

Long-Term Inhibition of the Glucagon Receptor with a Monoclonal Antibody in Mice Causes Sustained Improvement in Glycemic Control, with Reversible α -Cell Hyperplasia and Hyperglucagonemia

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ABSTRACT

Uncontrolled hepatic glucose output (HGO) contributes significantly to the pathological hyperglycemic state of patients with type 2 diabetes. Glucagon, through action on its receptor, stimulates HGO, thereby leading to increased glycemia. Antagonizing the glucagon signaling pathway represents an attractive therapeutic approach for the treatment of type 2 diabetes. We previously reported the generation and characterization of several high-affinity monoclonal antibodies (mAbs) targeting the glucagon receptor (GCGR). In the present study, we demonstrate that a 5-week treatment of diet-induced obese mice with mAb effectively normalized nonfasting blood glucose. Similar treatment also reduced fasting blood glucose without inducing hypoglycemia or other undesirable metabolic perturbations. In addition, no hypoglycemia was found in *db/db* mice that were

treated with a combination of insulin and mAb. Long-term treatment with the mAb caused dose-dependent hyperglucagonemia and minimal to mild α -cell hyperplasia in lean mice. There was no evidence of pancreatic α -cell neoplastic transformation in mice treated with mAb for as long as 18 weeks. Treatment-induced hyperglucagonemia and α -cell hyperplasia were reversible after treatment withdrawal for periods of 4 and 10 weeks, respectively. It is noteworthy that pancreatic β -cell function was preserved, as demonstrated by improved glucose tolerance throughout the 18-week treatment period. Our studies further support the concept that long-term inhibition of GCGR signaling by a mAb could be an effective approach for controlling diabetic hyperglycemia.

Increased glucose production is commonly associated with type 2 diabetes, and together with reduced glucose disposal and β -cell dysfunction, it contributes to the pathophysiology of the disease (Consoli et al., 1989). Several studies have suggested that increased hepatic gluconeogenesis and glycogenolysis are responsible for the overproduction of glucose in both the fasting and postprandial states of type 2 diabetics (Mitrakou et al., 1992; Singhal et al., 2002). Glucagon, a 29-amino acid peptide secreted by pancreatic α cells, plays an important role in controlling endogenous glucose production

under normal physiological conditions. By activating the glucagon receptor (GCGR), which is predominantly expressed in the liver and kidneys, glucagon stimulates adenylyl cyclase activity and phosphoinositol turnover (Christophe, 1996). These activities ultimately regulate the expression and activity of several key gluconeogenic and glycogenolytic enzymes including phosphoenolpyruvate carboxykinase (PEPCK), fructose-1,6-bisphosphatase (F1,6BPase), glucose-6-phosphatase (G6Pase), and glycogen phosphorylase (GP) (Jiang and Zhang, 2003). In addition, glucagon reduces glycogen synthesis in the liver (Ciudad et al., 1984). Glucagon has also been documented to be involved in other aspects of fuel homeostasis, including the inhibition of hepatic lipogenesis and stimulation of ketogenesis (Klain, 1977; Reed et al., 1984; Unger, 1985).

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ABBREVIATIONS: GCGR, glucagon receptor; PEPCK, phosphoenolpyruvate carboxykinase; F1,6BPase, fructose-1,6-bisphosphatase; G6Pase, glucose-6-phosphatase; GP, glycogen phosphorylase; HGO, hepatic glucose output; ASOs, antisense oligonucleotides; KO, knockout; mAb, monoclonal antibody; GLP-1, glucagon-like peptide-1; DIO, diet-induced obese; Q3D, every 3 days; Q5D, every 5 days; NEFA, nonesterified fatty acids; ANOVA, analysis of variance; SREBP-1c, sterol regulatory element-binding protein-1c.

In patients with type 2 diabetes, relative glucagon hypersecretion in the fasted state and a lack of suppression of postprandial glucagon secretion contribute to the increased rate of hepatic glucose output (HGO) characteristic of the pathogenesis of diabetic hyperglycemia (Müller et al., 1970; Dunning and Gerich, 2007). Accordingly, inhibition of GCGR activity represents a potential treatment approach for reducing the excess glucose production of diabetic patients.

Studies using rodent models suggest that antagonizing the glucagon/GCGR pathway may alleviate the hyperglycemia associated with type 2 diabetes. Mice with a genetic deletion of GCGR exhibit lower glucose levels and improved glucose tolerance without hypoglycemia (Parker et al., 2002; Gelling et al., 2003). Treatment with a glucagon-neutralizing antibody decreased the blood glucose levels of *ob/ob* mice (Sorensen et al., 2006). Furthermore, reduction in GCGR expression by antisense oligonucleotides (ASOs) ameliorated the diabetic syndrome in *db/db* mice and Zucker diabetic fatty rats by decreasing HGO and improving pancreatic β -cell function (Liang et al., 2004; Sloop et al., 2004). Finally, small molecule and peptidyl GCGR antagonists blocked exogenous glucagon-induced glucose elevation in both rodents and humans (Petersen and Sullivan, 2001; Qureshi et al., 2004). In addition to robust reduction in blood glucose levels, GCGR knockout (KO) mice and rodents treated with GCGR ASO also exhibited pancreatic α -cell hyperplasia and markedly increased levels of circulating glucagon (hyperglucagonemia), a compensatory response to decreased GCGR signaling (Parker et al., 2002; Gelling et al., 2003; Liang et al., 2004; Sloop et al., 2004). Increases in glucagon levels pose technical challenges for all therapeutic modalities aiming to block the glucagon signaling pathway.

We have recently described the generation and characterization of several high-affinity GCGR-antagonizing monoclonal antibodies (mAbs). These antibodies effectively improve glycemic control after a single injection in both mice and cynomolgus monkeys. It is noteworthy that a compensatory elevation of both glucagon and active glucagon-like peptide-1 (GLP-1) levels were also observed in the treated animals (Yan et al., 2009). Because glucagon plays an important role in controlling the hepatic energy status during metabolic stress such as fasting, several important questions need to be answered to evaluate the potential impact of anti-GCGR mAb treatment in longer-term studies. First, due to the compensatory oversecretion of glucagon associated with GCGR antagonism (Parker et al., 2002; Gelling et al., 2003; Liang et al., 2004; Sloop et al., 2004), it is necessary to determine whether the long-term treatment with anti-GCGR mAb leads to sustained lowering of blood glucose levels. Second, given that the glucagon pathway plays an important role in inducing blood glucose elevation during conditions of reduced glycemia, the risk of hypoglycemia during prolonged treatment or combined with insulin treatment needs to be assessed. Third, glucagon has been shown to inhibit hepatic lipogenesis by various mechanisms (Klain, 1977; Unger, 1985); however, GCGR KO mice were resistant to high fat diet-induced hepatic steatosis (Conarello et al., 2007). Therefore, it still remains unclear whether long-term blockade of glucagon signaling has beneficial or adverse consequences on hepatic lipid metabolism. Finally, pancreatic islet morphology and function need to be evaluated during the course of chronic treatment with GCGR mAb. In the present study, we inves-

tigated the effects of long-term treatment with a mouse anti-murine GCGR mAb on glucose homeostasis, hepatic lipid and glycogen contents, β -cell function, and α -cell responses in multiple mouse models.

Materials and Methods

Experimental Animals. All mouse studies were conducted at Amgen Inc., approved by the Institutional Animal Care and Use Committee, and experiments were performed in accordance with guidelines and regulations. C57BL/6 male mice were purchased from Charles River Laboratories, Inc. (Wilmington, MA). Mice were maintained on a 12-h light/dark cycle with free access to food and water. During the study, all blood samples taken for glucose measurement were collected from the retro-orbital sinus of nonanesthetized mice to minimize stress levels, and measurements were performed using a handheld glucometer (Lifescan, Inc., Milpitas, CA). Injections of compounds were performed between 7:00 and 9:00 AM on animals that had been fed ad libitum. Various animal models were used allowing us to address different questions. In particular, diet-induced obese (DIO) mice were used to address tachyphylaxis; DIO and *db/db* mice were used to assess hypoglycemia, and normal C57BL/6 mice were used to examine the long-term treatment effects on α -cell hyperplasia and β -cell function. C57BL/6 mice were chosen to avoid the diabetic complications that occur in most of the mouse diabetic models. Dose regimens were determined during our pilot studies. Depending on the duration of action and the chosen dose of our mAbs, animals received injections every 3 days (Q3D) or every 5 days (Q5D).

Generation and Characterization of GCGR mAbs. The generation and characterization of the anti-GCGR mAbs were described previously (Yan et al., 2009). mAb, a mouse IgG₁, was used for all chronic studies, and an analog mAb recombinantly expressed as human IgG₂ was used for the *db/db* mouse study. Phosphate-buffered saline was used as a vehicle for all studies, because our mAb was suspended in this buffer.

DIO Mouse Model. Groups of 16- to 17-week-old male C57BL/6 mice were fed a high-fat diet containing 60% of energy from fat enriched with saturated fatty acids (D12492; Research Diets, Inc., New Brunswick, NJ) for 12 to 13 weeks ($n = 8-12$ /group). Mice were sorted into groups having similar distributions based on body weight and blood glucose levels.

DIO Mice Treated for 5 Weeks to Assess Tachyphylaxis. To determine whether chronic administration by repeated dosing of antagonistic anti-GCGR antibodies would result in a sustained glucose-lowering effect in DIO mice, three groups of DIO mice received intraperitoneal injections of either vehicle (phosphate-buffered saline) or anti-GCGR mAb Q5D for 34 days. The groups treated with mAb received the first injection as a "loading" dose at 3 mg/kg i.p., followed by "maintenance" doses of either 0.3 or 3 mg/kg. Fed blood glucose levels were measured at time 0, 2, 24, and 34 days. Blood samples were collected before the treatment and at the end of the 34-day treatment. Plasma from the vehicle-treated and mAb-treated mice was analyzed for cholesterol, triglyceride, and nonesterified fatty acids (NEFA) levels.

DIO Mice Treated for 4 Weeks to Assess Hypoglycemia. To test the effects of chronic GCGR inhibition on fasting blood glucose levels and to assess hypoglycemic risk in an animal model of type 2 diabetes, DIO mice received intraperitoneal injections of either vehicle or mAb Q3D for 4 weeks at 1, 5, or 10 mg/kg. Two additional groups of age-matched and chow-fed animals were treated with vehicle or 10 mg/kg mAb Q3D. After 4 weeks of chronic treatment and 12 h after the last injection, the mice were fasted for 12 h. Fasting blood glucose levels were measured at the end of the 12-h fast. Blood and liver samples were collected from nonfasted mice 48 h after the last injection of vehicle or mAb, and plasma insulin, glucagon, active GLP-1, and ketone body levels were measured. Liver triglyceride and glycogen contents were also determined.

db/db Mice Treated for 5 Weeks with Insulin with Subsequent Addition of mAb to Assess Hypoglycemia. To further assess hypoglycemia in a more severe model of diabetes than DIO mice, *db/db* mice were used. In addition, this study design is more relevant to potential combination treatment approaches of patients.

Thirty-two 9-week-old *db/db* mice ($n = 8/\text{group}$) were sorted on similar blood glucose distributions of animals within each group. Two groups of 16 mice were treated for 5 weeks with either saline or insulin at 8 U/kg (Eli Lilly & Co., Indianapolis, IN) twice a day (BID) at 9:00 AM and 4:00 PM. At the end of the 5-week treatment with either saline or insulin at 8 U/kg, mice were randomized into four groups (vehicle/vehicle; vehicle/mAb; insulin/vehicle; insulin/mAb) and treated with either saline or mAb at 1 mg/kg. Blood glucose was monitored at 1, 4, 24, 48, 72, and 120 h after the mAb administration.

C57BL/6 Chronic Study. To avoid diabetic complications that occur in most of the mouse diabetic models, C57BL/6 mice were chosen for this long-term study. Eight-week-old C57BL/6 mice ($n = 6$ to 10/group) were sorted based on similar body weight distributions of animals within each group. Mice received intraperitoneal injections of either vehicle or mAb at 1 or 5 mg/kg on day 1, then Q3D for 3, 6, 9, 12, 15, or 18 weeks, and were then sacrificed at these different time points for islet morphology assessment and α -cell hyperplasia scoring. In addition, two sets of mice received intraperitoneal injections of vehicle or mAb at 1 or 5 mg/kg Q3D for 15 weeks. The treatment was stopped at 15 weeks, and the mice were allowed to recover for periods of either 6 or 10 weeks. Glucagon levels were measured and α -cell hyperplasia was scored.

Islet Morphology and α -Cell Hyperplasia Assessment. Body and pancreas weights were measured and pancreata were fixed in zinc formalin. The entire pancreas from each mouse was processed, embedded in paraffin, sectioned, and stained by using an immunohistochemical method to demonstrate glucagon-producing α -cells. These sections were examined by routine light microscopy for changes in α -cells. Hyperplasia of α -cells was scored using a semi-quantitative score, 0 to 4 scale, and scores were assigned in a "blinded" fashion, i.e., without knowledge of the treatment group. Glucagon was demonstrated by routine immunohistochemistry procedures. In brief, 5- μm sections of pancreata were deparaffinized and hydrated in deionized water. Sections were treated with CAS Block (Zymed Laboratories, South San Francisco, CA) and incubated with rabbit antibody to human glucagon (Dako North America, Inc., Carpinteria, CA) for 1 h at room temperature. Bound antibody was detected with biotin-labeled goat antibody to rabbit IgG (Vector Laboratories, Burlingame, CA). Sections were quenched with 3% H_2O_2 and covered with Avidin-Biotin horseradish peroxidase Complex (Vector Laboratories). Reaction sites were visualized with 3,3'-diaminobenzidine (Dako North America, Inc.). Sections were counterstained with hematoxylin.

β -Cell Function Assessment by Glucose Tolerance Test of C57BL/6 Mice. Eight-week-old C57BL/6 mice received intraperitoneal injections of either vehicle or mAb at 1 or 5 mg/kg on day 1 and then Q3D thereafter. Six, 12, and 18 weeks after starting treatment, intraperitoneal glucose tolerance tests were performed as follows: Mice were fasted for 12 h (2100–0900 h), a fasted blood glucose measurement was taken at ~0900 h (time 0), followed immediately by an intraperitoneal injection of D-glucose (2 g/kg b.wt.); blood glucose levels were measured again 30 and 90 min after the glucose bolus injection.

Plasma Constituent Measurements. Total plasma cholesterol, triglyceride, and NEFA were measured using an Olympus AU400e Chemistry Analyzer (Olympus America, Inc., Center Valley, PA). Insulin, glucagon, and active GLP-1 were measured using the Mouse Endocrine LINCoplex Kit (Millipore Corporation, Billerica, MA). Leptin levels were measured using the Mouse Adipokine LINCoplex Kit (Millipore Corporation). Plasma total ketone bodies were measured using the manual procedure for Wako Autokit Total Ketone Bodies (Wako Diagnostics, Richmond, VA). Plasma corticosterone levels were quantified using the ImmunoChem Double Antibody Cor-

ticosterone ^{125}I RIA Kit (MP Biomedicals, Irvine, CA), and the plasma epinephrine levels were quantified from samples pooled from each group by using the Epinephrine EIA kit (ALPCO Diagnostics, Salem, NH).

RNA Isolation and Real-Time Quantitative Reverse Transcription-Polymerase Chain Reaction. Total RNA was isolated from frozen liver tissue by using the TissueLyser apparatus and RNeasy Mini kit reagents (QIAGEN, Valencia, CA) according to the manufacturer's protocols. In brief, 50 to 100 mg of tissue was homogenized in tubes containing 5-mm stainless steel beads and lysis buffer at a frequency of 25 Hz for 2 min. Homogenate was subsequently collected and eluted in RNeasy columns. RNA concentrations were determined by using 260/280 UV spectrophotometry (Beckman Coulter, Fullerton, CA). RNA integrities were analyzed by using the ABI Prism 7900HT Sequence Detection System and SDS 2.1 software (Applied Biosystems, Foster City, CA). Reactions were performed in 20- μl volumes on 384-well plates containing TaqMan One Step PCR Master Mix reagents (Applied Biosystems), 175 nM of TaqMan probes, 350 nM of primers, and 20 to 40 ng of RNA per reaction. All samples were analyzed in quadruplicate measuring both the gene of interest and cyclophilin or β -actin as an internal control. Reactions were conducted as follows: 48°C for 30 min, 95°C for 10 min, then 40 cycles of 95°C for 15 s and 60°C for 1 min. Sequences of oligonucleotide primers and probes are shown in Table 1.

Liver Glycogen Content. Frozen liver samples were homogenized by using a TissueLyser with 5-mm stainless steel beads (QIAGEN) for 2 min at 25 Hz at a concentration of 0.1 g tissue per milliliter of HCl (0.03 mol/l). Homogenates (100 μl) were mixed with 400 μl of HCl (1.25 mol/l) and heated for 1 h at 100°C. Samples were centrifuged at 14,000 rpm, and the supernatant was diluted and assayed by using the Amplex Red Glucose Assay Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Samples were incubated with 100 μM Amplex Red reagent, 0.2 U/ml horseradish peroxidase, and 2 U/ml glucose oxidase for 30 min. Fluorescence was measured in excitation range of 530 to 560 nm and emission detection at 590 nm.

Liver Triglyceride Content. By using the TissueLyser apparatus (QIAGEN), liver samples were homogenized in 2:1 chloroform-methanol (v/v). One milliliter of the chloroform/methanol extract was then mixed with 200 μl of water and centrifuged at 3000g for 5 min. A biphasic system was obtained, the upper phase consisting of non-lipid substances and the lower phase containing the tissue lipids. The lower lipid phase was collected and concentrated by using a Speedvac (Thermo Fisher Scientific, Waltham, MA). The lipid pellet was dissolved in 60 μl of *tert*-butanol and 40 μl of Triton X-114 and methanol (2:1, v/v). After dilution, samples were assayed by using the Triglyceride Determination kit (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's protocols. In brief, samples were incubated in triglyceride working reagent and incubated for 15 min before absorbance was read at 540 nm.

Statistical Analysis. Statistical analyses were performed with StatView 5.0.1 (SAS Institute, Cary, NC) or GraphPad Prism version 5.0 (GraphPad Software Inc., La Jolla, CA) by using analysis of variance (ANOVA) for multiple comparison. Statistical testing of islet α -cell hyperplasia was performed by using an Armitage trend test from SAS Proc Freq (SAS Institute), comparing each treatment group versus the saline control group.

Results

Five-Week Treatment of DIO Mice with mAb Decreased Blood Glucose Levels. We recently described the generation and initial characterization of an anti-GCGR mAb, a high-affinity chimera with IC_{50} of ~25 nM against the mouse GCGR in cell-based signaling assays (Yan et al., 2009). To determine whether chronic administration by re-

TABLE 1. Primer and probe sequences for quantitative reverse transcription-polymerase chain reaction

Gene Name	Abbreviation	Forward Primer	Reverse Primer	Probe
Phosphoenolpyruvate carboxykinase, cytosolic isoform	PEPCK-C	5'-ATC CAG GGC AGC CTC GA-3'	5'-AGC ATT GCC TTC CAC GAA CT-3'	5'-AG CCT GCC CCA GGC AGT GAG G-3'
Fructose-1,6-bisphosphatase	F1,6Bpase	5'-CTG CAT GTT GTA CAT TCC TAG AAA CA-3'	5'-TTG GGC ATT GCA AAG CAT TA-3'	5'-CC TAA CAG CGT GGA TAG TTT CAC AGC-3'
Glycogen phosphorylase	GP	5'-CCA TTT ACC AGC TTG GAT TGG A-3'	5'-CCC AAG ACC GCC ATT GC-3'	5'-AA GGC CGG CAT CTT CTT CAA TTT CTT CTA ACT CT-3'
Glucose-6-phosphatase catalytic subunit	G6Pase	5'-GAA AGA AAA AGC CAA CGT ATG GA-3'	5'-GCA CAG CCC AGA ATC CCA-3'	5'-CC ACA AGA TGA CGT TCA AAC ACC GGA-3'
Glucagon receptor	GCGR	5'-TTT CCT GCC CCT GGT ACC TA-3'	5'-CGG GCC CAC ACC TCT TG-3'	5'-CC ACA AAG TGC AGC ACC GCC TAG TG-3'
Peroxisome proliferator-activated receptor γ	PPAR γ	5'-GCA GCA GGT TGT CTT GGA TGT-3'	5'-TCA GTG GAG ACC GCC CAG-3'	5'-CT TGC TGA ACG TGA AGC CCA TCG A-3'
Stearoyl CoA desaturase1	SCD-1	5'-CCT TCG ACT ACT CTG CCA GTG A-3'	5'-GCC ATG CAG TCG ATG AAG AA-3'	5'-AC CGC TGG CAC ATC AAC TTC ACC AC-3'
Diacylglycerol acyltransferase-1	DGAT1	5'-GGT GAA ACC ATG ATG GTC GAG-3'	5'-TCT GTA GGC ACC GTG CCA-3'	5'-CG CGC CAC TCA ACA CCC CG-3'
Acetyl-CoA carboxylase1	ACC1	5'-TGA GGT CAT CAC CAT CAG CCT-3'	5'-GTC CCA GCC GGA CAA GGT A-3'	5'-TT ACA TGC CGG GCC ATT GGT ATT GG-3'
Fatty acid synthase	FAS	5'-CGG GTG AGG AGG TTT ACA AAG-3'	5'-CAT GAC CTC GTG ATG AAC GTG T-3'	5'-CC GTC ACT TCC AGT TAG AGC AGG ACA AGC-3'
Sterol regulatory element-binding protein 1a	SREBP1a	5'-GCC GAG ATG TGC GAA CTG-3'	5'-GTC ACT GTC TTG GTT GTT GAT GAG-3'	5'-AC AGC GGT TTT GAA CGA CAT CGA AGA C-3'
Sterol regulatory element binding protein 1c	SREBP1c	5'-GGA GCC ATG GAT TGC ACA TT-3'	5'-TCA AAT AGG CCA GGG AAG TCA-3'	5'-AA GAC ATG CTT CAG CTT ATC AAC AAC CAA GAC A-3'

peated dosing of antagonistic anti-GCGR antibodies would result in a sustained glucose-lowering effect in DIO mice, an animal model that closely resembles those of human type 2 diabetes, DIO mice were treated with either vehicle or mAb Q5D for 34 days. As shown in Fig. 1, mAb treatment dose-dependently decreased fed blood glucose levels on days 2, 24, and 34 after initial treatment. It is noteworthy that repeated administration of the anti-GCGR antibody did not cause tachyphylaxis, as demonstrated by the sustained decrease of blood glucose levels in mAb-treated DIO mice (Fig. 1). Plasma parameters and body weight from vehicle and 3 mg/kg mAb-treated mice are shown in (Table 2). Body weight did not differ significantly between the vehicle and mAb-treated animals. Moreover, there were no statistically significant changes in plasma triglyceride levels compared with pretreatment levels. As expected, in animals with continued high-fat feeding, both vehicle-treated mice and GCGR mAb-treated mice demonstrated significant elevations in plasma cholesterol levels after 34 days of treatment. NEFA levels increased in the vehicle-treated mice, whereas they were unchanged in the GCGR mAb-treated mice.

Four-Week Treatment of DIO Mice with mAb Decreased Fasting Blood Glucose Levels without Inducing Hypoglycemia. Under normal physiological conditions, glucagon is secreted in response to decreasing glucose levels to prevent hypoglycemia. To test the effects of chronic GCGR inhibition on fasting blood glucose levels and to assess hypoglycemic risk in animal models of type 2 diabetes, four groups of DIO mice received injections of either vehicle or mAb (1, 5, or 10 mg/kg) Q3D for 4 weeks. In addition, two groups of age-matched chow-diet-fed mice were treated with either vehicle or mAb at 10 mg/kg Q3D for 4 weeks. At the end of the 4-week treatment, mice were fasted for 12 h, and fasting blood glucose levels were determined. As shown in Fig. 2A, chronic treatment of DIO mice with mAb significantly decreased fasting blood glucose levels in a dose-dependent manner ($P < 0.001$ for 5 and 10 mg/kg treatment groups). We previously conducted pilot experiments to establish a work-

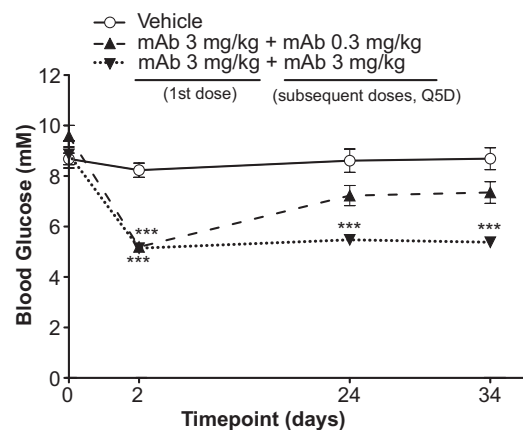


Fig. 1. Effect of mAb on fed blood glucose levels of DIO C57BL/6 mice treated for 34 days. Male C57BL/6 mice fed a high-fat diet for 12 to 13 weeks before the study were maintained on this diet throughout the treatment period. Mice were treated with either vehicle or 3 mg/kg mAb on day 0 and were treated thereafter Q5D with vehicle (0.3 or 3 mg/kg mAb). Injections of compounds were administered between 7:00 and 9:00 AM to animals that had been fed ad libitum. Blood glucose levels measured before and during the 5-week treatment period on days 2, 24, and 34 are plotted as the mean \pm S.E.M. $n = 9-12$ mice per group. ***, $P < 0.001$ versus vehicle-treated group.

TABLE 2

DIO C57BL/6 mice body weights and selected plasma components at baseline and after 5 weeks of treatment with vehicle or 3 mg/kg mAb administered Q5D

Injections of compounds were administered between 7:00 and 9:00 AM to animals that had been fed ad libitum.

	Control		3 mg/kg mAb	
	Pretreatment	Posttreatment	Pretreatment	Posttreatment
Body Weight (g)	49.9 ± 0.9	53.6 ± 1.0	50.0 ± 0.6	53.1 ± 1.0
Cholesterol (mg/dl)	223 ± 5.1	245 ± 5.0*	230 ± 8.3	269 ± 7.7*
Triglyceride (mg/dl)	121 ± 13	124 ± 8.5	130 ± 8.8	112 ± 4.4
NEFA (mEq/l)	1.19 ± 0.03	1.37 ± 0.03**	1.19 ± 0.05	1.18 ± 0.03

** $P < 0.01$ and *** $P < 0.001$, compared to pretreatment levels of the respective group. $n = 10$ per group.

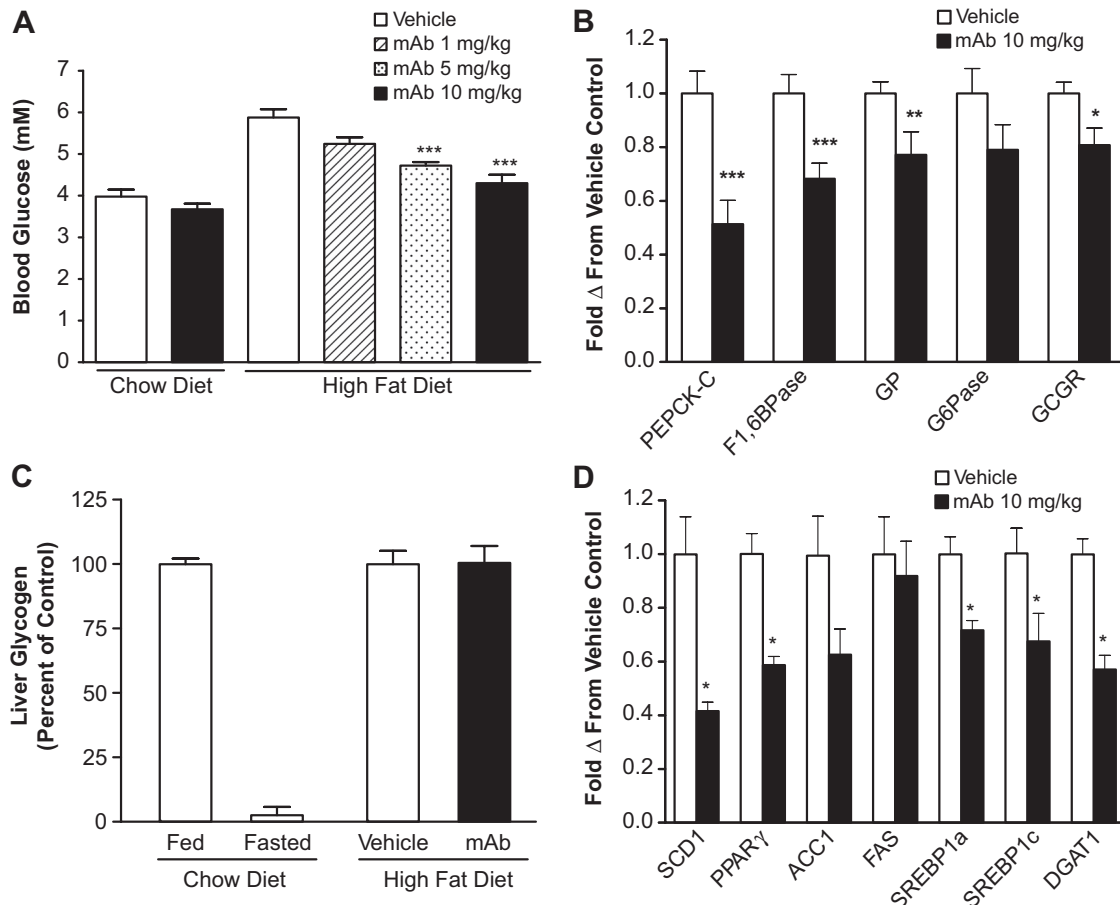


Fig. 2. Long-term effects of mAb on fasting blood glucose levels and liver glycogen content. A, male C57BL/6 mice fed a high-fat diet for 12 to 13 weeks before the study were maintained on this diet throughout the treatment period. Mice were treated Q3D with vehicle, 1 mg/kg, 5 mg/kg, or 10 mg/kg mAb. Two groups of C57BL/6 mice maintained on standard chow diet were also treated Q3D with vehicle or 10 mg/kg mAb. Injections of compounds were administered between 7:00 and 9:00 AM to animals that had been fed ad libitum. Mice were fasted for 12 h after 4 weeks of treatment. Fasting blood glucose levels are represented as the mean + S.E.M. $n = 5$ to 8 mice per group. ***, $P < 0.001$ versus DIO C57BL/6 vehicle-treated mice. B, effect of mAb on hepatic mRNA expression levels of select gluconeogenic and glycogenolytic genes. Quantitative reverse transcription-polymerase chain reaction was used to assess mRNA expression levels of PEPCK-C, F1,6BPase, GP, G6Pase, and GCGR in liver. Values are expressed as the fold change from vehicle control levels + S.E.M. for each gene. $n = 11$ for vehicle group and $n = 8$ for mAb group. *, $P < 0.05$, **, $P < 0.01$, and ***, $P < 0.001$ compared with vehicle-treated group. C, glycogen content of livers from fed and fasted lean C57BL/6 mice and glycogen content of livers of fed DIO mice from A. Values are plotted as the mean glycogen content expressed as the percentage of the glycogen content of untreated chow fed or high-fat diet fed, controls, respectively, + S.E.M. $n = 6$ mice per group. D, effects of mAb on hepatic mRNA expression levels of lipogenic genes. Quantitative reverse transcription-polymerase chain reaction was used to assess mRNA expression levels of stearoyl-CoA desaturase-1 (SCD1), peroxisome proliferator-activated receptor- γ (PPAR γ), acetyl-CoA carboxylase-1 (ACC1), fatty acid synthase (FAS), sterol regulatory element-binding protein 1a and 1c (SREBP1a, SREBP1c), and diacylglycerol acyltransferase-1 (DGAT1) in liver. Values are expressed as the fold change from vehicle control levels + S.E.M. for each gene. $n = 11$ for vehicle group and $n = 8$ for mAb group. *, $P < 0.05$.

ing definition of the hypoglycemic condition in mice (Yan et al., 2009). It is noteworthy that the high-dose (10 mg/kg) mAb treatment reduced fasting blood glucose levels of DIO mice to levels comparable to those of lean mice (Fig. 2A). In both

chow-diet-fed and DIO mice, repeated dosing of mAb did not induce hypoglycemia. Consistent with the level of blood glucose reduction observed, treatment of DIO mice with anti-GCGR mAb (10 mg/kg) for 4 weeks reduced the transcrip-

tional levels of key genes that regulate glucagon-mediated hepatic glucose production, including PEPCK, F1,6Bpase, and GP (Fig. 2B). It is interesting to note that hepatic GCGR mRNA was also reduced in the mAb-treated mice (Fig. 2B). Hepatic glycogen content was dramatically reduced in control chow-fed mice after a 12-h fast. In the fed state, liver glycogen content was not significantly different between the mAb-treated and the vehicle-treated DIO mice (Fig. 2C).

Plasma hormones and other metabolites involved in fuel homeostasis were measured in plasma collected from mice during a nonfasted state at the end of the 4-week study. Plasma insulin levels decreased in a statistically significant dose-dependent manner in the mAb-treated groups similar to the decrease in blood glucose levels (Table 3). Consistent with the observations made from the single-dose GCGR mAb studies (Yan et al., 2009), treatment of DIO mice for 4-weeks with mAb resulted in dose-dependent increases in both glucagon and active GLP-1 levels (Table 3). In the fed state, plasma ketone bodies, hepatic triglyceride content, and leptin levels were not significantly different between the anti-GCGR mAb-treated and the vehicle-treated mice (Table 3). Moreover, hepatic mRNA levels of several lipogenic genes including peroxisome proliferator-activated receptor γ , sterol regulatory element-binding protein-1a, sterol regulatory element-binding protein-1c (SREBP-1c), stearyl-CoA desaturase-1, and diacylglycerol acyltransferase 1 were significantly reduced in DIO mice treated with GCGR mAb compared to those of vehicle-treated mice (Fig. 2D).

Five-Week Treatment of *db/db* Mice with b.i.d. Insulin Followed by mAb Administration Decreased Blood Glucose Levels without Inducing Hypoglycemia. To continue assessment of hypoglycemia in a more severe model of diabetes than DIO mice, *db/db* mice were used. This study design was also designed to determine the relevance of a potential combination of treatment approaches. As expected, 8 U/kg insulin statistically significantly decreased blood glucose levels of *db/db* mice after 5 weeks of b.i.d. treatment (Fig. 3A). After 1 mg/kg mAb administration, blood glucose levels were significantly decreased in both groups of mice treated with either saline or insulin. Nevertheless, in either group none of the mice showed any sign of hypoglycemia (Fig. 3B). mAb and insulin had additive effects as observed by additional blood glucose lowering upon mAb treatment in mice pretreated with insulin for 5 weeks (Fig. 3B).

Chronic Treatment with mAb in C57BL/6 Mice Induced Mild α -Cell Hyperplasia That Is Reversible after Treatment Ends. Previous studies have demonstrated that GCGR KO mice and GCGR ASO-treated diabetic rodents display α -cell hyperplasia characterized by the presence of an increased number of glucagon-producing cells visualized by

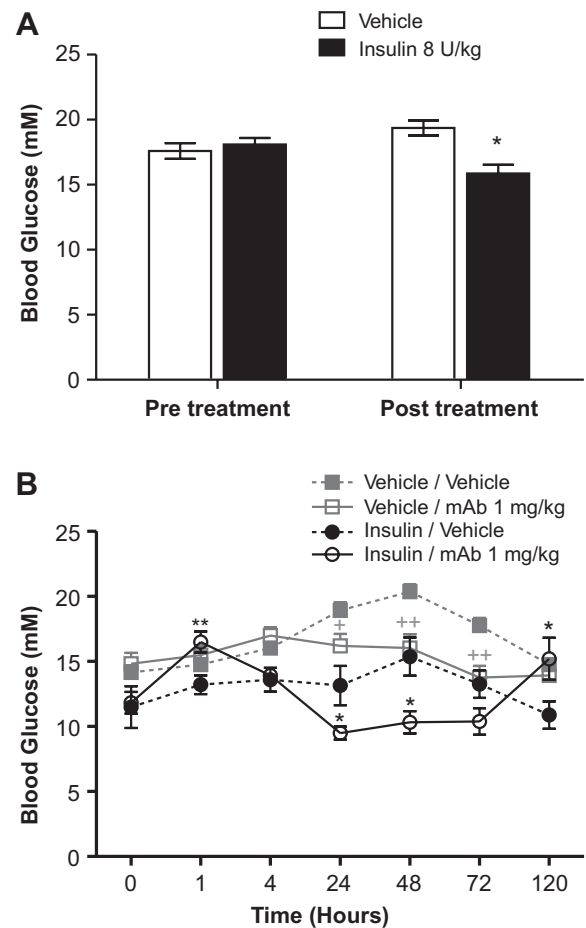


Fig. 3. Effects of 1 mg/kg mAb analog treatment after 5 weeks of insulin treatment on fasting blood glucose levels of *db/db* mice. A, male *db/db* mice were treated for 5 weeks with 8 U/kg insulin b.i.d. Blood glucose levels were measured before starting the vehicle or insulin treatment (pretreatment) and after 5 weeks of treatment (post-treatment). B, blood glucose levels of mice treated for the previous 5 weeks with either vehicle or insulin (Fig. 3A) and then treated with either vehicle or mAb at 1 mg/kg. Blood glucose levels were measured 1, 4, 24, 48, 72, and 120 h after the vehicle or mAb administration. Values are expressed as mean \pm S.E.M. $n = 8$ for each. *, $P < 0.05$, **, $P < 0.01$, +, $P < 0.05$, and ++, $P < 0.01$ compared with the vehicle-treated group.

immunostaining of pancreatic islets (Gelling et al., 2003; Liang et al., 2004; Sloop et al., 2004). In an ideal setting, diabetic rodent models would have been our first choice to perform our long-term study. Nevertheless, none of these models would have performed well for such an extended period. It is well known that some mouse models have β -cells capable of maintaining robust lifelong insulin secretion but already have hypertrophy and hyperplasia of pancreatic is-

TABLE 3

DIO C57BL/6 mice plasma chemistry and liver triglyceride contents after 4 weeks of treatment with vehicle (control) or mAb administered Q3D at 1 or 10 mg/kg

Injections of compounds were administered between 7:00 and 9:00 AM to animals that had been fed ad libitum.

	Control	<i>n</i>	1 mg/kg mAb	<i>n</i>	10 mg/kg mAb	<i>n</i>
Insulin (nmol/l)	28.7 \pm 4.9	11	14.7 \pm 4.5	11	10.8 \pm 3.6*	9
Glucagon (pmol/l)	49.4 \pm 13.7	10	85.2 \pm 23.1	11	606 \pm 143**	9
Active GLP-1 (pmol/l)	9.6 \pm 2.2	7	19.8 \pm 5.6	6	37.5 \pm 7.9**	8
Ketone bodies (μ mol/l)	51.2 \pm 4.0	11	54.5 \pm 5.5	11	41.8 \pm 4.0	9
Leptin (ng/ml)	17.7 \pm 1.8	11	22.9 \pm 3.8	11	20.4 \pm 3.4	9
Liver triglyceride (mg/g liver)	226.7 \pm 17.6	6	194.9 \pm 10.3	6	208.5 \pm 28.6	5

* $P < 0.05$ and ** $P < 0.01$, compared with the vehicle-treated group.

lets (Velasquez et al., 1990), and other models possess labile pancreatic β cells allowing only for transient insulin secretion (Srinivasan and Ramarao, 2007). To avoid changes of pancreatic status with the treatment period, C57BL/6 mice were chosen to address three important questions: 1) whether chronic treatment of mice with an antibody to GCGR would cause hyperplasia of α -cells; 2) whether treatment-induced α -cell hyperplasia would stabilize during the course of treatment; and 3) whether treatment-induced α -cell hyperplasia would be reversed after a treatment-free recovery phase. Multiple groups of 8-week-old male C57BL/6 mice received intraperitoneal injections of either vehicle or mAb at 1 or 5 mg/kg Q3D for periods of 3, 6, 9, 12, 15, or 18 weeks. In addition, separate sets of 8-week-old male C57BL/6 mice received intraperitoneal injections of vehicle or mAb at 1 or 5 mg/kg Q3D for 15 weeks. The treatment of these mice was stopped after 15 weeks, and mice were allowed to recover for periods of either 6 or 10 weeks.

Groups of mice treated for 3, 6, 9, 12, 15, or 18 weeks were euthanized at each time point of the treatment phase. Pancreas weights were determined, pancreas tissue was fixed, and glucagon was stained by immunohistochemistry to allow visualization of α -cells. As shown in Fig. 4, hyperplasia of α cells were scored semiquantitatively on a 0 to 4 scale [0 = none, 1 = minimal, 2 = mild, 3 = moderate, 4 = marked (i.e., the extent noted in the most severely affected GCGR KO mice)]. Figure 4, A–C, shows representative images of pancreata from vehicle-treated, 1 mg/kg and 5 mg/kg mAb treatment groups, respectively, mice after 18 weeks of treatment. As shown in Fig. 5A, treatment of mice with anti-GCGR mAb at doses of 1 and 5 mg/kg caused dose-dependent, minimal to

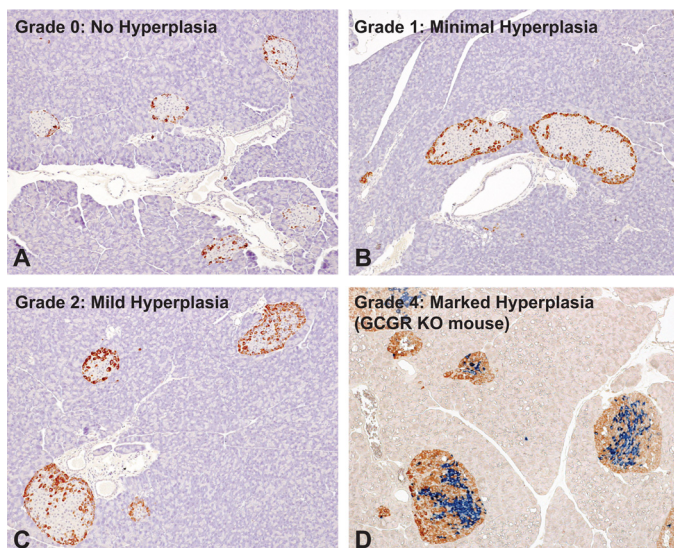


Fig. 4. Representative images of immunohistochemical staining and scoring of pancreas for hyperplasia of glucagon-producing α -cells. Sections were stained by using an immunohistochemical method to demonstrate glucagon-producing α -cells as described under *Materials and Methods*. These sections were examined by routine light microscopy for changes in α -cells. Hyperplasia of α -cells was scored without knowledge of treatment group by using a semiquantitative score, 0 to 4 scale. Representative images from observed scores from each group treated for 18 weeks with either vehicle or mAb at 1 or 5 mg/kg are shown at a magnification of 100 \times . As a reference for severity assessments, α -cell hyperplasia was scored in GCGR KO mice (A) Grade 0, no hyperplasia in vehicle-treated mouse (B) Grade 1, minimal hyperplasia in a mouse treated with mAb at 1 mg/kg. C, Grade 2, mild hyperplasia in a mouse treated with mAb at 5 mg/kg. D, Grade 4, marked hyperplasia (GCGR KO mouse).

mild hyperplasia of α -cells in the pancreas. This change was noted at the first time point evaluated (week 3). The peak hyperplasia severity score at each dose level (grade 1 at 1 mg/kg and grade 2 at 5 mg/kg) did not increase with time. However, the number of mice reaching this score at each dose level increased with increasing duration of treatment. For example, in the group treated with 5 mg/kg anti-GCGR mAb, 2 of 10 mice scored grade 2 after 3 weeks of treatment, and after 18 weeks of treatment 6 of 8 mice reached grade 2 α -cell hyperplasia. The severity of the α -cell hyperplasia did not progress beyond a severity score of 2, whereas GCGR KO mice displayed severity scores of 4 (Fig. 4D). There were no treatment effects on terminal body weight or pancreas weight when expressed as either absolute organ weight or pancreas weight as a percentage of terminal body weight (data not shown). In addition, after 18 weeks of treatment with either vehicle or 5 mg/kg mAb, plasma epinephrine and corticosterone levels, liver triglyceride, and glycogen contents did not differ between mice treated with the mAb and mice treated with vehicle (Table 4).

To test for reversibility of α -cell hyperplasia, sets of mice that received intraperitoneal injections of vehicle or mAb at 1 or 5 mg/kg were allowed to recover for either 6 or 10 weeks after 15 weeks of treatment. As shown in Fig. 5B, termination of treatment at week 15 resulted in complete reversal of α -cell hyperplasia in the 1 mg/kg group at the first recovery time point (week 6) and in a definite trend toward reversal in the 5 mg/kg group at both recovery time points (weeks 6 and 10).

Chronic Treatment with mAb Induced Hyperglucagonemia in C57BL/6 Mice and a Complete Reversal of Hyperglucagonemia Was Achieved after a 4-Week Washout Period. During the 18 weeks of Q3D intraperitoneal dosing with either vehicle or mAb at 1 or 5 mg/kg, plasma glucagon levels were monitored at each time point of the treatment phase to evaluate the effects of chronic treatment with GCGR antibody on hyperglucagonemia. As shown in Fig. 5C, a dose-dependent increase in glucagon levels was observed in mice treated with anti-GCGR antibody. This increase was noted at the first time point (week 3). Glucagon levels reached a plateau at week 9 and did not increase further with extended treatment.

To investigate whether this hyperglucagonemia was reversible upon treatment withdrawal, glucagon levels were monitored in mice that were treated with mAb for 15 weeks and allowed to recover. As expected, glucagon levels were elevated in a dose-dependent fashion in mice treated with mAb at 1 or 5 mg/kg for 15 weeks (Fig. 5D). After a 4-week washout period, blood samples were collected and plasma glucagon levels were determined. At the 4-week washout period, plasma glucagon levels had returned to normal for both doses of mAb (Fig. 5D). At the same time, blood glucose was identical among the 3 groups of mice (Table 4).

Chronic Treatment with mAb Preserved β -Cell Function in C57BL/6 Mice. Our current studies have demonstrated that chronic blockade of the GCGR signal with a mAb leads to α -cell hyperplasia in mice. To determine whether altered β - to α -cell ratio in islets would lead to a deterioration in β -cell function and consequently impaired glucose tolerance, intraperitoneal glucose challenges were conducted in C57BL/6 mice that had been treated with either vehicle or mAb at doses of 1 or 5 mg/kg Q3D for 6, 12, or 18

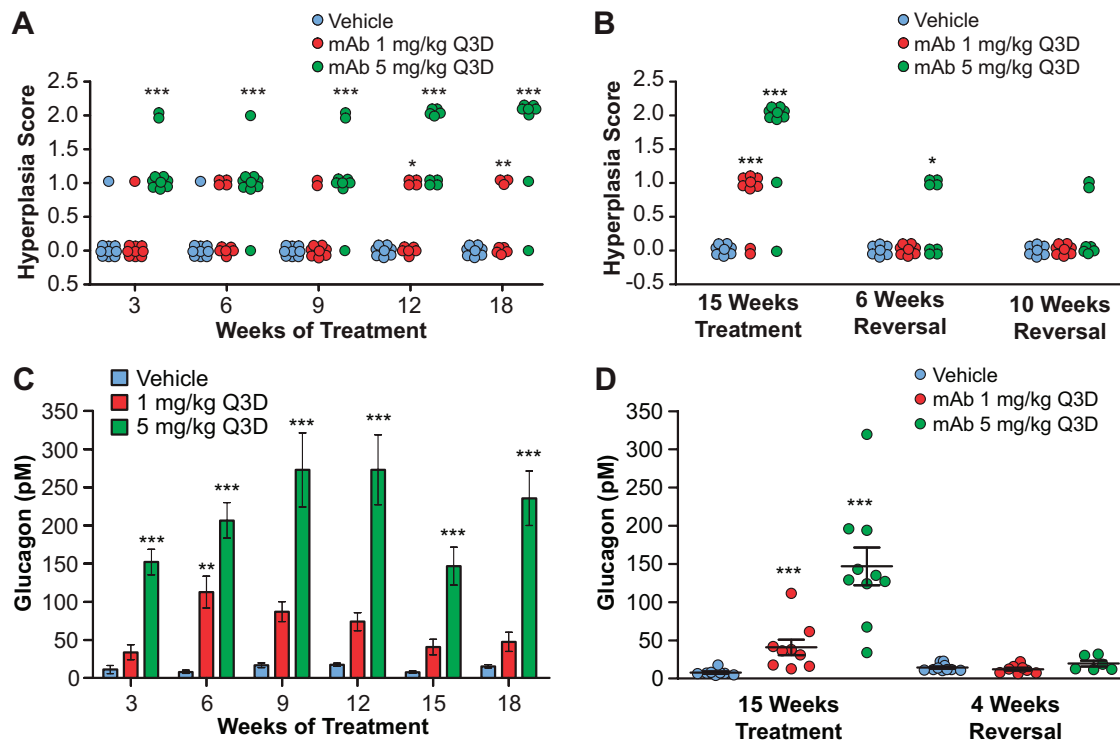


Fig. 5. Effect of mAb on α -cell hyperplasia and plasma glucagon levels. C57BL/6 mice were treated with either vehicle or mAb at 1 or 5 mg/kg administered Q3D. Pancreata were isolated after 3, 6, 9, 12, and 18 weeks of treatment. Blood glucose levels were measured in mice that had been treated for 15 weeks and in mice treated for 15 weeks and then withheld for 4 weeks. Injections of compounds were administered between 7:00 and 9:00 AM to animals that had been fed ad libitum. A, islet α -cell hyperplasia score of C57BL/6 mice throughout 18 weeks of mAb treatment. *, $P < 0.05$, **, $P < 0.01$, and ***, $P < 0.001$. B, three groups of mice were treated with either vehicle or mAb at 1 mg/kg or 5 mg/kg administered Q3D for 15 weeks. Pancreata were isolated after 15 weeks of treatment, and after 6 or 10 weeks discontinued treatment. Results are expressed as individual values ($n = 8-10$ /group). Statistical testing for significance was performed by using an Armitage trend test from SAS Proc Freq, comparing each treatment group versus vehicle-treated group at the appropriate time. *, $P < 0.05$, **, $P < 0.01$, and ***, $P < 0.001$. C, plasma glucagon levels of C57BL/6 mice throughout 18 weeks of vehicle or mAb treatment Q3D are displayed as the mean \pm S.E.M. $n = 7$ to 10 mice per group. Statistical testing was performed by using ANOVA with multiple comparison post-tests. **, $P < 0.01$ and ***, $P < 0.001$. D, glucagon levels in mice treated for 15 weeks and 4 weeks after treatment withdrawal. Individual glucagon levels of mice are plotted, and the mean \pm S.E.M. is shown. $n = 6$ to 10 mice per group. Statistical testing was performed by using unpaired t -tests of log-transformed data comparing the mAb-treated groups to the vehicle-treated group at the equivalent time point. ***, $P < 0.001$.

TABLE 4

Plasma epinephrine and corticosterone levels and liver triglyceride and glycogen contents of fed C57BL/6 mice treated with vehicle (control) or mAb at 1 or 5 mg/kg (Q3D) for 18 weeks

Blood glucose levels of C57BL/6 mice treated for 15 weeks and 4 weeks after treatment withdrawal. Injections of compounds were administered between 7:00 and 9:00 AM to animals that had been fed ad libitum.

	Control	<i>n</i>	1 mg/kg mAb	<i>n</i>	5 mg/kg mAb	<i>n</i>
Epinephrine (ng/ml)	9.1 \pm 0.6	8	8.3 \pm 0.1	7	8.3 \pm 0.6	8
Corticosterone (ng/ml)	4.6 \pm 1.2	8	13.2 \pm 9.9	8	12.5 \pm 4.9	8
Liver triglyceride (mg/g liver)	11.7 \pm 1.6	5	19.7 \pm 4.4	5	16.5 \pm 5.6	5
Liver glycogen (μ mol/g liver)	457.0 \pm 25.2	5	463.1 \pm 30.4	5	469.7 \pm 8.1	5
Blood glucose (mmol/l)	5.9 \pm 0.3	6	5.7 \pm 0.2	10	6.4 \pm 0.2	9

weeks (Fig. 6, A–C, respectively). As shown in Fig. 6, time 0 blood glucose levels were not significantly different between vehicle- and mAb-treated mice. After the glucose challenge, statistically significant improvements in glucose clearance and excursion were observed in mAb treated-mice in a dose-dependent manner (Fig. 6). This study provides evidence that β -cell function was preserved in the presence of α -cell hyperplasia after chronic treatment for up to 18 weeks with mAb.

Discussion

Our previous studies have demonstrated that acute treatment with a GCGR-neutralizing mAb improves glucose ho-

meostasis as well as induces compensatory over secretion of endogenous glucagon and active GLP-1 (Yan et al., 2009). In the current report, we have demonstrated that chronic administration of GCGR mAb in DIO mice resulted in sustained improvement in glycemic control, even in the presence of hyperglucagonemia. It is noteworthy that repeated dosing of DIO mice with mAb did not induce hypoglycemia or other unfavorable metabolic perturbations.

Elevation of HGO has been shown to contribute to the hyperglycemic state of type 2 diabetes. A number of pharmacological approaches have been pursued to inhibit hepatic glucose production. Direct targeting of key enzymes in glycogenolysis or gluconeogenesis, such as inhibitors of GP (Mar-

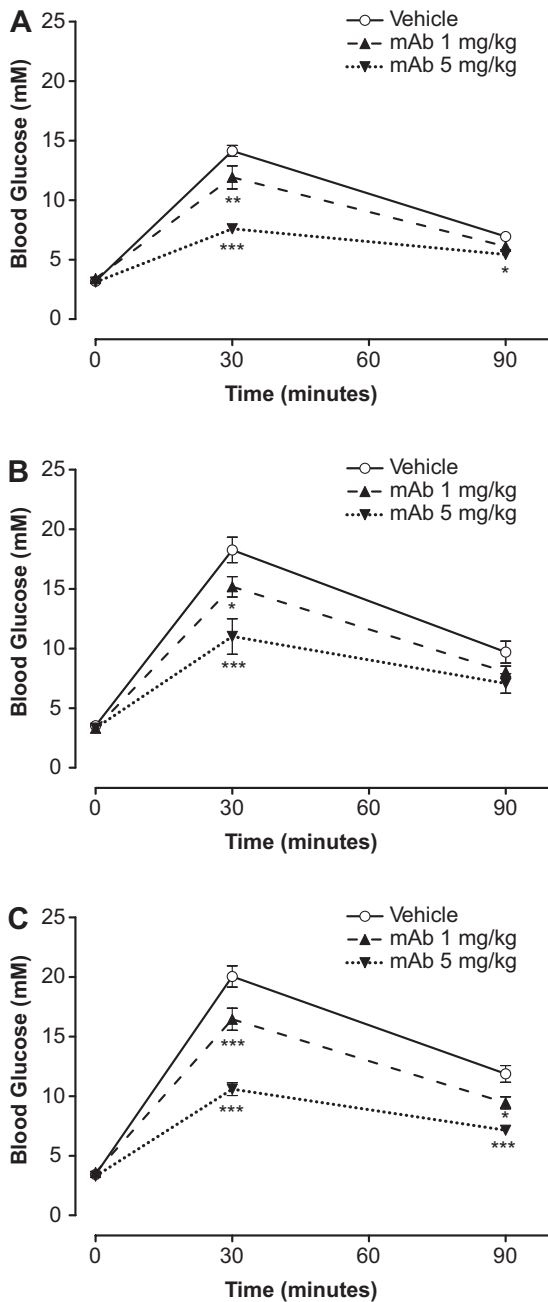


Fig. 6. Glucose tolerance tests after 6 weeks (A), 12 weeks (B), or 18 weeks (C) of treatment with either vehicle or mAb at 1 or 5 mg/kg administered Q3D. Injections of compounds were administered between 7:00 and 9:00 AM to animals that had been fed ad libitum. Blood glucose levels were measured before and 30 and 90 min after the glucose bolus injection (2 g/kg). Blood glucose levels are shown as the mean \pm S.E.M. Statistical testing was performed by using ANOVA with multiple comparison post-tests. $n = 7$ to 10 per group. *, $P < 0.05$, **, $P < 0.01$, and ***, $P < 0.001$ versus vehicle-treated group.

tin et al., 1998), G6Pase (Arion et al., 1998), and F1,6BPase has been explored (van Poelje et al., 2006). In a published report, a 2-week treatment of Zucker diabetic fatty rats with an F1,6BPase inhibitor attenuated hyperglycemia; however, serum lactate, triglycerides, and cholesterol levels increased (van Poelje et al., 2006). Blocking the glucagon pathway has been known to inhibit gluconeogenesis and glycogenolysis, two critical pathways in carbohydrate metabolism that generate glucose (Jiang and Zhang, 2003). In addition, glucagon

has also been reported to influence other aspects of fuel homeostasis including the mobilization of free fatty acids (FFA) from fat (Carlson et al., 1993), the inhibition of hepatic lipid synthesis by regulating the rate-limiting enzyme acetyl-CoA carboxylase 1 (Klain, 1977; Unger, 1985), and the enhancement of ketogenesis as a result of the increased demand for β -oxidation of mobilized free fatty acids (Keller et al., 1983; Reed et al., 1984). In cultured hepatocytes, transcription of SREBP-1c, a key activator of fatty acid synthesis in the liver, has been shown to be down-regulated by glucagon (Horton et al., 2002). Inhibition of the glucagon pathway could theoretically reverse the process and cause hepatic steatosis and hypertriglyceridemia. However, GCGR KO mice and mice treated with GCGR ASO did not show abnormality in lipid metabolism. In fact, GCGR-deficient mice have been shown to be resistant to high-fat diet-induced hepatic steatosis (Conarello et al., 2007). Furthermore, *db/db* mice treated with GCGR ASO for 4 weeks had reduced plasma triglyceride levels and decreased liver triglyceride content (Sloop et al., 2004). In our current study, we have demonstrated that chronic treatment of DIO or C57BL/6 mice with GCGR mAb did not cause significant changes in plasma lipids, hepatic triglyceride content, or leptin levels. Moreover, hepatic mRNA levels of several lipogenic genes were significantly reduced in DIO mice treated with GCGR mAb compared to those of vehicle-treated mice (Fig. 2). Plasma levels of ketone bodies (β -hydroxybutyrate and acetoacetate) were not significantly different between the GCGR mAb-treated and the vehicle-treated mice, suggesting that fatty acid oxidation was not significantly altered by the treatment.

Because glucagon promotes glycogenolysis (Jiang and Zhang, 2003), inhibition of the glucagon pathway may lead to excess glycogen accumulation in the liver. Once again, our data showed that the hepatic glycogen content of the DIO mice treated with mAb for 4 weeks or the C57BL/6 mice treated for 18 weeks were statistically indistinguishable from the control group. Our studies have demonstrated that long-term treatment of the GCGR mAb does not cause hypoglycemia. In C57BL/6 mice, blood glucose levels during mAb withdrawal were not increased even in presence of α -cell hyperplasia or in monkey when glucagon levels were still elevated (Yan et al., 2009). Moreover, the levels of corticosterone, a glucocorticoid that stimulates gluconeogenesis, were not elevated by the 5-week GCGR mAb treatment (data not shown). Epinephrine and corticosterone levels of C57BL/6 mice treated with mAb for 18 weeks were not different from the levels of mice treated with vehicle. It remains to be determined whether other hormonal and neural mechanisms compensate for decreased glucagon signaling to prevent hypoglycemia. In addition, mAb treatment of mice that had been chronically treated for 5-weeks with insulin b.i.d. did not induce hypoglycemia. Instead, the two compounds worked additively, because mAb treatment further decreased blood glucose levels from the levels that 8 U/kg insulin had achieved. Specific studies to evaluate recovery from hypoglycemia were not conducted, and, therefore, the effect of mAb on this critical issue remains undetermined.

Whereas 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 addi-

tional benefits due to increases in circulating levels of GLP-1 (Gelling et al., 2003; Liang et al., 2004; Sloop et al., 2004). In GCGR ASO-treated mice, plasma concentrations of active GLP-1 (7–36 amide and 7–37) were elevated ~10 fold (Sloop et al., 2004). In GCGR KO mice, the content of total GLP-1 in pancreatic extracts increased by >25-fold (Gelling et al., 2003). In the present study, plasma active GLP-1 levels were increased ~4 fold in DIO mice treated with GCGR mAb (10 mg/kg) for 4 weeks. These data provide evidence that GLP-1 actions contributed to the efficacy of the GCGR mAb in glycaemic control. These results suggest that the inhibition of GCGR signaling has dual benefits from both GCGR-mediated hepatic and GLP-1 receptor-mediated pancreatic effects.

Consistent with data from GCGR KO mice and GCGR ASO-treated mice, inhibiting GCGR signaling with mAb resulted in hyperglucagonemia and α -cell hypertrophy/hyperplasia. However, the severity of α -cell hyperplasia was lower than that observed in GCGR KO mice (Fig. 4). In fact, the maximal severity of hyperplasia observed at 3 weeks was mild and did not increase during the 18-week treatment phase. There was no evidence of pancreatic neoplasia in mice treated with GCGR-mAb for as long as 18 weeks. Both hyperglucagonemia and α -cell hyperplasia were reversible over time after the cessation of antibody treatment. The exact mechanism of the adaptive compensatory changes in pancreatic α -cells in response to reduced GCGR signaling is unknown and may be species dependent. It is noteworthy that there are differences in islet anatomy and function between species (Cabrera et al., 2006). For example, in response to the blockade of glucagon receptor signaling, rodents show increased α -cell proliferation, whereas primates display α -cell hypertrophy (Sloop et al., 2004; Bhanot et al., 2006). This scenario is reminiscent of the different responses to insulin demand between rodents and humans. Rodents meet insulin demand predominantly by β -cell proliferation, whereas humans provide more insulin by islet neogenesis, defined as new islet formation from exocrine ducts (Butler et al., 2003).

There is ample evidence indicating that a relative glucagon hypersecretion in the fasted state and inadequate suppression of postprandial glucagon levels contribute to the hyperglycemia characteristic of the diabetic state (Müller et al., 1970; Unger and Orci, 1975; Dunning and Gerich, 2007). It is not clear whether the number of glucagon receptors is altered in diabetic states in humans. It is noteworthy that GCGR mRNA expression in the livers of DIO mice treated with mAb for 4 weeks was slightly reduced. So far, GCGR antagonism achieved by either ASO or mAb has effectively ameliorated hyperglycemia in all rodent models tested (Liang et al., 2004; Sloop et al., 2004; Yan et al., 2009). Our present data have alleviated some specific concerns regarding potential metabolic perturbations associated with long-term inhibition of glucagon signaling in preclinical animal models. However, it remains to be seen whether the anti-GCGR mAb efficacy demonstrated in animal models can translate to clinical benefits in human studies.

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