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The glucagon-like peptide-2 receptor modulates islet adaptation to metabolic stress in the ob/ob mouse

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Abstract

Background & Aims: GLP-2 is a gut hormone that increases gut growth, reduces mucosal cell death and augments mesenteric blood flow and nutrient absorption. Exogenous GLP-2(1-33) also stimulates glucagon secretion and enhances gut barrier function with implications for susceptibility to systemic inflammation and subsequent metabolic dysregulation. We examined the importance of GLP-2R signaling for glucose homeostasis in multiple models of metabolic stress, diabetes and obesity. Methods: Body weight, islet function, glucose tolerance, and islet histology was studied in wildtype, high fat fed, lean diabetic, Glp2r-/- and ob/ob:Glp2r-/- mice. Results: GLP-2 did not stimulate glucagon secretion from isolated pancreatic islets in vitro, and exogenous GLP-2 had no effect on the glucagon response to insulin-induced hypoglycemia in vivo. Glp2r-/- mice exhibit no change in glycemia and plasma glucagon levels were similar in Glp2r-/- vs. Glp2r+/+ mice following hypoglycemia or following oral or intraperitoneal glucose challenge. Moreover, glucose homeostasis was comparable in Glp2r-/- vs. Glp2r+/+ mice fed a high fat diet for 5 months or following induction of streptozotocin-induced diabetes. In contrast, loss of the GLP-2R leads to increased glucagon secretion and α-cell mass, impaired intraperitoneal glucose tolerance, hyperglycemia, reduced β-cell mass, and decreased islet proliferation in ob/ob:Glp2r-/- mice. Conclusions: Our results demonstrate that although the GLP-2R is not critical for the stimulation or suppression of glucagon secretion or glucose homeostasis in normal or lean diabetic mice, elimination of GLP-2R signaling in obese mice impairs the normal islet adaptive response required to maintain glucose homeostasis.

Key words: Glucagon, GLP-2, GLP-1, inflammation, islets
Introduction

The control of glucose homeostasis is a tightly regulated process involving the interplay of gut and pancreatic hormones, gastric motility, insulin sensitivity, neural signals and regulation of hepatic glucose production. The gastrointestinal tract plays a key role in glucose homeostasis in both the fasted and fed states. During fasting, the gut may act as a gluconeogenic organ and contribute upwards of 20% of endogenous glucose production. In the postprandial state, the gut contributes to the regulation of glucose homeostasis by releasing multiple hormones, including the incretins glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP) \(^1\). Both GLP-1 and GIP stimulate insulin secretion yet exert contrasting effects on the pancreatic \(\alpha\)-cell and the regulation of glucagon secretion. GLP-1 is a potent inhibitor of glucagon secretion in normal subjects under euglycemic but not hypoglycemic conditions \(^2\). GLP-1 also decreases glucagon levels in patients with type 1 and 2 diabetes. While GLP-1 regulates glucagon secretion \(\textit{in vivo}\), the mechanisms through which GLP-1 regulates \(\alpha\)-cell function may be indirect, as the presence of the GLP-1 receptor (GLP-1R) on pancreatic \(\alpha\)-cells remains controversial \(^3,4\). Moreover, recent studies implicate a role for somatostatin as a mediator of the GLP-1-mediated inhibition of glucagon secretion via the somatostatin-2 receptor \(^5\).

Glucagon-like peptide-2 (GLP-2) is a 33 amino acid proglucagon-derived peptide structurally related to GLP-1. Exogenous administration of GLP-2 expands the surface area of the intestinal mucosal epithelium via stimulation of crypt cell proliferation and inhibition of apoptosis \(^6\). Additional actions of GLP-2 include the rapid stimulation of hexose transport \(^7\), inhibition of gastric emptying and acid secretion \(^8,9\), and augmentation of mesenteric blood flow \(^10,11\). The majority of GLP-2 actions appear to be indirect, as GLP-2 receptor (GLP-2R) expression has been localized to rare subsets of enteroendocrine cells, enteric neurons, and intestinal myofibroblasts \(^12-16\). The ability of GLP-2 to expand mucosal surface area and enhance nutrient absorption has prompted clinical evaluation of native GLP-2 and GLP-2 analogues in patients with enteral nutrient malabsorption due to short bowel syndrome. The available data suggest that GLP-2-treated subjects exhibit enhanced nutrient absorption without detectable changes in glucose homeostasis \(^17,18\).

Unlike GLP-1, GLP-2 has not been reported to modulate insulin secretion \(^19,20\). However, recent studies demonstrated that GLP-2 infusion results in stimulation of glucagon secretion \(\textit{in vivo}\). In healthy human volunteers, GLP-2(1-33) increased circulating glucagon levels in the fasted and fed state \(^21\) and perfusion of isolated rat pancreas with GLP-2 resulted in increased glucagon secretion with no effect on insulin or somatostatin secretion \(^22\). Consistent with a direct effect of GLP-2 in islets, GLP-2R mRNA transcripts were detected by real-time PCR and GLP-2R immunoreactivity was detected in rat and human pancreatic \(\alpha\)-cells \(^22\). Surprisingly, despite an increase in plasma glucagon levels, plasma glucose levels were unchanged following GLP-2 administration to normal healthy human subjects \(^21,23\). Thus, in humans and rats, acute GLP-2 infusion increases glucagon secretion without changes in glucose homeostasis.

GLP-2 has also been implicated as a mediator of gut permeability that in turn impacts the extent of endotoxemia and inflammation in mice with metabolic stress. Prebiotic treatment of high fat fed ob/ob mice reduced multiple parameters of inflammation, reduced gut permeability, and increased levels of GLP-2 \(^24\). Remarkably, a GLP-2R antagonist reversed many of the beneficial metabolic actions of the prebiotic, whereas therapy with GLP-2 reduced systemic and hepatic inflammation in ob/ob mice \(^24\). Taken together, these findings suggest that GLP-2 may be important for metabolic homeostasis and glucose metabolism either through regulation of glucagon secretion and/or control of inflammation and insulin action in models exemplified by the ob/ob mouse. Accordingly, we have now examined the role of the GLP-2R in normal, glucose-intolerant and diabetic mice. We show that endogenous GLP-2R signaling is not essential for control of glucagon secretion or glucose homeostasis in normal chow or high fat fed mice or in mice with streptozotocin-induced experimental diabetes. However, ob/ob:Glp2r-/- mice...
exhibited elevated levels of glucagon, ambient hyperglycemia, impaired intraperitoneal glucose tolerance and abnormal allocation of β- and α-cell lineages. Taken together, these findings suggest that the endogenous GLP-2R is required for the adaptation of the endocrine pancreas to metabolic stress.
Materials and Methods

Peptides & Reagents
Exendin-4 was purchased from California Peptide Research Inc. (Napa, CA). Humulin R insulin was from Eli Lilly (Toronto, ON). Synthetic human [Gly2] glucagon-like peptide-2 (h[Gly2]GLP-2) acetate was from Pepceuticals Ltd. (Nottingham, UK). Native GLP-2 was purchased from Bachem Inc. (Torrance, CA). Streptozotocin (STZ), Hanks Balanced Salt Solution (HBSS), Diprotin A, arginine, and TRI reagent were from Sigma (St. Louis, MO). The 45% kcal high fat diet was obtained from Research Diets (New Brunswick, NJ).

Animals
Wildtype (WT) C57BL/6 mice were obtained from Taconic (Germantown, NY). Glp2r-/- mice and littermate controls were generated at the Toronto General Hospital Animal Resource Centre and genotyped as previously described.25, 26 Ob/ob:Glp2r-/- mice and littermate controls were generated at the Toronto Centre for Phenogenomics by mating heterozygote ob/+ mice (Jackson Laboratories, Bar Harbor, Maine) to homozygote Glp2r-/- mice. Mice were genotyped using PCR from tail snip DNA for the Glp2r locus and for leptin using two PCR reactions, one mutant-specific and one wildtype-specific as previously described.27 Fat and lean mass were assessed using a whole body magnetic resonance analyzer (Echo Medical Systems, Houston, Texas). All animals were maintained under a 12 hour light/dark cycle and had free access to water and standard rodent chow unless otherwise specified. All animal protocols were approved by the Toronto General Hospital and Toronto Centre for Phenogenomics Animal Care Committee.

Glucagon secretion from pancreatic islets
Mouse islets were isolated from wildtype mice as described.28 Following isolation, pancreatic islets were stabilized for 2 hours in HBSS containing 8.3 mM glucose and stimulated with h[Gly2]GLP-2 (20 nM) or arginine (20 mM) for 30 minutes in the presence of 2.8, 8.3, or 16.8 mM glucose. Glucagon levels were measured using a Lincoplex endocrine assay (Millipore, Billerica, MA). Isolated pancreatic islets were obtained separately for RNA analysis.

Insulin and glucose tolerance tests
Insulin tolerance tests (ITT) were carried out in mice following a 5 hour fast using 1.2 U insulin/kg BW administered intraperitoneally. Glycemia was monitored for 4 hours following insulin administration from tail vein blood samples using a Contour glucometer (Bayer, Mississauga, ON). Blood samples for measurement of plasma glucagon were collected prior to, 20 min and 40 min after insulin injection. Oral and IP glucose tolerance (OGTT, IPGTT) tests were carried out following an overnight fast and administration of glucose (15% glucose, 1.5 mg/g body weight). Plasma samples were collected for measurement of plasma glucagon prior to glucose administration and 15 min (OGTT) or 20 min (IPGTT) after glucose challenge.

Streptozotocin-induced diabetes
Diabetes was induced in Glp2r-/- mice and littermate controls via a single injection of streptozotocin (STZ - 200mg/kg BW by intraperitoneal injection). STZ was prepared fresh directly before injections to mice in a 0.1M sodium citrate solution pH 5.5. Control mice were given 0.1M sodium citrate as the vehicle.

Feeding studies
For studies in high fat fed and STZ-diabetic mice, pre-weighed food was given to mice in individual cages and re-weighed 24 hours later. For the ob/ob:Glp2r experiments, mice were fasted overnight and food was then weighed 1, 2, 4, 8 and 24 hours following re-feeding.
**Immunostaining and histological analysis**
The pancreas was rapidly removed and a small fragment was immediately homogenized in TRI reagent and frozen for RNA analysis. The remainder was cut into approximately 10 pieces, fixed in 10% formalin for 48 hours and embedded in paraffin for histological analysis. Immunostaining was performed using a rabbit anti-insulin primary antibody (1:30 dilution; Dako, Glostrup, Denmark) followed by a biotinylated goat antirabbit secondary antibody (1:200 dilution; Vector Laboratories, Burlingame, CA) or rabbit anti-glucagon primary antibody (1:100 dilution, Cell Signalling, Beverley, MA) followed by an alkaline-phosphatase conjugated goat anti-rabbit secondary antibody (1:100 dilution, Zymed). Immunostained sections were scanned using the Scanscope Imagescope system at 20X magnification (Aperio Technologies, Vista, CA). The number of positive pixels indicative of insulin or glucagon staining was summed using an optimized positive pixel count algorithm and normalized per total islet area (square millimeters) for each mouse. Total alpha or beta cell mass was calculated by multiplying this value by the weight of the pancreas. Cell proliferation was assessed by counting the number of Ki67+ cells per pancreatic islet and normalizing to islet area (μm²) calculated using Aperio software.

**Real-time RT-PCR**
Total RNA was isolated using TRI reagent according to the manufacturer’s instructions and subjected to reverse transcription using Supercript II and random hexamers (Invitrogen, Carlsbad, CA). Real-time quantitative PCR was performed with the ABI Prism 7900 Sequence Detection System using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA) for proglucagon (Mm00801712_m1) and Glp2r (Mm01328477_m1). Relative mRNA expression was quantified using the 2^(-ΔΔCT) method, and 18S ribosomal RNA was analyzed as an endogenous control. RNA from islets was isolated using the RNaseasy mini kit according to the manufacturer’s instructions (Qiagen, Mississauga, ON) and subjected to reverse transcription as described above. The sequence for the 5’ and 3’ GLP-2R primers were as follows: [CTTCCTCGCCCTGCTTCT] and [CTCTCTTCCAGAATCTCCTCCA]. The generated PCR product was transferred to a nylon membrane after gel electrophoresis and hybridization was carried out using an internal primer [GCACACGCAATTACATCCAC] under standard conditions.

**Plasma and tissue metabolites and hormones**
Blood samples were collected by cardiac puncture or tail vein. For plasma preparation, blood samples were supplemented with trasylol, EDTA and diprotin A and centrifuged at 6,000 rpm at 4°C for 5 min. Quantification of plasma GLP-2 was carried out using the ALPCO enzyme immunoassay kit for mouse GLP-2 (Alpco Diagnostics, Salem, NH) according to the manufacturer’s instructions. Quantification of active GLP-1, glucagon and insulin from endpoint cardiac bleedings was carried out using a Meso Scale endocrine assay (Gaithersburg, Maryland) according to manufacturer’s instructions. Glucagon levels in plasma collected during ITT, OGTT and IPGTT, or supernatant from islets were measured using a Lincoplex endocrine assay (Millipore, Billerica, MA). Pancreatic insulin content was measured as previously described 29.

**Statistical Analyses**
All results are expressed as mean ± standard error of the mean. The Prism software package (version 4; GraphPad Software, La Jolla, CA) was used for statistical analyses. Statistical significance was established by student’s t-test or two-way ANOVA with a Bonferroni post-hoc analysis as appropriate. Statistical significance was defined as p<0.05.
Results

GLP-2 does not stimulate glucagon secretion in mice

We first assessed whether activation of GLP-2R signaling under conditions of hypoglycemia would further enhance glucagon secretion and lead to a more rapid or exaggerated glycemic recovery from insulin-induced hypoglycemia. Acute administration of the DPP-4-resistant GLP-2 receptor agonist h[Gly²]GLP-2[30] did not alter glucose excursion (Fig 1A) or plasma glucagon levels (Fig 1B) during an insulin tolerance test (ITT) in wildtype mice. In contrast, the GLP-1R agonist exendin-4 blunted the recovery of glucose and attenuated the plasma glucagon response to hypoglycemia (Figure 1A,B). Concomitant administration of h[Gly²]-GLP-2 had no effect on levels of glucose or glucagon in the presence or absence of exendin-4 (Figure 1A,B). We next determined whether chronic GLP-2R activation leads to changes in levels of glucose or glucagon by administering native GLP-2(1-33) to WT mice twice daily for 7 weeks. Plasma glucose levels increased significantly in GLP-2-treated mice (Figure 1C), however plasma glucagon levels were decreased in GLP-2-treated mice (Figure 1D). No significant changes in proglucagon or GLP-2R mRNA transcripts were observed in pancreas (Figure 1E,F) or jejunum of GLP-2-treated WT mice (Supplementary Figure 1A,B).

To examine whether the absence of the endogenous GLP-2 receptor was associated with changes in the acute regulation of glucagon secretion, we studied glucose homeostasis under conditions of hypo- or hyperglycemia in Glp2r-/- mice. Glucose excursion was comparable following an ITT, OGTT, or IPGTT in Glp2r-/- vs. Glp2r+/+ littermate control mice (Figure 2A,C,E). Plasma glucagon levels were higher 20 and 40 minutes following insulin challenge in Glp2r-/- compared to Glp2r+/+ mice (Figure 2B) but these trends failed to reach statistical significance. In contrast, plasma glucagon levels were similar in Glp2r-/- vs. Glp2r+/+ mice after oral or intraperitoneal glucose challenge (Figure 2D,F). Hence, loss of basal GLP-2R signaling does not perturb the control of glucagon secretion under a range of glucose levels.

Glp2r-/- mice are not protected from diet-induced obesity or glucose intolerance

As GLP-2 regulates barrier function and gut microbiota-associated systemic inflammation following high fat feeding in obese mice [24], we hypothesized that loss of the GLP-2R may predispose mice to enhanced inflammation and insulin resistance. To determine whether elimination of the murine Glp2r gene leads to abnormalities in glucose homeostasis in mice with metabolic stress [31], we fed Glp2r-/- and Glp2r+/+ littermate control mice a 45% kCal high fat diet (HFD) or a standard chow diet for 5 months. Body weight (Figure 3A) and fat mass (Supplementary Figure 2B) were significantly increased and food intake and lean body mass were decreased (Supplementary Figure 2A,C) in HFD mice, but no genotypic differences were observed in Glp2r+/+ vs. Glp2r-/- mice. Despite prolonged high fat feeding and expansion of adipose tissue mass, there was no difference in oral glucose tolerance in Glp2r+/+ vs. Glp2r-/- mice after 3 months on the standard vs. high fat diet (Figure 3B,C). Furthermore, although ambient glycemia was increased in high fat fed mice (compare Figure 3D with 3E), ambient, fed and fasted glucose levels measured after 4 months of HFD were comparable in Glp2r-/- vs. Glp2r+/+ mice (Figure 3E). Similarly, although β-cell mass increased as a result of high fat feeding, no differences were observed in β-cell mass (Figure 3F), or pancreatic or intestinal weights (Supplementary Figure 2D,E) in Glp2r+/+ vs. Glp2r-/- mice on a HFD.

GLP-2R signaling does not modify glucose homeostasis in lean diabetic mice

As the presence or absence of GLP-1R signaling modifies the susceptibility to apoptosis and the severity of hyperglycemia following STZ administration [32], we assessed whether β-cell injury and the severity of experimental diabetes would be modified by the loss of the GLP-2R. A single administration of STZ caused a rapid increase in blood glucose (fed and fasted) (Figure 4A,B), a decrease in body weight (Figure 4C), and an increase in food intake (Figure 4D). However no differences in these parameters were detected in Glp2r+/+ vs. Glp2r-/- mice. As partial attenuation of GLP-2 activity reduced intestinal
adaptation to experimental diabetes in rats, we assessed intestinal and pancreatic mass in diabetic mice. Intestinal and pancreas weight increased significantly in STZ-treated mice compared to vehicle-treated non-diabetic controls but these parameters were comparable in Glp2r+/+ vs. Glp2r/-/- mice (Figure 4 E,F).

**Loss of GLP-2R signaling modifies glucose homeostasis and islet adaptation in obese mice**

As STZ-induced diabetes is characterized by β-cell destruction associated with insulin deficiency and weight loss, we examined whether basal levels of GLP-2R signaling modified glucose homeostasis and glucagon secretion in a genetic model of obesity, inflammation, and insulin resistance via generation and analysis of obese ob/ob:Glp2r/-/- mice. Body weight (Figure 5A), lean and fat mass (Supplementary Figure 3A,B), food intake (Supplementary Figure 3C), energy expenditure and locomotion (Supplementary Figures 3E-H) were not different between ob/ob:Glp2r/-/- mice and littermate controls. Despite similar body weight (Figure 5A), fasting and fed glucose levels were significantly increased in ob/ob:Glp2r/-/- vs. ob/ob:Glp2r+/+ mice (Figure 5B) in association with modest increases in plasma glucagon in ob/ob:Glp2r/-/- mice (Figure 5C). Furthermore, pancreas weight was significantly increased in ob/ob:Glp2r/-/- mice (Supplementary Figure 3D). To understand the mechanism(s) contributing to increased glycemia and glucagon levels in ob/ob:Glp2r/-/- mice, we quantified α- and β-cell mass in mice of different genotypes. Histological analysis revealed significant increases in α-cell mass and decreased β-cell mass in ob/ob:Glp2r/-/- mice (Figure 5D,E). Immunohistochemistry for Ki-67, a marker of cell proliferation, demonstrated impaired islet cell proliferation despite the stimulus of more severe hyperglycemia in ob/ob:Glp2r/-/- mice (Figure 5F).

To further assess the functional metabolic phenotype of the ob/ob:Glp2r/-/- mouse, we performed glucose and insulin tolerance tests. Surprisingly, oral glucose tolerance was improved (Figure 6A) in association with increased levels of plasma insulin (Supplementary Figure 4A) and GLP-1 (Supplementary Figure 4C) in ob/ob:GLP-2R/-/- vs. ob/ob:GLP2R+/+ mice. Plasma glucagon levels did not change or trended lower after oral glucose with no genotype differences (Figure 6B). In contrast, and consistent with observations of ambient and fasting hyperglycemia and hyperglucagonemia (Figure 6D), intraperitoneal glucose tolerance was impaired (Figure 6C) without significant differences in levels of plasma insulin (Supplementary Fig 4B) or glucagon (Figure 6D) across genotypes. No difference in glucose excursion or recovery from hypoglycemia was detected following insulin tolerance testing, an indirect index of insulin sensitivity, in ob/ob:Glp2r/-/- vs ob/ob:Glp2r+/+ mice (Figure 6E). Intriguingly, plasma levels of GLP-1 (Supplementary Figure 4C), and GLP-2 (Supplementary Figure 4D) were significantly elevated in random fed ob/ob:Glp2r/-/- mice. Taken together, these findings demonstrate that GLP-2R signaling is important for control of islet cell proliferation, α- and β-cell mass and glucose homeostasis in the ob/ob genetic background.

We next examined whether activation of the GLP-2 receptor directly modulates glucagon secretion from isolated pancreatic islets. h[Gly2]GLP-2 had no effect on glucagon release from murine islets cultured at 2.8, 8.3 or 16.8 mM glucose, whereas glucagon secretion was significantly increased following exposure to arginine (Figure 7A). Moreover, Glp2r mRNA transcripts were undetectable in RNA from wild-type mouse islets (Figure 7B), whereas GLP-1R mRNA transcripts were abundant in the same islet RNA samples (Figure 7B-right panel). Reverse-transcriptase PCR using RNA from isolated pancreatic islets of wildtype and ob/ob mice followed by hybridization of the PCR products with a Glp2r-specific oligonucleotide probe did not detect Glp2r RNA transcripts in any of the samples (Figure 7C) while the GLP-1R was easily detected in the same islet samples (Figure 7D).
Discussion

The majority of studies of GLP-2 action have focused on its intestinotrophic and cytoprotective actions in the gastrointestinal tract. More recent experiments have suggested that GLP-2 receptor signaling may also influence glucose metabolism and insulin action. Studies in humans demonstrated that acute exogenous administration of native GLP-2(1-33) was associated with increased circulating levels of plasma glucagon. Exogenous administration of GLP-2(1-33) in healthy human volunteers increased circulating glucagon levels in both the fasting and postprandial state, with associated increases in levels of triglycerides and free fatty acids but without changes in gastric emptying. Moreover, GLP-2(1-33) increased glucagon levels in healthy humans without changes in circulating GLP-1, GIP, insulin or glucose. In contrast, there is no information about the effects of degradation-resistant GLP-2 analogs on glucagon secretion, and whether chronic GLP-2 administration perturbs glucagon or glucose homeostasis in humans has not been carefully examined.

The mechanisms underlying the GLP-2-dependent stimulation of glucagon secretion in human subjects remains unclear. The GLP-2 receptor was localized using immunohistochemistry to human and rat α-cells, and perfusion of rat islets with GLP-2(1-33) increased glucagon secretion, without changes in levels of somatostatin or insulin. Furthermore, GLP-2 attenuated the glucagonostatic actions of co-administered GLP-1 in perfused rat islets. In contrast, we were unable to detect GLP-2R mRNA transcripts in mouse islets, and h[Gly2]GLP-2 did not modify the inhibitory effects of exendin-4 on glucagon secretion in vivo. Hence, these observations illustrate differences in the actions of structurally distinct GLP-2 peptides in the mouse vs. the rat endocrine pancreas.

In an attempt to unmask a potential effect of enhanced or diminished GLP-2R signaling on the control of glucagon secretion, we studied glucagon levels and glucose homeostasis in lean and obese diabetic and non-diabetic mice under a diverse range of conditions, including chronic GLP-2(1-33) administration to normal mice, and during acute administration of h[Gly2]GLP-2 during oral and intraperitoneal glucose challenge, and insulin-induced hypoglycemia. Our experimental results demonstrated a lack of effect of acute exogenous h[Gly2]GLP-2 or chronic GLP-2(1-33) administration on murine glucagon secretion under conditions of hypoglycemia, normoglycemia, or hyperglycemia. Taken together, these findings are consistent with our lack of detection of the GLP-2R in murine islets and provide evidence that acute or chronic GLP-2R activation does not modify murine glucagon secretion.

As previous studies examined the consequences of acute GLP-2(1-33) administration on glucagon secretion, we have now ascertained the putative importance of endogenous basal GLP-2 signaling for islet function through analysis of glucose homeostasis and glucagon secretion in normal, high fat fed, and obese diabetic and non-diabetic mice under a diverse range of conditions, including chronic GLP-2(1-33) administration to normal mice, and during acute administration of h[Gly2]GLP-2 during oral and intraperitoneal glucose challenge, and insulin-induced hypoglycemia. Our experimental results revealed normal glucose tolerance, preservation of appropriate responses to hypoglycemia, and no evidence of abnormal glucagon secretion under conditions of hypo- or hyperglycemia in Glp2r-/- mice. Furthermore, induction of metabolic stress either through STZ-mediated β-cell destruction resulting in diabetes, weight loss and insulin deficiency, or via a high fat diet that classically induces insulin resistance, failed to unmask abnormalities in glucose homeostasis or glucagon secretion in Glp2r-/- mice. Hence, the available experimental evidence does not support a role for endogenous basal GLP-2R signaling in the control of glucose homeostasis or islet function under normal or diabetic conditions.

To further evaluate the metabolic importance of endogenous GLP-2R action, we generated ob/ob:Glp2r-/- mice. Recent studies of high fat fed ob/ob mice have implicated an essential role for GLP-2 in the transduction of bacteria-derived inflammatory signals to the systemic circulation via the control of gut permeability and barrier function. Prebiotic fed mice exhibited reduced permeability, increased levels of GLP-2, reduced systemic and hepatic inflammation, decreased circulating levels of LPS, and decreased markers of macrophage tissue infiltration. Furthermore, treatment of prebiotic-fed ob/ob mice with the GLP-2(3-33) antagonist diminished the prebiotic-induced reduction of endotoxemia. Conversely, treatment of ob/ob mice for 12 days with GLP-2(1-33) reduced plasma LPS and decreased levels of circulating proinflammatory cytokines as well as tissue markers of oxidative stress and...
macrophage inflammation, although insulin sensitivity and plasma levels of glucose, insulin or glucagon were not reported in these studies 24.

Hence, we wanted to determine whether loss of endogenous GLP-2R signaling predisposed mice to increased inflammation, and perhaps a reduction in insulin sensitivity leading to deterioration in glucose control. Genetic disruption of GLP-2R signaling in lean or ob/ob mice was not associated with significant changes in body weight and insulin sensitivity was comparable in ob/obGlp2r+/+ vs. ob/ob:Glp2r-/- mice. Nevertheless, ob/ob:Glp2r-/- mice exhibited significant increases in both fed and fasting blood glucose, and glucagon levels were significantly increased in ob/ob mice in the absence of the GLP-2R. Moreover, β-cell mass and islet cell proliferation were significantly reduced and α-cell mass was significantly increased in ob/ob:Glp2r-/- mice.

Although we did not detect definitive evidence for significant changes in gut permeability after 4 weeks of high fat feeding (Supplementary Figure 5) or in circulating markers of inflammation in ob/ob:Glp2r-/- mice (data not shown), we cannot exclude the possibility that developmental adaptation to loss of the GLP-2R may lead to upregulation of compensatory factors that maintain gut integrity and barrier function. Alternatively subtle differences in diet composition or the intestinal microbiome may also account for differences between our data and the findings reported by Cani et al 24. Intriguingly, recent evidence implicates systemic and islet inflammation in the pathophysiology of β-cell loss and dysfunction 34, and it remains possible that low grade systemic or localized islet inflammation contributed to the pathophysiology of reduced β-cell mass in ob/ob:Glp2r-/- mice. Similarly, the increase in pancreatic α-cell mass detected in ob/ob:Glp2r-/- mice may also reflect increased pro-inflammatory signals, as the proinflammatory cytokine interleukin-6 has been implicated in the pathophysiology of α-cell proliferation and enhanced glucagon secretion in experimental models of metabolic stress and diabetes 35.

In conclusion, although exogenous GLP-2 has no effect on glucagon secretion under normal conditions in normoglycemic, high fat fed or lean diabetic mice, loss of the GLP-2R leads to islet dysfunction characterized by exaggerated glucagon secretion, increased α-cell mass, hyperglycemia, reduced β-cell mass, and decreased islet proliferation in ob/ob:Glp2r-/- mice. Our findings are consistent with emerging evidence implicating a role for GLP-2 in the regulation of systemic and tissue inflammation 24,36, and suggest that further assessment of the link between the consequences of localized or systemic inflammation and GLP-2R signaling is clearly warranted.
References


Figure Legends

**Figure 1.** *Exogenous administration of GLP-2 does not stimulate glucagon secretion in mice.*
Glycemia (A) and glucagon levels (B) during an insulin tolerance test (1.2 U insulin /kg) in wildtype mice fasted for 5 h. Exendin-4 (24 nmol/kg) and/or h[Gly2]GLP-2 (0.25 mg/kg) were administered IP 10 minutes prior to insulin. (n=12). (C-F) Wildtype mice were injected with native GLP-2 for 7 weeks (5 µg/mouse twice daily). Glycemia (C) and plasma glucagon levels (D) were measured 15 minutes after the last peptide injection. Proglucagon (E) and GLP2R mRNA levels (F) were measured in whole pancreas using real time PCR and 18S as a control gene. (n=5-6) * = p<0.05, *** = p<0.001 compared to PBS-treated control.

**Figure 2.** *Endogenous GLP-2R signaling does not modulate glycemia or glucagon secretion during insulin or glucose tolerance tests.*
Glycemia (A) and glucagon levels (B) during an insulin tolerance test (1.2 U insulin /kg) in Glp2r-/- mice and Glp2r+/+ littermate controls fasted for 5h. Glycemia (C, E) and glucagon levels (D, F) during an intraperitoneal (C, D) or oral (E, F) glucose tolerance test in Glp2r-/- mice and littermate controls fasted overnight for 16 hours (n=3-6). No significant differences were observed between genotypes.

**Figure 3.** *Endogenous GLP-2R signaling does not modify glucose homeostasis under a high fat diet challenge.*
Glp2r-/- mice and littermate controls were fed a high fat (45% kcal from fat) or a standard chow diet for 5 months, starting at the age of 16 weeks. (A) Body weight is shown for up to 25 weeks on standard chow or high fat diet. Oral glucose tolerance was assessed in mice fed a standard rodent chow diet for 3 months (B) and in age-matched mice fed a high fat diet (C). Ambient, overnight fasted, and 1 hour re-fed glycemia of mice on standard chow diet (D) or high fat diet (E). (F) Beta cell mass for Glp2r-/- and littermate controls on standard chow or high fat diet. * = p<0.05, ** = p<0.01, *** = p<0.001 compared to standard chow fed mice.

**Figure 4.** *Endogenous GLP-2R signaling and STZ-induced diabetes.*
Morning blood glucose (A), fasting blood glucose (B), body weight (C), 24 hour food intake (D) and small intestine (E) and pancreas (F) weight of diabetic Glp2r-/- and Glp2r+/+ littermate controls. (n=6-9). * = p<0.05, *** = p<0.001 vs. vehicle-treated control.

**Figure 5.** *Role of GLP-2R signaling in the ob/ob mouse.*
Body weight (A), fasted and endpoint fed blood glucose levels (B), and plasma glucagon levels (C) are shown for 8-12 week old ob/ob:Glp2r-/- mouse and littermate controls. Alpha and beta cell mass (D), histology (E) and incidence of Ki-67+ cells (F) in islets of ob/ob:Glp2r-/- mouse and littermate ob/ob:Glp2r+/+ controls. (n=11-30). * = p<0.05, ** = p<0.01 vs. ob/ob:Glp2r+/+ control.

**Figure 6.** *Glucose tolerance and circulating glucagon levels in ob/ob:Glp2r-/- mice.*
Glycemia (A) and plasma glucagon levels (B) during an oral glucose tolerance test in ob/ob:Glp2r-/- mice and littermate controls. Area under the curve (AUC, 0-120 min) for ob/ob:Glp2r+/+ mice = 1708.6 and for ob/ob:Glp2r-/- = 1279.1, p=0.017 as assessed by student’s t-test. Glycemia (C), and plasma glucagon levels (D) following an intraperitoneal glucose tolerance test in ob/ob:Glp2r-/- mice and littermate controls. Area under the curve (AUC, 0-120min) for ob/ob:Glp2r+/+ mice = 2102.3 and for ob/ob:Glp2r-/- = 2749.2, p=0.012 as assessed by student’s t-test. Insulin tolerance test (E) in ob/ob:Glp2r-/- mice and littermate controls. (n=11-30). *=p<0.05, **=p<0.01 vs. ob/ob:Glp2r+/+ control.

**Figure 7.** *GLP-2R signaling does not regulate glucagon secretion from isolated pancreatic islets.*
(A) Mouse islets were cultured for 2h in HBSS containing 8.3 mM glucose, then stimulated with Gly2-GLP-2 (20 nM) or arginine (20 mM) for 30 min in the presence of 2.8, 8.3 or 16.8 mM glucose. Glucagon
levels in the supernatant were measured using a Lincoplex assay. (B) GLP2R mRNA levels measured in jejunum, whole pancreas, and islets and GLP-1R RNA was measured in islets prepared from wildtype mice. 18S was used as an internal control gene. (n=3)  (C) RT-PCR followed by hybridization of PCR products with a Glp2r-specific oligonucleotide probe for RNA from ob/ob islets (lanes 1 and 2), WT islets (lane 3), mouse jejunum RNA (lanes 4 and 5) and a Glp2r-/- jejunum RNA sample (lane 6). (D) RT-PCR for the mouse GLP-1 receptor (mGLP-1R) using RNA from ob/ob islets (lanes 1 and 2) or WT islets (lane 3) or negative control (lane 4). *=p<0.05, **= p<0.01 compared to control untreated islets.

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Figure 1

A

PBS
GLP-2
Ex-4
Ex-4 + GLP-2

Min
0
40
80
120
160
200
240

Glucose (mM)

B

PBS
Ex-4
GLP-2
Ex-4 + GLP-2

Min
-10
0
20
40

Plasma glucagon (pmol/L)

C

PBS
GLP-2

Glucose (mM)

D

Plasma glucagon (pmol/L)

E

Pancreas

Proglucagon mRNA expression (relative to 18S)

F

Pancreas

GLP2R mRNA expression (relative to 18S)
Figure 2

A

B

C

D

E

F

Glp2r+/+
Glp2r-/

Plasma glucagon (pmol/L)

Min Glucose (mM)

Plasma glucagon (pmol/L)

Min Glucose (mM)

Plasma glucagon (pmol/L)

Min Glucose (mM)
Figure 3

A

Body weight (g)

Wks on diet

Glp2r+/+
Glp2r+/
Glp2r-/
Glp2r-/

High fat diet
Standard chow

B

Glucose (mM)

Min Ambient Fast Refed

Glp2r+/+
Glp2r-/

High fat diet
Standard chow

C

D

E

F

Beta cell mass (mg)

Glucose (mM)

Glucose (mM)

Glucose (mM)

Standard chow
High fat diet

| Standard chow | Glp2r+/+ | Glp2r-/
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Figure 4

A. Glucose (mM) over Day 1-13 for Glp2r+/+ & Veh, Glp2r+/+ & STZ, Glp2r-/- & Veh, and Glp2r-/- & STZ.

B. Fasting glucose (mM) for Glp2r+/+ and Glp2r-/- with Veh and STZ.

C. Body weight (g) over Day 1-13 for Glp2r+/+ and Glp2r-/-.

D. 24-hour food intake (g) for Glp2r+/+ and Glp2r-/-.

E. Small intestinal weight (% BW) for Glp2r+/+ and Glp2r-/-.

F. Pancreas weight (% BW) for Glp2r+/+ and Glp2r-/-.
Figure 5

(A) Body weight (g) over weeks for ob/ob:Glp2r+/+, ob/ob:Glp2r+-, and ob/ob:Glp2r--.

(B) Glucose (mM) levels in Fasted and Fed conditions for ob/ob:Glp2r+/+, ob/ob:Glp2r+-, and ob/ob:Glp2r--.

(C) Plasma glucagon levels (pg/mL) for ob/ob:Glp2r+/+, ob/ob:Glp2r+-, and ob/ob:Glp2r--.

(D) Alpha cell mass (mg) and Beta cell mass (mg) for ob/ob:Glp2r+/+, ob/ob:Glp2r+-, and ob/ob:Glp2r--.

(E) Immunohistochemistry for Insulin and Glucagon in Ob/ob:Glp2r+/+ and Ob/ob:Glp2r--.

(F) Ki-67+ cells/um^2 area for ob/ob:Glp2r+/+ and ob/ob:Glp2r--.
Figure 7

A

Control   GLP2   Arginine

Glucose (mM)  2.8  8.3  16.8

Glucagon (pmol/L/10 islets/30min)

B

GLP2R mRNA expression (relative to 18S)

Jejunum  Pancreas  Islets  Islets

GLP1R mRNA expression (relative to 18S)

C

1  2  3  4  5  6

1.5 kb mGLP-2R

D

1  2  3  4

mGLP1R RT-PCR

1.5 kb
Supplementary Figure 1. Proglucagon (A) and GLP-2R (B) mRNA expression levels in jejunum of wildtype mice treated with PBS (white bars) or native GLP-2 (black bars) for 7 weeks.
Supplementary Figure 2. Food intake (A), body fat composition (B, C), pancreas (D) and small intestinal (E) weight in Glp2r-/- and Glp2r+/+ mice fed a 45% kCal high fat diet or standard chow for 5 months. * = p<0.05, ** = p<0.01 *** = p<0.001, vs standard chow fed group of same genotype.
Supplementary Figure 3. Body composition (A,B), 24 hour food intake (C), and pancreas weight (D) in ob/ob:Glp2r-/- mice and littermate controls. Oxygen consumption (E), carbon dioxide output (F), and locomotor activity (G,H) in ob/ob:Glp2r-/- mice and littermate controls. (I) Small intestinal weight of ob/ob:Glp2r-/- mice and littermate controls. *=p<0.05, vs. ob/ob:Glp2r+/+ littermate control.
**Supplementary Figure 4.** Plasma insulin during an oral (A) and IP (B) glucose tolerance test at fasted levels at time 0, 10 and 30 min following glucose load. Plasma active GLP-1 (C) and GLP-2 (D) levels from mice (n=2-30) *=p<0.05, ***=p<0.001 vs. ob/ob:Glp2r+/+ controls.
Supplementary Figure 5

Chronic high fat feeding was carried out to induce a proinflammatory state in ob/ob:Glp2r-/- mice and littermate ob/ob:Glp2r+/+ mice (60% high fat diet for 4 weeks). After 2 weeks on the high fat diet, in vivo gut permeability was assessed by gavaging fasted mice with fluorescent dextran-FITC (500mg/kg BW) and measuring dextran-FITC in plasma collected at 1 and 4 hours following oral gavage. No detectable changes in plasma levels of FITC dextran were observed between ob/ob:Glp2r+/+ and ob/ob:Glp2r-/- mice at either time point.