

# BASIC—ALIMENTARY TRACT

## Glucagon-Like Peptide-2 Receptor Modulates Islet Adaptation to Metabolic Stress in the ob/ob Mouse

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**BACKGROUND & AIMS:** Glucagon-like peptide-2 (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-2 receptor (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 were studied in wild-type, high-fat fed, lean diabetic, *Glp2r*<sup>-/-</sup> and ob/ob:*Glp2r*<sup>-/-</sup> 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*<sup>-/-</sup> mice exhibit no change in glycemia, and plasma glucagon levels were similar in *Glp2r*<sup>-/-</sup> and *Glp2r*<sup>+/+</sup> mice after hypoglycemia or after oral or intraperitoneal glucose challenge. Moreover, glucose homeostasis was comparable in *Glp2r*<sup>-/-</sup> and *Glp2r*<sup>+/+</sup> mice fed a high-fat diet for 5 months or after induction of streptozotocin-induced diabetes. In contrast, loss of the GLP-2R leads to increased glucagon secretion and  $\alpha$ -cell mass, impaired intraperitoneal glucose tolerance and hyperglycemia, reduced  $\beta$ -cell mass, and decreased islet proliferation in ob/ob:*Glp2r*<sup>-/-</sup> mice. **CONCLUSIONS:** Our results show 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.

**Keywords:** Glucagon; GLP-2; Glucagon-Like Peptide-1; GLP-1; Inflammation; Islets.

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 produc-

tion. 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 upward 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).<sup>1</sup> 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.<sup>2</sup> GLP-1 also decreases glucagon levels in patients with type 1 and 2 diabetes. Although GLP-1 regulates glucagon secretion 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.<sup>3,4</sup> Moreover, recent studies implicate a role for somatostatin as a mediator of the GLP-1-mediated inhibition of glucagon secretion by the somatostatin-2 receptor.<sup>5</sup>

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 by stimulation of crypt cell proliferation and inhibition of apoptosis.<sup>6</sup> Additional actions of GLP-2 include the rapid stimulation of hexose transport,<sup>7</sup> inhibition of gastric emptying and acid secretion,<sup>8,9</sup> and augmentation of mesenteric blood flow.<sup>10,11</sup> Most GLP-2 actions appear to be indirect,

*Abbreviations used in this paper:* BW, body weight; GIP, glucose-dependent insulinotropic peptide; GLP-1, glucagon-like peptide-1; GLP-1R, glucagon-like peptide-1 receptor; GLP-2, glucagon-like peptide-2; GLP-2R, glucagon-like peptide-2 receptor; HBSS, Hanks' balanced salt solution; HFD, high-fat diet; IPGTT, intraperitoneal glucose tolerance test; ITT, insulin tolerance test; OGTT, oral glucose tolerance test; PCR, polymerase chain reaction; RT, reverse transcription; STZ, streptozotocin; WT, wild-type.

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because GLP-2 receptor (GLP-2R) expression has been localized to rare subsets of enteroendocrine cells, enteric neurons, and intestinal myofibroblasts.<sup>12-16</sup> The ability of GLP-2 to expand mucosal surface area and to enhance nutrient absorption has prompted clinical evaluation of native GLP-2 and GLP-2 analogues in patients with enteric 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.<sup>17,18</sup>

Unlike GLP-1, GLP-2 has not been reported to modulate insulin secretion.<sup>19,20</sup> However, recent studies have shown that GLP-2 infusion results in stimulation of glucagon secretion in vivo. In healthy human volunteers, GLP-2(1-33) increased circulating glucagon levels in the fasted and fed state,<sup>21</sup> and perfusion of isolated rat pancreas with GLP-2 resulted in increased glucagon secretion with no effect on insulin or somatostatin secretion.<sup>22</sup> Consistent with a direct effect of GLP-2 in islets, GLP-2R mRNA transcripts were detected by real-time polymerase chain reaction (PCR), and GLP-2R immunoreactivity was detected in rat and human pancreatic  $\alpha$  cells.<sup>22</sup> Surprisingly, despite an increase in plasma glucagon levels, plasma glucose levels were unchanged after GLP-2 administration to normal healthy human subjects.<sup>21,23</sup> Thus, in human beings 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 affects 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.<sup>24</sup> 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.<sup>24</sup> 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 (STZ)-induced experimental diabetes. However, ob/ob:Glp2r<sup>-/-</sup> mice exhibited elevated levels of glucagon, ambient hyperglycemia, impaired intraperitoneal glucose tolerance, and abnormal allocation of  $\beta$ - and  $\alpha$ -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 and Reagents

Exendin-4 was purchased from California Peptide Research Inc (Napa, CA). Humulin R insulin was from Eli Lilly (Toronto, ON). Synthetic human [Gly<sup>2</sup>] glucagon-like peptide-2 (h[Gly<sup>2</sup>]GLP-2) acetate was from PeptideCals Ltd (Nottingham, United Kingdom). Native GLP-2 was purchased from Bachem Inc (Torrance, CA). STZ, Hanks' balanced salt solution (HBSS), Diprotin A, arginine, and TRI reagent were from Sigma (St. Louis, MO). The 45% kcal high-fat diet (HFD) was obtained from Research Diets (New Brunswick, NJ).

### Animals

Wild-type (WT) C57BL/6 mice were obtained from Taconic (Germantown, NY). *Glp2r*<sup>-/-</sup> mice and littermate controls were generated at the Toronto General Hospital Animal Resource Centre and genotyped as previously described.<sup>25,26</sup> Ob/ob:*Glp2r*<sup>-/-</sup> mice and littermate controls were generated at the Toronto Centre for Phenogenomics by mating heterozygote ob/+ mice (The Jackson Laboratory, Bar Harbor, Maine) to homozygote *Glp2r*<sup>-/-</sup> mice. Mice were genotyped with the use of PCR from tail snip DNA for the *Glp2r* locus<sup>25,26</sup> and for leptin with the use of 2 PCR reactions, one mutant-specific and one WT-specific as previously described.<sup>27</sup> Fat and lean mass were assessed with the use of a whole-body magnetic resonance analyzer (Echo Medical Systems, Houston, TX). 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 WT mice as described.<sup>28</sup> After isolation, pancreatic islets were stabilized for 2 hours in HBSS containing 8.3 mmol/L glucose and stimulated with h[Gly<sup>2</sup>]GLP-2 (20 nmol/L) or arginine (20 mmol/L) for 30 minutes in the presence of 2.8, 8.3, or 16.8 mmol/L glucose. Glucagon levels were measured with the use of a Lincoplex endocrine assay (Millipore, Billerica, MA). Isolated pancreatic islets were obtained separately for RNA analysis.

### Insulin and Glucose Tolerance Tests

Insulin tolerance tests (ITTs) were carried out in mice after a 5-hour fast with 1.2 U insulin/kg of body weight (BW) administered intraperitoneally. Glycemia was monitored for 4 hours after insulin administration from tail vein blood samples with a Contour glucometer (Bayer, Mississauga, ON). Blood samples for measurement of plasma glucagon were collected before and 20 minutes and 40 minutes after insulin injection. Oral and intraperitoneal glucose tolerance tests (OGTT, IPGTT)

were carried out after an overnight fast and administration of glucose (15% glucose, 1.5 mg/g BW). Plasma samples were collected for measurement of plasma glucagon before glucose administration and 15 minutes (OGTT) or 20 minutes (IPGTT) after glucose challenge.

### ***STZ-Induced Diabetes***

Diabetes was induced in *Glp2r*<sup>-/-</sup> mice and littermate controls by a single injection of STZ (200 mg/kg BW by intraperitoneal injection). STZ was prepared fresh directly before injections to mice in a 0.1 mol/L sodium citrate solution pH 5.5. Control mice were given 0.1 mol/L sodium citrate as the vehicle.

### ***Feeding Studies***

For studies in high-fat-fed and STZ-diabetic mice, preweighed food was given to mice in individual cages and reweighed 24 hours later. For the *ob/ob:Glp2r* experiments, mice were deprived of food overnight, and food was then weighed 1, 2, 4, 8, and 24 hours after refeeding.

### ***Immunostaining and Histologic Analyses***

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 histologic analysis. Immunostaining was performed with the use of a rabbit anti-insulin primary antibody (1:30 dilution; Dako, Glostrup, Denmark) followed by a biotinylated goat anti-rabbit secondary antibody (1:200 dilution; Vector Laboratories, Burlingame, CA) or rabbit anti-glucagon primary antibody (1:100 dilution; Cell Signalling, Beverly, MA) followed by an alkaline-phosphatase-conjugated goat anti-rabbit secondary antibody (1:100 dilution; Zymed Laboratories, South San Francisco, CA). Immunostained sections were scanned with the use of the Scanscope Imagescope system at 20 $\times$  magnification (Aperio Technologies, Vista, CA). The number of positive pixels indicative of insulin or glucagon staining was summed with the use of an optimized positive pixel count algorithm and normalized per total pancreas area (in mm<sup>2</sup>) 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<sup>+</sup> cells per pancreatic islet and normalizing to islet area ( $\mu$ m<sup>2</sup>) calculated with the use of Aperio software (Vista, CA).

### ***Real-Time RT-PCR***

Total RNA was isolated with TRI reagent according to the manufacturer's instructions and subjected to reverse transcription (RT) with the use of Superscript II and random hexamers (Invitrogen, Carlsbad, CA). Real-time quantitative PCR was performed with the ABI Prism 7900 Sequence Detection System with the use

of TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA) for proglucagon (Mm00801712\_m1) and *Glp2r* (Mm01329477\_m1). Relative mRNA expression was quantified with the 2<sup>- $\Delta$ CT</sup> method, and 18S ribosomal RNA was analyzed as an endogenous control. RNA from islets was isolated with the RNeasy mini kit according to the manufacturer's instructions (QIAGEN, Mississauga, ON) and subjected to RT as described above. The sequence for the 5' and 3' GLP-2R primers were as follows: [CTTCTCGCCCTGCTTCT] and [CTCTCTTCAGAATCTCCTCCA]. The generated PCR product was transferred to a nylon membrane after gel electrophoresis, and hybridization was carried out with an internal primer [GCACACGCAATTACATCCAC] under standard conditions.

### ***Plasma and Tissue Metabolites and Hormones***

Blood samples were collected by cardiac or tail vein puncture. For plasma preparation, blood samples were supplemented with Trasylol, EDTA, and Diprotin A and were centrifuged at 6000 rpm at 4°C for 5 minutes. Quantification of plasma GLP-2 was carried out with 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 end point cardiac bleedings was carried out with a Meso Scale endocrine assay (Gaithersburg, MD) according to the manufacturer's instructions. Glucagon levels in plasma collected during ITT, OGTT, and IPGTT or supernatant from islets was measured with the use of a Lincoplex endocrine assay (Millipore, Billerica, MA).<sup>29</sup>

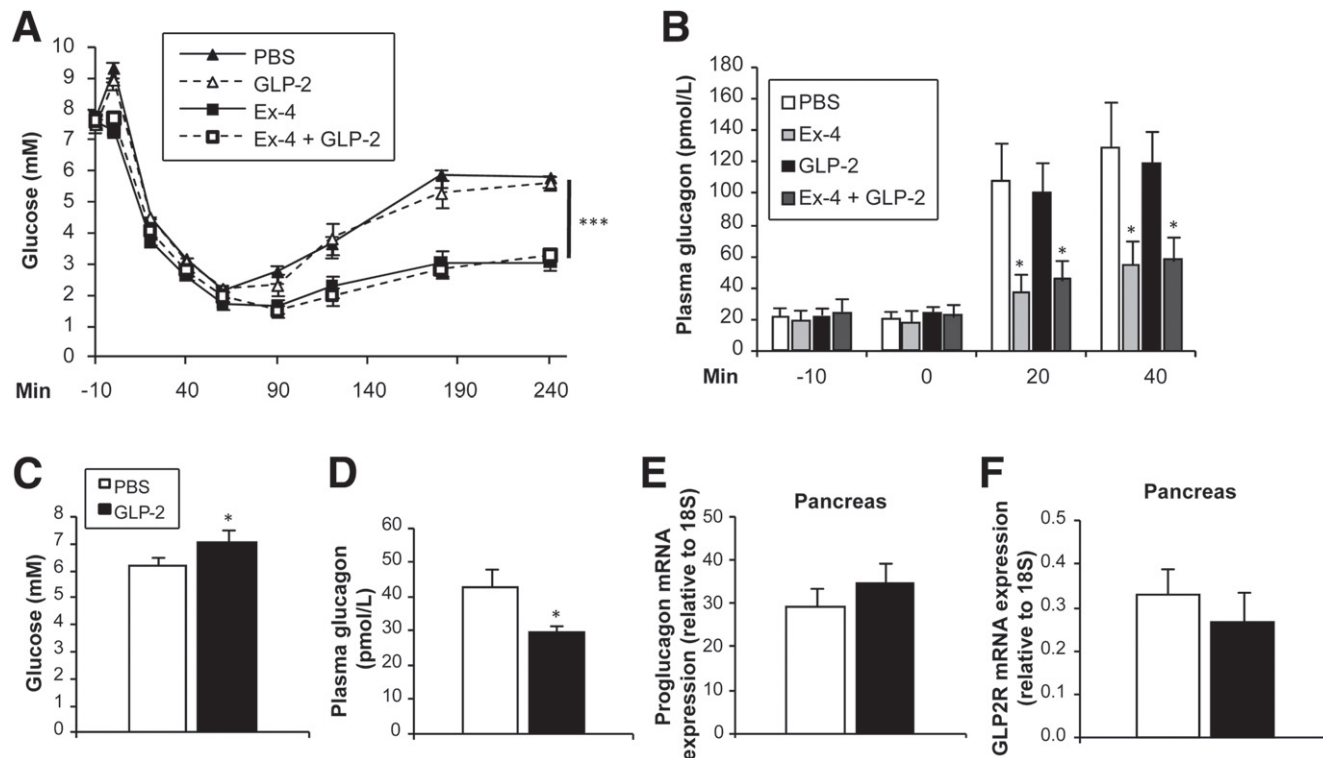
### ***Statistical Analyses***

All results are expressed as mean  $\pm$  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 *t* test or 2-way analysis of variance with a Bonferroni post hoc analysis as appropriate. Statistical significance was defined as *P* < .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 dipeptidyl peptidase 4-resistant GLP-2 receptor agonist h[Gly<sup>2</sup>]GLP-2<sup>30</sup> did not alter glucose excursion (Figure 1A) or plasma glucagon levels (Figure 1B) during an ITT in WT mice. In contrast, the GLP-1R agonist exendin-4 blunted the recovery of glucose and attenuated the plasma glucagon



**Figure 1.** Exogenous administration of GLP-2 does not stimulate glucagon secretion in mice. Glycemia (A) and glucagon (B) levels during an insulin tolerance test (1.2 U insulin/kg) in WT mice deprived of food for 5 hours. Exendin-4 (24 nmol/kg) and/or h[Gly<sup>2</sup>]GLP-2 (0.25 mg/kg) were administered intraperitoneally 10 minutes before insulin. (n = 12). (C–F) WT mice were injected with native GLP-2 for 7 weeks (5  $\mu$ g/mouse twice daily). Glycemia (C) and plasma glucagon (D) levels were measured 15 minutes after the last peptide injection. Proglucagon (E) and GLP2R mRNA (F) levels were measured in whole pancreas with the use of real-time PCR and 18S as a control gene (n = 5–6); \*P < .05, \*\*\*P < .001 compared with PBS-treated control.

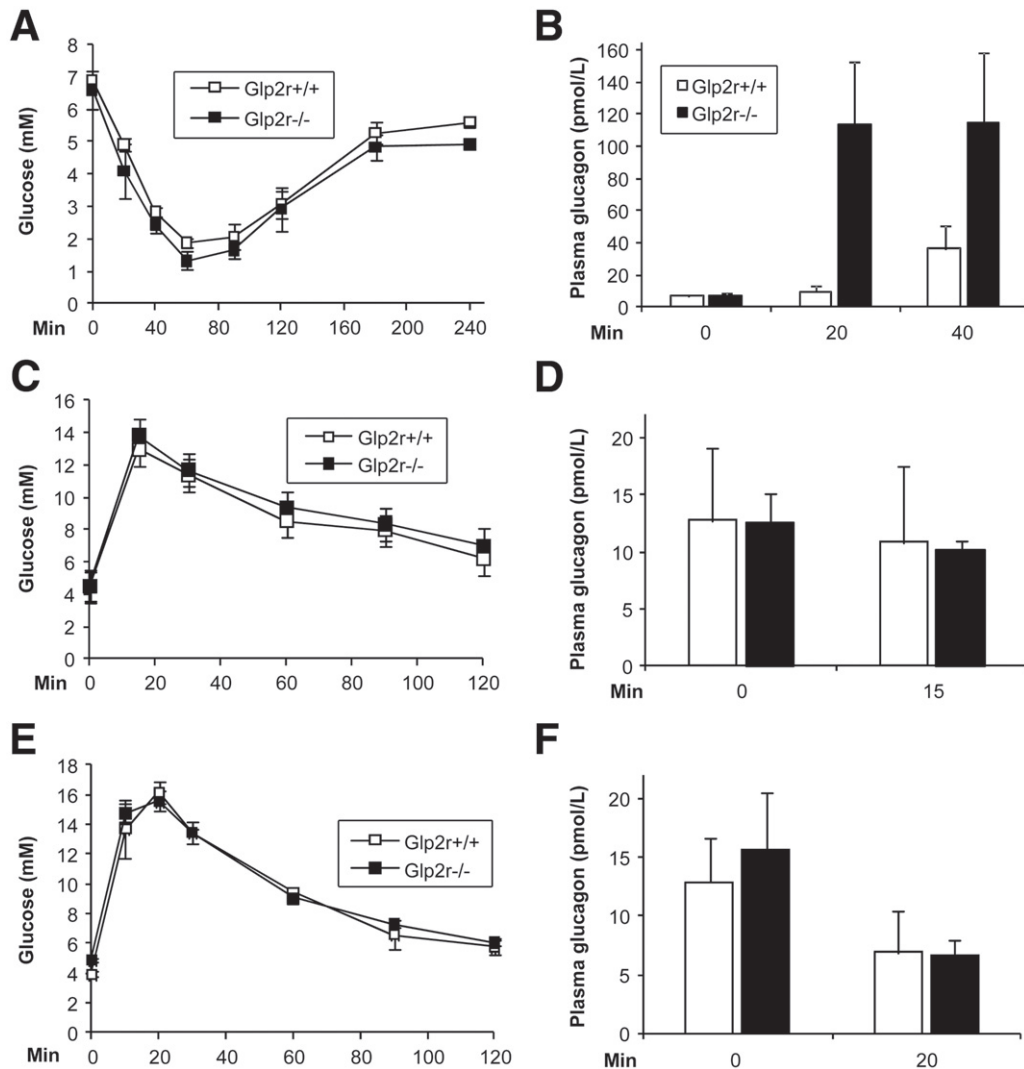
response to hypoglycemia (Figure 1A and B). Concomitant administration of h[Gly<sup>2</sup>]-GLP-2 had no effect on levels of glucose or glucagon in the presence or absence of exendin-4 (Figure 1A and B). We next determined whether chronic GLP-2R activation leads to changes in levels of glucose or glucagon 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 the pancreas (Figure 1E and F) or jejunum of GLP-2-treated WT mice (Supplementary Figure 1A and B).

To examine whether the absence of the endogenous GLP-2R was associated with changes in the acute regulation of glucagon secretion, we studied glucose homeostasis under conditions of hypoglycemia or hyperglycemia in *Glp2r*<sup>-/-</sup> mice. Glucose excursion was comparable after ITT, OGTT, or IPGTT in *Glp2r*<sup>-/-</sup> and *Glp2r*<sup>+/+</sup> littermate control mice (Figure 2A, C, and E). Plasma glucagon levels were higher 20 and 40 minutes after insulin challenge in *Glp2r*<sup>-/-</sup> mice compared with *Glp2r*<sup>+/+</sup> mice (Figure 2B), but these trends failed to reach statistical significance. In contrast, plasma glucagon levels were similar in *Glp2r*<sup>-/-</sup> and *Glp2r*<sup>+/+</sup> mice after oral or intra-

peritoneal glucose challenge (Figure 2D and F). Hence, loss of basal GLP-2R signaling does not perturb the control of glucagon secretion under a range of glucose levels.

### *Glp2r*<sup>-/-</sup> Mice Are Not Protected From Diet-Induced Obesity or Glucose Intolerance

Because GLP-2 regulates barrier function and gut microbiota-associated systemic inflammation after high-fat feeding in obese mice,<sup>24</sup> 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,<sup>31</sup> we fed *Glp2r*<sup>-/-</sup> and *Glp2r*<sup>+/+</sup> littermate control mice a 45% kcal 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 and C) in HFD mice, but no genotypic differences were observed in *Glp2r*<sup>+/+</sup> vs *Glp2r*<sup>-/-</sup> mice. Despite prolonged high-fat feeding and expansion of adipose tissue mass, no difference was observed in oral glucose tolerance in *Glp2r*<sup>+/+</sup> vs *Glp2r*<sup>-/-</sup> mice after 3 months on the standard vs HFD (Figure 3B and C). Furthermore, although ambient glycemia was increased in mice fed the



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

HFD (compare Figure 3D with E), ambient, fed, and fasted glucose levels measured after 4 months of HFD were comparable in *Glp2r<sup>-/-</sup>* and *Glp2r<sup>+/+</sup>* mice (Figure 3E). Similarly, although  $\beta$ -cell mass increased as a result of high-fat feeding, no differences were observed in  $\beta$ -cell mass (Figure 3F) or in pancreatic or intestinal weights (Supplementary Figure 2D and E) in *Glp2r<sup>+/+</sup>* and *Glp2r<sup>-/-</sup>* mice on an HFD.

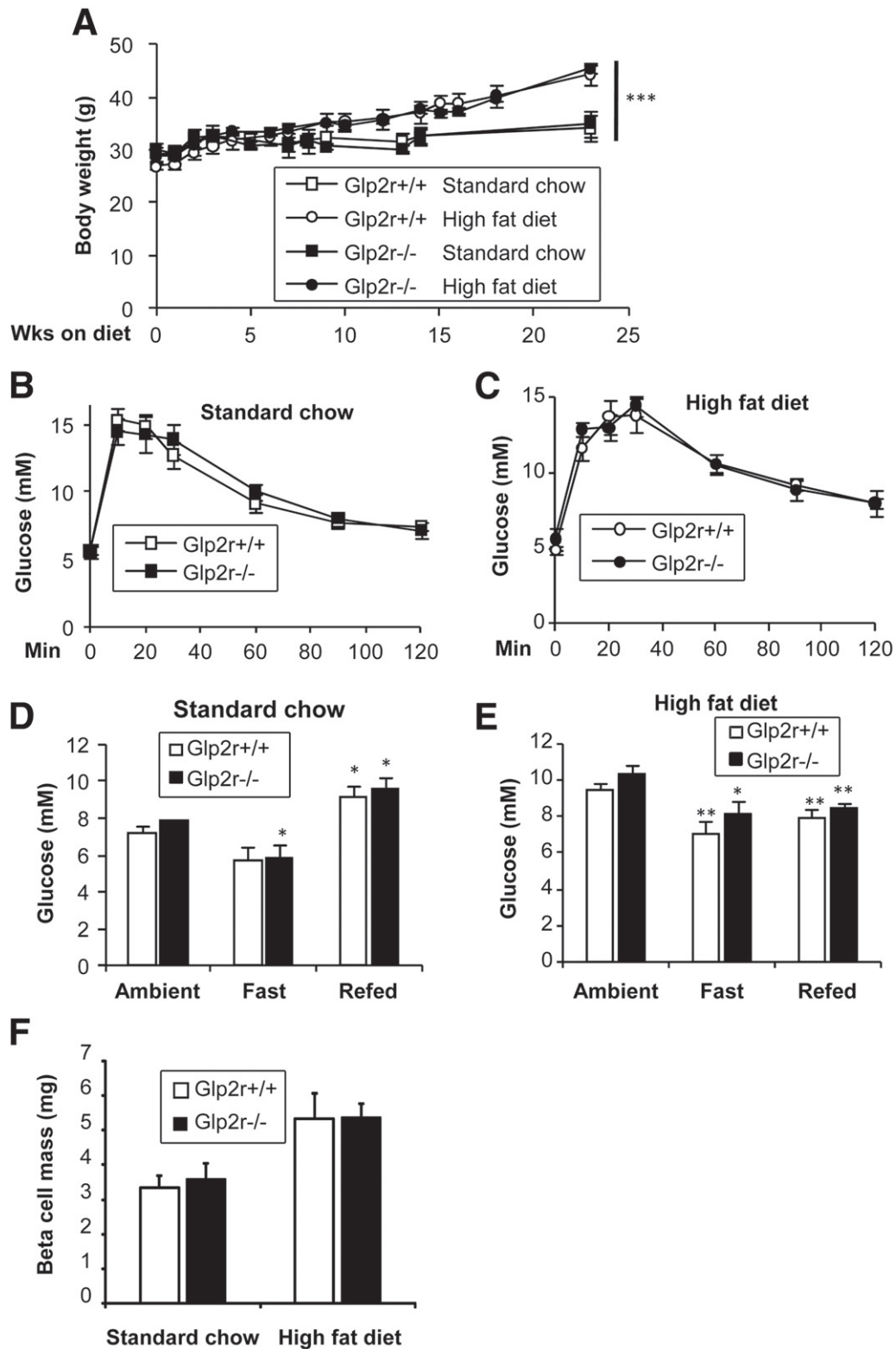
#### **GLP-2R Signaling Does Not Modify Glucose Homeostasis in Lean Diabetic Mice**

Because the presence or absence of GLP-1R signaling modifies the susceptibility to apoptosis and the severity of hyperglycemia after STZ administration,<sup>32</sup> we assessed whether  $\beta$ -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

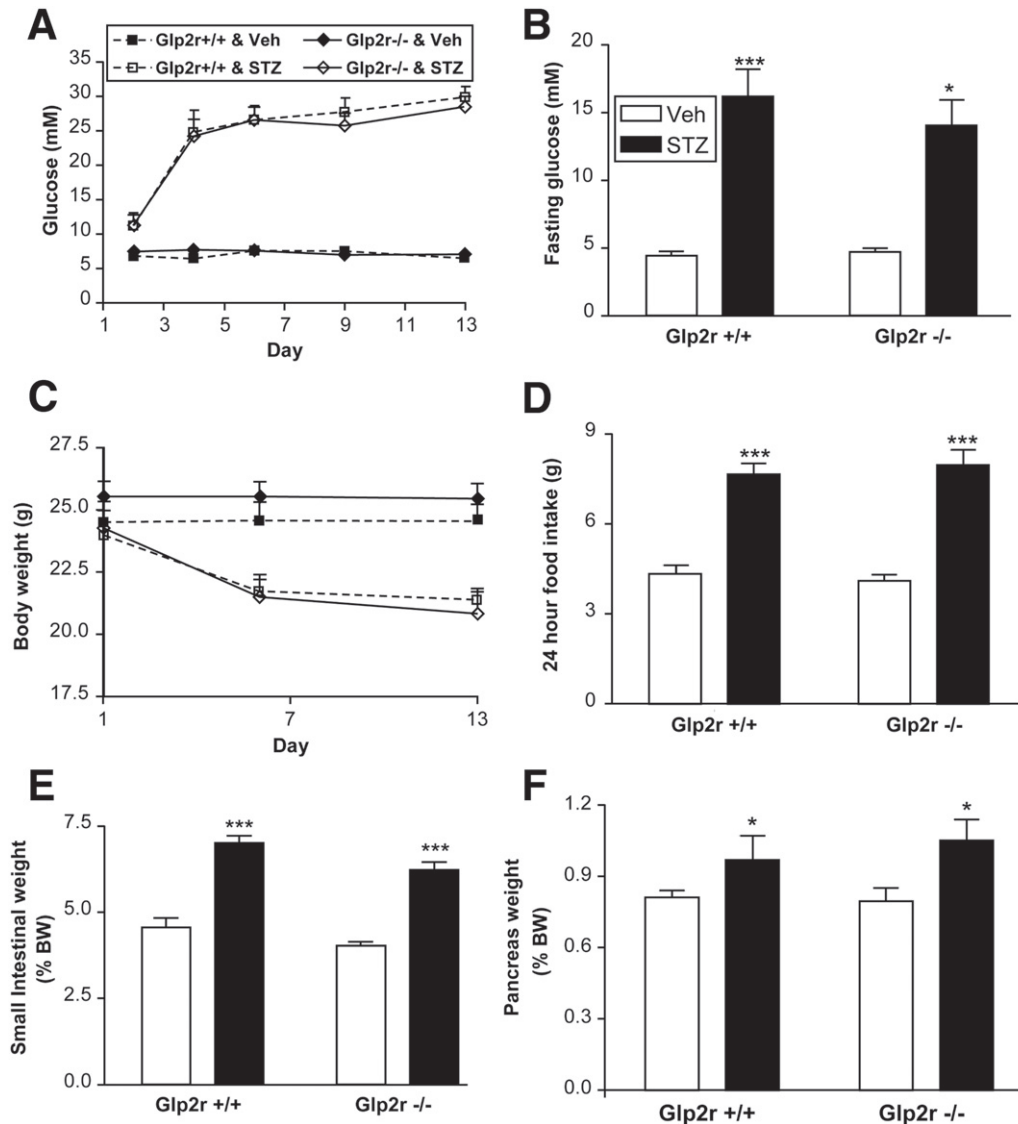
increase in blood glucose (fed and fasted) (Figure 4A and B), a decrease in body weight (Figure 4C), and an increase in food intake (Figure 4D). However, no differences in these parameters were detected in *Glp2r<sup>+/+</sup>* and *Glp2r<sup>-/-</sup>* mice. Because partial attenuation of GLP-2 activity reduced intestinal adaptation to experimental diabetes in rats,<sup>33</sup> we assessed intestinal and pancreatic mass in diabetic mice. Intestinal and pancreas weight increased significantly in STZ-treated mice compared with vehicle-treated nondiabetic controls, but these parameters were comparable in *Glp2r<sup>+/+</sup>* and *Glp2r<sup>-/-</sup>* mice (Figure 4E and F).

#### **Loss of GLP-2R Signaling Modifies Glucose Homeostasis and Islet Adaptation in Obese Mice**

Because STZ-induced diabetes is characterized by  $\beta$ -cell destruction associated with insulin deficiency and



**Figure 3.** Endogenous GLP-2R signaling does not modify glucose homeostasis under an HFD challenge. *Glp2r*<sup>-/-</sup> mice and littermate controls were fed an HFD (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 the standard chow diet or the HFD. Oral glucose tolerance was assessed in mice fed a standard rodent chow diet for 3 months (B) and in age-matched mice fed an HFD (C). Ambient, overnight fasted, and 10-hour refed glycemia of mice on standard chow diet (D) or HFD (E). (F)  $\beta$ -Cell mass for *Glp2r*<sup>-/-</sup> and littermate controls on standard chow diet or HFD. \* $P < .05$ , \*\* $P < .01$ , \*\*\* $P < .001$  compared with standard chow-fed mice.

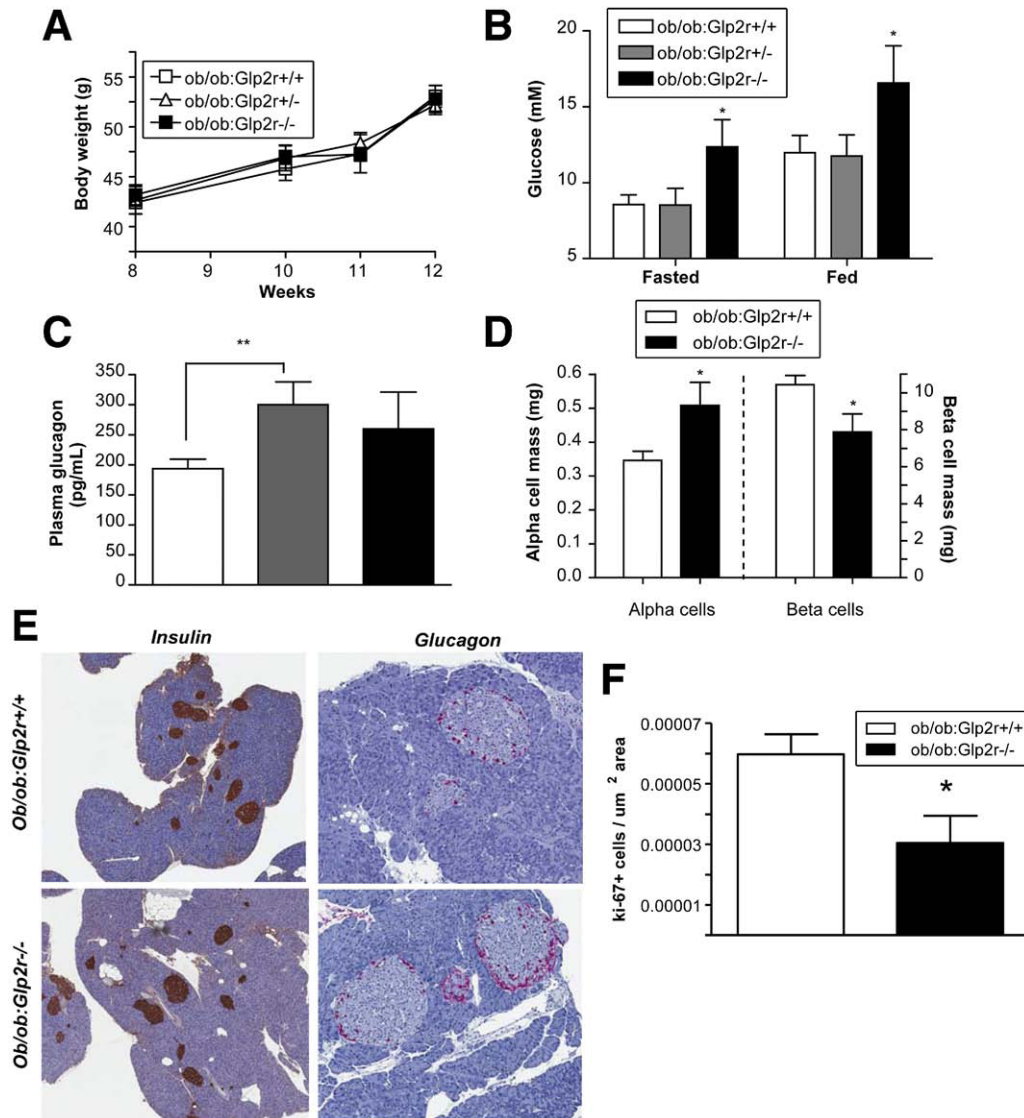


**Figure 4.** Endogenous GLP-2R signaling and STZ-induced diabetes. Morning blood glucose (A), fasting blood glucose (B), body weight (C), 24-hour food intake (D), and small intestine (E) and pancreas (F) weight of diabetic *Glp2r*<sup>-/-</sup> and *Glp2r*<sup>+/+</sup> littermate controls. (n = 6–9). \**P* < .05, \*\*\**P* < .001 vs vehicle-treated control.

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 through the generation and analysis of obese *ob/ob:Glp2r*<sup>-/-</sup> mice. Body weight (Figure 5A), lean and fat mass (Supplementary Figure 3A and B), food intake (Supplementary Figure 3C), energy expenditure, and locomotion (Supplementary Figures 3E–H) were not different between *ob/ob:Glp2r*<sup>-/-</sup> mice and littermate controls. Despite similar body weight (Figure 5A), fasting and fed glucose levels were significantly increased in *ob/ob:Glp2r*<sup>-/-</sup> vs *ob/ob:Glp2r*<sup>+/+</sup> mice (Figure 5B) in association with modest increases in plasma glucagon in *ob/ob:Glp2r*<sup>-/-</sup> mice (Figure 5C). Furthermore, pancreas weight was significantly increased in *ob/ob:Glp2r*<sup>-/-</sup> mice (Supplementary Figure 3D). To under-

stand the mechanism(s) contributing to increased glycemia and glucagon levels in *ob/ob:Glp2r*<sup>-/-</sup> mice, we quantified  $\alpha$ - and  $\beta$ -cell mass in mice of different genotypes. Histologic analysis showed significant increases in  $\alpha$ -cell mass and decreased  $\beta$ -cell mass in *ob/ob:Glp2r*<sup>-/-</sup> mice (Figure 5D and E). Immunohistochemistry for Ki-67, a marker of cell proliferation, showed impaired islet cell proliferation despite the stimulus of more severe hyperglycemia in *ob/ob:Glp2r*<sup>-/-</sup> mice (Figure 5F).

To further assess the functional metabolic phenotype of the *ob/ob:Glp2r*<sup>-/-</sup> 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:Glp2r*<sup>-/-</sup> vs *ob/ob:Glp2r*<sup>+/+</sup> mice. Plasma gluca-



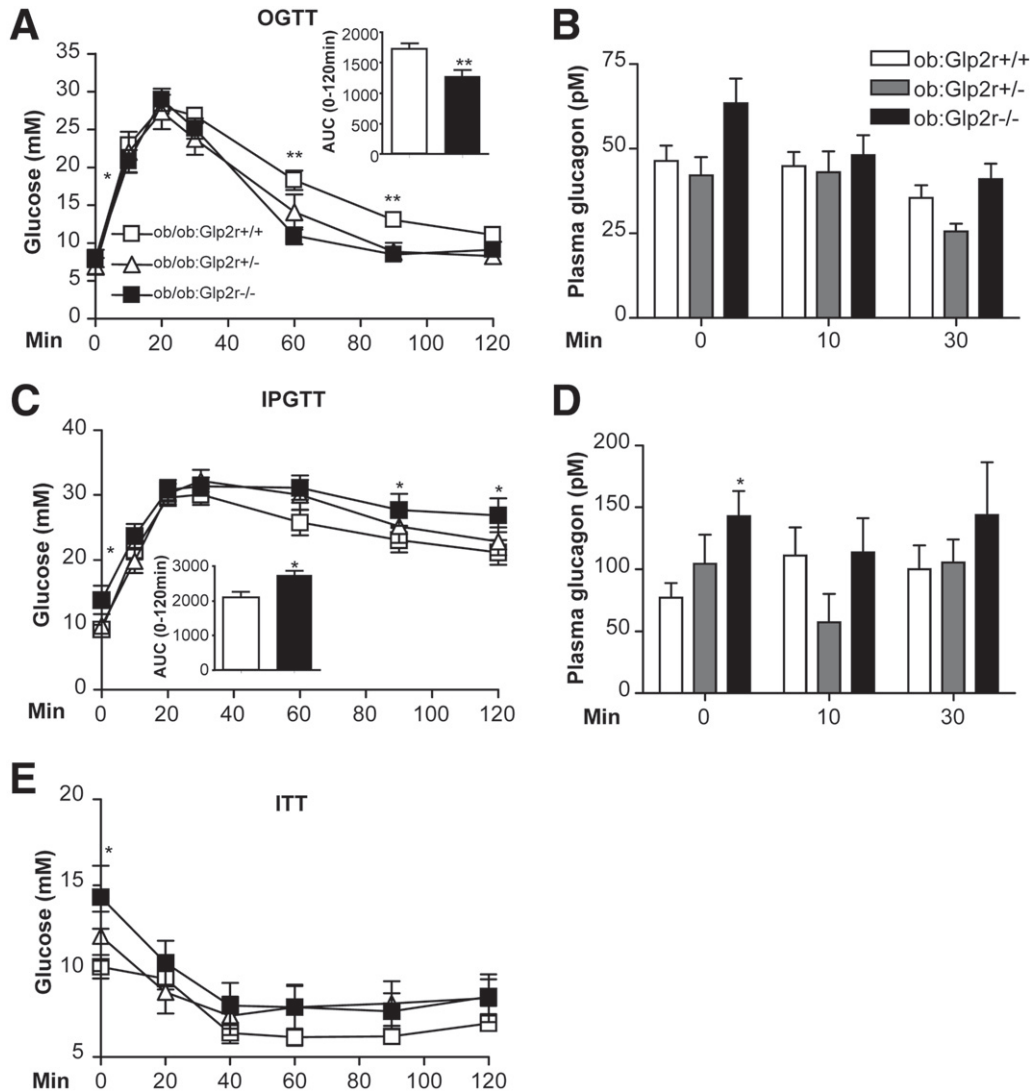
**Figure 5.** Role of GLP-2R signaling in the *ob/ob* mouse. Body weight (A), fasted and end point fed blood glucose levels (B), and plasma glucagon levels (C) are shown for 8- to 12-week-old *ob/ob:Glp2r<sup>-/-</sup>* mice and littermate controls. The  $\alpha$ - and  $\beta$ -cell mass (D), histology (E), and incidence of Ki-67<sup>+</sup> cells (F) in islets of *ob/ob:Glp2r<sup>-/-</sup>* mice and littermate *ob/ob:Glp2r<sup>+/+</sup>* controls (n = 11–30). \**P* < .05, \*\**P* < .01 vs *ob/ob:Glp2r<sup>+/+</sup>* control.

gon levels did not change 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 Figure 4B) or glucagon (Figure 6D) across genotypes. No difference in glucose excursion or recovery from hypoglycemia was detected after insulin tolerance testing, an indirect index of insulin sensitivity, in *ob/ob:Glp2r<sup>-/-</sup>* vs *ob/ob:Glp2r<sup>+/+</sup>* 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<sup>-/-</sup>* mice. Taken together, these findings show

that GLP-2R signaling is important for control of islet cell proliferation,  $\alpha$ - and  $\beta$ -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[Gly<sup>2</sup>]GLP-2 had no effect on glucagon release from murine islets cultured at 2.8, 8.3, or 16.8 mmol/L glucose, whereas glucagon secretion was significantly increased after exposure to arginine (Figure 7A). Moreover, *Glp2r* mRNA transcripts were undetectable in RNA from WT mouse islets (Figure 7B), whereas *Glp1r* mRNA transcripts were abundant in the same islet RNA samples (Figure 7B, right panel). Reverse transcriptase PCR with the use of RNA from isolated pancreatic islets of WT and *ob/ob* mice followed by hybridization





**Figure 6.** Glucose tolerance and circulating glucagon levels in *ob/ob:Glp2r<sup>-/-</sup>* mice. Glycemia (A) and plasma glucagon levels (B) during an OGTT in *ob/ob:Glp2r<sup>-/-</sup>* mice and littermate controls. Area-under-the-curve (AUC, 0–120 minutes) for *ob/ob:Glp2r<sup>+/+</sup>* mice = 1708.6 and for *ob/ob:Glp2r<sup>-/-</sup>* = 1279.1 ( $P = .017$ ) as assessed by Student *t* test. Glycemia (C) and plasma glucagon levels (D) after an IPGTT in *ob/ob:Glp2r<sup>-/-</sup>* mice and littermate controls. AUC (0–120 minutes) for *ob/ob:Glp2r<sup>+/+</sup>* mice = 2102.3 and for *ob/ob:Glp2r<sup>-/-</sup>* = 2749.2 ( $P = .012$ ) as assessed by Student *t* test. ITT (E) in *ob/ob:Glp2r<sup>-/-</sup>* mice and littermate controls ( $n = 11–30$ ). \* $P < .05$ , \*\* $P < .01$  vs *ob/ob:Glp2r<sup>+/+</sup>* control.

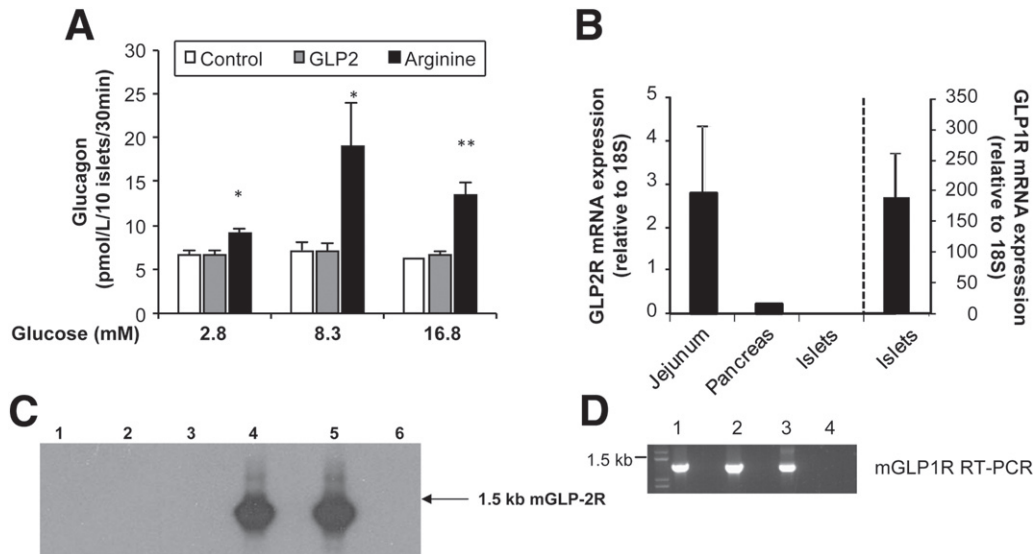
of the PCR products with a *Glp2r*-specific oligonucleotide probe did not detect *Glp2r* RNA transcripts in any of the samples (Figure 7C), whereas the GLP-1R was easily detected in the same islet samples (Figure 7D).

## Discussion

Most studies of GLP-2 action have focused on its intestinotrophic and cytoprotective actions in the gastrointestinal tract. More recent experiments have suggested that GLP-2R signaling may also influence glucose metabolism and insulin action. Studies in human beings have shown that acute exogenous administration of native GLP-2(1-33) was associated with increased circulating levels of plasma glucagon.<sup>21,23</sup> 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 the levels of triglycerides and free fatty acids<sup>21</sup> but without changes in gastric emptying. Moreover, GLP-2(1-33) increased glucagon levels in healthy human beings without changes in circulating GLP-1, GIP, insulin, or glucose.<sup>23</sup> In contrast, there is no information about the effects of degradation-resistant GLP-2 analogues on glucagon secretion, and whether chronic GLP-2 administration perturbs glucagon or glucose homeostasis in human beings has not been carefully examined.

The mechanisms underlying the GLP-2-dependent stimulation of glucagon secretion in human subjects remain unclear. The GLP-2R was localized with the use of immunohistochemistry to human and rat  $\alpha$  cells, and



**Figure 7.** GLP-2R signaling does not regulate glucagon secretion from isolated pancreatic islets. (A) Mouse islets were cultured for 2 hours in HBSS containing 8.3 mmol/L glucose, then stimulated with Gly<sup>2</sup>-GLP-2 (20 nmol/L) or arginine (20 mmol/L) for 30 minutes in the presence of 2.8, 8.3, or 16.8 mmol/L glucose. Glucagon levels in the supernatant were measured with a Lincoplex assay. (B) GLP2R mRNA levels were measured in jejunum, whole pancreas, and islets, and GLP-1R mRNA was measured in islets prepared from WT 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 using RNA from ob/ob islets (lanes 1 and 2), WT islets (lane 3), mouse jejunum RNA (lanes 4 and 5), and a *Glp2r*<sup>-/-</sup> jejunum RNA sample (lane 6). (D) RT-PCR for the mouse GLP-1 receptor (mGLP-1R) with the use of RNA from ob/ob islets (lanes 1 and 2) or WT islets (lane 3) or negative control (lane 4). \**P* < .05, \*\**P* < .01 compared with control untreated islets.

perfusion of rat islets with GLP-2(1-33) increased glucagon secretion without changes in levels of somatostatin or insulin.<sup>22</sup> Furthermore, GLP-2 attenuated the glucagonostatic actions of coadministered GLP-1 in perfused rat islets. In contrast, we were unable to detect GLP-2R mRNA transcripts in mouse islets, and h[Gly<sup>2</sup>]GLP-2 did not modify the inhibitory effects of exendin-4 on glucagon secretion in vivo. Hence, these observations show 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 nondiabetic mice under a diverse range of conditions, including chronic GLP-2(1-33) administration to normal mice, and during acute administration of h[Gly<sup>2</sup>]GLP-2 during oral and intraperitoneal glucose challenge, and insulin-induced hypoglycemia. Our experimental results have shown a lack of effect of acute exogenous h[Gly<sup>2</sup>]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.

Because 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 *Glp2r*<sup>+/+</sup> and *Glp2r*<sup>-/-</sup> mice. Our findings show normal glucose tolerance, preservation of appropriate responses to hypoglycemia, and no evidence of abnormal glucagon secretion under conditions of hypoglycemia or hyperglycemia in *Glp2r*<sup>-/-</sup> mice. Furthermore, induction of metabolic stress either through STZ-mediated  $\beta$ -cell destruction, resulting in diabetes, weight loss, and insulin deficiency, or by an HFD that classically induces insulin resistance<sup>31</sup> failed to unmask abnormalities in glucose homeostasis or glucagon secretion in *Glp2r*<sup>-/-</sup> 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*<sup>-/-</sup> 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.<sup>24</sup> 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.<sup>24</sup> 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.<sup>24</sup>

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/ob: *Glp2r*<sup>+/+</sup> and ob/ob: *Glp2r*<sup>-/-</sup> mice. Nevertheless, ob/ob: *Glp2r*<sup>-/-</sup> 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,  $\beta$ -cell mass and islet cell proliferation were significantly reduced and  $\alpha$ -cell mass was significantly increased in ob/ob: *Glp2r*<sup>-/-</sup> 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*<sup>-/-</sup> mice (data not shown), we cannot exclude the possibility that developmental adaptation to the loss of the GLP-2R may lead to up-regulation 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.<sup>24</sup> Intriguingly, recent evidence implicates systemic and islet inflammation in the pathophysiology of  $\beta$ -cell loss and dysfunction,<sup>34</sup> and it remains possible that low-grade systemic or localized islet inflammation contributed to the pathophysiology of reduced  $\beta$ -cell mass in ob/ob: *Glp2r*<sup>-/-</sup> mice. Similarly, the increase in pancreatic  $\alpha$ -cell mass detected in ob/ob: *Glp2r*<sup>-/-</sup> mice may also reflect increased proinflammatory signals, because the proinflammatory cytokine interleukin-6 has been implicated in the pathophysiology of  $\alpha$ -cell proliferation and enhanced glucagon secretion in experimental models of metabolic stress and diabetes.<sup>35</sup>

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  $\alpha$ -cell mass, hyperglycemia, reduced  $\beta$ -cell mass, and decreased islet proliferation in ob/ob: *Glp2r*<sup>-/-</sup> mice. Our findings are consistent with emerging evidence implicating a role for GLP-2 in the regulation of systemic and tissue inflammation<sup>24,36</sup> and suggest that further assessment of the link between the consequences of localized or systemic inflammation and GLP-2R signaling is clearly warranted.

## Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at [www.gastrojournal.org](http://www.gastrojournal.org), and at doi: [10.1053/j.gastro.2010.05.006](https://doi.org/10.1053/j.gastro.2010.05.006).

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#### Conflicts of interest

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