Glucagon receptor antagonist-mediated improvements in glycemic control are dependent on functional pancreatic GLP-1 receptor

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Gu W, Winters KA, Motani AS, Komorowski R, Zhang Y, Liu Q, Wu X, Rulifson IC, Sivits G Jr, Graham M, Yan H, Wang P, Moore S, Meng T, Lindberg RA, Véniant MM. Glucagon receptor antagonist-mediated improvements in glycemic control are dependent on functional pancreatic GLP-1GLP-1 receptor. Am J Physiol Endocrinol Metab 299: E624–E632, 2010. First published July 20, 2010; doi:10.1152/ajpendo.00102.2010.—Antagonism of the glucagon receptor (GCGR) is associated with increased circulating levels of glucagon-like peptide-1 (GLP-1). To investigate the contribution of GLP-1 to the antidiabetic actions of GCGR antagonism, we administered an anti-GCGR monoclonal antibody (mAb B) to wild-type mice and GLP-1 receptor knockout (GLP-1R KO) mice. Treatment of wild-type mice with mAb B lowered fasting blood glucose, improved glucose tolerance, and enhanced glucose-stimulated insulin secretion during an intraperitoneal glucose tolerance test (ipGTT). In contrast, treatment of GLP-1R KO mice with mAb B had little efficacy during an ipGTT. Furthermore, pretreatment with the GLP-1R antagonist exendin-(9–39) diminished the antihyperglycemic effects of mAb B in wild-type mice. To determine the mechanism whereby mAb B improves glucose tolerance, we generated a monoclonal antibody that specifically antagonizes the human GLP-1R. Using a human islet transplanted mouse model, we demonstrated that pancreatic islet GLP-1R signaling is required for the full efficacy of the GCGR antagonist. To identify the source of the elevated GLP-1 observed in GCGR mAb-treated mice, we measured active GLP-1 content in pancreas and intestine from db/db mice treated with anti-GCGR mAb for 8 wk. Elevated GLP-1 in GCGR mAb-treated mice was predominantly derived from increased pancreatic GLP-1 synthesis and processing. All together, these data show that pancreatic GLP-1 is a significant contributor to the glucose-lowering effects observed in response to GCGR antagonist treatment.

Studies performed with rodent models suggest that GCGR antagonism may alleviate hyperglycemia associated with type 2 diabetes. GCGR knockout (KO) mice and mice treated with GCGR antisense oligonucleotides (ASOs) exhibited improved fasting glucose, glucose tolerance, and pancreatic β-cell function (7, 14, 19, 21). Recently, we described the generation and characterization of several high-affinity GCGR-antagonizing antibodies, including mAb B and mAb Ac. These antibodies effectively improved glycemic control without causing hypoglycemia in both mice and cynomolgus monkeys (9, 26). Consistent with the observations made from the GCGR KO and GCGR ASO-treated mice, compensatory elevations of both glucagon and active glucagon-like peptide-1 (GLP-1) levels were observed in the treated animals (9, 26). GLP-1 is a hormone with multiple biological activities, including stimulation of glucose-dependent insulin secretion (GSIS), β-cell preservation, inhibition of gastric emptying, and glucagon secretion (5). It has been hypothesized that, in both GCGR KO and GCGR ASO-treated mice, a compensatory increase of GLP-1 production in islets may contribute to improved glucose homeostasis and β-cell function (7, 21, 22). However, whether the antidiabetic effects of GCGR antagonism are dependent on, or only associated with, enhanced levels of GLP-1 has not been clearly determined.

Several studies were performed to assess whether GLP-1 directly contributes to the GCGR antagonist-mediated improvement in glucose homeostasis. In the first study, we used wild-type and GLP-1R KO mice to evaluate the efficacy of the GCGR antagonist mAb B. In a follow up study, we extended our findings by administering the GLP-1R receptor antagonist exendin-(9–39) [Ex-(9–39)] to mAb B-treated mice. To gain mechanistic insight into the origin of the increased GLP-1 signal associated with GCGR antagonism, we generated an anti-human, GLP-1R-specific monoclonal antibody antagonist (hGLP-1R mAb). In human islet transplanted mice, the hGLP-1R mAb specifically reduced mAb Bc efficacy. Our findings demonstrate that full efficacy of the GCGR antagonist requires a functional pancreatic GLP-1 receptor (GLP-1R).

MATERIALS AND METHODS

Experimental animals. All mouse studies were conducted at Amgen Inc. and were performed in accordance with guidelines and regulations approved by the Institutional Animal Care and Use Committee. Mice were maintained on a 12:12-h light-dark cycle with free access to food and water. Administration of test articles was performed between 0700 and 0900 on animals that had been fed ad libitum. Various animal models and experimental designs were used to address a number of different physiological questions. Specifically, GLP-1R KO mice (from Dr. Daniel Drucker’s laboratory), fully back-crossed onto the C57BL/6 strain (currently maintained at...
Charles River Laboratories, San Diego, CA, and C57BL/6 (Charles River) mice were used to address the contribution of the GLP-1R to the blood glucose-lowering efficacy observed with GCGR antagonism; human islet-transplanted mice to determine the islet GLP-1R-dependent mechanism associated with the efficacy exhibited by GCGR antagonism; and db/db mice to investigate the sources of increased active GLP-1 in mice that had been treated with anti-GCGR antibody. Dose regimens were determined during pilot studies. Depending on the duration of action and the chosen dose of our anti-GCGR antibody, animals were injected every 3 days (Q3D) or every 5 days (Q5D). Male nude mice, 6–8 wk of age and female db/db mice, 5–6 wk of age were purchased from Harlan Laboratories (Indianapolis, IN).

Generation and characterization of anti-GCGR and anti-hGLP-1R antibodies. The generation and characterization of the anti-GCGR antibodies were described previously (26). Briefly, mAb B is a human IgG that antagonizes GCGR. In cells expressing recombinant mouse GCGR, mAb B inhibited glucagon-induced cAMP signal (IC50 ~1.8 nM). This antibody was used for acute in vivo pharmacology studies. To avoid potential immunogenicity, a mouse analog anti-GCGR mAb (mAb Ac) was used for the 3-wk study in db/db mice. In cell-based cAMP assays, mAb Ac exhibited an IC50 of 25 nM against the mouse GCGR. A similar chimera of mAb B (mAb Bc) was used for the generation of the hGLP-1R mAb. Phosphate-buffered saline (PBS) was used as the vehicle for all studies.

Anti-GCGR mAb effects in genetically engineered GLP-1R KO mice. Two groups of 13- to 14-wk-old male GLP-1R KO mice (n = 61 KO total; 32 received mAb B at 3 mg/kg) and two groups of 13- to 14-wk-old male C57BL/6 (n = 30/per group, n = 60 total; 30 received saline; and 30 received mAb B at 3 mg/kg) were sorted into groups having similar distributions based on body weight. These mice were injected intraperitoneally (ip) with either vehicle or mAb B at 10 mg/3.3 ml/kg 72 h before the ip glucose tolerance tests (ipGTT) were initiated. The ipGTT involved fasting the mice for 12 h (2100 to 0900) and measuring the fasting blood glucose from each mouse at ~0900 (time 0) for all 0 time points. Mice in 15-, 30-, or 90-min time point groups were injected ip with glucose (2 g/3.3 ml/kg body wt); blood glucose levels were measured at 15, 30, or 90 min after the glucose bolus injection using a hand-held glucometer (LifeScan, Milpitas, CA), and blood samples were collected at the same time points for insulin, glucagon, and active GLP-1 level determinations via liquid chromatography/tandem mass spectrometry (LC-MS/MS). Since mice with 15-min time points were used, only one time point per mouse was measured. Each data point represents the mean ± SE of 6–10 mice.

Anti-GCGR mAb effect in mice treated with the GLP-1R antagonist Ex-(9–39). Fifteen-week-old male C57BL/6 (n = 10/per group) mice were sorted into four groups having similar distributions based on body weight. These mice were injected ip first with either vehicle or mAb B at 10 mg/3.3 ml/kg and second with either vehicle (saline) or 10 µg/100 µl of mouse Ex-(9–39) (Bachem Americas, Torrance, CA) at 72 h and 20 min, respectively, before the initiation of the ipGTT. The Ex-(9–39) dose used to block the GLP-1R was determined in an independent pilot study. The ipGTT was performed as follows. Mice were fasted for 12 h (2100 to 0900), a fasted blood glucose measurement was taken at ~0900 (time 0), followed immediately by an ip injection of glucose (2 g/3.3 ml/kg body wt), and blood glucose levels were measured again 30 and 90 min after the glucose bolus injection.

hGLP-1R mAb activity assay. Control IgG or 10, 100, or 1,000 nM hGLP-1R antibody was added to cell lines expressing human or mouse GLP-1R, as previously described (15, 26). Cells were incubated at 37°C for 20 min before addition of serially diluted GLP-1-(7–36) amide (Bachem Americas) in the presence of 3-isobutyl-1-methylxanthine (Sigma-Aldrich, St. Louis, MO). Cells were stimulated at 37°C for 15 min. The AMP levels were quantified by homogeneous time-resolved fluorescence using the Cisbio cAMP Dynamic 2 kit (Cisbio US, Bedford, MA). The two-step protocol was followed per the manufacturer’s instructions.

Streptozotocin-induced diabetic mice. Male nude mice, 6–8 wk of age, were purchased from Harlan Laboratories and were aged to 14 wk to match the human islet-transplanted mice described in the next section. β-Cell ablation was induced in nude mice by streptozotocin (STZ, Sigma) injection (180 mg/kg body wt ip). Within 4 days of exhibiting elevated blood glucose levels, compromised mice (baseline glucose levels mean ± SE, 368 ± 19 mg/dl, n = 24) and a control non-STZ-injected group of age-matched mice (intact controls, baseline glucose 139 ± 3 mg/dl, n = 24) were randomized into treatment groups (n = 12) to receive either anti-GCGR antibody mAb Bc or vehicle. Mice were injected with mAb Bc (3 mg/10 ml/kg ip) or PBS (vehicle, 10 ml/kg ip). An ipGTT (2 g/10 ml/kg glucose ip after a 3-h fast, initiated at 0700) was then conducted 72 h after the injections of mAb Bc or vehicle. After the last time point of the ipGTT (90 min post-glucose), blood samples were collected by terminal decapitation without anesthesia into tubes containing EDTA and DPP-4 inhibitor (Millipore, Billerica, MA), final concentration 50 µM. Plasma was isolated and stored at −80°C for insulin, glucagon, and active GLP-1 level determinations.

Human islet transplantation. STZ-treated mice with a sustained blood glucose value of greater than 400 mg/dl were selected as recipients for transplantation. To maintain the health of the recipients until arrival of human islets, selected recipients were implanted subcutaneously with time-release insulin LinBits (LinShin Canada, Toronto, ON, Canada) according to the manufacturer’s instructions. Immediately before islet transplantation, the LinBits were removed from the upper back flank of the mice. To generate the human islet-transplanted mice, the kidney was exposed by left-flank incision under sterile conditions and was nicked at the inferior renal pole. PE-50 polyethylene tubing was used to deliver the human islets (2,500 islet equivalents purified from healthy donors, provided by Prodo Laboratories, Irvine, CA), as a small pellet, beneath the kidney capsule to the superior pole of the kidney. Following islet delivery, the nick was cauterized and the incision site sealed. Graft acceptance was defined as a prolonged period of euglycemia (<200 mg/dl) and the presence of human-specific insulin in the serum in response to glucose challenge. Immunohistological analysis of pancreas tissue upon harvest confirmed the absence of more than 90% of endogenous β-cells (before transplantation) in the human islet-transplanted mice due to the initial STZ treatment (data not shown).

Anti-GCGR mAb effect in human islet-transplanted mice. Human islet-transplanted mice and age-matched intact control (Intact, 14 wk of age) mice were subjected to an oral glucose tolerance test (OGTT, 4 g/10 ml/kg) after a 4-h fast (initiated at 0600). The area under the curve (AUC) for glucose was then used to randomize the mice into treatment groups (data not shown). To test the efficacy of the anti-GCGR antibody, human islet-transplanted and intact mice were injected with 3 mg/kg mAb Bc (3 mg/10 ml/kg ip) or PBS (vehicle, 10 ml/kg ip). An ipGTT (2 g/10 ml/kg glucose ip after a 3-h fast, initiated at 0700) was then conducted 72 h after the injections of anti-GCGR antibody or vehicle. After restoration of food and overnight recovery, blood samples were collected by tail nick into tubes containing EDTA and DPP-4 inhibitor (Millipore) at a final concentration of 50 µM. Plasma was isolated and stored at −80°C for insulin, glucagon, and active GLP-1 level determinations.

Effect of combined anti-GCGR mAb and hGLP-1R mAb in human islet-transplanted mice. The same groups of mice used for the previous study were allowed to recover for 29 days before initiation of the second series of experiments. Mice were then injected with anti-human GLP-1R antibody (30 mg/10 ml/kg ip) or PBS (vehicle, 10 ml/kg ip) on day 1. On day 2, all mice were injected with mAb Bc (3 mg/10 ml/kg ip). On day 5, an ipGTT was conducted as described above. Terminal blood samples were collected on day 9 and processed as described above.
GLP-1 contents in db/db mice chronically treated with anti-GCGR monoclonal antibody mAb Aec. Female db/db mice, 5–6 wk of age, were purchased from Harlan Laboratories. Mice were randomized and divided into two groups: mAb Aec treated (10 mg/kg ip; 1 injection per week) and PBS treated (equal volume; 1 ip injection per week) for 8 wk. At the time of harvest, the dorsal pancreas and proximal duodenum were immediately excised from euthanized mice, snap-frozen, and stored at −80°C until processing. Tissue was homogenized in 0.18 M HCl and 70% ethanol and incubated with shaking overnight at 4°C. Insoluble material was removed by centrifugation. In addition, plasma was collected for active GLP-1 level assessments. DPP-4 inhibitor and aprotinin were added to each blood sample to final concentrations of 100 μM and 85 μg/ml, respectively. The protein extracts and plasma were assessed for active GLP-1 content by use of the Mouse Endocrine Milliplex Kit (Millipore) according to the manufacturer’s instructions.

Plasma analysis. DPP-4 inhibitor and aprotinin were added to each blood sample collected to final concentrations of 100 μM and 85 μg/ml, respectively. Glucose levels were analyzed using a hand-held glucometer (LifeScan). Plasma insulin, active GLP-1, and glucagon levels were measured using the Human or Mouse Endocrine Milliplex Kit (Millipore) per the manufacturer’s protocol. Cross-reactivity of the active GLP-1 and glucagon immunoassays with other preproglucagon-derived peptides is undetectable.

**Quantitative real-time PCR.** After euthanasia by asphyxiation, the dorsal pancreas was removed from mice, immediately immersed in RNAlater (Ambion, Foster City, CA), and stored at −80°C until processed. Total RNA was extracted using TRizol Reagent (Invitrogen, Carlsbad, CA). The samples were further cleaned, and genomic DNA was removed using the RNeasy Plus Mini system (Qiagen, Valencia, CA). All protocols were followed according to the manufacturer’s instructions.

Quantitative real-time PCR was performed on an MX3000P Stratagene machine using Brilliant QRT-PCR and 1-Step reagents (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. All samples were analyzed in at least duplicate and corrected using mouse cyclophilin-A (NM_008907) as an internal standard. Mouse preproglucagon (NM_008907) gene sequences for probe and primers: probe: 5’-AGGGCACATTCACCAGCGACTACAGC-3’; primer forward: 5’-GAATGAAGACAAACGCCACTCA-3; primer reverse: 5’-GCGGGGAGTCCAGGTATT-3’. Statistical analysis. Statistical analyses were performed with StatView 5.0.1, GraphPad Prism version 5.01, or SAS version 9.1.3 by unpaired t-tests or using ANOVA for multiple comparisons.

**RESULTS**

Effects of anti-GCGR monoclonal antibody mAb B in wild-type C57BL/6 and GLP-1R KO mice during ipGTT. We (13) recently described several anti-GCGR antibodies that decreased blood glucose and improved glucose tolerance in two species (mouse and cynomolgus monkey). In addition, our previously published studies established the dosing protocols used for the current studies. Efficacy of anti-GCGR antibodies was accompanied by an increase in plasma GLP-1 levels (9, 26). To investigate the contribution of the GLP-1 pathway to the observed efficacy, ipGTTs were performed in GLP-1R KO and wild-type mice 72 h after treatment with either vehicle or mAb B. We chose to perform ipGTT rather than OGTT to reduce the incretin effect of endogenous GLP-1 release and to address the decreased rate of gastric emptying caused by elevated circulating GLP-1 levels, which we thought would confound our interpretations. Blood glucose, plasma insulin, glucagon, and active GLP-1 levels were measured at the indicated time points. Treatment with mAb B reduced fasting blood glucose in wild-type mice (Fig. 1A, t = 0 min, control 63 ± 7 mg/dl vs. mAb B 46 ± 2 mg/dl, P = 0.03). Moreover, mAb B significantly improved glucose clearance at each time point measured (Fig. 1A) in the wild-type mice. In contrast, only one time point (t = 30 min) showed a modest improvement in glucose clearance (Fig. 1A) with administration of mAb B to GLP-1R KO mice (P = 0.0015).

In this study, we performed only one blood collection per mouse to minimize variability in blood glucose and hormonal levels due to multiple bleeds. Each individual time point represents the average of a different set of individual mice. As such, an evaluation of AUC cannot be performed. Therefore, the data were analyzed using an analysis of variance (ANOVA) for each parameter. Since a statistically significant interaction between mouse line and treatment was observed (P = 0.0021), an additional analysis by genotype was performed. For glucose, there is a statistically significant treatment effect for the C57BL/6 mice (P < 0.0001) but not for the GLP-1R KO mice (P = 0.7051), where glucose is significantly lower for the mAb B-treated group. Compared with the vehicle-treated mice, a single administration of mAb B caused significant elevation in active GLP-1 levels in both wild-type and GLP-1R KO mice (Fig. 1B, P < 0.0001 for both treated groups). Of note, the wild-type mice treated with mAb B showed enhanced insulin release at t = 15 min, corresponding to the peak glucose concentration (Fig. 1C, P = 0.039) whereas the treated GLP-1R KO mice did not. Consistent with the action of GLP-1 in GSIS, there were no significant increases in insulin levels at other time points measured in treated wild-type mice where glucose levels were not elevated (Fig. 1C). As expected, glucagon levels were increased in both groups of mice treated with mAb B (wild-type and GLP-1R KO mice, Fig. 1D).

Effects of anti-GCGR mAb B in wild-type C57BL/6 mice treated with Ex-(9–39) during ipGTT. To ascertain whether the dramatic reduction of response to mAb B in GLP-1R KO mice is physiologically relevant and not caused by developmental defects, we used the GLP-1R antagonist Ex-(9–39), a pharmacological agent known to bind and antagonize mammalian GLP-1R (8). Treatment of wild-type mice with Ex-(9–39) 20 min prior to glucose challenge produced a significant increase in basal glucose [Fig. 2A; t = 0 min, vehicle 79 ± 3 mg/dl vs. Ex-(9–39) 105 ± 4 mg/dl, unpaired t-test, P < 0.001]. This glucose increment was sustained throughout the ipGTT. Moreover, treatment with Ex-(9–39) significantly reduced the mAb B-mediated improvement in basal glucose levels [Fig. 2A; t = 0 min, mAb B 68 ± 3 mg/dl vs. mAb B + Ex-(9–39) 97 ± 3 mg/dl, unpaired t-test, P < 0.0001]. Importantly, Ex-(9–39) treatment nearly abrogated all glucose reduction effects of mAb B treatment throughout the ipGTT. As shown in Fig. 2B, Ex-(9–39) did not change the glucose excursion AUC0–90 in the vehicle-treated group, however, Ex-(9–39) treatment significantly worsened the glucose excursion AUC0–90 of the mAb B-treated group (P < 0.01).

*In vitro characterization of the anti-human GLP-1R monoclonal antibody hGLP-1R mAb.* Functional specificity of the GLP-1R monoclonal antibody was evaluated in cell lines expressing recombinant human or mouse GLP-1R. The incubation of the hGLP-1R mAb at 100 and 1,000 nM, but not of 100 nM control IgG, induced a rightward shift of the GLP-1-stimulated dose-response curve in hGLP-1R-expressing cells (Fig. 3A). The estimated IC50 against the human GLP-1R is...
In contrast, as shown in Fig. 3B, the hGLP-1R mAb did not affect the GLP-1-stimulated cAMP accumulation in cells expressing the mouse GLP-1R, demonstrating the specific antagonistic activity toward the human GLP-1R.

Effects of anti-GCGR mAb Bc in human islet transplanted mice with and without human GLP-1R antagonist. To further assess the involvement of the local pancreatic GLP-1 pathway, a human-specific GLP-1R antagonistic mAb and human islet-transplanted mice were utilized. We first conducted pilot studies to evaluate the efficacy of mAb Bc, an anti-GCGR antibody, in moderately compromised, STZ-treated nude mice. For the STZ study, the mice were used within 4 days of reaching elevated blood glucose levels with an average of 370 mg/dl. This suggests that, although in decline, the β-cell pool remaining in these mice is not completely ablated at this early point of the STZ treatment but is less than what is observed in normal mice. We expected a reduction in efficacy of anti-GCGR mAb in STZ-treated mice; therefore, to preserve some efficacy, we used mice at this early stage of β-cell destruction.

Treatment of mice with STZ, a selective pancreatic β-cell toxin, did not result in significantly reduced insulin levels compared with intact mice 90 min post-glucose bolus in an ipGTT at this early point in the STZ-mediated progression of pancreatic β-cell destruction (Table 1). Single administration of mAb Bc (3 mg/kg) significantly improved glucose clearance during ipGTT in both groups (Fig. 4A and Table 1). Although administration of mAb Bc led to a reduction in glucose excursion AUC in both intact and STZ mice, the magnitude of the decrement was greater for the intact mice than for the STZ mice (102 and 63%, respectively) (Fig. 4B), suggesting that STZ treatment may have impaired but not eliminated the potentiation of GSIS induced by mAb Bc treatment. The residual efficacy of mAb B in our STZ-treated mice is consistent with the report published by Brand et al. (1), which demonstrated normalization of hyperglycemia by immunoneutralization of endogenous glucagon in moderately STZ-diabetic rats. Consistent with previous studies, treatment with mAb Bc caused elevations in glucagon and active GLP-1 levels (only reaching significance for GLP-1) in both intact and STZ mice compared with their respective control groups (Table 1). Insulin levels were not significantly different between the mAb Bc-treated and control groups at the end of the ipGTT (Table 1), analogous to the observations we made in C57BL/6 mice (Fig. 1C) at the 90-min time point.

In follow-up studies, we determined the efficacy of mAb Bc in human islet-transplanted mice during an ipGTT. In contrast to what is described in the previous study, the STZ-treated mice used for human islet transplant (HIT) were selected at a later time point after the STZ treatment was initiated. These mice had sustained elevated blood glucose levels greater than 400 mg/dl; in fact, most mice had blood glucose levels higher than 500 mg/dl. Notably, basal glucose levels were significantly lower in human islet-transplanted vehicle-treated mice compared with vehicle-treated STZ mice (Fig. 4A, 0 min; Fig. 4A, t = 0, HIT 78 ± 6 mg/dl vs. STZ 360 ± 25 mg/dl), demonstrating that insulin secreted from transplanted human islets contributed to reduced blood glucose levels in these mice. A single injection of mAb Bc (3 mg/kg) significantly improved blood glucose clearance and glucose excursion AUC during ipGTT in intact control mice (Fig. 4, C and D). In
human islet-transplanted mice, a single injection of mAb Bc (3 mg/kg) significantly improved blood glucose clearance at the 15- and 30-min time points, whereas the glucose excursion AUC trended lower during the ipGTT. The lack of statistical significance in the glucose excursion AUC decrement with treatment is likely due to the highly glucose-tolerant nature of the human islet-transplanted mice and the resulting small window available to demonstrate efficacy (as evidenced by the large difference in glucose AUC between vehicle-treated intact mice and human islet-transplanted mice). Consistent with previous study results, a single administration of mAb Bc resulted in increases in glucagon and active GLP-1 levels, not, however, reaching statistical significance compared with their respective control groups (Table 2). Under fed conditions, mAb Bc-treated intact mice had significantly reduced blood glucose levels, whereas insulin levels trended lower compared with control groups. Fed blood glucose levels trended lower and insulin levels were significantly reduced in human islet-transplanted mice (Table 2). This observation is consistent with elevated GLP-1 levels increasing insulin levels only under conditions of relative hyperglycemia.

After establishing the efficacy of mAb Bc in human islet-transplanted mice, we proceeded to investigate the contribution of pancreatic GLP-1 receptor signaling to the observed efficacy of GCGR antagonism. By utilizing a monoclonal antibody that specifically antagonizes human GLP-1R but not mouse GLP-1R, we selectively blocked islet GLP-1R in the human islet-transplanted mouse model. Interestingly, in the human islet-transplanted mouse model, when pancreatic GLP-1Rs were blocked by pretreatment with the anti-hGLP-1R mAb, the beneficial effects of mAb Bc treatment were significantly diminished during ipGTT (Fig. 5). In contrast, pretreatment with anti-human GLP-1R mAb in intact mice had no significant impact on mAb Bc-induced improvement during ipGTT (Fig. 5). This is consistent with the in vitro observation in which the hGLP-1R mAb does not inhibit mouse GLP-1R signaling.

GLP-1 content in db/db mice treated with anti-GCGR mAb Ac for 8 wk. We (26) recently described the generation and characterization of several anti-GCGR mAbs. A high-affinity chimera (mAb Ac) with an IC50 of ~25 nM against the mouse GCGR in cell-based signaling assays was used for chronic studies to avoid potential immunogenicity. To further determine the origin of the increased GLP-1 levels associated with...
anti-GCGR mAb treatment, db/db mice were treated with vehicle or mAb Ac for 8 wk. As expected, plasma active GLP-1 levels were significantly increased in mAb Ac-treated db/db mice (Fig. 6A). Compared with the control group, pancreatic active GLP-1 content increased dramatically in mAb Ac-treated mice (Fig. 6B). Consistent with the accumulation of the GLP-1 peptide, pancreatic preproglucagon mRNA was also dramatically upregulated (Fig. 6C). GLP-1 content in the gut was not significantly different between the vehicle-treated and mAb Ac-treated mice (Fig. 6D).

**DISCUSSION**

Although it is generally accepted that inhibition of the glucagon signaling pathway leads to reduced glycemia by suppressing glucose production in the liver, recent studies suggest that inhibition of glucagon signaling may have additional benefits due to an increase in circulating levels of GLP-1 (7, 14, 21). However, to what extent GLP-1 action contributes to the full efficacy of a GCGR antagonist on glycemic control remains to be determined. The present data demonstrate that inhibition of glucagon signaling may have additional benefits due to an increase in circulating levels of GLP-1 action, as minimal beneficial effects of anti-GCGR mAb treatment were detected in GLP-1R KO mice. In addition, blocking GLP-1R function by pretreatment with Ex-(9–39) confirmed the importance of GLP-1R-dependent pathways for the action of anti-GCGR mAb, since the presence of the GLP-1R antagonist diminished the majority of the beneficial effects observed with mAb treatment on both fasted blood glucose levels and glucose excursion following an ip glucose bolus. Finally, using a human GLP-1R-specific blocking antibody, we demonstrated that the efficacy of an anti-GCGR antibody is significantly abrogated in human islet-transplanted mice. Hence, using multiple experimental approaches including species-specific GLP-1R antagonism and genetic loss of GLP-1R action, we demonstrated that complete GCGR antagonist efficacy requires a functional pancreatic GLP-1 receptor.

Both GLP-1 and glucagon are encoded by the same preproglucagon gene but are posttranslationally processed in a cell type-specific manner by the predominant prohormone convertases. GLP-1 is produced from the preproglucagon peptide by prohormone convertase-1/3 (PC1/3) (5). The major site of GLP-1 production is in the enteroendocrine L-cells of the intestine, with a minor amount produced from pancreatic β-cells and the central nervous system (5, 10, 16). Previous

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**Table 1. Hormone analysis of intact and STZ-treated mice treated with vehicle or anti-GCGR monoclonal antibody mAb Bc**

<table>
<thead>
<tr>
<th>Model</th>
<th>Treatment</th>
<th>Blood Glucose, mg/dl</th>
<th>n</th>
<th>Insulin, ng/ml</th>
<th>n</th>
<th>Glucagon, ng/ml</th>
<th>n</th>
<th>Active GLP-1, pg/ml</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact</td>
<td>Vehicle</td>
<td>200 ± 20</td>
<td>12</td>
<td>0.56 ± 0.10</td>
<td>12</td>
<td>0.041 ± 0.008</td>
<td>12</td>
<td>31 ± 6</td>
<td>12</td>
</tr>
<tr>
<td>mAb Bc</td>
<td></td>
<td>90 ± 9*</td>
<td>12</td>
<td>0.55 ± 0.11</td>
<td>12</td>
<td>0.43 ± 0.10</td>
<td>12</td>
<td>260 ± 70***</td>
<td>12</td>
</tr>
<tr>
<td>STZ</td>
<td>Vehicle</td>
<td>530 ± 30</td>
<td>12</td>
<td>0.48 ± 0.07</td>
<td>12</td>
<td>0.058 ± 0.010</td>
<td>12</td>
<td>26 ± 4</td>
<td>12</td>
</tr>
<tr>
<td>mAb Bc</td>
<td></td>
<td>200 ± 40***</td>
<td>12</td>
<td>0.52 ± 0.05</td>
<td>12</td>
<td>0.34 ± 0.08</td>
<td>12</td>
<td>210 ± 50**</td>
<td>12</td>
</tr>
</tbody>
</table>

Values are means ± SE. STZ, streptozotocin; GCGR, glucagon receptor. Blood collections were performed 90 min post glucose bolus. *P < 0.05; **P < 0.01; ***P < 0.001 vs. vehicle-treated control group.
studies described by Gelling et al. (6) (GCGR KO) and Sloop et al. (20) (GCGR ASO), have shown that pancreatic amidated GLP-1 and islet active GLP-1 contents are increased with GCGR signaling suppression. In addition, GCGR antisense-treated animals also have increased preproglucagon mRNA expression. However, no changes in gut amidated and/or active GLP-1 content or preproglucagon mRNA levels were observed, even when the gut was sectioned into colon, ileum, and jejunum. Our experiments investigating the source of the elevated GLP-1 observed in GCGR mAb-treated mice yielded consistent results. Eight-week anti-GCGR mAb treatment of db/db mice increased the levels of preproglucagon mRNA transcripts and active GLP-1 peptide content in the pancreas but not in the small intestine (Fig. 6). We performed additional studies to measure the GLP-1 content from each segment of the entire intestine in mice treated with GCGR mAb acutely. We found there were no statistically significant differences in active GLP-1 content of respective intestine segments between vehicle, GLP-1 receptor agonist, or GCGR mAb-treated mice (data not shown). Our data confirm that, after both chronic and acute treatment with anti-GCGR antibody, GLP-1 expression is not increased in the small intestine. Although we did not directly confirm increased expression in the pancreas after acute dosing with anti-GCGR antibody, the sum of the data would suggest that pancreas-derived GLP-1 mediates the effects of anti-GCGR antibody both acutely and chronically.

Our work demonstrated increased pancreatic expression and processing of preproglucagon to GLP-1 following GCGR antagonist treatment; however, the exact cell type where the prohormone processing occurred remains to be determined. Several lines of evidence support the hypothesis that pancreatic α-cells are likely responsible for the increased processing and release of GLP-1. First, our work and others’ (8, 13) demonstrate a compensatory α-cell hyperplasia following GCGR antagonism. Second, literature data show that pancreatic α-cells express PC1/3 and are capable of producing active GLP-1. For example, in a publication by Nie et al. (18), the authors describe immunohistochemical characterization of PC2 and PC1/3 expression in α- and β-cells of control and STZ-treated rats. Both PC1/3 and PC2 expression increased in α-cells of STZ-treated rats, which correlated with the increased immunocytochemical staining of GLP-1_{1–37} and GLP-1_{1–37} in α-cells. A separate publication by Varnell et al. (24) demonstrated GLP-1 immunoreactivities stored with glucagon in the core of the secretory granule of the pancreatic α-cell. Therefore, it is reasonable to hypothesize that, in mice treated with GCGR mAb chronically, a compensatory increase in GLP-1 synthesis and processing coupled with the observed expansion of the α-cell population could lead to increased circulating GLP-1 levels.

The primary component of the GLP-1-mediated reduction of postprandial glucose levels has been shown to occur through stimulation of insulin release via GLP-1R activation on pancreatic β-cells in a glucose-dependent manner (5). Augmented peak insulin levels of anti-GCGR mAb B-treated C57BL/6 mice coincides with the peak glucose level during an ipGTT (Fig. 1, A and C). Under conditions of normoglycemia, one would not expect to observe elevated insulin levels in response to GLP-1R activation. In fact, we observed slight reductions of insulin levels in anti-GCGR mAb-treated mice under nonfasted conditions. However, GLP-1 receptors are expressed in many other tissues, including the stomach, lung, brain, heart, and nerve terminals in the hepatic portal vein (12, 23). Thus, the function of GLP-1 is not limited to the islets. The extrapancreatic actions of GLP-1 include inhibition of gastric emptying, regulation of appetite and satiety, suppression of glucose production, and promotion of hepatic glucose uptake (2, 4, 20, 23, 25, 27).

We previously reported that in mice and monkeys, GCGR mAb treatment improved glucose tolerance and was accompanied by an increased plasma active GLP-1 [GLP-1_{1–36}-amide and GLP-1_{7–37}] concentration (9, 26). We now further extend these findings by demonstrating that single-dose
GCGR mAb treatment improved glucose tolerance in early-stage STZ-treated mice, (Fig. 4A). The relative loss of β-cells in the STZ model contributes to the attenuated efficacy of the anti-GCGR antibody in this model compared with intact mice. The GCGR mAb exhibited superior glucose reduction efficacy in intact mice compared with early-stage STZ-treated mice (102 and 63% glucose AUC reduction, respectively, relative to control mice), suggesting that full efficacy of the GCGR mAb is dependent on β-cell health and function. In addition, the GCGR mAb-mediated elevations of active GLP-1 levels are comparable in intact mice and STZ mice, suggesting that β-cell injury induced by STZ had no impact on GLP-1 synthesis and processing (Table 1).

Blocking the glucagon signaling pathway with anti-GCGR mAb treatment in wild-type mice led to elevated basal plasma active GLP-1 levels (Fig. 1B). Following a glucose challenge, an amplified early-phase insulin spike was observed in these wild-type mice (Fig. 1C, t = 15 min), coinciding with reduced glucose excursion (Fig. 1A) and suggesting that GLP-1-mediated enhancement of GSIS may contribute to the GCGR mAb efficacy. Treatment of STZ-treated and control nude mice with anti-GCGR mAb also led to increased active GLP-1 levels and reduced glucose excursions during ipGTT; however, the extent of improved glucose disposal of the STZ-treated mice was slightly reduced compared with control nude mice (Fig. 4, A and B). This could be explained by STZ-induced impairment but not total abrogation of the β-cell response to GLP-1-augmented glucose-dependent insulin secretion. Since we did not collect blood at each time point during the ipGTT, we were unable to determine whether there were transient increases in plasma insulin levels of anti-GCGR-treated mice similar to that of the C57BL/6 treated mice to support this hypothesis. However, consistent with this hypothesis, although mAb B treatment of GLP-1R KO mice resulted in increased basal plasma active GLP-1 levels (Fig. 1B), it had no impact on insulin secretion or overall glucose clearance during ipGTT (Fig. 1A).

While our data demonstrate that a significant proportion of the effect of GCGR antagonism is mediated by the actions of GLP-1R, not all of the improvements are mediated through the GLP-1R. For example, at the 30-min time point of the ipGTT in the GLP-1R KO mice (Fig. 1A), there was a statistically significant reduction in the blood glucose level following GCGR mAb administration. Presumably this effect was due to the suppression of glucagon signaling alone. In a wild-type mouse, glucagon secretion would normally be inhibited by GLP-1-potentiated insulin secretion and 2) activation of the α-cell GLP-1R (3). In addition, the GLP-1 antagonist Ex-(9–39) failed to completely abolish the efficacy of GCGR mAb treatment during ipGTT (Fig. 2A). It is documented that in type 2 diabetes the relative hyperglucagonemia is linked to increased hepatic glucose output. In a study conducted by Sørensen et al. (22), immunoneutralization of endogenous glucagon reduced hepatic glucose output and increased the rate of hepatic glycogen synthesis in ob/ob mice, both pathways known to be regulated by glucagon signaling. We are currently conducting additional studies to compare the pharmacological effects of a GLP-1R agonist with a GCGR mAb. Further investigation will help us to understand the benefits and the limitations of these potential therapeutic agents.

To further determine the extent to which improved glucose tolerance by GCGR antagonism reflects enhanced GLP-1 signaling on the pancreatic β-cells vs. the contribution of extrapancreatic GLP-1 action, we used a human GLP-1R-specific antagonist in the human islet-transplanted mouse model. Selective antagonism of GLP-1 action in the transplanted human islets by hGLP-1R mAb attenuated the observed beneficial effect of anti-GCGR mAb on glucose tolerance (Fig. 5). Thus, our data identify islet GLP-1R-dependent mechanisms as critical mediators of GCGR antagonist action.

In conclusion, GCGR antagonism improved glucose homeostasis in mice through a mechanism requiring a functional GLP-1 receptor. We further demonstrated that an important
physiological mechanism responsible for anti-GCGR mAb-mediated increase of glucose tolerance involved the pancreatic GLP-1 signaling pathway. Finally, GCGR antagonism resulted in compensatory overproduction of GLP-1 likely from pancreatic α-cells. This locally produced GLP-1 may play an important role in intraislet regulation and maintenance of β-cell function.

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DISCLOSURES

No conflicts of interest are reported by the authors.

REFERENCES


