genomics 15:620.

[45] Baron, M., Veres, A., Wolock, S.L., Faust, A.L., Gaugjoux, R., Vetere, A., et al., 2016. A single-cell transcriptomic map of the human and mouse pancreas reveals inter- and intra-cell population structure. Cell Systems 3:346—360 e344.

[46] Guo, K., McMinn, J.E., Ludwig, T., Yu, Y.H., Yang, G., Chen, L., et al., 2007. Disruption of peripheral leptin signaling in mice results in hyperleptinemia without associated metabolic abnormalities. Endocrinology 148:3987—3997.

[47] Knight, Z.A., Haman, K.S., Greenberg, M.L., Friedman, J.M., 2010. Hyperleptinemia is required for the development of leptin resistance. PloS One 5: e11376.