β₁-Adrenergic receptor deficiency in ghrelin-expressing cells causes hypoglycemia in susceptible individuals

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Ghrelin is an orexigenic gastric peptide hormone secreted when caloric intake is limited. Ghrelin also regulates blood glucose, as emphasized by the hypoglycemia that is induced by caloric restriction in mouse models of deficient ghrelin signaling. Here, we hypothesized that activation of β₁-adrenergic receptors (β₁ARs) localized to ghrelin cells is required for caloric restriction–associated ghrelin release and the ensuing protective glucoregulatory response. In mice lacking the β₁AR specifically in ghrelin-expressing cells, ghrelin secretion was markedly blunted, resulting in profound hypoglycemia and prevalent mortality upon severe caloric restriction. Replacement of ghrelin blocked the effects of caloric restriction in β₁AR-deficient mice. We also determined that treating calorically restricted juvenile WT mice with beta blockers led to reduced plasma ghrelin and hypoglycemia, the latter of which is similar to the life-threatening, fasting-induced hypoglycemia observed in infants treated with beta blockers. These findings highlight the critical functions of ghrelin in preventing hypoglycemia and promoting survival during severe caloric restriction and the requirement for ghrelin cell–expressed β₁ARs in these processes. Moreover, these results indicate a potential role for ghrelin in mediating beta blocker–associated hypoglycemia in susceptible individuals, such as young children.

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neuron terminals, blocks the overnight fast–induced increase in plasma ghrelin, as does the β1-adrenergic receptor (β1AR) blocker atenolol (34). Importantly, β1AR is the most highly expressed of all nonodorant GPCRs within ghrelin cells (~25-fold higher than the next), the most highly expressed of the adrenergic receptors within ghrelin cells, and the only small-molecule neurotransmitter receptor enriched in ghrelin cells as compared with non-ghrelin gastric mucosal cells (34, 35, 37).

Given this β1AR expression data and stimulation of ghrelin secretion by catecholamines, we designed the current study to test the hypothesis that activation of β1ARs is required to engage the protective glucoregulatory functions of the ghrelin system.

Results

Generation of mice with ghrelin cell–specific deletion of β1ARs. The majority of the mice generated previously using a germline β1AR-KO (Adrb11/1) approach were reported to die prenatally (38). Here, we selectively deleted β1AR from ghrelin-expressing cells. To do so, a conditional Adrb1–KO (Adrb1fl/fl) mouse line was first developed by flanking the single exon Adrb1 gene with inserted loxP sites (Figure 1), followed by its genetic cross with our previously reported and validated ghrelin-Cre line that expresses Cre recombinase selectively in ghrelin cells (35, 39). As demonstrated previously, and together with further immunohistochemical and quantitative reverse transcriptase PCR (qPCR) authentication of the ghrelin-Cre line performed here (see Supplemental Methods; Supplemental Figures 1 and 2; supplemental material available online with this article; doi:10.1172/JCI86270DS1), cells with Cre activity include more than 95% of ghrelin cells in the gastric mucosa (from which most circulating ghrelin in adults emanates [refs. 40–44]) and a majority (~69%) of the highly dispersed, far less numerous population of ghrelin cells in the duodenum (35). Other cells with Cre activity include Leydig cells of the testis, epithelial cells of the epididymis, and scarce cells within some pancreatic islets, all of which correspond to known ghrelin cell distribution patterns (44–49). Germline transmission of the recombinant loxP-flanked Adrb1 gene was validated by PCR analysis of genomic DNA obtained by tail snips (Supplemental Figure 3, A and B). Four experimental groups were generated: β1ARfl/fl ghrelin-CreTg– mice (referred to herein as GC-β1AR–/– mice) with ghrelin cell–selective deletion of β1AR and 3 control genotypes (WT control: β1ARWT/WT ghrelin-CreTg+, referred to herein as β1ARWT/WT);Cre recombinase control: β1ARWT/WT ghrelin-CreTg–; floxed β1AR control: β1ARWT/WT ghrelin-CreTg–, referred to herein as β1ARfl/fl/G-CreTg–; and floxed β1AR control: β1ARWT/WT ghrelin-CreTg–, referred to herein as β1ARfl/fl/G-CreTg–. Cre-mediated deletion of the β1AR gene selectively in ghrelin cells from GC-β1AR–/– mice was confirmed by comparing Adrb1 mRNA levels (as determined by qPCR) in FACS-purified gastric ghrelin cells obtained from GC-β1AR–/– mice with Adrb1 mRNA levels in FACS-purified gastric ghrelin cells obtained from β1ARWT/WT mice (Adrb1 mRNA levels in FACS-purified gastric ghrelin cells obtained from β1ARWT/WT control mice, after those mice had been placed on a ghrelin humanized Renilla reniformis GFP (ghrelin-hrGFP) reporter background (37). As compared with gastric ghrelin cells from control mice, those from GC-β1AR–/– mice had a 73.5% reduction in Adrb1 mRNA levels (qPCR) authentication of the ghrelin-Cre line performed here (see Supplemental Methods; Supplemental Figures 1 and 2; supplemental material available online with this article; doi:10.1172/JCI86270DS1), cells with Cre activity include more than 95% of ghrelin cells in the gastric mucosa (from which most circulating ghrelin in adults emanates [refs. 40–44]) and a majority (~69%) of the highly dispersed, far less numerous population of ghrelin cells in the duodenum (35). Other cells with Cre activity include Leydig cells of the testis, epithelial cells of the epididymis, and scarce cells within some pancreatic islets, all of which correspond to known ghrelin cell distribution patterns (44–49). Germline transmission of the recombinant loxP-flanked Adrb1 gene was validated by PCR analysis of genomic DNA obtained by tail snips (Supplemental Figure 3, A and B). Four experimental groups were generated: β1ARfl/fl ghrelin-CreTg– mice (referred to herein as GC-β1AR–/– mice) with ghrelin cell–selective deletion of β1AR and 3 control genotypes (WT control: β1ARWT/WT ghrelin-CreTg+, referred to herein as β1ARWT/WT);Cre recombinase control: β1ARWT/WT ghrelin-CreTg–; floxed β1AR control: β1ARWT/WT ghrelin-CreTg–, referred to herein as β1ARfl/fl/G-CreTg–; and floxed β1AR control: β1ARWT/WT ghrelin-CreTg–, referred to herein as β1ARfl/fl/G-CreTg–. Cre-mediated deletion of the β1AR gene selectively in ghrelin cells from GC-β1AR–/– mice was confirmed by comparing Adrb1 mRNA levels (as determined by qPCR) in FACS-purified gastric ghrelin cells obtained from GC-β1AR–/– mice with Adrb1 mRNA levels in FACS-purified gastric ghrelin cells obtained from β1ARWT/WT control mice, after those mice had been placed on a ghrelin humanized Renilla reniformis GFP (ghrelin-hrGFP) reporter background (37). As compared with gastric ghrelin cells from control mice, those from GC-β1AR–/– mice had a 73.5% reduction in Adrb1
mRNA expression levels (Supplemental Figure 3C). Adrb1 expression was unaffected in a set of 7 tissues outside the gastrointestinal tract (Supplemental Table 1).

Ghrelin cell–selective β1AR deletion lowers plasma ghrelin and fasting blood glucose levels. Plasma ghrelin normally increases upon fasting. To study the in vivo impact of β1AR deletion in ghrelin cells on ghrelin secretion and blood glucose levels, we measured plasma ghrelin and blood glucose before and after a 24-hour fast in 8-week-old GC-β1AR−/− mice. These mice showed, on average, 4.5-fold lower ad libitum–fed and 5.8-fold lower fasted plasma acyl-ghrelin and norepinephrine levels when compared with levels in the control groups (Figure 2, A and D). Plasma total ghrelin levels in GC-β1AR−/− mice were, on average, 2.2-fold lower in ad libitum–fed and 3.6-fold lower in fasted conditions when compared with levels in the control groups (Figure 2, B and E). These in vivo results were corroborated by ghrelin secretion experiments using ex vivo primary gastric mucosal cell cultures derived from ad libitum–fed control groups or GC-β1AR−/− mice treated for 6 hours with or without 10 μM norepinephrine (NE). (G) Acyl-ghrelin concentrations in media from primary gastric mucosal cell cultures derived from ad libitum–fed control groups or GC-β1AR−/− mice treated for 6 hours with or without 10 μM norepinephrine (NE), n = 6–9 wells each. *P < 0.05 and ****P < 0.001, for significant increases in ghrelin secretion with norepinephrine treatment compared with vehicle treatment within the same genotype and significant decreases in ghrelin secretion in cultures with GC-β1AR−/− mice compared with control groups, as analyzed by 2-way ANOVA, followed by Holm-Sidak’s post-hoc multiple comparisons test. All values are expressed as the mean ± SEM. w/w, mice with WT β1AR genes; fl/fl, mice homozygous for the loxP-flanked β1AR gene; −, absence of the ghrelin-Cre Tg; +, presence of the ghrelin-Cre Tg.

The plasma ghrelin levels achieved by ghrelin cell–selective β1AR deletion were similar to those achieved by global β1AR blockade or deletion: pharmacological inhibition of β1AR by twice–daily administration of atenolol for 3 days reduced plasma acyl-ghrelin in ad libitum–fed WT mice by 3.2-fold and markedly blunted the usual rise in plasma ghrelin induced by a 24-hour fast (Supplemental Figure 5), as reported previously (34). The accompanying blood glucose values of these fasted 6-week-old mice were unaffected by atenolol. Also, we generated limited numbers of global β1AR-KO mice (Z-β1AR−) by crossing the β1AR0/0 mice with Zp3-Cre mice (51) (see Supplemental Methods for a detailed description). The few surviving Z-β1AR−/− mice, which we confirmed as having global β1AR deletion (Supplemental Figure 6A) and which were phenotypically similar to WT mice in overall BW and appearance, demonstrated lower basal and fasted plasma acyl- and total ghrelin levels (Supplemental Figure 6B).

Ghrelin deficiency in GC-β1AR−/− mice does not greatly impact food intake, BW, or adiposity. To determine the effect of ghrelin cell β1AR deletion–induced blunting of ghrelin secretion on food intake and BW, we fed female GC-β1AR−/− mice standard chow or an HFD (42% kcal from fat) for 16 weeks. Ad libitum–fed and fasted acyl-
ure 3A). Chow-fed GC-β₁AR⁻/⁻ mice demonstrated reduced gastric mucosal preproghrelin mRNA expression, although this observation was significant only in the fasted condition (Figure 3B). Preproghrelin mRNA expression levels were not different among the experimental groups fed an HFD (Supplemental Figure 8). No differences in weekly food intake or BW were observed in GC-β₁AR⁻/⁻ mice (Figure 3, C–F). Likewise, body composition and body length were similar at the end of the study (Supplemental Figure 9).

ghrelin levels were lower in the female GC-β₁AR⁻/⁻ mice than were levels in control mice at various time points over the 16-week period (Figure 3A; Supplemental Figure 7), similar to our observation in standard chow–fed 8-week-old male mice (Figure 2). While control groups fed an HFD for 16 weeks had reduced fasted plasma ghrelin levels compared with mice fed standard chow, HFD feeding in GC-β₁AR⁻/⁻ mice did not alter plasma ghrelin levels compared with levels in the standard chow–fed GC-β₁AR⁺/⁺ mice (Figure 3A). Chow-fed GC-β₁AR⁻/⁻ mice demonstrated reduced gastric mucosal preproghrelin mRNA expression, although this observation was significant only in the fasted condition (Figure 3B). Preproghrelin mRNA expression levels were not different among the experimental groups fed an HFD (Supplemental Figure 8). No differences in weekly food intake or BW were observed in GC-β₁AR⁻/⁻ mice (Figure 3, C–F). Likewise, body composition and body length were similar at the end of the study (Supplemental Figure 9).
As GHSR antagonist has been shown to reduce food intake in acutely fasted WT mice (13), we also tested whether blunted fasting-induced ghrelin secretion in GC-βAR−/− mice has a similar effect. Rebound food intake following a 24-hour fast of 8-week-old male mice that had been maintained on a standard chow diet was measured. GC-βAR−/− mice ingested less standard chow in the 24 hours following fasting than did the β1AR+/−/− G-Cre Tg WT control group mice (Figure 4).

Severe (60%) caloric restriction of GC-βAR−/− mice induces frank hypoglycemia and increases mortality. To test the functional significance of ghrelin cell βARs during a more severe state of energy insufficiency, we subjected singly housed 8-week-old GC-βAR−/− mice to 60% caloric restriction (by feeding them 40% of their usual daily calories) for 6 successive days, using a previously reported protocol (39, 52-54). BW declines in GC-βAR−/− mice were similar to those in control groups (Supplemental Figure 10). Although plasma acyl-ghrelin levels rose in GC-βAR−/− mice to 60% caloric restriction (by feeding them 40% of their usual daily calories) for 6 successive days, using a previously reported protocol (39, 52-54). BW declines in GC-βAR−/− mice were similar to those in control groups (Supplemental Figure 10). Although plasma acyl-ghrelin levels rose in GC-βAR−/− mice to 60% caloric restriction (by feeding them 40% of their usual daily calories) for 6 successive days, using a previously reported protocol (39, 52-54). BW declines in GC-βAR−/− mice were similar to those in control groups (Supplemental Figure 10). Although plasma acyl-ghrelin levels rose in GC-βAR−/− mice to 60% caloric restriction (by feeding them 40% of their usual daily calories) for 6 successive days, using a previously reported protocol (39, 52-54). BW declines in GC-βAR−/− mice were similar to those in control groups (Supplemental Figure 10). Although plasma acyl-ghrelin levels rose in GC-βAR−/− mice to 60% caloric restriction (by feeding them 40% of their usual daily calories) for 6 successive days, using a previously reported protocol (39, 52-54). BW declines in GC-βAR−/− mice were similar to those in control groups (Supplemental Figure 10). Although plasma acyl-ghrelin levels rose in GC-βAR−/− mice to 60% caloric restriction (by feeding them 40% of their usual daily calories) for 6 successive days, using a previously reported protocol (39, 52-54). BW declines in GC-βAR−/− mice were similar to those in control groups (Supplemental Figure 10). Although plasma acyl-ghrelin levels rose in GC-βAR−/− mice to 60% caloric restriction (by feeding them 40% of their usual daily calories) for 6 successive days, using a previously reported protocol (39, 52-54). BW declines in GC-βAR−/− mice were similar to those in control groups (Supplemental Figure 10). Although plasma acyl-ghrelin levels rose in GC-βAR−/− mice to 60% caloric restriction (by feeding them 40% of their usual daily calories) for 6 successive days, using a previously reported protocol (39, 52-54). BW declines in GC-βAR−/− mice were similar to those in control groups (Supplemental Figure 10). Although plasma acyl-ghrelin levels rose in GC-βAR−/− mice to 60% caloric restriction (by feeding them 40% of their usual daily calories) for 6 successive days, using a previously reported protocol (39, 52-54). BW declines in GC-βAR−/− mice were similar to those in control groups (Supplemental Figure 10). Although plasma acyl-ghrelin levels rose in GC-βAR−/− mice to 60% caloric restriction (by feeding them 40% of their usual daily calories) for 6 successive days, using a previously reported protocol (39, 52-54). BW declines in GC-βAR−/− mice were similar to those in control groups (Supplemental Figure 10). Although plasma acyl-ghrelin levels rose in GC-βAR−/− mice to 60% caloric restriction (by feeding them 40% of their usual daily calories) for 6 successive days, using a previously reported protocol (39, 52-54). BW declines in GC-βAR−/− mice were similar to those in control groups (Supplemental Figure 10). Although plasma acyl-ghrelin levels rose in GC-βAR−/− mice to 60% caloric restriction (by feeding them 40% of their usual daily calories) for 6 successive days, using a previously reported protocol (39, 52-54). BW declines in GC-βAR−/− mice were similar to those in control groups (Supplemental Figure 10). Although plasma acyl-ghrelin levels rose in GC-βAR−/− mice to 60% caloric restriction (by feeding them 40% of their usual daily calories) for 6 successive days, using a previously reported protocol (39, 52-54). BW declines in GC-βAR−/− mice were similar to those in control groups (Supplemental Figure 10). Although plasma acyl-ghrelin levels rose in GC-βAR−/− mice to 60% caloric restriction (by feeding them 40% of their usual daily calories) for 6 successive days, using a previously reported protocol (39, 52-54). BW declines in GC-βAR−/− mice were similar to those in control groups (Supplemental Figure 10). Although plasma acyl-ghrelin levels rose in GC-βAR−/− mice to 60% caloric restriction (by feeding them 40% of their usual daily calories) for 6 successive days, using a previously reported protocol (39, 52-54).

To confirm the role of reduced ghrelin secretion in the marked hypoglycemia and increased mortality experienced by GC-βAR−/− mice, a separate cohort of GC-βAR−/− mice was administered 4 mg/kg/day acyl-ghrelin s.c. via an osmotic minipump during the severe caloric restriction period. Ghrelin infusion, which increased plasma ghrelin to a level slightly higher than that attained physiologically in control mice during severe caloric restriction (Figures 5, A and D), not only increased blood glucose in ad libitum-fed GC-βAR−/− mice (day 0 in Figure 5E) but also prevented the fall in blood glucose in GC-βAR−/− mice upon severe caloric restriction and resulted in 100% survival (Figure 5, E and F).

Administration of atenolol to juvenile mice induces hypoglycemia accompanied by reduced plasma ghrelin levels. Beta blockers are widely prescribed drugs to treat cardiac failure and hypertension. While hypoglycemia unawareness is a concern in adult diabetic subjects treated with beta blockers (55), life-threatening hypoglycemic episodes are not an infrequent occurrence in fasted infants and toddlers treated with beta blockers, e.g., for infantile hemangioma and arrhythmias (56-59). To test whether beta blocker-induced hypoglycemia in young patients could be related to reductions in plasma ghrelin levels, we designed a preclinical model in which 3-week-old WT mice were administered atenolol or vehicle twice daily for 3 days. Plasma acyl- and total ghrelin levels measured in fed conditions or after a 24-hour fast were both significantly reduced on the third day with atenolol administration (Figure 6, A and B). Atenolol treatment significantly lowered blood glucose (in many cases to below 50 mg/dl) when measured after fasting, but not in the fed state (Figure 6C). Plasma acyl-ghrelin levels positively correlated with blood glucose levels in these fasted 3-week-old mice (Figure 6D). The correlation was significantly influenced more by the vehicle-treated mice than by the atenolol-treated mice (which, as a group, had lower ghrelin levels), suggesting a threshold concentration of ghrelin above which its protective glucoregulatory functions are active. Plasma insulin levels did not differ with atenolol treatment in the fasted 3-week-old mice, but plasma IGF-1 was significantly lower with atenolol treatment (Supplemental Figure 12). The reduction in blood glucose and IGF-1 levels with atenolol treatment was accompanied by induction of the hepatic gluconeogenic genes encoding peroxisome proliferative activated receptor γ, coactivator 1α (PGC1α [Pparcg1a]), phosphoenolpyruvate carboxykinase (1 [PEPCK [Pck1]], and glucose-6-phosphatase (6GP (G6p)) (Supplemental Figure 13). Other hepatic genes encoding for the gluconeogenic enzymes hepatic nuclear factor 4a (2 (HNF4α [Hnf4a]) and pyruvate carboxylase (PCX [Pcx]) and the glycogenolytic enzyme liver glycogen phosphorylase (PYGL [Pygl]) did not change with atenolol treatment. Although plasma IGF-1 was reduced by atenolol, mRNA levels of IGF1 and insulin-like growth factor–binding protein 1 (Igfbipl) were unchanged (Supplemental Figures 12 and 13). Unlike in fasted 3-week-old mice, neither fed 3-week-old mice (Figure 6C) nor fed or fasted 6-week-old mice (Supplemental Figure 5) had atenolol-induced reductions in plasma ghrelin levels associated with hypoglycemia.
Discussion

Until now, advances in our understanding of β₁AR biology, including as it regards ghrelin secretion, have been limited mainly to pharmacological approaches because of the poor survival rates of germline β₁AR-KO mice (38). The new conditional β₁AR-KO mouse line reported here has enabled us to test the functional significance of ghrelin cell β₁ARs, including their effects on ghrelin secretion, blood glucose regulation, survival response to a starvation-like state, food intake, and BW. Using GC-β₁AR–/– mice, which reproduce and survive normally, we now provide genetic evidence of required ghrelin cell β₁AR expression to regulate basal ghrelin release and to maintain preproghrelin gene transcription and stimulate ghrelin secretion in response to caloric restriction. Furthermore, we reveal the required roles for ghrelin cell β₁ARs in preventing falls in blood glucose levels during caloric restriction (including preventing the development of marked hypoglycemia in both severely calorie-restricted adult mice and acutely fasted young mice), in supporting survival in the setting of a starvation-like state, and in encouraging rebound food intake following a short-term fast.

There are several noteworthy discussion points and implications of these results. Hypoglycemia was the most conspicuous phenotype resulting from the abrogated ghrelin secretion in GC-β₁AR–/– mice, developing within just a few days of exposure to severe caloric restriction and probably contributing to the pronounced mortality. This protocol, which rapidly and markedly depletes fat stores and emulates a starvation state (28), has the same effect in mice that lack ghrelin (54), GOAT (52, 60), or GHSR (53) and in mice with targeted ghrelin cell degradation (39). However, the reproducibility of this effect and the dependency of ghrelin secretion in this starvation model on ghrelin cell β₁AR expression were not expected, especially since low glucose can itself stimulate ghrelin secretion (26). Notably, although this effect of low glucose is observed in cultured gastric ghrelin cells (26), the same phenomenon when occurring in vivo seems unable to mount a ghrelin secretory response of sufficient magnitude to prevent further blood glucose falls and preserve life. We conclude that β₁AR-driven ghrelin secretion induced by caloric restriction is protective, particularly in starvation-like states during which it prevents marked hypoglycemia and sustains life.

Given our results with severely calorie-restricted GC-β₁AR–/– mice and overnight-fasted young WT mice administered atenolol, we also propose that unintended blockade of ghrelin cell β₁ARs in beta blocker–treated individuals and the ensuing reduction in plasma ghrelin might, in a permissive setting, predispose them to...
severe hypoglycemia and death (Figure 7). As mentioned above, life-threatening hypoglycemia is a well-recognized adverse effect of beta blocker therapy in young children (57, 58, 61). Several cases of hypoglycemia in newborns and toddlers with hemangioma treated with the nonselective beta blocker propranolol have been reported and most often occurred when there was poor oral intake such as during an overnight fast or concomitant infection (56). These cases have prompted the establishment of a standardized consensus–derived set of best practices for propranolol-treated such as during an overnight fast or concomitant infection (56).

As mentioned in the Introduction, ghrelin has at its disposal several potential downstream effector hormones and tissues through which it may act to sustain blood glucose levels in fasted states. One of its key glucoregulatory mediators is GH, for which ghrelin serves as a potent secretagogue during caloric restriction (1, 52). Whereas plasma GH progressively rises in WT mice subjected to a week of 60% caloric restriction, peaking just prior to the addition of food, this rise is blunted in similarly treated GOAT–/– mice, which exhibit an approximately 2-fold reduction in plasma GH accompanying hypoglycemia when measured on the final day of 60% caloric restriction (52, 54, 60). In the absence of ghrelin, fat-depleted mice with limited access to food, as achieved using the 60% caloric restriction protocol, are unable to maintain sufficient rates of gluconeogenesis, which is attributed to its GH secretion which in the case of GOAT–/– and ghrelin–/– mice, which exhibit an approximately 2-fold reduction in plasma GH accompanying hypoglycemia when measured on the final day of 60% caloric restriction (52, 54, 60).

The previous studies with GOAT–/– and ghrelin–/– mice revealed a reduced generation of substrates for gluconeogenesis during 60% caloric restriction, which in the case of GOAT–/– mice was associated with reduced hepatic autophagy (54, 60). Those previous studies also suggested that ghrelin’s protective effects on blood glucose and survival during 60% caloric restriction are directly attributable to its GH secretagogue activity and in turn (via a non-IGF-1–signaling mechanism) to stimulation of hepatic autophagy, together with other as-yet undiscovered processes, to supply substrates for gluconeogenesis (54, 60). In the absence of ghrelin, fat-depleted mice with limited access to food, as achieved using the 60% caloric restriction protocol, are unable to maintain sufficient rates of gluconeogenesis, even though there is an attempt via upregulation of gluconeogenic enzyme gene expression (e.g., PEPCK and G6Pase) (52). While the
severely calorie-restricted GC-β1AR–/– mice in the current study did not demonstrate reduced GH levels, as had been detected previously in GOAT–/– and ghrelin–/– mice, unlike the previous studies, in which the blood samples for GH estimation were collected from all mice that underwent the severe caloric restriction protocol (including those that were in a moribund state due to hypoglycemia) (52), here, samples were not taken from moribund animals and instead only included those from mice that survived the entire 6 days of the protocol. This also explains the reduced sample size of plasma GH values from GC-β1AR–/– mice (Supplemental Figure 4E). Had samples been obtained from all GC-β1AR–/– mice in the severe caloric restriction study, including those that did not survive (just prior to their demise), we believe that an impaired GH response as a result of ghrelin secretion deficiency would become evident, similar to what was observed in the previous studies.

A blunted GH pathway response as a result of lowered ghrelin levels, though, was detected here in the fastest 3-week-old C57BL/6N mice treated with atenolol. In particular, following the 24-hour fast, lowered plasma IGF-1 accompanied the lowered blood glucose and lowered ghrelin levels in mice treated with atenolol as compared with levels in controls. Thus, despite likely differences in the amounts of stored glucose, stored fat, and potential circulating gluconeogenic substrates in 24-hour fasted 3-week-old mice versus adult mice exposed to a week-long, fat-depleting 60% caloric restriction protocol, in both scenarios, hypoglycemia results from an experimentally induced insufficient ghrelin response and a resulting insufficient GH response (albeit seemingly normally mediated by IGF-1 in the former scenario versus hepatic phosphorylated-STAT5 in the latter scenario) (60). Also of note, while gene expression of the hepatic gluconeogenic enzymes PGC1α, PEPCK, and G6P was induced here in the fastest, atenolol-treated 3-week-old mice, it is apparent that this likely compensatory response is nonetheless inadequate to prevent hypoglycemia from developing. Similarly, gluconeogenesis previously had been determined to be insufficient in 60% calorie-restricted GOAT–/– mice, despite upregulation of PEPCK and G6P, presumably due to an insufficient availability of gluconeogenic substrates (52).

Ghrelin also has the capacity to influence blood glucose by stimulating food intake, which was experimentally restricted in the current study, and by raising circulating glucocorticoids and/or reducing insulin sensitivity (23, 50, 66), which were not specifically examined here. Furthermore, ghrelin can modify the secretion of several pancreatic islet hormones that regulate blood glucose including the reduction of insulin secretion and stimulation of glucagon secretion (via either direct actions on pancreatic β cells [21, 23, 67] and α cells [24] or indirect actions on hypothalamic AgRP neurons [53] or somatostatin-secreting pancreatic δ cells [68]). However, insulin and glucagon levels in GC-β1AR–/– mice subjected to the 60% caloric restriction protocol were like those in similarly treated control groups. These results, along with similar observations in 60% calorie-restricted GOAT–/– mice (52), suggest that ghrelin modulation of insulin or glucagon levels may not be a significant contributor to ghrelin’s actions to sustain blood glucose in states of severe caloric restriction. Rather, these effects on insulin and glucagon probably play a more prominent role in ghrelin’s glucoregulatory efficacy in other scenarios. For instance, previously, we have demonstrated exaggerated drops in plasma glucose associated with exaggerated drops in blood glucose (albeit not frank hypoglycemia) in overnight-fasted GHSR-deficient mice as compared with levels in WT mice (24, 53). The unaltered insulin and glucagon levels observed here also suggest that the scarce expression of ghrelin-Cre within pancreatic islets did not alter the overall usual sympathetic influence on islet hormone release during hypoglycemia (69, 70).

It is also noteworthy that signals from the autonomic nervous system so prominently impact ghrelin secretion, especially given the inherent nutrient-sensing, enteroendocrine nature of most ghrelin cells (71, 72). While recognition by ghrelin cells of dietary metabolites — and, in particular, their sensing of low glucose levels — could drive the modest increase in plasma ghrelin levels observed in GC-β1AR–/– mice upon a 24-hour fast or severe caloric restriction, the levels achieved are far lower than those in mice with intact ghrelin cell β1AR signaling. Thus, it could be that the major impact of nutrients such as glucose on ghrelin secretion is inhibitory and, instead, that signals from the brain — such as those carried by the sympathetic nerves — serve as the main stimulatory regulators of ghrelin release. The entrainment of preprandial rises in plasma ghrelin levels to set meal schedules.
Here, mice lacking ghrelin cell
being a compensatory response to prevent exaggerated BW gain
are usually lower in diet-induced obesity, which is often touted as
or body composition. Of related interest, plasma ghrelin levels
tion did not impact ad libitum feeding responses, BW regulation,
food intake following a 24-hour fast, suggesting a modest role for
ghrelin cells, the β
1AR signaling in
specific role of catecholaminergic input from the sympathetic
nervous system in β
1AR-driven ghrelin secretion, as opposed to
catecholamines produced by the adrenal glands, is supported by
studies showing failed induction of ghrelin secretion by parenter-
al administration of epinephrine to achieve levels mirroring
those seen in severe stress (29).

Another important topic of discussion relates to the physi-
ological role of secreted ghrelin in eating and BW. There is a
significant dichotomy in the results obtained using ghrelin gain-
of-function models as compared with and among ghrelin system
loss-of-function studies. As such, while administered ghrelin
stimulates food intake, increases BW, and promotes adiposity (5,
14, 78), and rats carrying the above-described gain-of-function
GHSR-Q343X isoform exhibit increased adiposity when older
and improved BW maintenance during chronic caloric restriction
(25), many studies with recombinant mice lacking a functional
ghrelin system show no or only modest feeding and/or BW phe-
notypes (4, 39, 52, 79–83), while others suggest that intact ghrelin
signaling is required for normal eating and BW responses, espe-
cially to hedonically rewarding diets (13, 79, 82). Here, deletion
of ghrelin cell β
1AR-dependent ghrelin secretion blunted rebound
food intake following a 24-hour fast, suggesting a modest role for
β
1AR-mediated increases in endogenous ghrelin secretion in the
usual feeding response to a short-term fast. However, this dele-
tion did not impact ad libitum feeding responses, BW regulation,
or body composition. Of related interest, plasma ghrelin levels
are usually lower in diet-induced obesity, which is often touted as
being a compensatory response to prevent exaggerated BW gain
(11). Here, mice lacking ghrelin cell β
1ARs failed to further reduce
plasma ghrelin levels in response to chronic HFD feeding, sug-
gesting that obesity-related reductions in plasma ghrelin levels
are mediated by reduced sympathetic drive or, as demonstrated
previously using cultured gastric mucosal cells from diet-induced
obese mice (27), are mediated by a reduced responsiveness of
ghrelin cells to sympathetic stimulation. Regardless, in the cur-
rent study, the HFD-fed GC-β
1AR−/− mice did not diverge in BW
from that of control animals, despite divergent ghrelin secretion
responses to HFD feeding.

As final discussion points, the present study also demonstrat-
ed a differential magnitude of acyl-ghrelin versus total ghrelin
reductions due to ghrelin cell–selective β
1AR deletion, which we
speculate could result from differential effects on acyl-ghrelin ver-
sus desacyl-ghrelin release or alterations to GOAT activity and/or
other enzymes responsible for ghrelin biosynthesis, maturation, or
degradation (2, 3). Additionally, it should be noted that, besides
being a powerful tool to investigate the role of β
1AR signaling in
ghrelin cells, the β
1AR−/− line reported here should prove valuable
in future studies of other important β
1AR-mediated processes, such as cardiac function, smooth muscle tone, the hypoglycemia
counterregulatory response, and adipose tissue physiology. Also,
given our findings in calorie-restricted GC-β
1AR−/− mice, we would
predict that other conditions associated with enhanced sympa-
thetic drive, such as psychosocial stress and cold exposure — both
of which have been shown to be associated with increased plasma
ghrelin levels — may involve robust β
1AR-dependent ghrelin secre-
tion responses (50, 84–87) on which the full physiological respons-
es to those conditions may depend. Furthermore, the occurrence
of hypoglycemia in calorie-restricted GC-β
1AR−/− mice and the
prevention thereof by ghrelin administration suggest that closer
blood glucose monitoring might be prudent in cachectic individu-
als if beta blocker therapy becomes indicated, just as has already
been recommended for children with infanticile hemangiomata
with propranolol, for whom we now propose that reduced
ghrelin secretion contributes to the beta blocker–associated hypo-
glycemia. Finally, our current results can be added to the accumu-
ating evidence suggesting that the ghrelin system has only subtle
effects on eating and BW when food is readily available and plen-
tful, but that it nonetheless remains essential for the maintenance
of life-sustainable glycemic levels when energy stores approach
those of starvation states.

Methods
Detailed experimental procedures for standard animal techniques,
qPCR, and cell culture are described in the Supplemental Methods.

Animal procedures. Mice were housed under standard laboratory
conditions and were provided ad libitum access to standard chow
(Harlan Teklad 2016, with an energy density of 3 kcal/g, of which 12%
of kcal are derived from fat) and water unless otherwise specified.

Generation of β
1ARfl/fl mice. A mouse line containing a recombinant
β
1AR gene (Adrb1) flanked by loxP sites was generated by BAC recom-
bination techniques in EL250 and EL350 cells (Figure 1) using previ-
ously described methods (88, 89). Briefly, a 111,024-bp BAC clone
(BMQ-52i16) containing the entire Adrb1 gene was obtained from
Source Bioscience and transformed into EL350 cells. To generate the
targeting construct, a 4,210-bp fragment containing the complete
Adrb1 coding sequence and homology arms was cloned into a com-
mercially available pGEM vector (Promega), which had been altered
to contain the thymidine kinase gene (used for negative selection
of ES cell clones). A previously described loxP-neomycin resistance
gene (Neo)-loxP cassette from pL452 was inserted 607 bp upstream
of the Adrb1 start codon using homologous recombination, followed
by removal of Neo-loxP by arabinose induction of Cre recombinase
(90). Next, another loxP-frt-Neo-frt cassette from pL451 was inserted
517 bp downstream of the Adrb1 stop codon. These loxP insertion sites
were chosen on the basis of a comparison of the 5′- and 3′-UTR regions
of the Adrb1 gene in an array of mammalian species and were within
regions with the least homology near the start and stop codons. The
resulting targeting construct was prepared and used for electropora-
tion into 129/SvEvTac (SM-1 cells) by the UT Southwestern Medical
Center Transgenic Technology Core Facility. Correctly targeted ES
cell clones were identified by Southern blot and PCR analyses. Three
embryonic stem (ES) cell clones were selected for blastocyst injection,
and germline transmission was established for 2 clones. The result-
ing pups were backcrossed with C57BL/6N mice for 3 generations. To

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remove the Neo-Frt sequence, the lines were then crossed with “Flp1 recombinase” mice [B6.Cg-Tg(ActFLPe)9205Dym/J; The Jackson Laboratory] that had been backcrossed for more than 10 generations onto the C57BL/6N background in our colony. One of the lines was chosen for subsequent breeding to generate experimental mice. Furthermore, to demonstrate germline transmission of the loxP-flanked Adrb1 allele, heterozygous βAR<sup>WT/lox</sup> mice were crossed with each other, yielding the predicted numbers of mice with 2 copies of the loxP-flanked Adrb1 allele, mice with 2 copies of the WT Adrb1 allele, and heterozygotes, as determined using a PCR strategy on genomic DNA of resulting pups (Supplemental Figure 3).

Generation of mice with ghrelin cell-selective βAR deletion. Ghrelin-Cre mice (35, 39) on a pure C57BL/6N genetic background were crossed with heterozygous βAR<sup>WT/lox</sup> mice to generate breeders harboring 1 copy of the loxP-flanked Adrb1 allele and 1 copy of the ghrelin-Cre<sup>Tg</sup> (G-Cre<sup>Tg</sup>). These mice were bred with heterozygous Adrb1<sup>WT/lox</sup> mice to generate the 4 study groups, which included mice containing 2 loxP-flanked Adrb1 alleles or 2 WT Adrb1 alleles, all with or without 1 copy of the ghrelin-Cre<sup>Tg</sup>. The primers used in the study to validate the genotype of all the genetically engineered mice are listed in Supplemental Table 2.

Long-term feeding studies. Female mice were fed either standard chow or an HFD (Harlan Teklad TD88137, with an energy density of 4.5 kcal/g, of which 42% of kcal are derived from fat) for 16 weeks. Fed plasma acyl-ghrelin levels were sampled at 4 and 12 weeks of the study, and 24-hour-fasted acyl-ghrelin levels were sampled at 8 and 16 weeks of the study. After 16 weeks, body composition analyses were performed using an EchoMRI-100 (Echo Medical Systems), body (nose-to-anus) length was measured under anesthesia using a ruler, and then half the cohort was sacrificed in an ad libitum–fed state, while the other half was sacrificed after a 24-hour fast. Gastric mucosal cells were harvested from excised stomachs (see Supplemental Methods) and were then treated with RNA-STAT 60 (Tel-Test Inc.) for preproghrelin mRNA quantification.

Severe (60%) caloric restriction protocol. Eight-week-old male mice were provided access to 40% of their usual daily calories in the form of their usual diet, as described previously, for 6 days (52, 53). All mice were individually housed during the study and acclimatized for 1 week before starting the caloric restriction protocol. During the acclimatization period, daily food intake was measured for 5 days to determine the mean usual daily caloric intake for each mouse. At the start of the experiment, the percentage of body fat mass of the mice as determined using the EchoMRI-100 system was between 8% and 10%. Mice subjected to this protocol typically experience a drop in body fat mined using the EchoMRI-100 system was between 8% and 10%.

The samples were immediately centrifuged at 4°C at 1,500 g for 15 minutes, and HCl was added to the supernatant to achieve a final concentration of 0.1N (for stabilization of acyl-ghrelin). Processed samples were stored at –80°C in small aliquots until analysis of acyl-ghrelin or total ghrelin levels. Ghrelin concentrations in the plasma and cell culture media were determined by using ELISA kits from Millipore-Merk (catalog EZRGRA-90K for acyl-ghrelin and catalog EZRGRT-91K for total ghrelin). The endpoint calorimetric assays were performed using a PowerWave XS Microplate spectrophotometer and KC4 Junior software (BioTek Instruments).

Isolation of primary gastric mucosal cells. Primary gastric mucosal cells were isolated by a combined enzymatic and mechanical dispersion method as described previously (26, 34, 35) and in more detail in the Supplemental Methods.

Atenolol administration. Atenolol (10 mg/kg BW i.p.) or its vehicle (2 mM HCl) was administered over 3 days to 3- or 6-week-old male WT C57BL/6N mice as described previously (34). Briefly, this occurred at 7 am and 7 pm on days 1 and 2 and at 7 am on day 3. For the 6-week-old mice, blood was collected at 8:30 am on day 2 (ad libitum–fed condition), after which the same mice were fasted for 24 hours and blood again collected at 8:30 am on day 3 (24-hour–fasted condition). For 3-week-old mice, the same dosing regimen was followed, but blood collections for the ad libitum–fed and 24-hour–fasted conditions were performed in separate cohorts of mice on day 3, one cohort of which was not fasted and the other cohort of which was fasted, respectively.

Statistics. All data are expressed as the mean ± SEM. Two-tailed statistical analysis and graph preparations were performed using GraphPad Prism 6.0 (GraphPad Software). A Student’s t test, 1-way ANOVA, 2-way ANOVA, or repeated-measures 2-way ANOVA, followed by the appropriate post-hoc comparison test, was used to test for significant differences among test groups. Survival curves were calculated by the Kaplan-Meier estimate method and compared using the Mantel-Cox log-rank test. The strength of the linear relationship between 2 sets of variables was compared by Pearson’s correlation coefficient. Outliers were detected by Grubb’s test. P values of less than 0.05 were considered statistically significant.

Study approval. All animal procedures and use of mice were approved by the IACUC of UT Southwestern Medical Center.

Author contributions
BKM, SOL, and JMZ conceptualized the experiments; BKM, SOL, PV, and CH performed the experiments and analyzed data; BKM, SOL, and JMZ wrote the manuscript; BKM and JMZ secured funding; and JMZ supervised the research activity.

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