Identification of Glucagon-Like Peptide 1 (GLP-1) Actions Essential for Glucose Homeostasis in Mice With Disruption of GLP-1 Receptor Signaling

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Glucagon-like peptide-1 (GLP-1) acts to control blood glucose via multiple mechanisms, including regulation of insulin and glucagon secretion, gastric emptying, satiety, and peripheral insulin sensitivity. However, the relative importance of these actions for regulation of blood glucose remains unclear. We demonstrate here a gene dosage effect for the incretin action of GLP-1, as heterozygous GLP-1R +/- mice exhibit an abnormal glycemic response to oral glucose challenge in association with reduced circulating levels of glucose-stimulated insulin. In contrast, GLP-1 signaling is not required for normal control of fasting and postabsorptive glucagon levels, and no significant changes were detected in the tissue content of pancreatic and intestinal proglucagon mRNA, glucagon-like immunoreactivity, or GLP-1 in GLP-1R -/- or +/- mice. Despite the demonstration that GLP-1 stimulates proinsulin gene transcription, pancreatic insulin mRNA transcripts were similar in wild-type and GLP-1R +/- mice. Furthermore, despite suggestions that GLP-1 regulates peripheral glucose disposal, whole-body glucose utilization was similar in wild-type and GLP-1R +/- mice under both basal and hyperinsulinemic conditions. These observations demonstrate that of the numerous physiological activities ascribed to GLP-1, only the incretin effect on pancreatic β-cells appears essential for regulation of glucose homeostasis in vivo. Diabetes 47:632–639, 1998

The observation that glucose administered via the gastrointestinal tract promotes a greater increase in insulin secretion than does glucose infused intravenously fostered research into the identity of specific gut-derived incretins, factors released by the intestine that potentiate glucose-dependent insulin secretion (1,2). One of the first incretins to be identified and characterized, glucagon-dependent insulinotropic polypeptide (GIP), is a 42-amino-acid peptide synthesized in and secreted from the proximal duodenum and jejunum. GIP stimulates glucose-dependent insulin secretion in rodents and humans. Blockade of GIP receptors with GIP peptide antagonists or immunoneutralization of GIP in rat intestinal extracts results in partial attenuation of the insulin response to glucose ingestion (3,4), consistent with the importance of GIP as a physiological incretin (5).

Interest in using an incretin-like molecule for enhancement of insulin secretion in patients with diabetes has recently focused on GLP-1, as the insulinotropic properties of GIP may be diminished in human subjects with diabetes (6). GLP-1, a product of the proglucagon gene, is released from intestinal L-cells in both the small and large intestine and potentiates glucose-dependent insulin secretion from the pancreatic β-cell both in vitro and in vivo (7–10). GLP-1 stimulates insulin secretion and lowers blood glucose in both normal subjects and patients with NIDDM (11,12). Remarkably, GLP-1 infusion also lowered blood glucose in patients with IDDM, suggesting a role for actions of GLP-1 independent of its effects on the pancreatic β-cell (12). Subsequent studies demonstrated that GLP-1 inhibits glucagon secretion (13,14), delays gastric emptying (15), and may promote glucose uptake or disposal in peripheral tissues (16–18), actions that would also contribute to lowering blood glucose in vivo.

The majority of GLP-1 actions are believed to be transduced by a single GLP-1 receptor (GLP-1R), originally cloned from pancreatic β-cells (19). To elucidate whether all of the actions currently attributed to GLP-1 are mediated by the known pancreatic GLP-1 receptor, studies with specific GLP-1 receptor antagonists would be extremely useful. The observation that a truncated lizard GLP-1–related peptide, exendin(9–39), binds to the GLP-1 receptor and functions as a GLP-1 antagonist (20) provided an important experimental approach for analysis of the short-term consequences of acute GLP-1 receptor blockade in vivo (21). Experiments with exendin(9–39) have provided strong evidence in support of a key role for GLP-1 as a physiologically relevant incretin in the control of insulin secretion and blood glucose (22–24). Nevertheless, recent data suggest that exendin(9–39) also binds to the GIP receptor (25,26) and hence may not be a pure GLP-1 receptor antagonist, complicating the interpretation of data derived from the use of exendin(9–39) in vivo.

A complementary approach for elucidating the biological importance of GLP-1 action involves the generation and analysis of mice with a null mutation in the GLP-1 receptor gene (27). GLP-1R -/- mice are viable and reproduce normally, permitting analysis of the physiological consequences of the complete disruption of GLP-1 signaling. Surprisingly, despite the potent inhibitory effects of GLP-1 on food and water...

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intake in the central nervous system (28–30), GLP-1R −/− mice are not obese and do not manifest defects in food intake (27), consistent with a possible redundant role for GLP-1 in the control of satiety. In contrast, after an oral glucose challenge, GLP-1R −/− mice exhibit increased levels of blood glucose in association with decreased levels of circulating insulin. GLP-1R −/− mice also exhibit fasting hyperglycemia and elevated levels of blood glucose after an intraperitoneal glucose challenge (27). Taken together, these observations provide strong evidence supporting a key role for GLP-1 in the control of blood glucose.

The pleiotropic activities of GLP-1 suggest that disruption of multiple physiological pathways, and not just glucose-stimulated insulin secretion, might contribute to the development of glucose intolerance in GLP-1R −/− mice. For example, if GLP-1 is essential for inhibitory control of gastric emptying (15,31), accelerated transit of nutrients from the stomach to the small intestine in GLP-1R −/− mice might explain, in part, the elevated blood glucose levels observed following oral glucose challenge (27). Nevertheless, as both fasting glucose and glycemic excursion after intraperitoneal glucose challenge are abnormal in GLP-1R −/− mice, mechanisms other than abnormal gastric emptying must account for glucose intolerance in GLP-1R −/− mice (27). Considering that GLP-1 inhibits glucagon secretion from the islets (13,32,33), loss of GLP-1 action on the islet A-cell might be associated with increased glucagon secretion that could also contribute to the development of hyperglycemia. Furthermore, GLP-1 might also lower blood glucose through peripheral effects on glucose uptake or hepatic glucose production (16,17). To address the relative importance of GLP-1 actions, we now present the results of more detailed characterization of mice with mutations in one or both GLP-1 receptor alleles.

RESEARCH DESIGN AND METHODS

Animal handling and propagation of mouse lines. GLP-1R −/− mice and age- and sex-matched control CD1 mice were maintained in the Toronto Hospital Animal Facility and raised on normal rat diet, as previously described (27). Mice were ~3 months old at the time of analysis, with the exception of the mice used for glucose-clamp studies, which were 12 months old. Animals were cared for according to guidelines established by the Animal Care Committee of the Toronto Hospital. For confirmation of heterozygote +/− and homozygous −/− genotype, genomic DNA prepared from tail snips was analyzed by Southern blotting.

RNA isolation and Northern blot analysis. Mice were anesthetized by CO2. The pancreas and small intestine were immediately removed, and total cellular RNA was isolated by the acid-guanidinium thiocyanate method (34). In some experiments, mRNA was isolated from the first 5 cm of small intestine distal to the duodenum and ileum samples from the last 5 cm of small intestine proximal to the colon. Total RNA (20 μg) was electrophoresed on a 1% (wt/vol) agarose-formaldehyde gel; the gel was stained with ethidium bromide to assess the integrity and migration of the RNA species, after which the RNA was transferred onto a nylon membrane (Nytran Plus; Schleicher and Schuell, Keene, NH) by capillary transfer. The RNA was fixed to the membrane by exposure to ultraviolet light in a Stratalinker UV Crosslinker (Stratagene, La Jolla, CA) and prehybridized and hybridized as previously described (35). For control to loading and transfer efficiency, the blots were rehybridized with a labeled cDNA for 18S rRNA.

Reversed phase extraction and analysis of peptides. The small intestine was removed and cleaned, and intestinal segments (5 cm duodenum and 5 cm distal ileum) and pancreas were homogenized twice in 5 ml extraction medium (1N HCl containing 5% [vol/vol] formic acid, 1% [vol/vol] trifluoroacetic acid, and 1% [wt/vol] NaCl) at 4°C. Peptides and small proteins were desorbed from the extracts by passage twice through a cartridge of C18 silica (Waters Associates, Milford, MA). Absorbed peptides were then eluted with 4 ml of 80% (vol/vol) isopropanol containing 0.1% (vol/vol) trifluoroacetic acid. The eluant was stored at −70°C before radioimmunoassay (RIA). RIA for glucagon-like immunoreactive peptides (GLIs = glucigenin + oxyntomodulin + glucagon) and immunoreactive GLP-I were carried out using two different antisera: K4023 ( Biospecific, Emeryville, CA) and GLP-1 R antisera, as described previously (36,37).

Plasma insulin and glucagon RIAs. Mice were killed after fasting (14–16 h) or 30 or 60 min following oral administration of glucose (1.5 mg glucose per gram body weight). Mice were anesthetized with CO2 and exsanguinated by cardiac puncture. Venous blood (800 μl) was collected by cardiac puncture into 80 μl of Trasylol:EDTA:Diprotin A (5,000 KIU/ml:32 mmol/l:0.1 nmol/l). The plasma was separated by centrifugation and stored at −80°C until assayed. Plasma insulin levels were determined in duplicate using an insulin RIA kit (Linco Research, St. Louis, MO) with rat insulin as a standard. Immunoreactive glucagon (IRG) levels were determined by RIA using antiserum 04A (Dr. R.H. Unger, Dallas, TX) that recognizes the free COOH-terminal end of glucagon (36,38).

Conditions for mice studied by hyperinsulinemic clamp. Control mice were sex-, weight-, and age-matched (1 year old) CD-1 mice obtained from Charles Rivers Laboratories (Wilmington, MA). The mice were fed Purina 5001 Laboratory Rodent Diet (23% protein, 5.5% fat, 49% carbohydrate, 5.3% fiber, 6.9% ash) and were housed in an approved, temperature-controlled facility on a 6:00 a.m.-6:00 p.m. light/dark cycle with access to water ad libitum. All protocols were approved in advance by the Washington University Animal Studies Committee.

Hyperinsulinemic clamp. Clamp experiments were carried out as previously described (39) with the following modifications. After a basal period of 60 min, control and experimental (GLP-1R −/−) mice were injected with insulin (27). Blood samples for determination of specific activity were taken at 5 or 20 mU · kg−1 · min−1 was started. Dextrose was begun at the same time, and the infusion rate was varied to maintain the blood glucose at ~8.9 mmol/l, the average glucose level in a feeding, wild-type, conscious mouse in our laboratory (40). Blood samples for determination of specific activity were taken 15 min before and at the end of the experimental period, when the blood glucose was in steady state. Blood glucose was measured using 5 μl of whole blood in the Hemocue glucose meter (Malmö, Sweden). Specific activity of glucose in whole blood was determined as described (39). The rate of appearance of glucose (Ra), which equals the rate of total-body glucose utilization (Rd) when the blood glucose is in steady state, was calculated by dividing the infusion rate of 3H glucose by the specific activity at the same time.

Statistical analysis. Statistical significance was calculated by analysis of variance using a SAS program (Statistical Analysis Systems, Cary, NC) for IBM computers. Data for clamp studies were analyzed using two-tailed t tests calculated by StatView 4.51 software (Abacus Concepts, Berkeley, CA).

RESULTS

Characterization of glucose tolerance in 6- to 8-week-old GLP-1R −/− mice previously demonstrated abnormal responses to both oral and intraperitoneal glucose challenges, in association with reduced levels of circulating insulin (27). To determine if similar abnormalities in incretin function were present in older mice, we next analyzed glucose-stimulated insulin levels in 3-month-old GLP-1R −/− male mice. These experiments demonstrated a significant reduction in plasma insulin after oral glucose challenge in the complete absence of GLP-1 signaling (Fig. 1A). Comparable reductions in glucose-stimulated insulin secretion have been consistently observed in older GLP-1R −/− mice up to 16 months of age (L.A.S., unpublished observations). To determine whether a partial reduction of GLP-1R expression was also associated with defective regulation of blood glucose, oral glucose tolerance and insulin levels were examined in GLP-1R −/− heterozygotes. Remarkably, oral glucose tolerance was clearly abnormal in GLP-1R −/+ mice, in association with a significant reduction in levels of glucose-stimulated insulin (Fig. 1B and C). A trend toward higher fasting blood glucose was also observed in GLP-1R −/− compared with −/+ control mice (Fig. 1D), although this difference was not statistically significant (P = 0.06 and 0.22 for −/+ vs. −/− female and male mice, respectively).

The effects of GLP-1 on regulation of blood glucose are complex and include stimulation of insulin secretion, inhibition of glucagon secretion, and potential modulation of glucose uptake and peripheral insulin sensitivity. Defective regulation of gastric emptying, in the absence of other metabolic
perturbations, is unlikely to explain the major phenotypic consequences of eliminating GLP-1 action, as GLP-1R –/– mice exhibit fasting hyperglycemia and abnormal glycemic excursion following intraperitoneal glucose challenge (27). To determine whether the increased blood glucose observed in GLP-1R –/– mice was attributable in part to increased levels of circulating glucagon, we measured the circulating and tissue levels of IGR in control and both GLP-1R +/- and GLP-1R –/– mice (Figs. 2 and 3). Despite the known effect of GLP-1 on inhibition of glucagon secretion, we did not detect increased levels of fasting glucagon in GLP-1R –/– mice (Fig. 2). Furthermore, glucagon levels were appropriately suppressed after oral glucose challenge in both wild-type and GLP-1R –/– mice (Fig. 2). These observations demonstrate that hyperglycemia associated with complete disruption of GLP-1 signaling is not attributable to increased levels of fasting glucagon. Furthermore, absence of the inhibitory action of GLP-1 on the pancreatic A-cell does not affect glucose-mediated suppression of circulating glucagon in vivo.

To determine whether loss of GLP-1 signaling is associated with abnormalities of proglucagon-derived peptide (PGDP) biosynthesis, we measured tissue levels of GLI and GLP-1 in the pancreas and intestine of wild-type, GLP-1R +/- and/GLP-1R –/– mice. Pancreatic GLI, but not GLP-1, was significantly higher in GLP-1R –/– mice compared with age-matched wild-type mice (Fig. 3). There was a trend toward increased levels of GLI in the duodenum and colon of GLP-1R –/– mice, but the difference did not reach statistical significance in the du-
denum, and the levels of GLP-I in duodenum and colon were similar for both wild-type and GLP-1R –/– mice (Fig. 3). The levels of both GLI and immunoreactive GLP-I were lower in the ileum of GLP-1R –/– mice, and the reduced ileal GLP-1 content was statistically significant (P < 0.05).

As previous studies have demonstrated that GLP-1 stimulates insulin gene expression, we examined the levels of insulin mRNA transcripts in wild-type and GLP-1R –/– mice. A representative Northern blot analysis is shown in Fig. 4A. A small decrease in levels of insulin mRNA transcripts was detected in some but not all GLP-1R –/– mice, suggesting that intact GLP-1 signaling is not associated with significant decreases in the levels of fasting insulin mRNA. No consistent change in the levels of pancreatic proglucagon mRNA transcripts was observed in GLP-1R –/– compared with wild-type control mice (Fig. 4A). Similarly, no significant differences in the relative levels of proglucagon or GIP mRNA transcripts were detected in RNA prepared from the jejunum or ileum of wild-type or GLP-1R –/– mice (Fig. 4B).

Because GLP-1 has been shown to exert peripheral effects on glucose disposal in vivo, we examined GLP-1R –/– mice for potential changes in glucose utilization under different physiological conditions. The basal anesthetized blood glucose and the basal tracer-determined rate of whole-body glucose utilization were not different (P = 0.9237 and 0.6563, respectively) in the GLP-1R –/– mice compared with age- and weight-matched CD1 control mice derived from the same genetic background (weights: 48.7 ± 2.1 vs. 49.7 ± 4.9 g for wild-type and GLP-1R –/– mice, respectively, P = 0.8611, Fig. 5). The blood glucose was maintained at ~160 mg/dl (8.9 mmol/l) during the hyperinsulinemic clamp periods (Fig. 5A). After establishment of hyperinsulinemia produced by infusion of 5 or 20 mU · kg⁻¹ · min⁻¹ of insulin, the rates of whole-body glucose utilization did not differ significantly (P = 0.5216 and 0.6019, respectively) between the GLP-1R –/– and control mice, although there was a tendency for the GLP-1R –/– mice to be more sensitive to insulin (Fig. 5B). The glucose infusion rates required to maintain the blood glucose at clamp levels were 15.8 ± 4.4 vs. 20.7 ± 4.4 mg · kg⁻¹ · min⁻¹ in wild-type and GLP-1R –/– mice, respectively, in the 5 mU · kg⁻¹ · min⁻¹ insulin clamp experiments, and 35.6 ± 1.1 and 39.3 ± 6.6 mg · kg⁻¹ · min⁻¹ in the same groups in the 20 mU · kg⁻¹ · min⁻¹ insulin clamp experiments.
DISCUSSION

After experiments demonstrating that GLP-1 potently stimulated glucose-dependent insulin secretion, initial concepts of GLP-1 action focused on the pancreatic β-cell. Subsequent studies demonstrated that incubation of GLP-1 with islet cell lines increased proinsulin gene transcription and mRNA transcripts (41), likely due in part to stimulation of β-cell cAMP accumulation (7). Although glucose-stimulated insulin secretion is clearly abnormal in GLP-1R –/– mice, we did not detect significant differences in the fasting levels of proinsulin mRNA transcripts in GLP-1R –/– mice. These observations suggest that GLP-1 signaling is essential for the normal β-cell secretory response to glucose. However, the control of proinsulin gene expression may exhibit considerably more plasticity, and maintenance of basal levels of proinsulin mRNA in the fasting state is not dependent on intact GLP-1 signaling in vivo.

The demonstration that GLP-1R +/+ mice also exhibit glucose intolerance and diminished glucose-stimulated insulin levels provides indirect evidence suggesting that even reduced GLP-1R expression may compromise β-cell function. These data imply that a reduction in GLP-1R signaling below a critical threshold level is not sufficiently compensated for by other insulin secretagogues, such as glucose alone or GIP, resulting in a subnormal β-cell response to glucose. Although we did observe slightly higher levels of fasting blood glucose levels in GLP-1R +/- mice, these differences were not statistically significant, perhaps because of the number of mice studied. In contrast, complete loss of GLP-1R function is associated with the development of fasting hyperglycemia in GLP-1R –/– mice (27). Taken together, the detection of a gene dosage phenotype for β-cell GLP-1R function in heterozygous GLP-1R +/- mice provides new evidence supporting the physiological importance of GLP-1 for regulation of glucose-stimulated insulin secretion in vivo.

Several studies have shown that GLP-1 inhibits glucagon secretion in rodents (13), normal human subjects (14), and patients with diabetes (42,43). The effects of GLP-1 on glucagon secretion may be indirect, via insulin secretion, or...
GLP-1R –/– mice, despite concomitant reductions in glucose- that disruption of GLP-1 signaling might lead to derepression of glucagon biosynthesis and secretion, as well as increased levels of circulating glucagon that contribute to the development of hyperglycemia in GLP-1R –/– mice. In contrast, fasting glucagon levels were normal and glucagon levels were appropriately suppressed following oral glucose loading in GLP-1R –/– mice, despite concomitant reductions in glucose-stimulated insulin secretion. These data indicate that glucose-stimulated glucagon suppression is not dependent on intact GLP-1 signaling in vivo, demonstrating redundancy in the pancreatic α-cell response to glucose.

The lack of changes in circulating glucagon in GLP-1R –/– mice might be explained by compensatory changes in proglucagon biosynthesis. Despite the normal levels of circulating glucagon, a small but significant increase in pancreatic GIP content was observed in GLP-1R –/– mice, consistent with disruption of GLP-1 inhibition of pancreatic proglucagon biosynthesis. GLP-1–mediated suppression of glucagon secretion and islet glucagon content has been previously reported in studies using isolated rat islets in vitro (47). In contrast, GLP-1 had no effect on proglucagon mRNA transcripts in isolated rat islets (47), consistent with our observations that the relative levels of proglucagon mRNA transcripts were normal in the pancreas and intestine of GLP-1R –/– mice. Intriguingly, a decrease in the tissue content of PGDPs was observed in the ileum, and this decrease was statistically significant for immunoreactive GLP-1. The physiological basis for this observation remains unclear; although GLP-1 receptors have been localized to the small intestine (48,49), the specific intestinal cell type(s) that expresses the GLP-1 receptor in the ileum has not yet been identified. The extremely low circulating levels of GLP-1 in the mouse preclude attempts at ascertaining whether the increased levels of ileal GLP-1 are also associated with corresponding changes in the levels of circulating GLP-1 in vivo.

The effects of GLP-1 on glucose uptake and disposal in tissues such as liver, adipose tissue, and muscle remain unclear. GLP-1 enhanced insulin-mediated glucose uptake in 3T3-L1 adipocytes (50) and isolated rat adipocytes (51), and it increased glucose utilization and incorporation into glycogen in both muscle and liver of normal and diabetic animals (18,52,53). GLP-1 infusion in six healthy subjects receiving intravenous glucose infusion enhanced both insulin-dependent glucose disappearance and glucose effectiveness, and GLP-1 appeared to enhance glucose disappearance in test subjects receiving a 50-g fat meal, suggesting a direct or indirect effect of GLP-1 on glucose disposal (16). In contrast, studies with healthy human subjects receiving somatostatin infusion to inhibit endogenous insulin secretion demonstrated no effect of GLP-1 on glucose elimination (54); and infusion of GLP-1 into subjects with well-controlled NIDDM did not affect insulin sensitivity during a hyperinsulinemic euglycemic clamp (43). These latter observations are consistent with our data demonstrating no change in whole-body glucose utilization during hyperinsulinemic clamp studies in GLP-1R –/– mice. Taken together, the data suggest that fasting hyperglycemia and glucose intolerance in mice with a disruption of the pancreatic GLP-1 receptor are unlikely to be attributable to significant changes in insulin sensitivity or glucose utilization.

As GLP-1 is predominantly considered important for control of blood glucose in the postabsorptive state, the finding of fasting hyperglycemia in mice with GLP-1R disruption (27) is perhaps unexpected. GLP-1 infusion has been shown to lower fasting plasma glucose in healthy normal subjects (14) and patients with NIDDM (55,56), and this reduction in fasting glucose is generally attributable to improved β-cell function. Although GLP-1R –/– mice exhibit fasting hyperglycemia, fasting insulin levels do not appear to be increased, as shown here and in Scrocchi et al. (27). Furthermore, because we did not detect significant increases in fasting glucagon levels or changes in insulin sensitivity, the mechanism responsible for fasting hyperglycemia in GLP-1R –/– mice remains unexplained. Further analysis of the actions of GLP-1 for control of fasting hyperglycemia in normal and GLP-1R –/– mice appears warranted.

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REFERENCES


L.A. SCROCCHI AND ASSOCIATES


Author Queries (please see Q in margin and underlined text)

Q1: As meant—in one or both (original had “in or both”)?
Q2: Please indicate who made these unpublished observations.
Q3: Should this be three or four knockout mice?

The editor separated 2A and 2B into figs. 2 and 3 for clarity. Citations of figures in text were changed to go along with this, but you may want to double check that they still refer to the correct figures.

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