Disordered glucagon secretion contributes to the symptoms of diabetes, and reduced glucagon action is known to improve glucose homeostasis. In mice, genetic deletion of the glucagon receptor (Gcgr) results in increased levels of the insulinotropic hormone glucagon-like peptide 1 (GLP-1), which may contribute to the alterations in glucose homeostasis observed in Gcgr−/− mice. Here, we assessed the contribution of GLP-1 receptor (GLP-1R) signaling to the phenotype of Gcgr−/− mice by generating Gcgr−/−Glp1r−/− mice. Although insulin sensitivity was similar in all genotypes, fasting glucose was increased in Gcgr−/−Glp1r−/− mice. Elimination of the Glp1r normalizes gastric emptying and impaired intraportal glucose tolerance in Gcgr−/− mice. Unexpectedly, deletion of Glp1r in Gcgr−/− mice did not alter the improved oral glucose tolerance and increased insulin secretion characteristic of that genotype. Although Gcgr−/−Glp1r−/− islets exhibited increased sensitivity to the incretin glucose-dependent insulinotropic polypeptide (GIP), mice lacking both Glp1r and the GIP receptor (Gipr) maintained preservation of the enteroinsular axis following reduction of Gcgr signaling. Moreover, Gcgr−/−Glp1r−/− islets expressed increased levels of the cholecystokinin A receptor (Cckar) and G protein–coupled receptor 119 (Gpr119) mRNA transcripts, and Gcgr−/−Glp1r−/− mice exhibited increased sensitivity to exogenous CCK and the GPR119 agonist AR231453. Our data reveal extensive functional plasticity in the enteroinsular axis via induction of compensatory mechanisms that control nutrient-dependent regulation of insulin secretion.

**Introduction**

Glucagon, a 29-amino-acid peptide hormone, is produced in pancreatic α cells and, together with insulin, plays a key role in regulating hepatic glucose production, serving as the first line of defense against hypoglycemia (1). Disordered control of glucagon secretion is observed in type 2 diabetes mellitus (T2DM) (2–4) and together with insulin deficiency or resistance results in metabolic derangements characteristic of T2DM (5). Glucagon exerts its actions through a class B family G protein–coupled receptor (GPCR), the glucagon receptor (Gcgr), that is expressed not only in its actions through a class B family G protein–coupled receptor (GPCR), the glucagon receptor (Gcgr), that is expressed not only in diabetes (16–23). The importance of glucagon action has also been examined via generation and characterization of the Gcgr−/− mouse. Surprisingly, mice with complete germline disruption of Gcgr are viable and grow normally (24, 25). Gcgr−/− mice exhibit normal body weight, food intake, and energy expenditure, yet display improved glucose tolerance, enhanced β cell function and insulin sensitivity, resistance to streptozotocin-induced (STZ-induced) diabetes, and delayed gastric emptying (25–27). These favorable phenotypes support reduction of glucagon action for the therapy of T2DM.

Interpretation of metabolic derangements arising from loss of Gcgr signaling is complicated by the concomitant finding of markedly increased circulating levels of glucagon-like peptide 1 (GLP-1) in Gcgr−/− mice (25). GLP-1, a related proglucagon-derived peptide normally secreted from enteroendocrine L cells, produces many of the actions described in Gcgr−/− mice. The increased circulating levels of GLP-1 in Gcgr−/− mice likely reflect increased production of bioactive GLP-1 in the Gcgr−/− pancreas (25). As GLP-1 improves β cell function and glucose tolerance, reduces gastric emptying, and promotes islet cell proliferation and resistance to STZ-induced diabetes, attribution of precise mechanisms responsible for the improved metabolic phenotype in Gcgr−/− mice is challenging. We now demonstrate that a functional GLP-1 receptor (GLP-1R) is essential for control of fasting and ambient glycemia, enhancement of glucose-stimulated insulin secretion following i.p. glucose challenge, and inhibition of gastric emptying in Gcgr−/− mice. Unexpectedly, Gcgr−/−Glp1r−/− mice continued to exhibit improved oral glucose tolerance and enhanced β cell function despite loss of two key insulinotropic β cell receptors. Although loss of GLP-1 action is classically compensated for by upregulation of the other major incretin, glucose-dependent insulinotropic polypeptide (GIP) (28), we demonstrate that reduction of Gcgr expression in mice with combined inactivation of both
Glp1r and the GIP receptor (Gipr) genes continue to be associated with preservation of oral glucose tolerance and enhanced insulin secretion. Disruption of the classical (GLP-1R and GIPR) entero-insular axis was associated with enhanced expression and activity of the cholecystokinin A receptor (Cckar) and G protein–coupled receptor 119 (Gpr119), functionally related insulinotropic receptors that respond to nutrient-sensitive signals. These findings reveal considerable plasticity in the incretin-related mechanisms regulating β cell function and glucose homeostasis.

**Results**

Glp1r is not required for pancreas enlargement or α cell hyperplasia in Gcgr−/− mice. Body weight, food intake, physical activity, and energy expenditure were comparable in WT, single incretin receptor knockout, and Gcgr−/−Glp1r−/− mice (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI43615DS1). As Gcgr−/− mice exhibit increased pancreatic mass and marked islet and α cell hyperplasia (25) and GLP-1R activation promotes expansion of islet and pancreatic mass (29–31), we assessed the contribution of Glp1r to the development of these abnormalities in Gcgr−/− mice. Consistent with previous findings, Gcgr−/− mice exhibited very high circulating levels of GLP-1 (Supplemental Figure 2A), significantly increased pancreas weight, and an approximately 4-fold increase in islet area (Figure 1); however, islet area and pancreatic mass remained significantly increased to a similar extent in Gcgr−/−Glp1r−/− mice (Figure 1, A and B). Immunohistochemical analysis revealed that the increased islet area was predominantly due to α cell hyperplasia, with most Gcgr−/− and Gcgr−/−Glp1r−/− islets containing a core of β cells surrounded by an expanded mantle of hyperplastic α cells (Figure 1C). Hence, Glp1r is not required for development of increased pancreatic mass and islet hyperplasia following loss of Gcgr action.

Disruption of Glp1r leads to increased fasting glycemia in Gcgr−/− mice. Both fasting and random glucose were reduced in Gcgr−/− mice (Figure 2, A and B), consistent with the central role of glucagon in the maintenance of euglycemia (25). Basal GLP-1R signaling also regulates fasting glycemia (32), classically through suppression of glucagon secretion (33). Surprisingly, a significant increase in fasting glucose was observed in Gcgr−/−Glp1r−/− compared with Gcgr−/− mice (Figure 2A) and elimination of the Glp1r normalized random-fed glycemia in Gcgr−/−Glp1r−/− mice (Figure 2B). Hence, loss of Glp1r substantially attenuates improvements in both ambient and fasting glycemia in Gcgr−/− mice (25).

Elimination of Glp1r reverses improvements in i.p. glucose tolerance in Gcgr−/− mice. To elucidate the contribution of enhanced GLP-1

![Figure 1](http://www.jci.org)
receptor signaling to improved β cell function and glucose tolerance in Gcgr–/– mice (25), we first assessed clearance of i.p. glucose in mice of different genotypes. Intraperitoneal glucose tolerance was significantly enhanced and plasma insulin levels increased in Gcgr–/– mice; conversely, i.p. glucose tolerance was impaired in Glp1r–/– mice (Figure 3A), consistent with previous studies (25, 32). Furthermore, disruption of Glp1r in Gcgr–/– mice reversed the improvements in i.p. glucose tolerance and normalized plasma insulin levels in Gcgr–/–Glp1r–/– mice (Figure 3A and B), whereas insulin sensitivity, approximated by insulin tolerance, was comparable among all genotypes (Figure 3C). Hence, the elevated levels of GLP-1 leading to increased GLP-1R signaling is primarily responsible for enhanced β cell function and improved glucose clearance after i.p. glucose challenge in Gcgr–/– mice.

The GLP-1 receptor mediates reduced gastric emptying; however, oral glucose tolerance remains improved independent of Glp1r in Gcgr–/– mice. As GLP-1–mediated reduction in gastric emptying may substantially account for the improved oral glucose tolerance in Gcgr–/– mice (25, 26), we quantified gastric emptying with two complementary methods. Both liquid-phase gastric emptying, assessed via measurement of plasma acetaminophen levels, and solid-phase gastric emptying were significantly reduced in Gcgr–/– mice and normalized in Gcgr–/–Glp1r–/– mice (Figure 4, A and B). We hypothesized that normalization of gastric emptying would be associated with deterioration of oral glucose tolerance in Gcgr–/–Glp1r–/– mice (25). Unfortunately, oral glucose tolerance remained significantly improved in Gcgr–/–Glp1r–/– mice to an extent comparable to that in Gcgr–/– mice alone (Figure 4, C and D). Furthermore, in contrast to the normalization of plasma insulin levels seen following i.p. glucose challenge in Gcgr–/–Glp1r–/– versus Gcgr–/– mice (Figure 3B), Gcgr–/–Glp1r–/– mice continued to exhibit significantly improved levels of plasma insulin following oral glucose challenge (Figure 4D).

Islets from Gcgr–/–Glp1r–/– mice display increased sensitivity to GIP. As Glp1r–/– mice exhibit enhanced GIP secretion and increased sensitivity to GIP (28), we explored whether GIP-related mechanisms underlie the enhanced enteral glucose-stimulated insulin secretion in Gcgr–/–Glp1r–/– mice. Although levels of GIP were modestly elevated in Glp1r–/– mice, GIP levels were not significantly increased in Gcgr–/–Glp1r–/– mice (Supplemental Figure 2B). We next examined control of insulin secretion from WT, Glp1r–/–, Gcgr–/–, and Gcgr–/–Glp1r–/– islets. No significant differences across genotypes were detected in response to 16.7 mM glucose with a modest but nonsignificant reduction in insulin secretion observed with Gcgr–/–Glp1r–/– islets (Figure 5A). Consistent with the loss of the Glp1r, the insulinotropic response to exendin-4 was absent in Glp1r–/– and Gcgr–/–Glp1r–/– islets (Figure 5A). Although GIP sensitivity was not enhanced in Glp1r–/– or Gcgr–/–Glp1r–/– islets, the insulinotropic response to GIP was significantly increased in Gcgr–/–Glp1r–/– islets (Figure 5A).

To explore the selectivity of the enhanced response to GIP, we tested a range of other insulin secretagogues. In contrast to the

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**Figure 2**
Glp1r controls fasting and fed glycemia in Gcgr–/– mice. (A) Blood glucose following 5 or 16 hours of fasting in 8- to 12-week-old WT, Gcgr–/–, Glp1r–/–, and Gcgr–/–Glp1r–/– mice (n = 5–30 mice per genotype). (B) Weekly random-fed blood glucose levels in 8- to 20-week-old Gcgr–/–Glp1r–/–, Gcgr–/–, Glp1r–/–, and littermate control WT mice (n = 3–20 mice per group). Values are expressed as mean ± SEM. *P < 0.05, Gcgr–/– versus Gcgr–/–Glp1r–/– mice; †P < 0.05, Gcgr–/– versus WT mice; ‡P < 0.05, Glp1r–/– versus WT mice; ††P < 0.05, Glp1r–/– versus Gcgr–/–Glp1r–/– mice.

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**Figure 3**
Glp1r controls fasting and fed glycemia in Gcgr–/– mice. (A) Blood glucose following 5 or 16 hours of fasting in 8- to 12-week-old WT, Gcgr–/–, Glp1r–/–, and Gcgr–/–Glp1r–/– mice (n = 5–30 mice per genotype). (B) Weekly random-fed blood glucose levels in 8- to 20-week-old Gcgr–/–Glp1r–/–, Gcgr–/–, Glp1r–/–, and littermate control WT mice (n = 3–20 mice per group). Values are expressed as mean ± SEM. *P < 0.05, Gcgr–/– versus Gcgr–/–Glp1r–/– mice; †P < 0.05, Gcgr–/– versus WT mice; ‡P < 0.05, Glp1r–/– versus WT mice; ††P < 0.05, Glp1r–/– versus Gcgr–/–Glp1r–/– mice.
enhanced response to GIP, Gcgr<sup>−/−</sup>Glpr<sup>−/−</sup> islets exhibited a normal response to pituitary adenylyl cyclase–activating peptide (PACAP) but significantly reduced insulin secretory responses to tolbutamide and l-arginine (Figure 5B). Consistent with the increased GIP sensitivity demonstrated for insulin secretion (Figure 5A), Gcgr<sup>−/−</sup>Glpr<sup>−/−</sup> islets also exhibited enhanced cAMP accumulation in response to GIP (Figure 5C). To evaluate GIP sensitivity in vivo, we administered i.p. glucose in the presence or absence of submaximal doses of exogenous GIP to WT, Glp1r<sup>−/−</sup>, Gcgr<sup>−/−</sup>, and Gcgr<sup>−/−</sup>Glpr<sup>−/−</sup> mice following ASO treatment (Figure 7, A and B, respectively). WT mice treated with Gcgr ASOs showed improved i.p. glucose tolerance and increased insulin levels following i.p. glucose challenge (Figure 7C). In contrast, DIRKO mice treated with Gcgr ASOs showed no improvement in glucose tolerance or insulin levels following i.p. glucose challenge (Figure 7D). WT mice treated with Gcgr ASOs also showed improved oral glucose tolerance and significantly higher plasma insulin levels than vehicle-treated controls (Figure 7E). Remarkably, despite loss of both classical incretin receptors, DIRKO mice treated with Gcgr ASOs also exhibited improved oral sensitivity in Gcgr<sup>−/−</sup>Glpr<sup>−/−</sup> islets. To more rigorously test this hypothesis, we reduced Gcgr expression using ASOs in mice lacking both functional incretin receptors, i.e., Glp1r<sup>−/−</sup>Gipr<sup>−/−</sup> (double incretin receptor knockout [DIRKO]) mice (34). Hepatic Gcgr mRNA transcripts were markedly decreased and plasma levels of GLP-1 progressively increased in WT and DIRKO mice following ASO treatment (Figure 7, A and B, respectively). WT mice treated with Gcgr ASOs showed improved i.p. glucose tolerance and increased insulin levels following i.p. glucose challenge (Figure 7C). In contrast, DIRKO mice treated with Glp1r ASOs showed no improvement in glucose tolerance or insulin levels following i.p. glucose challenge (Figure 7D). WT mice treated with Glp1r ASOs also showed improved oral glucose tolerance and significantly higher plasma insulin levels than vehicle-treated controls (Figure 7E). Remarkably, despite loss of both classical incretin receptors, DIRKO mice treated with Glp1r ASOs also exhibited improved oral
Glucose tolerance and significantly increased plasma insulin levels (Figure 7F). Hence, preferential improvement of glucose tolerance and enhanced β cell function following glucose administration in the gut can be achieved despite loss of both incretin receptors.

To identify mechanisms responsible for improvement of oral glucose tolerance and β cell function despite absence of both GLP-1 and GIP receptors, we assessed the expression of insulinotropic receptors in islets from (a) DIRKO mice treated with Gcgr ASOs and (b) Gcgr–/– Glp1r–/– mice. Remarkably, levels of mRNA transcripts for the insulinotropic receptors gastrin-releasing peptide receptor (Grpr), Cckar, and Gpr119 were significantly increased in islet RNA from Gcgr–/– Glp1r–/– mice (Figure 8A). Similarly Gpr119 and Cckar mRNA transcripts were also significantly increased in islet RNA from DIRKO mice following Gcgr ASO administration (Figure 8B). These findings raised the possibility that increased activity of related insulinotropic receptors may compensate for the loss of GLP-1 and GIP action on islet β cells.

Gcgr–/– Glp1r–/– mice display increased sensitivity to Gpr119 and Cckar agonists. To assess the functional significance of increased islet receptor expression, we carried out glucose tolerance tests in the presence or absence of exogenous GRP, CCK, PACAP, and the GPR119 agonist AR231453 (35) in WT, Gcgr–/–, Glp1r–/–, and Gcgr–/– Glp1r–/– mice. We did not detect enhanced sensitivity to exogenous GRP or PACAP (Supplemental Figure 4 and Supplemental Figure 5, A–D). However, although AR231453 failed to improve i.p. glucose tolerance in WT, Gcgr–/–, or Glp1r–/– mice, a robust improvement in glucose tolerance and marked stimulation of plasma insulin levels were observed following AR231453 administration in Gcgr–/– Glp1r–/– mice (Figure 9, A–D, and Supplemental Figure 6, A–D). Similarly, doses of CCK that failed to improve glucose tolerance in WT or single Gcgr–/– or Glp1r–/– mice produced a significant reduction in glycemic excursion and significantly elevated plasma insulin levels in Gcgr–/– Glp1r–/– mice, consistent with increased sensitivity to CCK (Figure 10, A–D, and Supplemental Figure 7, A–D).

**Discussion**

The central importance of glucagon action for the maintenance of euglycemia, together with observations that glucagon secretion may be inappropriately increased in many subjects with T2DM, has fostered great interest in reduction of glucagon action for the treatment of T2DM. Indeed, multiple therapeutic modalities, including insulin, amylin analogs, GLP-1R agonists, and dipeptidyl peptidase-4 inhibitors, exert their anti-diabetic actions in part through suppression of glucagon secretion (36). The Gcgr–/– phenotype — healthy mice with reduced fasting and postprandial glycemia (25), resistance to diet-induced obesity, and enhanced β cell function and survival (26) — further sup-

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**Figure 4**

Glp1r mediates reduced gastric emptying but not improved oral glucose tolerance in Gcgr–/– mice. (A) Liquid-phase gastric emptying (as determined by the appearance of acetaminophen in the circulation after 15 minutes) in 10- to 11-week-old mice (n = 4–14 mice per group). (B) Solid-phase gastric emptying in 20-week-old mice (n = 4–10 mice per group). Values are expressed as mean ± SEM. *P < 0.05, Gcgr–/– versus Gcgr–/– Glp1r–/– mice; †P < 0.05, Gcgr–/– versus WT mice. (C) Blood glucose levels during an OGTT in 10- to 11-week-old mice (n = 11–22 mice per group). (D) Area under the glucose curve and plasma insulin levels 0 and 15 minutes following oral glucose challenge (n = 4–9 mice per group). Values are expressed as mean ± SEM. In C and D: *P < 0.05, Gcgr–/– versus WT littermate control mice; †P < 0.05, Gcgr–/– Glp1r–/– versus WT mice; ‡P < 0.05, Glp1r–/– versus Gcgr–/– Glp1r–/– littermate control mice; ††P < 0.05, Glp1r–/– versus WT littermate control mice.
ports the concept of reducing glucagon receptor signaling for treatment of T2DM. Our studies clearly show that a substantial component of the improved metabolic phenotype of Gcgr−/− mice, including control of fasting and fed glycemia, reduced gastric emptying, improved i.p. glucose tolerance, and enhanced β cell function, reflects concomitant upregulation of GLP-1 action in Gcgr−/− mice (Figure 11). Although incretin action classically controls postprandial glucose excursion, considerable evidence supports a role for GLP-1 in the regulation of fasting glucose. GLP-1 receptor agonists reduce fasting glucose in human subjects, in association with reduced levels of circulating glucagon (37). Moreover, the GLP-1R antagonist exendin-4 (Ex-4, 10 nM), [d-Ala²]GIP (GIP, 10 nM), PACAP (10 nM), tolbutamide (Tol, 100 μM), or l-arginine (L-arg, 10 mM) for 1 hour at 37°C. Levels of insulin in the secretion medium were normalized to levels of islet insulin content and are expressed as a fold change in secretion relative to WT high-glucose treatment. Insulin content values averaged approximately 30–40 ng/islet for GIP mice and 15–25 ng/islet for Gcgr−/− and WT mice (n = 3 mice per group). Data shown are representative of 2–3 independent experiments, each with 3 replicates per condition. (C) Total cellular and media cAMP in islets from WT, Gip1r−/−, Gcgr−/−, and Gcgr−/−Gip1r−/− mice was quantified following treatment of the islets with 0, 1, 3, or 10 nM [d-Ala²]GIP. Levels of cAMP in the secretion medium were normalized to levels of islet insulin content and are expressed as a fold change in islet cAMP levels relative to WT high-glucose treatment (n = 3 mice per group). Values are expressed as mean ± SEM. §P < 0.05, Gip1r−/− versus Gcgr−/−Gip1r−/− mice; †P < 0.05, Gcgr−/− versus Gcgr−/−Gip1r−/− mice; ‡P < 0.05, Gcgr−/−Gip1r−/− versus WT mice; *P < 0.05, Gip1r−/− versus WT mice.

Figure 5 Function of GPCRs in isolated islets. Islet insulin secretion was assessed by preincubation of islets in KRB for 60 minutes at 2.8 mM glucose at 37°C before distribution in batches of 10 islets per condition into wells containing 16.7 mM glucose with or without (A) exendin-4 (Ex-4, 10 nM), [d-Ala²]GIP (GIP, 10 nM), (B) PACAP (10 nM), tolbutamide (Tol, 100 μM), or l-arginine (L-arg, 10 mM) for 1 hour at 37°C. Levels of insulin in the secretion medium were normalized to levels of islet insulin content and are expressed as a fold change in insulin secretion relative to WT high-glucose treatment. Insulin content values averaged approximately 30–40 ng/islet for Gip1r−/− and WT mice and 15–25 ng/islet for Gcgr−/− and Gcgr−/−Gip1r−/− mice (n = 3 mice per group). Data shown are representative of 2–3 independent experiments, each with 3 replicates per condition. (C) Total cellular and media cAMP in islets from WT, Gip1r−/−, Gcgr−/−, and Gcgr−/−Gip1r−/− mice was quantified following treatment of the islets with 0, 1, 3, or 10 nM [d-Ala²]GIP. Levels of cAMP in the secretion medium were normalized to levels of islet insulin content and are expressed as a fold change in islet cAMP levels relative to WT high-glucose treatment (n = 3 mice per group). Values are expressed as mean ± SEM. §P < 0.05, Gip1r−/− versus Gcgr−/−Gip1r−/− mice; †P < 0.05, Gcgr−/− versus Gcgr−/−Gip1r−/− mice; ‡P < 0.05, Gcgr−/−Gip1r−/− versus WT mice; *P < 0.05, Gip1r−/− versus WT mice.
mediate pancreatic or islet/α cell proliferation in Gcgr−/− mice. As Gcgr−/− mice are born with normal pancreatic mass and only modest α cell hyperplasia (25, 42), the precise mechanisms linking loss of the Gcgr to marked postnatal expansion of the exocrine and endocrine islet compartments remain unclear.

The finding of reduced gastric emptying in Gcgr−/− mice (26) might be explained by enhanced action of multiple proglucagon-derived peptides. GLP-1, oxyntomodulin, and to a lesser extent GLP-2 inhibit gastric emptying (43, 44), and GLP-1 and oxyntomodulin exert their actions on gastric emptying through the GLP-1 receptor. Unexpectedly, however, despite normalization of gastric emptying in Gcgr−/−Glp1r−/− mice, oral glucose tolerance remained substantially improved in Gcgr−/−Glp1r−/− mice, despite elimination of GLP-1R action. Moreover, levels of circulating insulin remained significantly increased following oral glucose challenge despite loss of GLP-1R action in Gcgr−/−Glp1r−/− mice. These findings implicate sustained enhancement of β cell function as a mechanism underlying persistent improvement in oral glucose tolerance despite loss of GLP-1R action in Gcgr−/−Glp1r−/− mice.

The demonstration that the Gcgr−/−Glp1r−/− β cell maintains enhanced insulin secretion in response to oral glucose was unexpected given the previous demonstration of selectively impaired β cell function in Gcgr−/− mice compared with age- and sex-matched WT C57BL/6 mice (27) and the multiple lines of evidence supporting important roles for both GLP-1 and glucagon in the control of glucose-stimulated insulin secretion. Glucagon directly stimulates insulin secretion in the rat pancreas independent of GLP-1 receptor action (45), and transgenic Gcgr expression in murine β cells significantly augments glucose-stimulated insulin secretion and reduces random glycemia in RIP-Gcgr transgenic mice (46). Moreover, the glucagon antagonist des-His1-[Glu9]-glucagon-amide significantly reduced glucose-stimulated insulin release in human islets (47). Similarly, GLP-1 induces β cell glucose competence, upregulates insulin biosynthesis and secretion, and markedly enhances glucose sensitivity and glucose-stimulated insulin secretion even in poorly responsive diabetic β cells (48). Hence, given the results of previous observations reporting impaired β cell function in Gcgr−/− islets (27), we initially predicted that a further deterioration in β cell function would ensue following removal of the GLP-1R signaling system in Gcgr−/− mice.

Surprisingly, however, elimination of Glp1r in Gcgr−/− mice did not lead to deterioration in glycemic excursion after oral glucose loading in Gcgr−/−Glp1r−/− mice. The preferential preservation of improved oral glucose tolerance and enhanced insulin secretion following glucose administration via the gastrointestinal tract, despite functional elimination of both the Gcgr and Glp1r, strongly implicates the existence of one or more gut-derived compensatory factors that augment β cell function in an “incretin-like” manner in Gcgr−/−Glp1r−/− mice. As compensatory upregulation of GIP action has been described in Glp1r−/− mice, we postulated that upregulation of the GIP-Gipr axis might similarly explain preservation of improved oral glucose tolerance in mice following loss of glucagon and GLP-1 action. Although circulating levels of GIP and levels of GIP RNA transcripts were

Figure 6  
Gcgr−/−Glp1r−/− mice exhibit enhanced sensitivity to [d-Ala2]GIP. An IPGTT was performed in 20- to 22-week-old (A) WT, (B) Gcgr−/−, (C) Glp1r−/−, and (D) Gcgr−/−Glp1r−/− mice following treatment with 1 nmol/kg [d-Ala2]GIP or saline (vehicle [Veh]). Insets depict the area under the glucose excursion curve (AUC) in mM×min and plasma insulin levels at 0 and 15 minutes following glucose challenge (n = 5–8). Values are expressed as mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, [d-Ala2]GIP–treated versus saline-treated group.

The Journal of Clinical Investigation http://www.jci.org Volume 121 Number 5 May 2011

1923
not increased in islets from \( \text{Gcgr}^{-/-}\text{Glp1r}^{-/-} \) mice, we detected increased insulin secretion and enhanced cAMP accumulation following treatment of \( \text{Gcgr}^{-/-}\text{Glp1r}^{-/-} \) islets with GIP, consistent with increased GIP sensitivity. Furthermore, glucose tolerance was significantly improved and plasma insulin levels were markedly increased following administration of GIP in vivo, further supporting enhanced sensitivity to GIP as a compensatory mechanism augmenting \( \beta \) cell function in \( \text{Gcgr}^{-/-}\text{Glp1r}^{-/-} \) mice. Unexpectedly, however, we continued to observe preferential improvement of oral glucose tolerance and \( \beta \) cell function in DIRKO mice treated with ASOs to reduce Gcgr expression, implying the existence of additional incretin-like mechanisms compensating for the lack of insulinotropic activity normally subserved by the glucagon, GLP-1, and GIP receptors. Our data demonstrating increased islet expression and functional activity of \( \text{Cckar} \) and \( \text{Gpr119} \) mRNA transcripts from \( \text{Gcgr}^{-/-}\text{Glp1r}^{-/-} \) mice, and in islets from DIRKO mice treated with Gcgr ASOs, reveal a mechanism compensating for the loss of insulinotropic incretin receptors (Figure 11B). The finding of greatly enhanced sensitivity in vivo (improved glucose tolerance and plasma insulin levels) to exogenous ligands for both the \( \text{Cckar} \) and \( \text{Gpr119} \) receptors is consistent with an important role for these receptors in maintaining an incretin response to enteral glucose administration despite loss of GLP-1 and GIP action.

It is notable that the putative ligands for both \( \text{Cckar} \), namely CCK, and \( \text{Gpr119} \), principally lipid-derived amides such as oleoyle-
thanolamide and N-oleoyldopamine, would be expected to increase significantly following oral, but not i.p., glucose ingestion, consistent with our findings of enhanced oral, but not i.p., glucose clearance in Gcgr−/−Glp1r−/− mice. Intriguingly, β cells from DIRKO mice appeared to compensate for loss of incretin receptor and Ggr expression by similar induction of Cckar and Gpr119 expression, extending the findings of islet incretin receptor plasticity to a second related, yet genetically distinct model.

Studies of islet adaptation classically carried out in the context of β cell injury and regeneration, pregnancy, or high-fat feeding have revealed numerous changes in islet gene expression thought to be linked to the need to expand β cell mass and/or enhance β cell function in response to insulin resistance (49–51). Hence, the concept of β cell plasticity is reasonably well established under physiological conditions requiring enhanced functional β cell mass. There is considerably less information concerning the potential plasticity of the gut-islet axis in circumstances associated with impairment or disruption of classic incretin receptor signals normally emanating from the GLP-1 and GIP receptors. Our observations made using two distinct genetic models, the Gcgr−/−Glp1r−/− mouse and the DIRKO mouse treated with Gcgr ASOs, reveal a heretofore unrecognized capacity for β cell adaptation to loss of insulinotropic receptor signaling (Figure 11). Intriguingly, upregulation of Cckar and Gpr119 expression was also observed in murine islets from pregnant mice, although the functional importance of these findings was not ascertained (51). Our findings may explain why the metabolic phenotype arising from loss of one or both incretin receptors is comparatively mild (32, 34, 52) and identify new models for exploring the expanding importance of incretin-related mechanisms that potentiate β cell function following oral nutrient ingestion.

Figure 8
Expression of insulinotropic GPCRs in islets. (A) Islets were isolated from WT, Gcgr−/−, Glp1r−/−, and Gcgr−/−Glp1r−/− mice, followed by isolation of mRNA for real-time PCR of basal levels of transcripts encoding Gipr, Pacapr, Gpr40, Gpr, Cckar, and Gpr119. (B) Islets were isolated from WT or DIRKO mice following 6 injections of vehicle or 25 mg/kg Gcgr ASOs, and mRNA levels of Gpr, Gpr119, and Cckar were determined. Levels of transcripts were normalized to levels of cyclophilin for each RNA sample, n = 4 mice per genotype. Values are expressed as mean ± SEM. /*P < 0.05, Gcgr−/− versus Gcgr−/−Glp1r−/− mice; †P < 0.05, Gcgr−/− versus Gcgr−/−Glp1r−/− mice; ‡P < 0.05, Gcgr−/−Glp1r−/− versus WT mice; §P < 0.01, WT Gcgr ASO– vs DIRKO Gcgr ASO–treated mice; ¶P < 0.01, WT saline- versus DIRKO Gcgr ASO–treated mice; †P < 0.01, DIRKO saline- versus DIRKO Gcgr ASO–treated mice.

Methods
Animal studies. Gcgr−/−Glp1r−/− double knockout (DKO) mice were generated by crossing double heterozygote Glp1r−/− and Gcgr−/− mice to obtain littermate Gcgr−/−Glp1r−/− (WT), Gcgr−/− and Glp1r−/− single knockouts, and Gcgr−/−Glp1r−/− DKO mice. Gpr119−/− mice (34) were provided by Y. Seino (Kansai Electric Power Hospital, Osaka, Japan) and used to generate DIRKO mice as previously described (52). All mice used in these studies were male and were housed up to 5 per cage under a light/dark cycle of 12 hours in the Toronto Centre for Phenogenomics animal facility, with free access to food and water except where noted. All procedures were conducted according to protocols and guidelines approved by the Toronto Centre for Phenogenomics Animal Care Committee. For confirmation of genotypes, genomic DNA prepared from tail snips was analyzed by PCR and Southern blotting as described previously (25, 32).

Treatment of mice with Gcgr ASOs. DIRKO and WT mice (11–13 weeks old) were given subcutaneous injections of Gcgr ASOs (Sigma-Aldrich, ISIS 180475, hybridizes to bases 1348–1367 of mouse GCGR sequence M96674.1) at a dose of 25 mg/kg every 3.5 days for a maximum of 6 injections (22). Intraperitoneal glucose tolerance tests (IPGTTs) were performed after administration of 3 injections of the ASOs, and oral glucose tolerance tests (OGTTs) were performed after administration of 4 injections of the ASOs. Mice were euthanized after administration of 6 injections of the ASOs, and islets and livers were obtained and examined for gene expression. DIRKO and WT mice treated with the Gcgr ASOs were fasted for 6 hours prior to the glucose tolerance tests.

Treatment of mice with peptides and agonists. Gcgr−/−Glp1r−/− mice and littermate controls were treated orally 30 minutes prior to IPGTT with vehicle (80% polyethylene glycol (PEG) 400, 10% Tween 80, and 10% ethanol) or the Gpr119 agonist AR231453 (5 mg/kg and 20 mg/kg, Arena Pharmaceuticals) (35). Gcgr−/−Glp1r−/− mice and littermate controls were injected
i.p. with PBS vehicle, [α-ALA]GIP (1 or 2 nmol/kg) (CHI Scientific), GRP (20 nmol/kg) (Bachem), CCK-8 (9 μg/kg or 18 μg/kg), or PACAP-38 (1.3 nmol/kg), immediately prior to IPGTT (Sigma-Aldrich).

Assessment of food intake. Sixteen- to 18 week-old mice were fasted overnight (16–18 hours), weighed, and then placed in individual cages containing pre-weighed rodent chow, with free access to water. Food was reweighed after 1, 2, 4, 8, and 24 hours, and food intake was expressed as grams consumed per gram of body weight.

Indirect calorimetry. Eight- to 10-week-old mice were placed in individual metabolic chambers, with free access to food and water. Oxygen consumption, CO₂ production, and total and ambulatory activity were determined by indirect calorimetry using an Oxymax System (Columbus Instruments) as described previously (34).

Pancreas weight and histological analysis. Pancreata from 20- to 24-week-old male mice were weighed, fixed in 10% neutral buffered formalin solution for 48 hours, and then embedded in paraffin. For assessment of islet area and histology, pancreatic sections were immunostained for insulin and/or glucagon, followed by scanning using the ScanScope CS system (Aperio Technologies) at ×20 magnification (53). Digital images were analyzed with ScanScope software (Aperio Technologies). Percent islet area was calculated as the sum of the total cross-sectional area of β and α cells/total pancreas area multiplied by 100.

Glucose tolerance and measurement of plasma metabolites. Eight- to 11-week-old male mice were fasted overnight (16–18 hours), and glucose (1.5 mg/g body weight) was administered orally (through a gavage tube) or via injection into the peritoneal cavity (IPGTT). Blood samples were drawn from the tail vein at 0, 15, 30, 60, 90, and 120 minutes after glucose administration, and blood glucose levels were measured using a Glucometer Elite blood glucose meter (Ascensia; Bayer HealthCare). For plasma insulin determinations, blood samples (100 μl) were drawn from the tail vein during the 0- and 15-minute time periods following glucose administration in a heparinized tube. Plasma was separated by centrifugation at 4 °C and stored at −80 °C until assayed. Plasma was assayed for insulin using a mouse insulin ELISA kit (Alpco). Plasma GLP-1 levels were measured using a mouse/rat total GLP-1 assay kit (Meso Scale Discovery). Plasma GIP levels were measured using a mouse/rat total GIP ELISA kit (Linco). Plasma levels of active GLP-1 and glucagon were measured using a mouse Milliplex endocrine assay (Millipore).

Insulin tolerance test. Twelve- to 13-week-old male mice were fasted for 5 hours and given 0.7 U/kg insulin (Humulin R, 100 U/ml; Lilly) by i.p. injection. Blood samples for blood glucose determination were drawn from the tail vein at 0, 20, 40, 60, 90, 120, and 180 minutes following insulin administration.

Gastric emptying. Gastric emptying was assessed using two protocols. For measurement of solid-phase gastric emptying, 12-week-old mice were fasted in individual cages overnight (16 hours) and allowed access to a pre-measured amount of rodent chow for 1 hour. Food intake was determined by reweighing the rodent chow after 1 hour of refeding. The stomach was isolated and gastric contents retrieved and weighed. The gastric emptying was determined using the following calculation: gastric emptying (%) = (1 – [stomach content wet weight/food intake]) × 100 (as described previously; ref. 4). In the second protocol, liquid-phase gastric emptying was assessed using the acetaminophen absorption test (54). Ten- to 11-week-old mice were fasted overnight and administered a solution containing acetaminophen at a dose of 100 mg/kg by gavage. Tail vein blood (50 μl) was collected into heparinized tubes at 0 and 15 minutes after acetaminophen
administration. Plasma was separated by centrifugation at 4°C and stored at −20°C until measurement of acetaminophen levels using an enzymatic-spectrophotometric assay (Diagnostic Chemicals Ltd.).

Islet isolation. After CO₂ euthanasia, pancreata from mice were inflated via the pancreatic duct with collagenase type V (0.7 mg/ml in HBSS), excised, and digested at 37°C for 10–15 minutes. The resulting digest was washed twice with cold HBSS (containing 0.25% wt/vol BSA), and islets were separated using a Histopaque density gradient (Sigma-Aldrich). The interface containing islets was removed and washed with HBSS plus BSA, and islets were resuspended in RPMI medium containing 10% FBS, 2 mM L-glutamine, 11 mM glucose, 100 U/ml penicillin, and 100 μg/ml streptomycin. After 4 hours of incubation at 37°C, islets were handpicked into fresh RPMI (containing 5.6 mM glucose) and allowed to recover overnight. Islets with preserved architectural integrity were either used for insulin secretion experiments or washed twice in PBS before being lysed by QIAshredder columns for RNA extraction using the RNeasy Micro Kit (QIAGEN). For insulin secretion, islets were preincubated in Krebs-Ringer buffer (KRB) containing 0.1% BSA, 10 mM HEPES (pH 7.4), and 2.8 mM glucose for 60 minutes. Batches of 10 islets were distributed into wells containing 0.5 ml KRB with either 2.8 or 16.7 mM glucose, with or without [d-Ala²]GIP, 1,3, or 10 nM [d-Ala²]GIP for 1 hour at 37°C. Reactions were terminated by the addition ice-cold absolute ethanol, and cell extracts were collected and stored at −80°C until measurement of cAMP using a cAMP RIA kit according to the manufacturer's instructions (BioMedical Technologies). cAMP levels were normalized to insulin content.

Gut RNA isolation. For gut mRNA analyses, 16- to 18-week-old mice were fasted in individual cages overnight (16 hours) and allowed access to a premeasured amount of rodent chow for 1 hour. Subsequently, duodenums were isolated and snap frozen. RNA isolation was carried out using TRIzol extraction (Invitrogen).

RNA analyses. Following RNA isolation, first-strand complementary DNA was synthesized from total RNA using the SuperScript III reverse transcriptase synthesis system (Invitrogen) and random hexamers. Real-time PCR was performed with the ABI Prism 7900 Sequence Detection System using TaqMan Gene Expression Assays and TaqMan Universal PCR Master Mix (Applied Biosystems). Levels of RNA transcripts were quantified using the 2−ΔΔCt method normalized to peptidyl-propyl isomerase A (cyclophilin).

Statistics. Results are presented as mean ± SEM. Statistical significance was determined using 1-way or 2-way ANOVA with Bonferroni post-hoc tests (as appropriate) using GraphPad Prism 4.0 (GraphPad Software Inc.). Statistical significance was noted when P values were less than 0.05.

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Figure 10

Gcgr–/–Glp1r–/– mice exhibit increased sensitivity to CCK. An IPGTT was performed in 22- to 24-week-old (A) WT, (B) Gcgr–/–, (C) Glp1r–/–, and (D) Gcgr–/–Glp1r–/– mice following treatment with 9 μg/kg of CCK-8 or vehicle. Insets depict the area under the glucose excursion curve (AUC) in mM.min and plasma insulin levels at 0 and 15 minutes following glucose challenge (n = 5–8). Values are expressed as mean ± SEM. *P < 0.05, **P < 0.01, ***P <0.001, CCK-8– versus saline-treated mice.
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Figure 11
Schematic of proposed model. (A) Loss of Gcgr in the liver markedly elevates plasma GLP-1 levels, which promotes glucose-stimulated insulin secretion during i.p. glucose challenge and inhibits gastric emptying in Gcgr−/− mice. (B) Loss of Glp1r in the Gcgr−/− mice results in preservation of the enteroinsular axis via compensatory mechanisms such as upregulation of Prf119 and Ccakr action in β cells, independent of the increase in GIP sensitivity.