Introduction

Ghrelin is a peptide hormone first isolated from the stomach and reported in 1999 (1). Following the unique posttranslational addition of an acyl group by ghrelin O-acyltransferase (GOAT), ghrelin can bind and activate the G protein-coupled growth hormone secretagogue receptor (GHSR), which is the only known ghrelin receptor (1–3). It is via CNS and/or peripheral GHSRs that ghrelin influences growth hormone (GH) secretion, mood, gastrointestinal motility, hippocampal neurogenesis, and many other processes (4–10). Actions of unmodified ghrelin (desacyl-ghrelin), the modes thereof, and the potential significance of the acyl-ghrelin/desacyl-ghrelin ratio are less well described (4). Of all processes influenced by the ghrelin system, eating and BW have received the most attention. Ghrelin is unique among gut hormones in that it is orexigenic, potently stimulating eating (5, 9). Plasma ghrelin rises before meals, after food deprivation, and after weight loss; it falls after eating and is low in most forms of obesity (9, 11). When the action of fasting-induced elevated ghrelin is blocked pharmacologically in mice, rebound overeating is blunted, and when the action of weight loss-associated elevated ghrelin is similarly blocked, as in a rat cancer cachexia model, worsened anorexia and hastened death have been described (12, 13). Exogenous infusions of ghrelin or GHSR agonists similarly increase eating and, in addition, lower energy expenditure and upregulate the expression of lipogenic and fat storage-promoting enzymes (5, 14–17). Ghrelin also shifts food preference toward high-fat diets (HFDs), shifts fuel preference away from metabolic utilization of fat, and engages several food reward behaviors (5, 13, 18, 19). Collectively, these actions are likely contributors to the ability of administered ghrelin to increase BW in normal subjects and maintain BW or delay weight loss in cachectic subjects (5, 15, 17, 20).

The ghrelin system also regulates and is regulated by blood glucose. Suggesting a role for ghrelin in modulating glycemia, ghrelin administration has been shown to increase blood glucose acutely, which may occur via its known actions to lower insulin levels, attenuate insulin sensitivity, stimulate glucagon secretion, induce GH secretion, raise glucocorticoid levels, and/or increase food intake (1, 5, 21–24). Also, both ad libitum–fed and Tg mice with hyperghrelinemia due to ghrelin promoter–driven expression of simian virus 40 large T antigen and overnight-fasted rats carrying a gain-of-function GHSR mutation (the GHSR-Q343X isoform, which is associated with a reduced ghrelin-induced GHSR internalization and enhanced sensitivity to administered ghrelin) exhibit higher blood glucose levels than do WT control animals (24, 25). Ex vivo studies with primary cultures of dispersed mouse gastric mucosal cells, approximately 0.3% to 1% of which include ghrelin cells, demonstrate that ghrelin cells can directly sense glucose, enhancing ghrelin release upon exposure to glucose concentrations representative of hypoglycemia and reducing ghrelin release when exposed to high glucose levels (26, 27). Not only does exposure of ghrelin cells to low glucose levels stimulate ghrelin release, but increased sympathetic tone, as occurs during the usual counterregulatory response to hypoglycemia, could also stimulate ghrelin secretion (26, 28). Supportive of this assertion, ghrelin secretion increases when sympathetic nerves are stimulated artificially or when adrenergic agents are infused locally into the gastric submucosa (29, 30). Norepinephrine, epinephrine, and isoproterenol all stimulate ghrelin secretion from ghrelinoma cell lines and from primary cultures of gastric mucosal cells (26, 31–36). Reserpine, which depletes norepinephrine from sympathetic

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 β-adrenergic receptor (βAR) blocker atenolol (34). Importantly, βAR 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 βAR expression data and stimulation of ghrelin secretion by catecholamines, we designed the current study to test the hypothesis that activation of βARs is required to engage the protective glucoregulatory functions of the ghrelin system.

Results

Generation of mice with ghrelin cell–specific deletion of βARs. The majority of the mice generated previously using a germline βAR-KO (Adrb1fl/fl) approach were reported to die prenatally (38). Here, we selectively deleted βAR from ghrelin-expressing cells. To do so, a conditional Adrb1-KO (Adrb1loxP/loxP) 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 immuno histochemical 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 gastric mucosal cells (34, 35, 37).

Figure 1. Generation of conditional βAR-KO mice and mice with ghrelin cell–selective βAR deletion. Schematic diagrams show the homologous recombination targeting strategy to flank the βAR (Adrb1) coding region with loxP sites, thus creating conditional βAR-KO mice. Binding sites for Southern blot probes used to detect correctly targeted genomic DNA (after restriction digestion with SphI) are depicted. Crosses first with Flp1 recombinase mice to remove the frt-Neo-frt cassette that had been included in the targeting construct to find neomycin-resistant ES cell clones and then with ghrelin-Cre mice result in Cre recombinase–mediated removal of the βAR coding sequence selectively in ghrelin cells.
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 βAR deletion lowers plasma ghrelin and fasting blood glucose levels.** Plasma ghrelin normally increases upon fasting. To study the in vivo impact of βAR 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-βAR+/– mice. These mice showed, on average, 4.5-fold lower ad libitum–fed and 5.8-fold lower fasted plasma acyl-ghrelin levels when compared with levels in the control groups (Figure 2, A and D). Plasma total ghrelin levels in GC-βAR+/– 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-βAR+/– 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 from GC-βAR+/– 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 βAR genes; fl/fl, mice homozygous for the loxP-flanked βAR gene; −, absence of the ghrelin-Cre Tg; +, presence of the ghrelin-Cre Tg.

The plasma ghrelin levels achieved by ghrelin cell–selective βAR deletion were similar to those achieved by global βAR blockade or deletion: pharmacological inhibition of βAR 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 βAR-KO mice (Z-βAR–/–) by crossing the βAR+Tg with Zp3-Cre mice (51) (see Supplemental Methods for a detailed description). The few surviving Z-βAR–/– mice, which we confirmed as Zp3-Cre mice (Supplemental Figure 4), showed reduced basal and fasted plasma acyl- and total ghrelin levels (Supplemental Figure 6).

**Ghrelin deficiency in GC-βAR–/– mice does not greatly impact food intake, BW, or adiposity.** To determine the effect of ghrelin cell βAR deletion–induced blunting of ghrelin secretion on food intake and BW, we fed female GC-βAR–/– mice standard chow or an HFD (42% kcal from fat) for 16 weeks. Ad libitum–fed and fasted acyl-
ure 3). 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 β1ARw/w/G-CreTg−/− 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 (7.4-fold), this increase was smaller than the 8.8- to 10.3-fold increases observed in the control groups, and the final level achieved was over 80% lower than that in the control groups (Figure 5A). This reduced plasma ghrelin level in GC-βAR−/− mice was accompanied by frank hypoglycemia (nadir = 45.3 ± 3.7 mg/dl) — while similarly treated control groups retained normoglycemia (nadir = 76.3 ± 5.8 mg/dl) — and increased mortality (36%) compared with the vehicle-treated mice (Supplemental Figure 12). The reduction in blood glucose levels with atenolol treatment was accompanied by induction of hypoglycemia unawareness (nadir = 45.3 ± 3.7 mg/dl) and increases mortality (36%) compared with the vehicle-treated mice (Supplemental Figure 12). The correlation was seemingly 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 levels with atenolol treatment was accompanied by induction of the hepatic gluconeogenic genes encoding peroxisome proliferative activated receptor γ, coactivator 1 α (PGC1α [Ppargc1a]), phosphoenolpyruvate carboxykinase 1 (PCK1 [Pck1]), and glucose-6-phosphatase (G6Pase [G6pc]) (Supplemental Figure 13). Other hepatic genes encoding for the gluconeogenic enzymes hepatic nuclear factor 4, α (HNF4α [Hnf4α]) and pyruvate carboxylase (PCX [Pcx]) and the glycolgenetic enzyme liver glycogen phosphorylase (PYGL [Ppygl]) 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 (Igfbp1) 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.
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 dependence 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 newborns (64) or in cases of exercise-induced hypoglycemia in beta blocker–treated individuals (65).

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, 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). The lower GH and blood glucose levels are both preventable by continuous ghrelin infusion or by continuous GH infusion during the caloric restriction (52). 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 undetermined 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 fasted 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 fasted, 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 glucagon 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
(73) also suggests the importance of higher-order inputs to ghrelin cells from the brain in stimulating ghrelin secretion. Another related observation is the significant elevation of plasma norepinephrine levels in fasted GC-βAR+/− mice. This could be due to a loss of usual ghrelin inhibition on sympathetic drive, as parenteral and intracerebroventricular administration of ghrelin is known to reduce plasma norepinephrine levels and inhibit sympathetic activity (74–76), or to a compensatory increase in sympathoadrenal drive in an attempt to boost ghrelin levels or in response to the low fasted blood glucose levels (77). Parenthetically, the specific role of catecholaminergic input from the sympathetic nervous system in βAR-driven ghrelin secretion, as opposed to catecholamines produced by the adrenal glands, is supported by studies showing failed induction of ghrelin secretion by parenteral administration of epinephrine to achieve levels mirroring those seen in severe stress (29).

Another important topic of discussion relates to the physiological 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 phenotypes (4, 39, 52, 79–83), while others suggest that intact ghrelin signaling is required for normal eating and BW responses, especially to hedonically rewarding diets (13, 79, 82). Here, deletion of ghrelin cell βAR-dependent ghrelin secretion blunted rebound food intake following a 24-hour fast, suggesting a modest role for βAR-mediated increases in endogenous ghrelin secretion in the usual feeding response to a short-term fast. However, this deletion 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 or body composition. Of related interest, plasma ghrelin levels are usually lower in diet-induced obesity, which is often touted as

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 βAR−/− mice. A mouse line containing a recombinant βAR gene (Adrb1) flanked by loxp sites was generated by BAC recombinase techniques in EL250 and EL350 cells (Figure 1) using previously described methods (88, 89). Briefly, a 111,024-bp BAC clone (BMQ-5216) 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 commercially available pGEM vector (Promega), which had been altered to contain the thymidine kinase gene (used for negative selection of ES cell clones). A previously describedloxP-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, anotherloxP-frt-Neo-frt cassette from pL451 was inserted 517 bp downstream of the Adrb1 stop codon. TheseloxP 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 electroporation 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 resulting pups were backcrossed with C57BL/6N mice for 3 generations. To
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 germine transmission of the loxp-flanked Adrb1 allele, heterozygous β,ARlox/Δ 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 β,AR deletion. Ghrelin-Cre mice (35, 39) on a pure C57BL/6N genetic background were crossed with heterozygous β,ARlox/Δ mice to generate breeders harboring 1 copy of the loxp-flanked Adrb1 allele and 1 copy of the ghrelin-CreTg (G-CreTgΔ). These mice were bred with heterozygous Adrb1lox/Δ mice to generate the 4 study groups, which included mice harboring 2 loxp-flanked Adrb1 alleles or 2 WT Adrb1 alleles, all with or without 1 copy of the ghrelin-CreTg. The primers used in the study to validate the genotypes 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 percentage to less than 2% after only 2 days (52, 53). We entered 25 GC-β,AR Cre mice, 22 β,AR Cre/G-CreΔ mice, 15 β,AR Cre/G-CreΔ mice, and 21 β,AR Cre/G-CreΔ mice into this study, although noted that 9, 2, 2, and 2 mice, respectively, had died by the sixth day of 60% caloric restriction (Figure 5C), leading us to discontinue the protocol on the sixth day instead of waiting 7 days or more, as had previously been published (39, 52–54, 60).

Determination of blood glucose and ghrelin levels. Blood samples were collected by a quick superficial temporal vein (submandibular) bleed into EDTA-coated microtubes containing a protease inhibitor, p-hydroxymercubenzoic acid (final concentration 1 mM), kept on ice. Blood glucose concentrations were measured using the hand-held AlphaTRAK 2 (Abbott Animal Health) blood glucose monitoring system. 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-Merck (catalog EZRGRA-90K for acyl-ghrelin and catalog EZRGRT-91K for total ghrelin). The endpoint caloricimetric 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 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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