Hypoglycemic Effect of Combined Ghrelin and Glucagon Receptor Blockade

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Glucagon receptor (GcgR) blockade has been proposed as an alternative to insulin monotherapy for treating type 1 diabetes since deletion or inhibition of GcgRs corrects hyperglycemia in models of diabetes. The factors regulating glycemia in a setting devoid of insulin and glucagon function remain unclear but may include the hormone ghrelin. Not only is ghrelin release controlled by glucose but also ghrelin has many actions that can raise or reduce falls in blood glucose level. Here, we tested the hypothesis that ghrelin rises to prevent hypoglycemia in the absence of glucagon function. Both GcgR knockout (*Gcgr*$^{-/-}$) mice and *db/db* mice that were administered GcgR monoclonal antibody displayed lower blood glucose levels accompanied by elevated plasma ghrelin levels. Although treatment with the pancreatic β-cell toxin streptozotocin induced hyperglycemia and raised plasma ghrelin levels in wild-type mice, hyperglycemia was averted in similarly treated *Gcgr*$^{-/-}$ mice and the plasma ghrelin level was further increased. Notably, administration of a ghrelin receptor antagonist further reduced blood glucose levels into the markedly hypoglycemic range in overnight-fasted, streptozotocin-treated *Gcgr*$^{-/-}$ mice. A lowered blood glucose level also was observed in overnight-fasted, streptozotocin-treated ghrelin receptor–null mice that were administered GcgR monoclonal antibody. These data suggest that when glucagon activity is blocked in the setting of type 1 diabetes, the plasma ghrelin level rises, preventing hypoglycemia.

Blood glucose level is normally tightly regulated by the opposing actions of insulin and glucagon. Recent studies (1) suggest that dissolution of the usual paracrine restraint of pancreatic α-cell function by insulin and the resulting aberrant hyperglucagonemia mediates the hyperglycemia and altered metabolic state of insulin-requiring diabetes. This hypothesis is supported by observations in streptozotocin (STZ)-treated glucagon receptor (GcgR) knockout (*Gcgr*$^{-/-}$) mice, which display normal glucose tolerance despite being insulin deficient due to STZ-induced pancreatic β-cell destruction (2–4). Furthermore, blockade of GcgR function, neutralization of glucagon, and suppression of hyperglucagonemia all can prevent and/or correct hyperglycemia in models of and patients with diabetes (5–8). Although not universally supported (9,10), this glucagon-focused theory opens a new avenue of targeting GcgRs for diabetes treatment.

In settings lacking both glucagon and insulin actions, the signals regulating blood glucose are unclear but may involve ghrelin. Ghrelin is a peptide hormone secreted primarily by specialized endocrine ghrelin cells within the gastric mucosa (11). After acylation by ghrelin-*O*-acyl transferase, ghrelin can bind to its receptor GHSR (growth hormone [GH] secretagogue receptor [i.e., ghrelin receptor]) to stimulate GH secretion and food intake (11). Ghrelin also regulates blood glucose, having at its disposal several targets with which to influence glucose handling. These include not only stimulatory effects on food intake and GH secretion, but also decreases in insulin secretion and insulin sensitivity and increases in circulating levels of glucocorticoids, glucagon release, hepatic gluconeogenesis, and hepatic autophagy, all of which can raise glycemia (11–16). Notably, ghrelin administration or transgenic overexpression raises blood

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glucose levels, whereas genetic deletion or pharmacologic blockage of key elements of the ghrelin system lowers blood glucose levels (13–15,17–19). Indeed, mice lacking the usual ghrelin response to marked caloric restriction develop severe hypoglycemia and experience increased mortality (12,16,20). Interestingly, plasma ghrelin levels also are negatively regulated by blood glucose, presumably because of effects on ghrelin secretion (21–23).

Thus, ghrelin both is regulated by glucose and regulates glycemia, with protective effects to maintain normoglycemia becoming apparent during marked caloric restriction. The close association of ghrelin with blood glucose positions it as a key regulator of glycemia in the settings of absent glucagon action or combined absent glucagon plus insulin actions, as would occur in patients with type 1 diabetes should anti-GcgR agents proceed to market. Here, we investigated the effects of genetic and pharmacologic GcgR blockade with or without STZ-induced pancreatic β-cell destruction on ghrelin and, in turn, the effects of manipulating this ghrelin response on glycemia.

RESEARCH DESIGN AND METHODS

Animals

All animal experiments were approved by the University of Texas Southwestern Medical Center Institutional Animal Care and Use Committee. *Gcgr*$^{-/-}$ (24) and wild-type (*Gcgr*$^{+/+}$) littermates are on a C57BL/6J background, unless indicated. *Ghsr*-null and wild-type littermates are on a C57BL/6N background. *db/db* (BKS.Gg-*Dock7*$^{m+/+}$*Lepr*^{db}/J) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were housed under a 12-h dark/light cycle with free access to water and a standard chow diet (2016 Teklad Global 16% protein diet; Envigo, Indianapolis, IN) for C57BL/6N and *Ghsr*-null mice or 2019 Teklad Global 19% protein extruded diet (Envigo) for *db/db*, *Gcgr*$^{-/-}$, and wild-type littermates, except as indicated. Male mice were studied.

Blood Collection for Ghrelin and Blood Glucose Measurement

Unless otherwise specified, samples were collected between 10:00 and 11:30 A.M. by quick superficial temporal vein bleed into EDTA-coated microtubes containing *p*-hydroxymercuribenzoic acid (final concentration 1 mmol/L; Sigma-Aldrich, St. Louis, MO) kept on ice and HCl was added to the plasma after centrifugation to achieve a final concentration of 0.1 nmol/L, as described (20). Blood glucose levels were measured using a Bayer CONTOUR Blood Glucose Monitoring System.

Primary Gastric Mucosal Cell Culture

Primary cultures of gastric mucosal cells were prepared from 8- to 14-week-old male C57BL/6N mice, as described previously (20,21).

Quantitative RT-PCR Assay

Total RNA isolation and cDNA synthesis were performed as described previously (20). Quantitative PCR was performed using an Applied Biosystems ViiA 7 Real-Time PCR System and TaqMan primer/probe chemistry using the gene-specific primers listed in Supplementary Table 1.

Immunohistochemisty

Tissue preparation and immunohistochemistry were performed as described previously (13,25).

Atenolol Administration Protocol

Atenolol was administered to 12- to 18-week-old *Gcgr*$^{-/-}$ mice and wild-type littermates as described previously (20,26). Mice were injected with atenolol (10 mg/kg body weight [BW] i.p.) or its vehicle (2 mmol/L HCl) twice daily for 3 days (at 7:30 A.M. and 7:30 P.M.). Blood was collected at 9:00 A.M. on day 2 (ad libitum fed condition), and the mice were fasted for 16 h beginning at 5:00 P.M. on day 2. On day 3, the mice were dosed with atenolol or vehicle at 7:30 A.M. while still fasting, and blood was collected at 9:00 A.M. (fasted condition).

GcgR Monoclonal Antibody Administration Protocol

Hyperglycemic (blood glucose concentration 275–400 mg/dL) *db/db* mice matched for blood glucose level were randomized to receive either a single weekly injection (5 mg/kg BW s.c.) of monoclonal antibody (mAb) B (a fully human GcgR mAb developed by REMD Biotherapeutics, Inc., Camarillo, CA) (6,27) or vehicle. The vehicle-treated group was further divided into an ad libitum fed group or a pair-fed group, in which they were given an amount of food daily that matched the daily food intake of the mAb-administered mice. Blood glucose and plasma ghrelin levels were measured at 9 weeks of age, 5 days after the last weekly injection, at 10:00 A.M.

The *Ghsr*-null mice were administered STZ (150 mg/kg i.p.) and then treated with a single injection of mAb B (10 mg/kg s.c.) or vehicle when the blood glucose concentration reached between 275 and 450 mg/dL. Blood glucose levels were measured 4 days after treatment at 10:00 A.M. and again 1 day later after a 24-h fast.

GHSR Antagonist Administration Protocol

GHSR antagonist ([*D*-Lys^3]-GHRP-6) or vehicle (water) was administered at a dose (200 nmol/L/30 g BW i.p.) described previously (17) twice daily for 5 consecutive days to *Gcgr*$^{-/-}$ mice that had or had not previously received STZ treatment (150 mg/kg i.p.). On the fourth day of drug administration, the mice were fasted overnight (12 h), after which the last injection of [*D*-Lys^3]-GHRP-6 was immediately administered. Thereafter, blood glucose levels were monitored by tail bleed at the indicated time points while the mice were continued under fasting condition. Of note, [*D*-Lys^3]-GHRP-6, although developed and widely used as a GHSR antagonist, has some off-target effects on other receptors (28).

Determination of Ghrelin and Insulin Levels

Ghrelin concentrations in plasma and cell culture media were determined by ELISA (catalog #EZRGRA-90K for acyl-ghrelin, catalog #EZRGRT-91K for total ghrelin; Merck Millipore). Plasma insulin was analyzed using an ultrasensitive mouse Insulin ELISA kit (catalog #90080; Crystal Chem, Downers Grove, IL). The end point calorimetric assays were performed using a BioTek PowerWave XS Microplate spectrophotometer and KC4 junior software.

Statistical Analyses

All data are expressed as the mean ± SEM. All statistical analyses and graph preparations were performed using GraphPad Prism version 6.0. Student *t* test, one-way ANOVA, and two-way ANOVA followed by post hoc comparison tests were used to test for significant differences among test groups, as indicated in the figure legends. Data with significant unequal variance assessed using Bartlett's test were log transformed to perform ANOVAs. The strength of the linear relationship between two sets of variables was compared by Pearson correlation coefficient. Outliers were detected by Grubb test. P values <0.05 were considered statistically significant, and P values ≥ 0.05 and <0.1 were considered to be evidence of statistical trends.

RESULTS

Blood Glucose Negatively Regulates Plasma Ghrelin

To confirm and extend previous observations showing glucose suppression of plasma ghrelin in rats and humans (22,23), we measured circulating ghrelin levels 30 min after gavage administration of D-glucose (2 g/kg BW) or saline in 24-h–fasted 8- to 12-week-old C57BL/6N mice, at which time blood glucose levels peak (29). Oral glucose not only raised blood glucose levels but also significantly reduced plasma acyl-ghrelin levels (Fig. 1*A* and *B*). Acyl-ghrelin negatively correlated with blood glucose (Fig. 1*C*). Replicating our previous findings that were suggestive of direct modulation of ghrelin secretion by glucose (21), we found that glucose can directly regulate ghrelin secretion from ex vivo primary cultures of dispersed gastric mucosal cells derived from C57BL/6N mice (Fig. 1*D*), with a glucose concentration of 10 mmol/L reducing ghrelin secretion by 17.6 ± 2.6% and a concentration of 1 mmol/L glucose increasing it by 150.4 ± 6.5% as compared with cells cultured in normoglycemic-like conditions (5 mmol/L glucose). Exposure to culture media with no glucose increased ghrelin secretion even further, by 253.1 ± 12.1% compared with that observed with a glucose concentration of 5 mmol/L. These results suggest that the ghrelin secretion machinery within

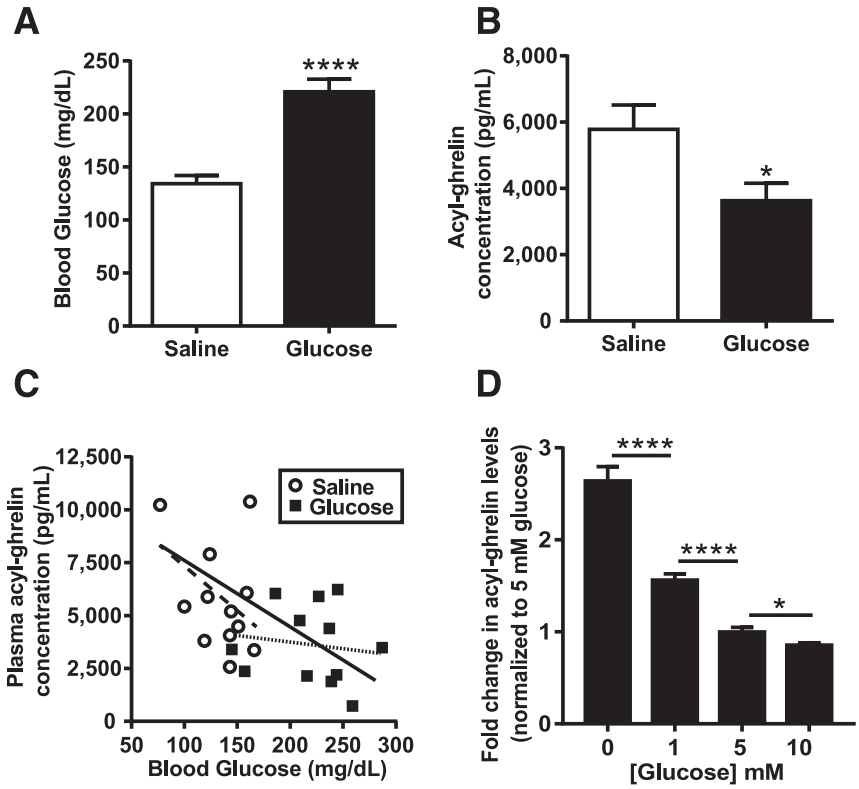

Figure 1—Glucose inhibits ghrelin secretion in vivo and ex vivo. Blood glucose levels (*A*) and plasma acyl-ghrelin concentrations (*B*) in fasted C57BL/6N mice gavaged with isotonic saline solution or D-glucose (2 g/kg BW), $n = 12$ each. Data were analyzed by Student unpaired *t* test. *$P < 0.05$; ****$P < 0.001$; significant difference between the saline and glucose administered groups. *C*: Scatter plot showing a significant negative correlation between blood glucose levels and acyl-ghrelin concentrations in those mice ($P = 0.02$; correlation coefficient $r = -0.5$). The solid line indicates the linear regression fit of all data points, the dashed line indicates the linear regression fit of data points from mice administered saline, and the dotted line indicates the linear regression fit of data points from mice administered D-glucose. *D*: Acyl-ghrelin concentrations measured in culture media of primary gastric mucosal cells after 6-h incubation in varying concentrations of D-glucose, $n = 9$ (three wells from three independent experiments), data normalized to mean acyl-ghrelin levels observed upon incubation in 5 mmol/L glucose. *$P < 0.05$; ****$P < 0.001$; significant difference between the different treatment conditions analyzed by one-way ANOVA followed by Tukey post hoc analysis. Values are expressed as the mean ± SEM.

ghrelin cells is sensitively tuned to blood glucose in the hypoglycemic range.

Plasma Acyl-Ghrelin and Total Ghrelin Are Elevated in *Gcgr*$^{-/-}$ Mice

Based on the above results, we hypothesized that *Gcgr*$^{-/-}$ mice, which demonstrate lower than usual blood glucose levels throughout the day (24), have high circulating ghrelin levels. Blood glucose levels were significantly lower in 8- to 10-week-old *Gcgr*$^{-/-}$ mice in both ad libitum fed and overnight-fasted conditions when compared with wild-type (*Gcgr*$^{+/+}$) littermates (Fig. 2*A* and *D*). This was accompanied by significantly elevated circulating concentrations of acyl- and total ghrelin in ad libitum fed (2.9-fold and 1.7-fold, respectively) and overnight-fasted (2.2-fold and 1.3-fold, respectively) conditions (Fig. 2*B*, *C*, *E*, and *F*). Acyl-ghrelin levels observed in ad libitum fed *Gcgr*$^{-/-}$ and *Gcgr*$^{+/+}$ mice on a C57BL/6J background (Fig. 2) were similar to those observed in mice on a mixed C57BL/6J and C57BL/6N background (data not shown). *Gcgr*$^{-/-}$ mice did not demonstrate changes in gastric ghrelin cell number, but preproghrelin mRNA (*Ghrl*) expression was elevated in gastric mucosal cell lysates (Supplementary Fig. 1). A much lower level of preproghrelin mRNA expression was observed in the pancreata of *Gcgr*$^{+/+}$ mice, which did not differ from that in *Gcgr*$^{-/-}$ mice (Supplementary Fig. 2). Ghrelin immunoreactivity was undetectable in pancreatic islets of both 10-week-old *Gcgr*$^{-/-}$ and *Gcgr*$^{+/+}$ mice (data not shown). These results suggest that the increase in plasma ghrelin in *Gcgr*$^{-/-}$ mice is not likely contributed to by alterations to pancreatic islets (a major site of ghrelin production in the fetal period) (15) but does involve elevated ghrelin biosynthesis within the stomach. In contrast, preproglucagon mRNA expression, which was higher in the pancreata of *Gcgr*$^{+/+}$ mice compared with their gastric mucosa, was exponentially elevated by GcgR deletion. Insulin I and Insulin II mRNA expression levels were unchanged by GcgR deletion (Supplementary Figs. 1 and 2).

Oral Administration of Glucose Reverses the Elevation of Plasma Ghrelin in *Gcgr*$^{-/-}$ Mice

To determine whether the low blood glucose levels evident in *Gcgr*$^{-/-}$ mice contributes to their elevated plasma ghrelin levels, we tested whether glucose regulation of plasma ghrelin is preserved in *Gcgr*$^{-/-}$ mice. Overnight-fasted 12- to 14-week-old *Gcgr*$^{-/-}$ mice and wild-type littermates were administered glucose (2 g/kg BW) by oral gavage, increasing blood glucose levels in both groups (Fig. 3*A*). The increase in blood glucose levels, which was lower in the *Gcgr*$^{-/-}$ mice, was accompanied by significant falls in acyl- and total ghrelin levels in both genotypes when compared with control mice administered saline (Fig. 3*B* and *C*), suggesting maintained

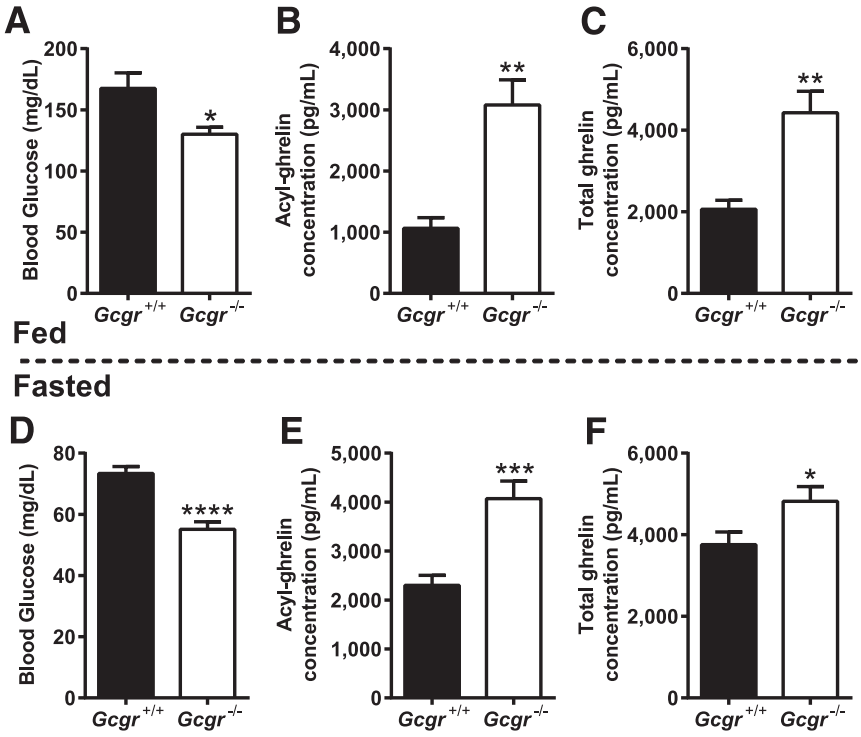

Figure 2—Reduced blood glucose in *Gcgr*$^{-/-}$ mice is accompanied by elevated plasma ghrelin levels in both ad libitum fed and overnight-fasted conditions. Blood glucose (*A* and *D*), plasma acyl-ghrelin (*B* and *E*), and total ghrelin (*C* and *F*) levels in *Gcgr*$^{-/-}$ mice are compared with those from wild-type mice (*Gcgr*$^{+/+}$) in ad libitum fed conditions (*A*–*C*) and after a 16-h overnight fast (*D*–*F*). *$P < 0.05$; **$P < 0.01$; ***$P < 0.005$; ****$P < 0.001$; significant difference in parameters in *Gcgr*$^{-/-}$ mice compared with *Gcgr*$^{+/+}$ mice, when analyzed by Student unpaired *t* test. n = 6–9. Values are expressed as the mean ± SEM.

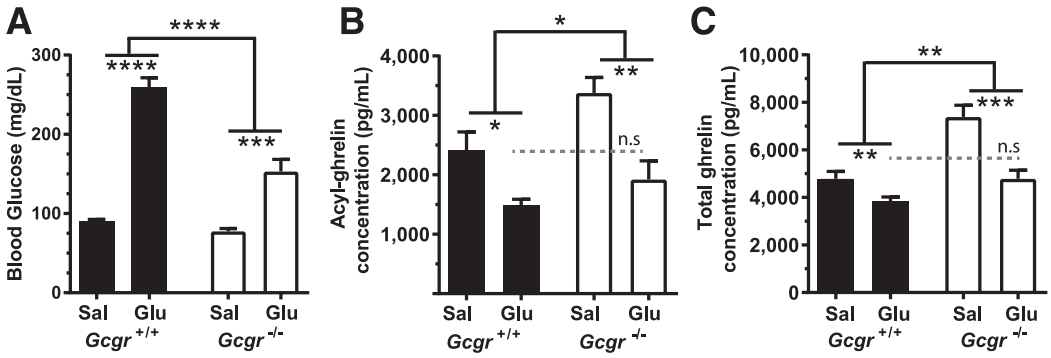

Figure 3—Oral glucose administration normalizes the higher plasma ghrelin levels in *Gcgr*$^{-/-}$ mice. *A*: Blood glucose levels in overnight-fasted mice gavaged with D-glucose (2 g/kg BW; Glu) or saline (Sal) as control in *Gcgr*$^{+/+}$ and *Gcgr*$^{-/-}$ mice. Corresponding acyl-ghrelin (*B*) and total ghrelin (*C*) levels. All the parameters were analyzed by two-way ANOVA followed by Tukey post hoc analysis. **P* < 0.05; ***P* < 0.01; ****P* < 0.005; *****P* < 0.001; significant difference in parameters due to genotype or administration of D-glucose compared with saline. No significant difference was observed in plasma acyl- and total ghrelin levels between the *Gcgr*$^{+/+}$ and *Gcgr*$^{-/-}$ mice after glucose administration (indicated by gray dashed line). *n* = 10–15. Values are expressed as the mean ± SEM.

glucose regulation of plasma ghrelin upon GcgR deletion. Although baseline ghrelin levels were higher in *Gcgr*$^{-/-}$ mice, similar to previous cohorts (Fig. 2), they fell to statistically similar levels in both *Gcgr*$^{-/-}$ and wild-type mice after glucose administration.

We also tested whether glucagon could directly influence ghrelin secretion. Using ex vivo primary cultures of dispersed gastric mucosal cells from adult C57BL/6N mice, we demonstrated that glucagon failed to induce any significant changes in ghrelin secretion (Supplementary Fig. 3).

Sympathetic Regulation of Ghrelin Secretion Is Preserved in *Gcgr*$^{-/-}$ Mice

Catecholamines released from sympathetic neurons also stimulate ghrelin secretion from ghrelin cells, contributing substantively to the usual rise in plasma ghrelin levels induced by caloric restriction (20,26). To better understand whether the increased plasma ghrelin levels in *Gcgr*$^{-/-}$ mice involves activation of the sympathetic system, we administered 8- to 12-week-old *Gcgr*$^{-/-}$ mice and wild-type littermates either the β-blocker atenolol or vehicle (10 mg/kg BW i.p.) every 12 h for 3 successive days. Atenolol did not impact ad libitum fed or fasted blood glucose levels (Fig. 4*A* and *D*). However, atenolol treatment significantly reduced plasma ghrelin levels in ad libitum fed and overnight-fasted conditions in both wild-type and *Gcgr*$^{-/-}$ mice (Fig. 4*B*, *C*, *E*, and *F*), just as had previously been demonstrated in wild-type mice (20,26).

STZ Treatment Further Elevates Plasma Ghrelin in *Gcgr*$^{-/-}$ Mice

As mentioned, *Gcgr*$^{-/-}$ mice are resistant to the hyperglycemia usually induced by STZ (2,4). Furthermore, plasma ghrelin becomes elevated in the STZ model of type 1 diabetes, whereas GHSR knockout mice and mice administered GHSR antagonist ([*D*-Lys^3]-GHRP-6) display ameliorated STZ-induced hyperphagia and hyperglycemia (30–32). Thus, we next assessed changes to plasma ghrelin levels in STZ-treated *Gcgr*$^{-/-}$ mice. Similar to previous studies, STZ administration (two doses of 150 mg/kg BW i.p., given 3 days apart) induced hyperglycemia (blood glucose concentration 467.9 ± 47.3 mg/dL) in 10- to 12-week-old ad libitum fed *Gcgr*$^{+/+}$ mice when measured 8 days after the first STZ dose; age-matched ad libitum fed *Gcgr*$^{-/-}$ mice receiving the same STZ treatment did not develop hyperglycemia (blood glucose concentration 119.5 ± 11.3 mg/dL) (Fig. 5*A*) despite comparable degrees of pancreatic β-cell ablation (Supplementary Fig. 4*A*) and similar falls in plasma insulin (Supplementary Fig. 4*B*). *Gcgr*$^{-/-}$ and *Gcgr*$^{+/+}$ mice demonstrated similar BW loss when measured 8 days after STZ treatment (Fig. 5*B*). Also, as shown previously, ad libitum fed STZ-treated wild-type mice developed higher plasma acyl-ghrelin levels than vehicle-treated mice (Fig. 5*C*). Extending that finding, STZ further increased the already higher plasma acyl-ghrelin level in *Gcgr*$^{-/-}$ mice (Fig. 5*C*). No statistically significant increases in total ghrelin were observed after STZ administration in either wild-type or *Gcgr*$^{-/-}$ mice (Fig. 5*D*).

GcgR mAb Restores Blood Glucose and Increases Plasma Ghrelin in *db/db* Mice

Similar to GcgR deletion, the administration of a GcgR mAb B lowers blood glucose levels in type 1 diabetes models (6). Here, we studied the effect of GcgR blockade in the hyperglycemic, functional leptin receptor–deficient *db/db* mouse model by administering mAb B or vehicle. mAb B corrected the hyperglycemia observed in ad libitum fed *db/db* mice (Fig. 6*A*). Plasma insulin was similar in the two groups (Supplementary Fig. 5*A*). mAb B increased plasma acyl-ghrelin levels about threefold (Fig. 6*B*). mAb B also lowered BW and food intake (Supplementary Fig. 5*B–D*). Pair feeding vehicle-treated mice to match the reduced food intake of mAb B–treated mice prevented the development of a higher BW (Supplementary Fig. 6*C*), as observed in ad libitum fed vehicle-treated mice (Supplementary Fig. 5*B*), but mAb B treatment nonetheless still reduced blood glucose and raised acyl-ghrelin levels in comparison (Supplementary

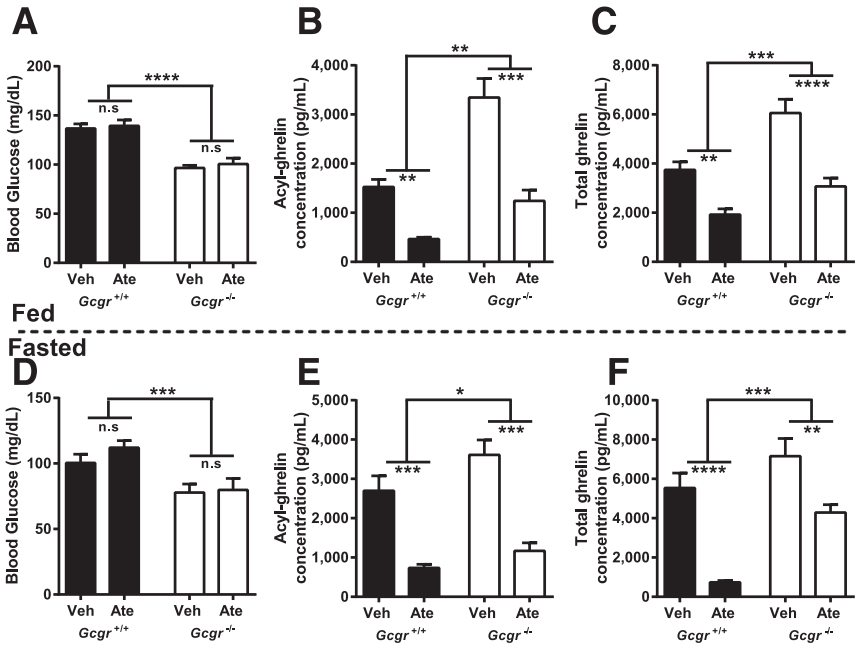

Figure 4—Pharmacologic blockade of β_1-adrenergic receptor blunts the elevation of plasma ghrelin levels in *Gcgr*$^{-/-}$ mice. Blood glucose (*A*), plasma acyl-ghrelin (*B*), and total ghrelin (*C*) levels in ad libitum fed *Gcgr*$^{+/+}$ or *Gcgr*$^{-/-}$ mice administered the β_1-adrenergic receptor–selective blocker atenolol (10 mg/kg BW i.p. every 12 h) or vehicle. Blood was collected on day 2 of atenolol administration to measure the parameters in the ad libitum fed state. Blood glucose (*D*), plasma acyl-ghrelin (*E*), and total ghrelin (*F*) levels in the same vehicle- or atenolol-treated *Gcgr*$^{+/+}$ or *Gcgr*$^{-/-}$ mice after an overnight fast. Blood was collected on day 3 of atenolol administration to measure the parameters in the overnight-fasted state. All the parameters were analyzed by two-way ANOVA followed by Tukey post hoc analysis. **P* < 0.05; ***P* < 0.01; ****P* < 0.005; *****P* < 0.001; significant difference in parameters due to genotype or administration of atenolol compared with vehicle. *n* = 6 or 7. Values are expressed as the mean ± SEM. Ate, atenolol; Veh, vehicle.

Fig. 6). These results suggest that the altered blood glucose and plasma ghrelin levels associated with mAb B are independent of its effects to reduce feeding.

Elevated Plasma Ghrelin Functions to Prevent Further Falls in Blood Glucose in STZ-Treated *Gcgr*$^{-/-}$ Mice

Of interest, in both the current STZ and *db*/*db* models and in previous studies using the STZ model and the NOD type 1 diabetes mouse model, GcgR genetic deletion or pharmacologic blockade with mAb B reduced glycemia to normal levels as opposed to hypoglycemic levels in ad libitum fed animals (4,6). Given the known function of raised ghrelin to prevent hypoglycemia from developing during long-term caloric restriction (12,20), we hypothesized that the increase in ghrelin herein observed in *Gcgr*$^{-/-}$ mice serves to prevent hypoglycemia. To test that possibility, we inhibited ghrelin action in STZ-treated 8- 10-week-old *Gcgr*$^{-/-}$ mice on a mixed C57BL/6N and C57BL/6J background by administering the GHSR antagonist [*D*-Lys^3]-GHRP-6 (200 nmol/L/30 g BW i.p. daily for 5 successive days) (Fig. 7*A*), using a previously reported protocol (17). When administered [*D*-Lys^3]-GHRP-6, STZ-treated *Gcgr*$^{-/-}$ mice became severely hypoglycemic after a 23-h fast compared with STZ-treated *Gcgr*$^{-/-}$ mice administered vehicle (Fig. 7*B*). A glucose-lowering effect also was observed in fasted *Gcgr*$^{-/-}$ mice without prior STZ treatment when subjected to a similar [*D*-Lys^3]-GHRP-6 injection protocol (Supplementary Fig. 7).

To complement those studies, STZ-treated *Ghsr*-null mice and wild-type littermates were administered GcgR mAb B or vehicle. mAb B lowered both fed (Supplementary Fig. 8*A*) and fasting (Fig. 7*C*) blood glucose levels in both wild-type and *Ghsr*-null mice. Importantly, just as observed in fasted STZ-treated *Gcgr*$^{-/-}$ mice in which GHSR antagonist dropped blood glucose levels from the low normal range into the hypoglycemic range (Fig. 7*B*), fasted STZ-treated *Ghsr*-null mice also exhibited a drop in blood glucose levels from the low normal range into the hypoglycemic range after the administration of GcgR mAb B (Fig. 7*C*). The degree of hypoglycemia induced by mAb B was slightly greater in *Ghsr*-null mice than in wild-type mice (Fig. 7*C*). There was no effect of treatment or genotype on BW, although food intake was lower in both genotypes with mAb B treatment (Supplementary Fig. 8*B* and *C*).

DISCUSSION

The current studies were designed to further our understanding of the role ghrelin plays in mediating blood glucose in the specific environment of blocked glucagon action. This setting of blocked—or more broadly, blocked, neutralized, deficient, or absent—glucagon action is relevant in light of a proposed significant effect of unopposed glucagon action in the occurrence of both type 1 diabetes– and type 2 diabetes–related hyperglycemia and, as such, the development of potential pharmacologic agents targeting

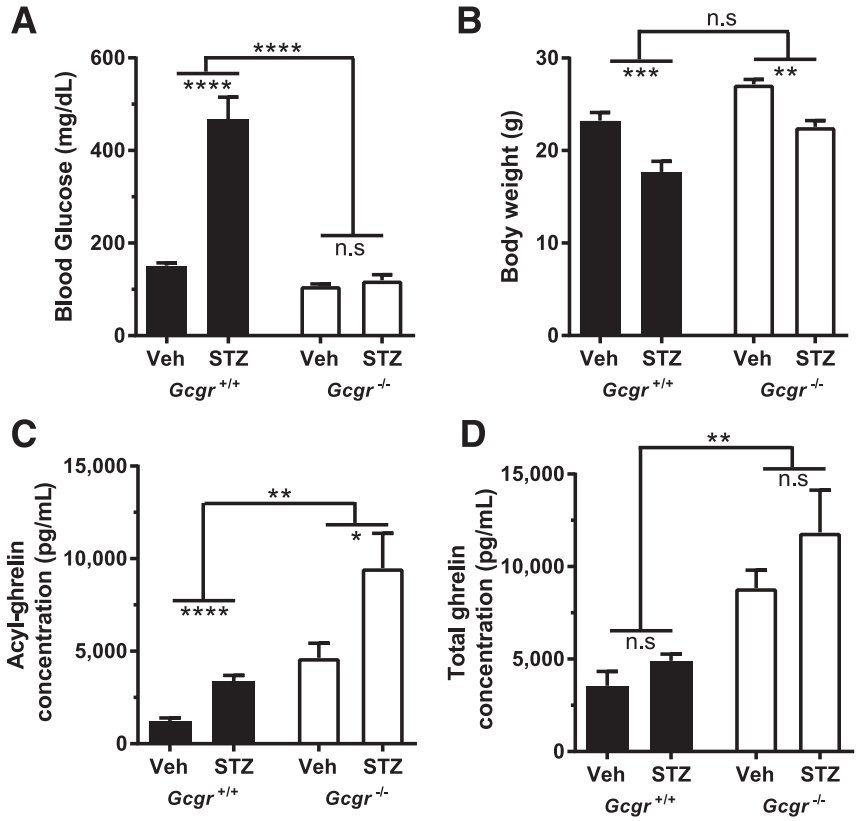

Figure 5—STZ treatment does not induce hyperglycemia in $Gcgr^{-/-}$ mice but further increases plasma acyl-ghrelin levels. Blood glucose (*A*) and BW (*B*) in $Gcgr^{+/+}$ or $Gcgr^{-/-}$ mice measured 8 days after the administration of two doses of STZ (150 mg/kg BW i.p.) 3 days apart. Corresponding plasma acyl-ghrelin (*C*) and total ghrelin (*D*) levels in the STZ-treated $Gcgr^{+/+}$ or $Gcgr^{-/-}$ mice. All the parameters were analyzed by two-way ANOVA followed by Tukey post hoc analysis. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.005$; $^{****}P < 0.001$; significant difference in parameters due to genotype or STZ treatment compared with vehicle. n = 7–9. Values are expressed as the mean ± SEM. Veh, vehicle.

glucagon action as novel treatments for diabetes (5–8). Indeed, confirming and extending previous work, the blockade of glucagon action, as achieved experimentally using genetic deletion of GcgR or by the administration of GcgR mAb B, was shown here to normalize blood glucose levels under conditions that otherwise are associated with hyperglycemia (STZ treatment or in *db/db* mice, respectively) (4,6). Excitingly, we now demonstrate that under these conditions of blocked glucagon action, plasma ghrelin becomes markedly elevated and that preventing the action of this raised ghrelin level exaggerates the blood glucose–lowering effect of blocked glucagon action, resulting in marked hypoglycemia in the setting of both absent insulin and glucagon function (fasted, STZ-treated, GHSR antagonist–administered $Gcgr^{-/-}$ mice and fasted, STZ-treated, GcgR mAb–administered *Ghsr*-null mice). Thus, these studies suggest that ghrelin rises in the setting of blocked glucagon action to prevent hypoglycemia. When viewed together with experiments demonstrating the regulation of ghrelin secretion by glucose, the regulation by ghrelin of many well-established hypoglycemia counterregulatory hormones, and an essential action of ghrelin in preventing marked life-threatening hypoglycemia during conditions mimicking a starvation state, our new results further emphasize the important role that ghrelin plays in numerous settings as an endogenous antihypoglycemic agent.

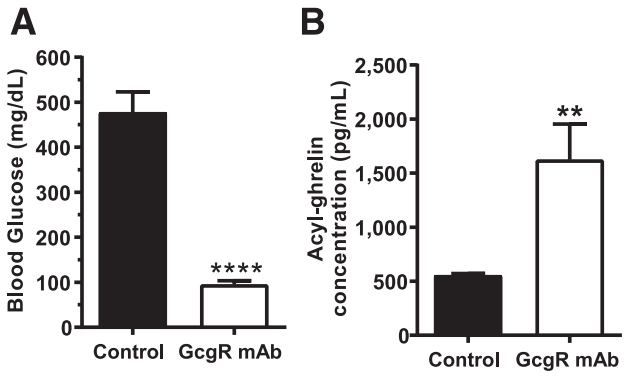

Figure 6—Treatment with GcgR mAb reduces blood glucose levels and increases plasma ghrelin levels in *db/db* mice. Blood glucose (*A*) and plasma acyl-ghrelin (*B*) levels measured in ad libitum fed *db/db* mice 5 days after treatment with vehicle or mAb to block GcgR function. $^{**}P < 0.01$; $^{****}P < 0.001$; significant difference in blood glucose and plasma acyl-ghrelin in *db/db* mice administered GcgR mAb or placebo when analyzed by Student unpaired t test. n = 6 in each group. Values are expressed as the mean ± SEM.

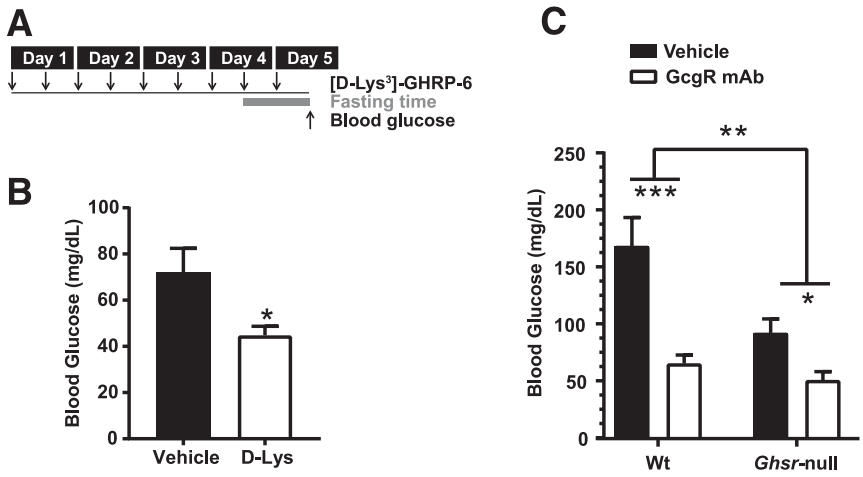

Figure 7—Lack of GHSR signaling in STZ-treated *Gcgr*$^{-/-}$ mice increases susceptibility to hypoglycemia during fasting. *A*: Schematic of the experiment shows the GHSR antagonist ([*D*-Lys^3]-GHRP-6) administration regimen (every 12 h for 5 days, as indicated by down-facing arrows). The last dose of [*D*-Lys^3]-GHRP-6 or vehicle (saline) was administered after an overnight 12-h fast. *B*: Blood glucose levels measured from the tail vein in the *Gcgr*$^{-/-}$ mice 11 h after administration of the last dose of [*D*-Lys^3]-GHRP-6 or vehicle (saline), by which time the animals had been fasted for 23 h. $n = 5$ in each group. *$P < 0.05$; significantly lower blood glucose levels with [*D*-Lys^3]-GHRP-6 treatment compared with vehicle treatment. Data were analyzed by Student unpaired *t* test. *C*: Fasted blood glucose levels in STZ-administered wild-type and *Ghsr*-null mice treated with GcgR mAb B or vehicle, measured on day 5 after treatment. $n = 5$–6. *$P < 0.05$; **$P < 0.01$; ***$P < 0.005$; significant difference in blood glucose levels due to genotype or GcgR mAb treatment. Data were analyzed by repeated-measures two-way ANOVA followed by Tukey post hoc analysis. Values are expressed as the mean ± SEM. D-Lys, [*D*-Lys^3]-GHRP-6; Wt, wild type.

The clinical significance of these findings reaffirming the antihypoglycemic actions of ghrelin, including in the setting of GcgR blockade, are predicted to become highly apparent if GcgR antagonists or mAbs proceed to market as antidiabetic therapies. Such agents already have shown promise in preclinical and initial clinical studies (6,27,33). Although experimentally in ad libitum fed states, STZ-treated *Gcgr*$^{-/-}$ mice maintain normoglycemia and *db*/*db* mice and NOD mice develop normoglycemia when treated with GcgR mAb (4,6) (Figs. 5–7), we would expect the blood glucose responses to therapies targeting glucagon action to be much more heterogeneous, with the hyperglycemia of some treated individuals not correcting fully. As such, we would envision the addition of agents targeting the ghrelin system, such as GHSR antagonists, as being viable, potential add-on (combined) therapies to those targeting glucagon action for the treatment of many individuals with type 1 diabetes. On the other hand, should appropriate glycemia be achieved with these GcgR antagonists, caution should be exercised not to combine this class of drugs with drugs that block GHSR function or that are expected to attenuate acyl-ghrelin secretion, such as β-blockers (20) or ghrelin-*O*-acyl transferase inhibitors (11). Also, if hypoglycemia is observed during GcgR blockade, we might want to test the efficacy of administering ghrelin or a ghrelin mimetic to individuals who are unable to consume simple sugars by mouth to correct the low blood glucose level, thus replacing the standard glucagon emergency kit, which presumably would not be as effective because of the anti-GcgR agent.

The current study also provides further insight regarding controls of ghrelin secretion, which is known to include several hormones (including glucagon, insulin, and glucagon-like peptide 1 [GLP-1]), nutrients (including glucose) and nutrient metabolites, and neurotransmitters (11,25). Studies reporting the influence of glucagon on ghrelin secretion have been inconsistent in the literature. Although glucagon enhanced ghrelin release from primary cultures of neonatal rat gastric mucosal cells and when infused into the arterial vasculature of an isolated rat stomach (34,35), it did not affect ghrelin release when infused directly into the gastric submucosa of rats or when applied to primary cultures of FACS-purified mouse gastric ghrelin cells from ghrelin-GFP mice or to two different immortalized ghrelinoma cell lines (26,36,37). Clinical studies have demonstrated either inhibition of ghrelin secretion and/or reduction of plasma ghrelin by glucagon (38–40) or no significant effect of glucagon administration on plasma ghrelin (41). Here, there was no direct modulation by glucagon of ghrelin secretion (Supplementary Fig. 3). Neither were plasma ghrelin levels lower in *Gcgr*$^{-/-}$ mice or GcgR mAb-treated *db*/*db* mice, as might be predicted if a purported stimulatory signal for ghrelin release were absent or blocked. Altogether, we interpret our new results along with previous findings of the very low expression of *Gcgr* in FACS-purified mouse ghrelin cells and ghrelinoma cell lines (25,37) as suggesting that glucagon has a minimal direct effect on ghrelin cells to modulate ghrelin secretion. This finding is relevant to the overall interplay between ghrelin and glucagon. For instance, we previously demonstrated (13,42) that ghrelin can increase glucagon release via direct effects on pancreatic α-cells and/or via indirect effects on hypothalamic AgRP neurons, and others have suggested

(43,44) that the modulatory effects of ghrelin on glucagon secretion may instead occur via engagement of GHSRs enriched on somatostatin-secreting pancreatic δ-cells. Despite the capacity of ghrelin to raise plasma glucagon levels (13,42), our current data support the conclusion that the antihypoglycemic actions of ghrelin do not require an intact glucagon system. Furthermore, although the current study does not directly address the mechanisms by which the ghrelin system prevents exaggerated falls in blood glucose in *Gcgr*$^{-/-}$ mice, these mechanisms likely do not include stimulation of either glucagon release or food intake (because the exaggerated reduction in blood glucose induced by GHSR antagonist in STZ-treated *Gcgr*$^{-/-}$ mice was observed in fasted conditions, in which food intake is not a factor).

Although the loss of glucagon action on ghrelin cells does not appear to be directly responsible for the increased plasma ghrelin levels observed in *Gcgr*$^{-/-}$ mice, the current study does hint at some other potentially relevant factors. For instance, plasma ghrelin in *Gcgr*$^{-/-}$ mice was responsive to atenolol treatment, just as in wild-type mice, suggesting the retention of sympathetic regulation of ghrelin secretion (20). There could also be a direct or indirect effect of the lowered blood glucose levels in *Gcgr*$^{-/-}$ mice in stimulating the higher plasma ghrelin levels (Fig. 1*D*) (21). Indeed, primary gastric mucosal cell cultures taken from STZ-treated mice retained responsiveness to both glucose and 2-deoxy-D-glucose (Supplementary Fig. 9*A* and *B*), as observed previously in cells from non–STZ-treated mice (21). Furthermore, in the combined setting of GcgR genetic deletion plus STZ treatment, in which we found plasma ghrelin levels to be higher than in either setting alone, it is likely that the absence of insulin additionally increases plasma ghrelin levels (Fig. 5). Indeed, insulin receptor expression analyses, insulin clamp studies in humans, and secretion studies using primary cultures of gastric mucosal cells suggest that insulin has a direct inhibitory effect on ghrelin release (21,45,46) (Supplementary Fig. 9*A*). The latter studies also suggest that the direct inhibitory effects of insulin on ghrelin release from ghrelin cells become progressively less sensitive in higher glucose environments (21) (Supplementary Fig. 9*A*). Thus, it is not unexpected for ghrelin to rise after STZ treatment, which is known to destroy pancreatic β-cells and thus create a hyperglycemic, insulin-deficient environment. The higher plasma ghrelin level, however, is unlikely to be due to direct influences of either GH (known to be higher in STZ diabetic mouse models) (47) (Supplementary Fig. 9*C*) or STZ on ghrelin cells (Supplementary Fig. 9*D*).

Notably, not all methods to block, delete, or neutralize glucagon signaling prevent and/or correct hyperglycemia in individuals with diabetes. For instance, diphtheria toxin–induced pancreatic α-cell ablation did not correct hyperglycemia in STZ-treated animals, although presumably this persistent hyperglycemia resulted from the persistence of modest or near-normal glucagonemia even after near total ablation of pancreatic α-cells (9,10). Neither did the administration of glucagon mAb or GcgR antagonist to STZ-treated wild-type mice correct hyperglycemia (9). It is important to note that in the latter study the reagents targeting the glucagon system were administered 20–28 days after STZ, whereas in the current study and others in which improvements in blood glucose were observed (2,4,6,48), glucagon signaling was eliminated genetically or by immunoneutralization typically 2–4 days after STZ administration at lower blood glucose levels (~350 mg/dL) and before the onset of ketoacidosis. Taken together, these results suggest that GcgR antagonist therapy to correct hyperglycemia in type 1 diabetes would likely be more effective when initiated before the onset of severe diabetic ketoacidosis.

Similarly, *Gcgr* genetic deletion is not effective in preventing hyperglycemia in all models of type 1 diabetes. In particular, genetic deletion of insulin (49) or the use of diphtheria toxin to induce near complete ablation of β-cells (50) in *Gcgr*$^{-/-}$ mice nonetheless induced full-blown diabetes. Thus, it has been suggested that a retained low level of insulin, as occurs in the STZ type 1 diabetes model, may be permissive for the blood glucose–lowering effect observed with GcgR antagonism.

As a final point of discussion, compensatory pancreatic α-cell hyperplasia and a resulting marked elevation in plasma glucagon levels develop in *Gcgr*$^{-/-}$ mice, mice administered GcgR antagonist (51), and humans with loss-of-function *Gcgr* mutations (Supplementary Fig. 4*A*) (4,24,33). In addition, *Gcgr*$^{-/-}$ mice exhibit both high plasma GLP-1 and fibroblast growth factor 21 levels, both of which have been implicated in protecting STZ-treated *Gcgr*$^{-/-}$ mice from developing hyperglycemia (3,24,48). Rodent models and humans in which GcgR mAb or GcgR antagonists have been administered also exhibit high plasma GLP-1 levels (5,27,51,52). Although GLP-1 has been shown to suppress ghrelin secretion in human subjects indirectly via stimulating insulin secretion (53), the higher ghrelin levels found within *Gcgr*$^{-/-}$ mice (Fig. 2) despite the presence of high GLP-1 levels (24) suggests that the purported inhibitory effect of GLP-1 on ghrelin secretion is not dominant in this setting. The increase in plasma ghrelin levels may, however, contribute to the higher glucagon and GLP-1 levels observed in these mice (24) because ghrelin not only can stimulate glucagon release from α-cells (13) but also (and perhaps unexpectedly given the overall effect of ghrelin to raise blood glucose or prevent falls in blood glucose levels) can stimulate GLP-1 release from L cells (54). Furthermore, within α-cell lines, ghrelin activates extracellular signal–regulated kinase signaling (13), which in other cell systems contributes to cell proliferation. Thus, it is plausible that high ghrelin levels could directly stimulate α-cell proliferation via activation of extracellular signal–regulated kinase signaling. Contribution by ghrelin to the plasma elevation of glucagon and GLP-1 and/or to α-cell hyperplasia in *Gcgr*$^{-/-}$ mice or in humans with loss-of-function GcgR mutations (4,24,33) are interesting possibilities that should be explored, especially in light of the proposed important roles

of GLP-1 hypersecretion in mediating the improved glycemia observed in STZ-treated *Gcgr*$^{-/-}$ mice (9).

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References

1. Unger RH, Orci L. Paracrinology of islets and the paracrinopathy of diabetes. Proc Natl Acad Sci U S A 2010;107:16009–16012
2. Lee Y, Berglund ED, Wang MY, et al. Metabolic manifestations of insulin deficiency do not occur without glucagon action. Proc Natl Acad Sci U S A 2012;109:14972–14976
3. Conarello SL, Jiang G, Mu J, et al. Glucagon receptor knockout mice are resistant to diet-induced obesity and streptozotocin-mediated beta cell loss and hyperglycaemia. Diabetologia 2007;50:142–150
4. Lee Y, Wang MY, Du XQ, Charron MJ, Unger RH. Glucagon receptor knockout prevents insulin-deficient type 1 diabetes in mice. Diabetes 2011;60:391–397
5. Sloop KW, Cao JX, Siesky AM, et al. Hepatic and glucagon-like peptide-1-mediated reversal of diabetes by glucagon receptor antisense oligonucleotide inhibitors. J Clin Invest 2004;113:1571–1581
6. Wang MY, Yan H, Shi Z, et al. Glucagon receptor antibody completely suppresses type 1 diabetes phenotype without insulin by disrupting a novel diabetogenic pathway. Proc Natl Acad Sci U S A 2015;112:2503–2508
7. O'Harte FP, Franklin ZJ, Irwin N. Two novel glucagon receptor antagonists prove effective therapeutic agents in high-fat-fed and obese diabetic mice. Diabetes Obes Metab 2014;16:1214–1222
8. Brand CL, Rolin B, Jørgensen PN, Svendsen I, Kristensen JS, Holst JJ. Immunoneutralization of endogenous glucagon with monoclonal glucagon antibody normalizes hyperglycaemia in moderately streptozotocin-diabetic rats. Diabetologia 1994;37:985–993
9. Steenberg VR, Jensen SM, Pedersen J, et al. Acute disruption of glucagon secretion or action does not improve glucose tolerance in an insulin-deficient mouse model of diabetes. Diabetologia 2016;59:363–370
10. Thorel F, Damond N, Chera S, et al. Normal glucagon signaling and β-cell function after near-total α-cell ablation in adult mice. Diabetes 2011;60:2872–2882
11. Müller TD, Nogueiras R, Andermann ML, et al. Ghrelin. Mol Metab 2015;4:437–460
12. Zhao TJ, Liang G, Li RL, et al. Ghrelin O-acyltransferase (GOAT) is essential for growth hormone-mediated survival of calorie-restricted mice. Proc Natl Acad Sci U S A 2010;107:7467–7472
13. Chuang JC, Sakata I, Kohno D, et al. Ghrelin directly stimulates glucagon secretion from pancreatic alpha-cells. Mol Endocrinol 2011;25:1600–1611
14. Tong J, Prigeon RL, Davis HW, et al. Ghrelin suppresses glucose-stimulated insulin secretion and deteriorates glucose tolerance in healthy humans. Diabetes 2010;59:2145–2151
15. Dezaki K, Hosoda H, Kakei M, et al. Endogenous ghrelin in pancreatic islets restricts insulin release by attenuating $Ca2^{+}$ signaling in beta-cells: implication in the glycemic control in rodents. Diabetes 2004;53:3142–3151
16. Zhang Y, Fang F, Goldstein JL, Brown MS, Zhao TJ. Reduced autophagy in livers of fasted, fat-depleted, ghrelin-deficient mice: reversal by growth hormone. Proc Natl Acad Sci U S A 2015;112:1226–1231
17. Brial F, Lussier CR, Belleville K, Sarret P, Boudreau F. Ghrelin inhibition restores glucose homeostasis in hepatocyte nuclear factor-1α (MODY3)-deficient mice. Diabetes 2015;64:3314–3320
18. Sun Y, Asnicar M, Saha PK, Chan L, Smith RG. Ablation of ghrelin improves the diabetic but not obese phenotype of ob/ob mice. Cell Metab 2006;3:379–386
19. Bewick GA, Kent A, Campbell D, et al. Mice with hyperghrelinemia are hyperphagic and glucose intolerant and have reduced leptin sensitivity. Diabetes 2009;58:840–846
20. Mani BK, Osborne-Lawrence S, Vijayaraghavan P, Hepler C, Zigman JM. β1-Adrenergic receptor deficiency in ghrelin-expressing cells causes hypoglycemia in susceptible individuals. J Clin Invest 2016;126:3467–3478
21. Sakata I, Park WM, Walker AK, et al. Glucose-mediated control of ghrelin release from primary cultures of gastric mucosal cells. Am J Physiol Endocrinol Metab 2012;302:E1300–E1310
22. Greenman Y, Golani N, Gilad S, Yaron M, Limor R, Stern N. Ghrelin secretion is modulated in a nutrient- and gender-specific manner. Clin Endocrinol (Oxf) 2004;60:382–388
23. Tschöp M, Smiley DL, Heiman ML. Ghrelin induces adiposity in rodents. Nature 2000;407:908–913
24. Gelling RW, Du XQ, Dichmann DS, et al. Lower blood glucose, hyperglucagonemia, and pancreatic alpha cell hyperplasia in glucagon receptor knockout mice. Proc Natl Acad Sci U S A 2003;100:1438–1443
25. Engelstoft MS, Park WM, Sakata I, et al. Seven transmembrane G protein-coupled receptor repertoire of gastric ghrelin cells. Mol Metab 2013;2:376–392
26. Zhao TJ, Sakata I, Li RL, et al. Ghrelin secretion stimulated by β1-adrenergic receptors in cultured ghrelinoma cells and in fasted mice. Proc Natl Acad Sci U S A 2010;107:15868–15873
27. Yan H, Gu W, Yang J, et al. Fully human monoclonal antibodies antagonizing the glucagon receptor improve glucose homeostasis in mice and monkeys. J Pharmacol Exp Ther 2009;329:102–111
28. Patel K, Dixit VD, Lee JH, et al. Identification of ghrelin receptor blocker, D-[Lys3] GHRP-6 as a CXCR4 receptor antagonist. Int J Biol Sci 2012;8:108–117
29. Bowe JE, Franklin ZJ, Hauge-Evans AC, King AJ, Persaud SJ, Jones PM. Metabolic phenotyping guidelines: assessing glucose homeostasis in rodent models. J Endocrinol 2014;222:G13–G25
30. Dong J, Peeters TL, De Smet B, et al. Role of endogenous ghrelin in the hyperphagia of mice with streptozotocin-induced diabetes. Endocrinology 2006;147:2634–2642
31. Ishii S, Kamegai J, Tamura H, Shimizu T, Sugihara H, Oikawa S. Role of ghrelin in streptozotocin-induced diabetic hyperphagia. Endocrinology 2002;143:4934–4937
32. Verhulst PJ, De Smet B, Saels I, et al. Role of ghrelin in the relationship between hyperphagia and accelerated gastric emptying in diabetic mice. Gastroenterology 2008;135:1267–1276

33. Bagger JI, Knop FK, Holst JJ, Vilsbøll T. Glucagon antagonism as a potential therapeutic target in type 2 diabetes. Diabetes Obes Metab 2011;13:965–971
34. Gagnon J, Anini Y. Glucagon stimulates ghrelin secretion through the activation of MAPK and EPAC and potentiates the effect of norepinephrine. Endocrinology 2013; 154:666–674
35. Kamegai J, Tamura H, Shimizu T, Ishii S, Sugihara H, Oikawa S. Effects of insulin, leptin, and glucagon on ghrelin secretion from isolated perfused rat stomach. Regul Pept 2004;119:77–81
36. de la Cour CD, Norlén P, Håkanson R. Secretion of ghrelin from rat stomach ghrelin cells in response to local microinfusion of candidate messenger compounds: a microdialysis study. Regul Pept 2007;143:118–126
37. Lu X, Zhao X, Feng J, et al. Postprandial inhibition of gastric ghrelin secretion by long-chain fatty acid through GPR120 in isolated gastric ghrelin cells and mice. Am J Physiol Gastrointest Liver Physiol 2012;303:G367–G376
38. Arafat MA, Otto B, Rochlitz H, et al. Glucagon inhibits ghrelin secretion in humans. Eur J Endocrinol 2005;153:397–402
39. Soule S, Pemberton C, Hunt P, Cole D, Raudsepp S, Inder W. Prandial regulation of ghrelin secretion in humans: does glucagon contribute to the preprandial increase in circulating ghrelin? Clin Endocrinol (Oxf) 2005;63:412–417
40. Hirsh D, Heinrichs C, Leenders B, Wong AC, Cummings DE, Chanoine JP. Ghrelin is suppressed by glucagon and does not mediate glucagon-related growth hormone release. Horm Res 2005;63:111–118
41. Broglio F, Gottero C, Prodam F, et al. Ghrelin secretion is inhibited by glucose load and insulin-induced hypoglycaemia but unaffected by glucagon and arginine in humans. Clin Endocrinol (Oxf) 2004;61:503–509
42. Wang Q, Liu C, Uchida A, et al. Arcuate AgRP neurons mediate orexigenic and glucoregulatory actions of ghrelin. Mol Metab 2013;3:64–72
43. Adriaenssens AE, Svendsen B, Lam BY, et al. Transcriptomic profiling of pancreatic alpha, beta and delta cell populations identifies delta cells as a principal target for ghrelin in mouse islets. Diabetologia 2016;59:2156–2165
44. DiGruccio MR, Mawla AM, Donaldson CJ, et al. Comprehensive alpha, beta and delta cell transcriptomes reveal that ghrelin selectively activates delta cells and promotes somatostatin release from pancreatic islets. Mol Metab 2016;5: 449–458
45. Flanagan DE, Evans ML, Monsod TP, et al. The influence of insulin on circulating ghrelin. Am J Physiol Endocrinol Metab 2003;284:E313–E316
46. Gagnon J, Anini Y. Insulin and norepinephrine regulate ghrelin secretion from a rat primary stomach cell culture. Endocrinology 2012;153:3646–3656
47. Johansen PB, Segev Y, Landau D, Phillip M, Flyvbjerg A. Growth hormone (GH) hypersecretion and GH receptor resistance in streptozotocin diabetic mice in response to a GH secretagogue. Exp Diabesity Res 2003;4:73–81
48. Omar BA, Andersen B, Hald J, Raun K, Nishimura E, Ahrén B. Fibroblast growth factor 21 (FGF21) and glucagon-like peptide 1 contribute to diabetes resistance in glucagon receptor-deficient mice. Diabetes 2014;63:101–110
49. Neumann UH, Ho JS, Mojibian M, Covey SD, Charron MJ, Kieffer TJ. Glucagon receptor gene deletion in insulin knockout mice modestly reduces blood glucose and ketones but does not promote survival. Mol Metab 2016;5:731–736
50. Damond N, Thorel F, Moyers JS, et al. Blockade of glucagon signaling prevents or reverses diabetes onset only if residual β-cells persist. Elife 2016;5:e13828
51. Gu W, Yan H, Winters KA, et al. Long-term inhibition of the glucagon receptor with a monoclonal antibody in mice causes sustained improvement in glycemic control, with reversible alpha-cell hyperplasia and hyperglucagonemia. J Pharmacol Exp Ther 2009;331:871–881
52. Guan HP, Yang X, Lu K, et al. Glucagon receptor antagonism induces increased cholesterol absorption. J Lipid Res 2015;56:2183–2195
53. Hagemann D, Holst JJ, Gethmann A, Banasch M, Schmidt WE, Meier JJ. Glucagon-like peptide 1 (GLP-1) suppresses ghrelin levels in humans via increased insulin secretion. Regul Pept 2007;143:64–68
54. Gagnon J, Baggio LL, Drucker DJ, Brubaker PL. Ghrelin Is a Novel Regulator of GLP-1 Secretion. Diabetes 2015;64:1513–1521