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Ghrelin Directly Stimulates Glucagon Secretion from Pancreatic α-Cells

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Previous work has demonstrated that the peptide hormone ghrelin raises blood glucose. Such has been attributed to ghrelin's ability to enhance GH secretion, restrict insulin release, and/or reduce insulin sensitivity. Ghrelin's reported effects on glucagon have been inconsistent. Here, both animal- and cell-based systems were used to determine the role of glucagon in mediating ghrelin's effects on blood glucose. The tissue and cell distribution of ghrelin receptors (GHSR) was evaluated by quantitative PCR and histochemistry. Plasma glucagon levels were determined following acute acyl-ghrelin injections and in pharmacological and/or transgenic mouse models of ghrelin overexpression and GHSR deletion. Isolated mouse islets and the α-cell lines αTC1 and InR1G9 were used to evaluate ghrelin's effects on glucagon secretion and the role of calcium and ERK in this activity. GHSR mRNA was abundantly expressed in mouse islets and colocalized with glucagon in α-cells. Elevation of acyl-ghrelin acutely (after sc administration, such that physiologically relevant plasma ghrelin levels were achieved) and chronically (by slow-releasing osmotic pumps and as observed in transgenic mice harboring ghrelinomas) led to higher plasma glucagon and increased blood glucose. Conversely, genetic GHSR deletion was associated with lower plasma glucagon and reduced fasting blood glucose. Acyl-ghrelin increased glucagon secretion in a dose-dependent manner from mouse islets and α-cell lines, in a manner requiring elevation of intracellular calcium and phosphorylation of ERK. Our study shows that ghrelin's regulation of blood glucose involves direct stimulation of glucagon secretion from α-cells and introduces the ghrelin-glucagon axis as an important mechanism controlling glycemia under fasting conditions. **(*Molecular Endocrinology* 25: 0000–0000, 2011)**

The peptide hormone ghrelin has many actions, including effects on GH secretion, eating-related behaviors, and body weight (BW) (1, 2). Several studies also implicate ghrelin as an important regulator of whole-body glucose homeostasis. For instance, ghrelin administration to rodents increases fasting blood glucose in a dose-dependent manner, lowers insulin levels, and attenuates insulin responses during glucose tolerance testing (3, 4). Similar ghrelin-mediated effects on blood glucose and/or insulin release have been demonstrated in isolated rodent islets and pancreata, in ghrelin-overexpressing mice, and in humans (3–7). Conversely, ghrelin receptor (GHSR) deletion lowers blood glucose and enhances insulin sensitivity, as most recently demonstrated using glucose tolerance and pyruvate tolerance tests and hyperglycemic and hyperinsulinemic-euglycemic clamp assays (8–10). Low circulating ghrelin has been associated with elevated fasting insulin and insulin resistance in humans (11, 12). Also, mice lacking both leptin and ghrelin show marked improvements in the insulin-resistant phenotype characteristic of leptin deficiency (13). Alone, ghrelin deletion improves glucose tolerance and increases glucose-stimulated insulin secretion from isolated islets (4, 14). Strikingly, mice lacking ghrelin *O*-acyltransferase (GOAT), the en-

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Abbreviations: BW, Body weight; $[Ca^{2+}]_i$, cytosolic calcium concentration; FBS, fetal bovine serum; GHSR, ghrelin receptor; GOAT, ghrelin *O*-acyltransferase; IHC, immunohistochemistry; ISHH, *in situ* hybridization histochemistry; qPCR, quantitative PCR; SAB, secretion assay buffer.

zyme responsible for ghrelin's unique posttranslational acylation, show a progressive decline in fasting blood glucose to the point of near death after only 7 d of 60% calorie restriction (15). Administration of either acyl-ghrelin or GH to GOAT-deficient mice normalizes blood glucose and prevents death under these conditions, thus suggesting an essential role for ghrelin, and, at least in part, for ghrelin-induced increases in GH release, in maintaining a minimum blood glucose level to allow the survival of severely calorie-restricted mice (15).

In addition to the aforementioned studies demonstrating effects of ghrelin on insulin sensitivity and GH secretion, most other studies have attributed ghrelin's effects on glucose to direct actions on pancreatic β-cells. For instance, ghrelin reduces glucose-stimulated insulin secretion from β-cells isolated from rat islets and from INS-1E and MIN6 β-cell lines (3, 4, 7, 16). GHSR mRNA expression in INS-1E and MIN6 cells and several human insulinomas has been demonstrated by RT-PCR (7, 17). Weak GHSR immunoreactivity has been observed in the central, β-cell-predominated region of rat islets, although the same study demonstrated stronger GHSR immunoreactivity within the islet periphery, populated mainly by glucagon-secreting α-cells (18). Interestingly, exposure of isolated islets or pancreata to antighrelin antiserum or to GHSR antagonists enhances glucose-stimulated insulin secretion, suggesting that at least some of ghrelin's effects on insulin release are due to local effects of islet-derived ghrelin (3).

The possibility that ghrelin's overall effects on blood glucose may be influenced by changes to glucagon also has been investigated, although the responses observed have been inconsistent. Certain reports indicate no effect of ghrelin on glucagon release. For instance, ghrelin failed to modify basal glucagon release from rat pancreata or that stimulated by L-arginine (19, 20). Neither did single iv nor 2 h-long ghrelin infusions to human subjects significantly change plasma glucagon (21–24). Other studies have demonstrated either elevated or decreased plasma glucagon after ghrelin exposure. For instance, ghrelin stimulated nitric oxide-dependent glucagon release from isolated rat islets (25). Similarly, ghrelin stimulated glucagon release in a dose-dependent manner, from isolated mouse islets in the presence of a range of glucose concentrations, although in the same report, ghrelin limited the efficacies of several known glucagon secretagogues *in vivo* (26). Ghrelin's actions on glucagon release from isolated rodent islets also were shown to be pronounced only in islets isolated from the splenic region of the pancreas (27). Ghrelin transiently and acutely increased glucagon levels in lactating cows, although it slightly decreased glucagon levels in nonlactating cows (28). Also, ghrelin stimulated glucagon release from pancreas fragments of streptozotocin-induced diabetic rats (20).

We undertook the current study to more fully examine the role of glucagon as a mediator of ghrelin's effects on blood glucose. Also, we investigated whether ghrelin's effects include direct actions via GHSR located on pancreatic α-cells, which has not previously been investigated, and the mechanism for the ghrelin-glucagon interaction.

Results

GHSR expression is enriched in mouse pancreatic α-cells

Various methods have been used to localize GHSR expression to islets and to tumors and cell lines derived from the endocrine pancreas (1, 7, 17, 18, 29, 30). To confirm and extend these results, we used quantitative PCR (qPCR) and *in situ* hybridization histochemistry (ISHH) to identify cells within mouse islets and islet-derived cell lines that express GHSR. Isolated islets demonstrated much higher levels of functional (type 1a) GHSR mRNA than did hypothalami or several peripheral organs (islet $C_T = 25.6$, Fig. 1A). Among the tissues and cell lines examined, the cultured αTC1 α-cell line showed the highest GHSR mRNA expression ($C_T = 22.1$), whereas both β-cell lines tested, βTC6 and MIN6, showed GHSR transcript levels less than 1% those of αTC1 cells (βTC6, $C_T = 28.6$; MIN6, $C_T = 31.9$). GHSR mRNA levels in islets and αTC1 cells were lower under high glucose (17.5 mM) conditions, suggesting that islet GHSR expression, including that within α-cells, is subject to regulation by ambient glucose and that the ghrelin-GHSR axis may be more responsive in islets under low-glucose conditions (Fig. 1, B and C). Immunohistochemistry (IHC) for glucagon and insulin on sections of pancreas from wild-type (Fig. 1D) and GHSR-null mice (Fig. 1E) was done together with GHSR ISHH (Fig. 1, F and H for wild-type and G and I for GHSR-null mice) on adjacent sections and supports the qPCR data demonstrating more abundant GHSR within α-cells than β-cells.

Ghrelin increases plasma glucagon and glucose

Because GHSR mRNA was abundantly expressed in pancreatic α-cells, we next used pharmacological and genetic approaches to test whether ghrelin modulates glucose homeostasis by directly acting on α-cells to regulate glucagon secretion. First, we administered acyl-ghrelin sc to adult male C57BL6/J mice at doses ranging from 0–2 mg/kg BW. The 2 mg/kg BW dose was chosen because it is known to acutely induce food intake and certain antidepressant-like and food-reward behaviors (31). Here,

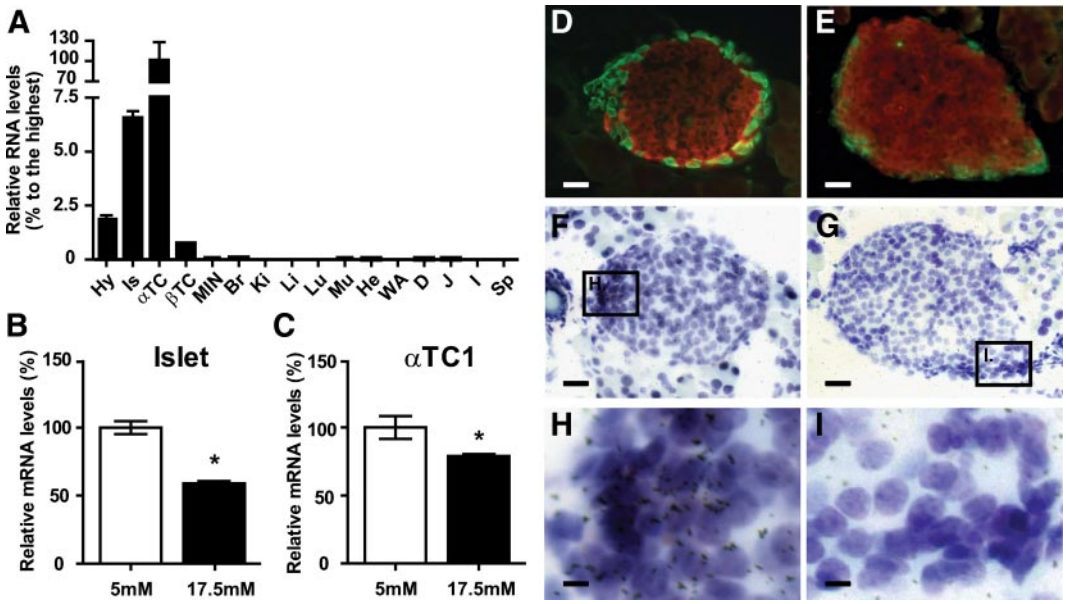

FIG. 1. GHSR is abundantly expressed in pancreatic α-cells and is down-regulated by glucose. A, The α-cell line, αTC1, expresses the highest level of GHSR mRNA among a panel of mouse tissues and cultured cells when examined by qPCR (tissue abbreviations are as follows: Hy, hypothalamus; Is, islet; αTC, αTC1 cell line; βTC, βTC6 cell line; MIN, MIN6 cell line; Br, brain; Ki, kidney; Li, liver; Lu, lung; Mu, muscle; He, heart; WA, white adipose; D, duodenum; J, jejunum; I, ileum; Sp, spleen. GHSR mRNA levels are decreased in panel B, mouse islets, and panel C, αTC1 cells, upon 16 h culture in high glucose (n = 3; *, $P < 0.05$ by two-tailed Student's *t* test). D and E, Representative IHC (D and E) and ISHH (F–I) photomicrographs. Black silver granules, representing the binding of GHSR antisense riboprobes, can be detected at a higher density within the α-cell-enriched periphery [with glucagon-immunoreactivity (*green*)] of wild-type islets as compared with the more centrally located β-cells with insulin immunoreactivity (*red*) (D, F, and H). Only background GHSR riboprobe signal was observed in islets from GHSR-null mice (E, G, and I). *Scale bars*, 50 μm in panels D–G; 10 μm in panels H and I.

circulating acyl-ghrelin levels achieved after 30 min using the 0.1–0.25 mg/kg BW doses of sc administered ghrelin (Fig. 2A) approached those induced physiologically with a 10-d chronic stress protocol (592 ± 106 pg/ml, Chuang J.-C., and J. M. Zigman, unpublished observations), upon a 16-h fast (343.7 ± 41.7 pg/ml; see Fig. 4) or upon a 24-h

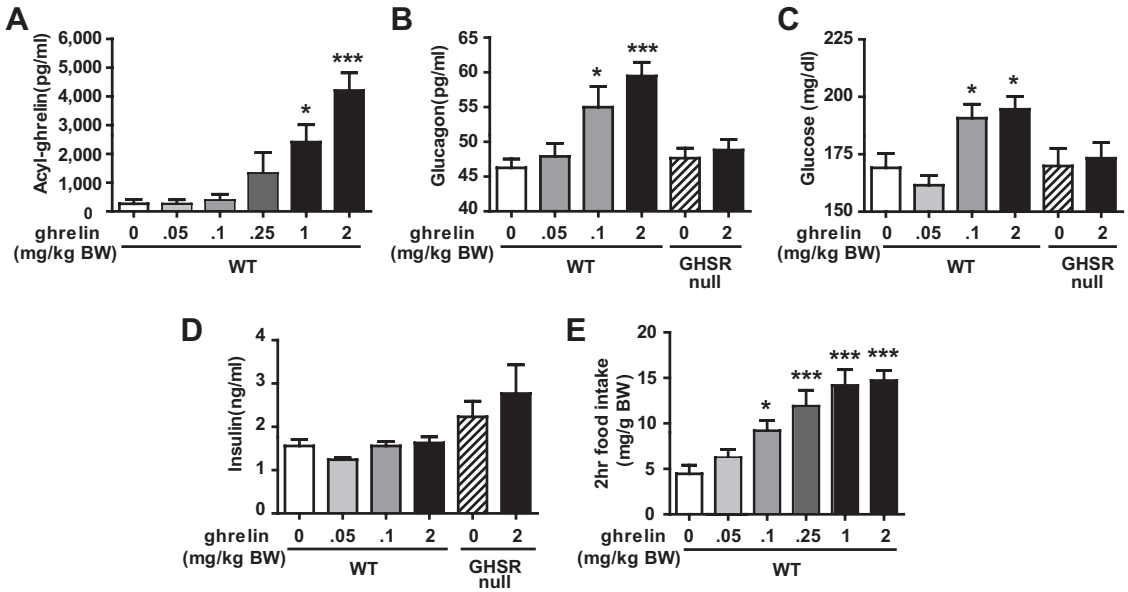

FIG. 2. Injections of acyl-ghrelin raise plasma glucagon levels acutely and in a dose-dependent manner. A–C, Blood samples were collected from mice 30 min after sc administration of various doses of saline or acyl-ghrelin to C57BL6/J mice (panel A, n = 3 per dose, with no access to food during that 30 min) or to both GHSR-null and wild-type (WT) littermates (panels B–D, n = 6–12 per dose, with no access to food during that 30 min), and then assayed for acyl-ghrelin (A), glucagon (B), glucose (C), and insulin (D). E, A separate cohort of mice (n = 5–10 per dose) was similarly treated in the presence of standard rodent diet to assess the acute orexigenic capacity of these acyl-ghrelin doses. The effects of ghrelin doses on plasma levels of acyl-ghrelin, glucagon, glucose, and insulin and on acute food intake in wild-type mice were analyzed by one-way ANOVA followed by Dunnett's *post hoc* test, whereas the same effects in GHSR-null mice were analyzed by two-tailed Student's *t* test. *, $P < 0.05$; ***, $P < 0.001$.

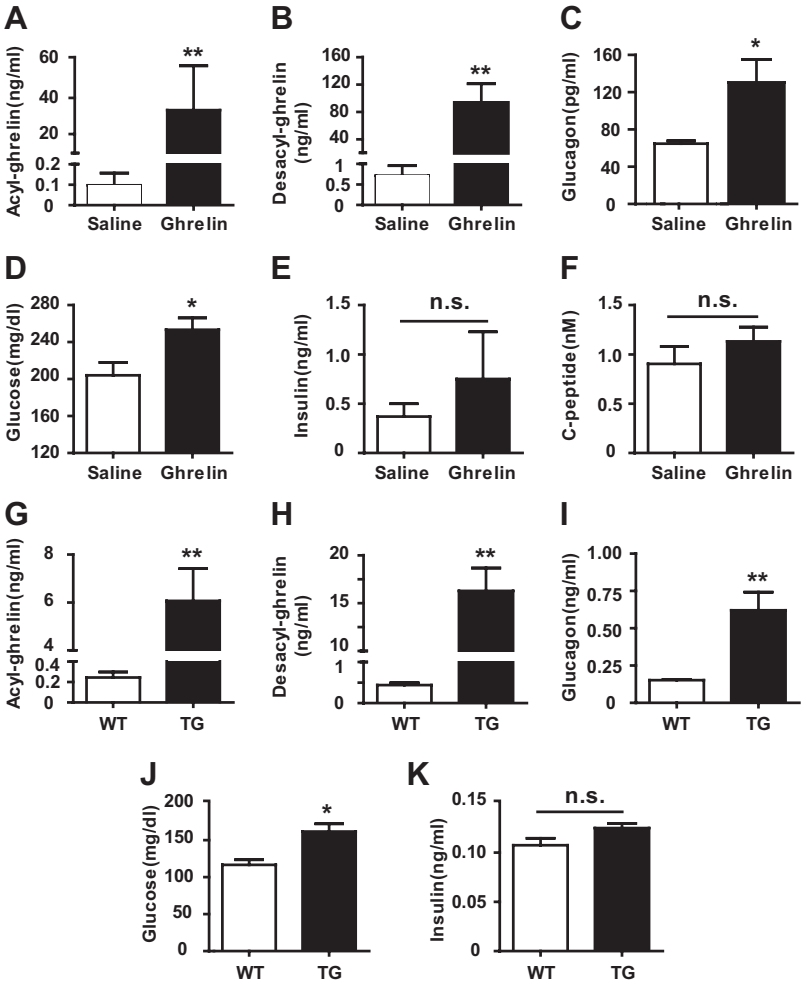

FIG. 3. Plasma glucagon and glucose are higher in mice receiving chronic sc delivery of ghrelin and in transgenic mice that overexpress ghrelin. A–F, Blood samples were collected from C57BL6/J mice 14 d after implantation with osmotic minipumps that delivered saline (n = 5) or acyl-ghrelin (n = 5). G–K, Blood was collected from wild-type mice (WT, n = 9) or transgenic littermates that overexpress ghrelin (TG, n = 9). Plasma was then analyzed for acyl-ghrelin (panels A and G), desacyl-ghrelin (panels B and H), glucagon (panels C and I), glucose (panels D and J), insulin (panels E and K), and C-peptide (panel F). Two-tailed Student's *t* test were used for these analyses. n.s., Not significant; $P < 0.05$; *, $P < 0.05$; **, $P < 0.001$.

fast [1169.9 ± 27.3 pg/ml, (32)]. Acyl-ghrelin increased plasma glucagon in a dose-dependent manner within 30 min of its administration, with significant effects present with both the 0.1 and 2.0 mg/kg doses (Fig. 2B). Blood glucose also was elevated at 30 min using these same doses of sc administered acyl-ghrelin; however, no effect on insulin levels was observed (Fig. 2, C and D). This same range of acyl-ghrelin doses was shown in a separate experiment to stimulate intake of freely-available regular chow in a dose-dependent manner (Fig. 2E). As expected, acute sc acyl-ghrelin delivery had no effect on plasma glucagon, glucose, or insulin in GHSR-null littermates (Fig. 2, B and D).

Next, we administered acyl-ghrelin sc to adult male C57BL6/J mice using osmotic minipumps at a dose of 4 mg/kg BW per day, resulting in constant elevations of acyl-ghrelin to levels 300-fold higher than in control mice receiving saline (as early as 2 d after minipump implantation) (Fig. 3A, data not shown); desacyl-ghrelin also was elevated using this technique (Fig. 3B). After 14 d of continuous sc delivery of acyl-ghrelin, plasma glucagon and glucose were both elevated (Fig. 3, C and D), but there were no significant effects on insulin or C-peptide (Fig. 3, E and F).

Also, we examined glucagon and glucose levels in a recently described transgenic mouse line with marked expansion of ghrelin cell numbers, as manifested by ghrelinomas in aged mice, and marked hyperghrelinemia resulting from ghrelin promoter-driven expression of simian virus 40 large T antigen (32) (Fig. 3, G and H). Plasma glucagon and glucose levels were higher in *ad libitum*-fed 20-wk-old transgenic ghrelin-overexpressing mice, whereas insulin levels were unaffected (Fig. 3, I–K). Of note, ghrelin-secreting cell lines derived from gastric and pancreatic islet ghrelinomas taken from aged transgenic ghrelin-overexpressing mice lack any detectable glucagon mRNA (32).

GHSR-null mice have lower plasma glucagon and glucose

Next, we determined glucagon and glucose levels in mice lacking GHSR expression (8). GHSR-null and wild-type littermates contained equivalent levels of fasting plasma acyl- and desacyl-ghrelin (Fig. 4, A and B). However, compared with wild-type littermates, GHSR-null mice displayed lower plasma glucagon and glucose when fasted 16 h (Fig. 4, C and D). Plasma insulin and C-peptide levels were unaffected (Fig. 4, E and F). This was despite similar islet glucagon mRNA content (Fig. 4G), similar general islet morphology (data not shown), and similar pancreatic glucagon and insulin contents (Fig. 4, H and I) for GHSR-null and wild-type littermates.

Ghrelin enhances glucagon secretion from islets and from pancreatic α-cell lines

To address whether the stimulatory effect of ghrelin on glucagon secretion reflects direct action on α-cells, we assessed *in vitro* effects of acyl-ghrelin on islets and two α-cell lines. As had been shown previously (26, 27), acyl-

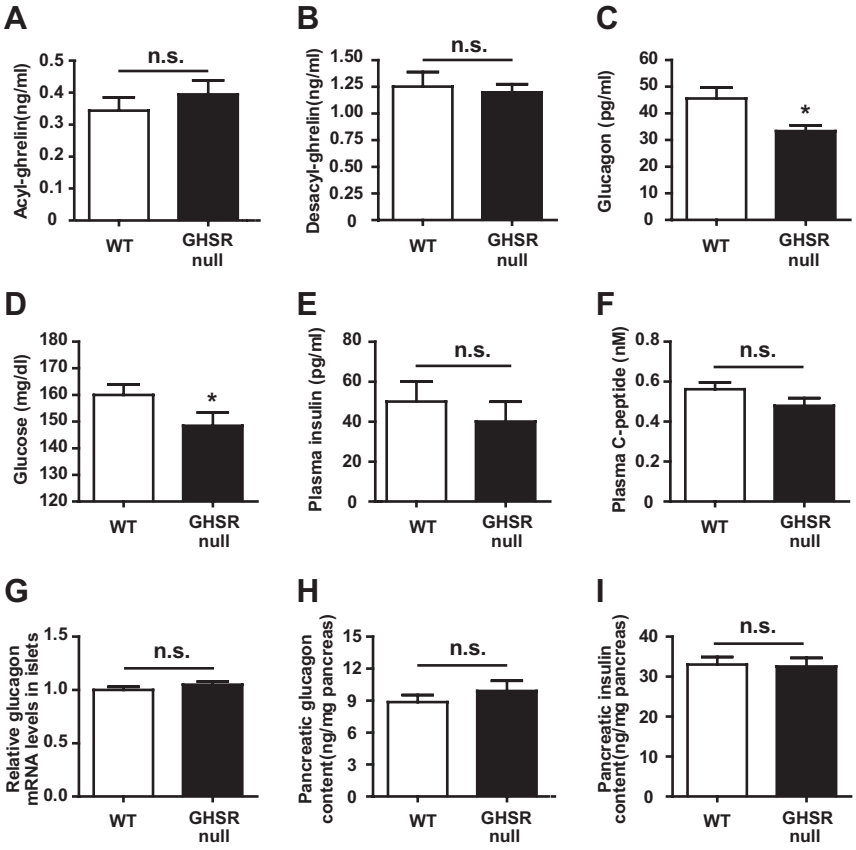

FIG. 4. Effects of genetic deletion of GHSR on plasma glucagon, glucose, and related parameters. Blood samples were collected from wild-type mice (WT) or GHSR-null littermates after a 16-h fast, and plasma was then analyzed for acyl-ghrelin (A), des-acyl-ghrelin (B), glucagon (C), glucose (D), insulin (E), and C-peptide (F). Plasma glucagon and glucose were significantly lower in GHSR-null mice. This was despite a lack of difference in glucagon mRNA levels from isolated islets (G) or in pancreatic pools of glucagon (H) or insulin (I). Two-tailed Student's *t* tests were used for these analyses (n = 8, n.s., not significant; *, $P < 0.05$).

ghrelin stimulated glucagon release from islets freshly isolated from the splenic lobes of C57BL6/J pancreata (Fig. 5A). No stimulation of glucagon release by ghrelin was observed from similarly prepared islets of GHSR-null littermates (Fig. 5B). Ghrelin also simultaneously reduced insulin release from C57BL6/J islets (Fig. 5C) but not from GHSR-null (Fig. 5D) islets. Furthermore, acyl-ghrelin increased glucagon secretion in a dose-dependent manner from αTC1 cells in medium containing 5 mM glucose (Fig. 5E). Similar results were observed in InR1G9 cells (Fig. 5F).

Mechanisms involved in ghrelin-mediated glucagon secretion

Regulation of α-cell intracellular signaling events mediating glucagon release is incompletely understood (33). To address whether intracellular calcium fluxes mediate ghrelin-enhanced glucagon release, we examined cytosolic calcium concentration ($[Ca^{2+}]_i$) changes within αTC1 cells. Fura-2 calcium imaging was used, with the ratio of Fura-2 emission intensity at 340 nm/380 nm as a measure of $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was increased by acyl-ghrelin in αTC1 cells in a dose-dependent manner (Fig. 6, A and B). Addition of EGTA, a Ca^{2+} chelator, or nifedipine, an L-type calcium channel antagonist, to the medium of αTC1 cells blocked acyl-ghrelin-induced glucagon secretion (Fig. 6C).

Next, we tested whether ghrelin-mediated ERK activation is required for glucagon secretion. Previously, ghrelin was shown to activate ERK in several cell types that express GHSR endogenously or via transfection (34–38). Here, exposure of αTC1 cells to acyl-ghrelin resulted in a dose-dependent (Fig. 7A) and rapid (Fig. 7B) phosphorylation of ERK. Two different specific inhibitors of ERK phosphorylation, U0126 (39) and PD98059 (40), blocked acyl-ghrelin-induced phosphorylation of ERK in αTC1 cells (Fig. 7C) and InR1G9 cells (data not shown), and this resulted in a failure by acyl-ghrelin to enhance glucagon secretion (Fig. 7D).

Discussion

Here, we focused on the potential role of glucagon in ghrelin-mediated effects on glycemic control. We used qPCR and histochemistry to confirm abundant GHSR expression within glucagon-producing α-cells and immortalized α-cell lines, thus suggesting the ability of ghrelin to directly engage α-cells. This finding supports previous studies demonstrating GHSR mRNA within a pancreatic α-cell line (TCa-9) and in a human glucagonoma (7, 17). Acyl-ghrelin administered sc acutely elevated plasma glucagon in C57BL6/J mice within 30 min, at a range of doses, including two (0.1 mg/kg BW and 0.25 mg/kg BW) in which the average circulating levels of acyl-ghrelin achieved were very close to those achieved physiologically in wild-type mice upon a 16-h fast and upon a 24-h fast (32). Chronic elevation of plasma ghrelin to supraphysiological levels, by either osmotic pump administration or in transgenic mice containing ghrelinomas, also raised plasma glucagon and blood glucose. Conversely, glucagon and blood glucose levels in fasted GHSR-null mice were shown to be significantly lower than those in fasted wild-type mice. These studies support previous work demonstrating a positive correla-

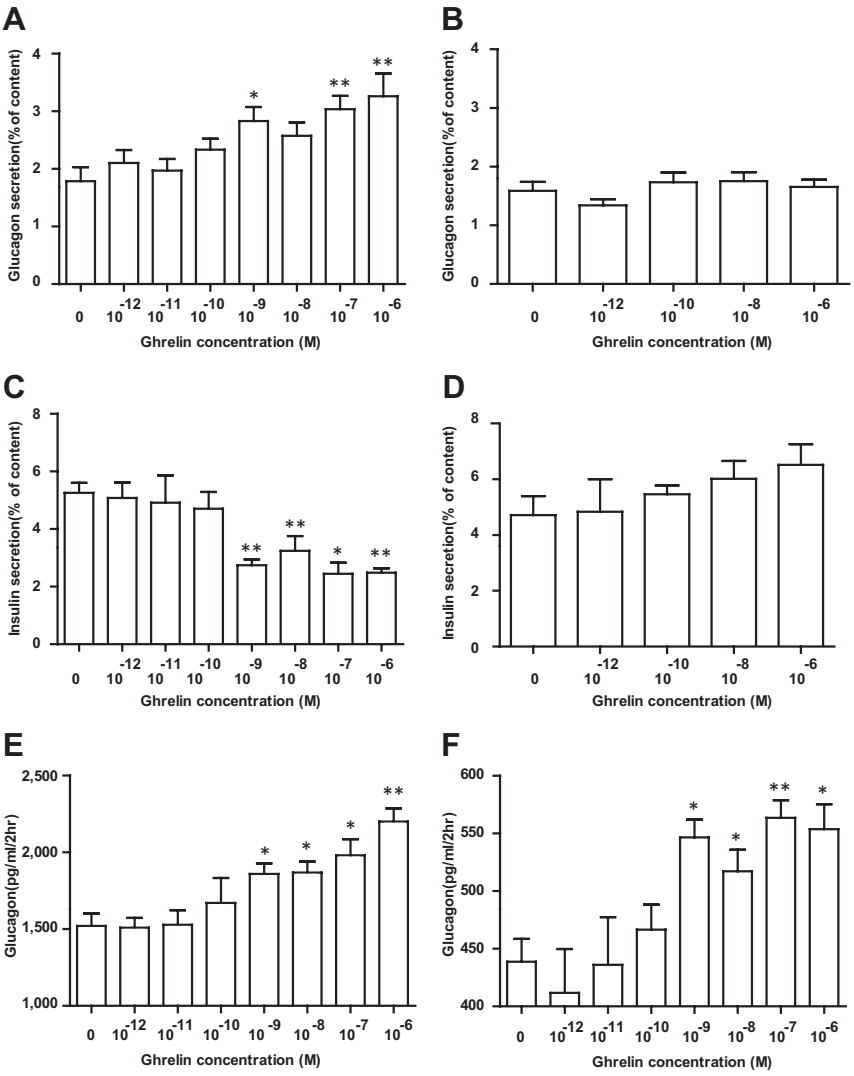

FIG. 5. Ghrelin increases glucagon secretion in a dose-dependent manner from isolated islets and from cultured α-cells and decreases insulin secretion from isolated islets. Glucagon secretion (A and B) and glucose-stimulated insulin secretion (C and D) from islets freshly isolated from the splenic portions of wild-type (A and C) and GHSR-null (B and D) pancreata were assessed after a 60-min incubation in media containing 12 mM glucose together with different concentrations of ghrelin. Similarly, glucagon secretion from two different pancreatic α-cell lines, αTC1 cells (E) and InR1G9 cells (F), was assessed after a 2-h incubation in media containing 5 mM glucose together with different concentrations of ghrelin. Ghrelin concentrations as low as 10^{-9} M significantly enhanced glucagon secretion from the isolated wild-type islets and two α-cell lines and significantly reduced glucose-stimulated insulin secretion from the isolated wild-type islets. The effect of ghrelin concentration on glucagon and insulin secretion was analyzed by one-way ANOVA followed by Dunnett's *post hoc* test (n = 6 wells containing 10 size-matched islets per well, or n = 8 wells containing 5×10^5 αTC1 cells per well, or n = 6 wells containing 2×10^5 InR1G9 cells per well, per each tested ghrelin concentration;
*, $P < 0.05$; **, $P < 0.01$.

tion between ghrelin and blood glucose levels and furthermore suggest that at least an indirect interaction of ghrelin with α-cells exists to help explain ghrelin's effects on glycemia.

Two different types of *in vitro* model systems, *i.e.* isolated mouse islets and cultured α-cell lines, were used to investigate whether acyl-ghrelin can act locally on α-cells to stimulate glucagon release. We used ghrelin to stimulate glucagon release from islets isolated from C57BL6/J mice, as had been previously demonstrated in both mouse and rat islets (25–27); ghrelin-induced glucagon release was not observed in isolated GHSR-null mouse islets. Also, our demonstration that acyl-ghrelin increases glucagon secretion in a dose-dependent manner from cultured α-cell lines, in a manner requiring elevation of intracellular calcium and phosphorylation of ERK, illustrates for the first time the ability of ghrelin to act directly on α-cell line models to stimulate glucagon release. This suggests that direct action of ghrelin, an appetite-regulating hormone secreted from the gastrointestinal tract under conditions of energy insufficiency and before set meals, on α-cells likely modulates glucagon secretion *in vivo*. In this regard, ghrelin may be considered to be a "glucretin", perhaps working in concert with the well-described gut incretins to couple changes in energy availability to a coordinated glucagon and insulin response to processes that would otherwise lead to altered whole-body glucose homeostasis.

There are several noteworthy discussion points and implications to these results. The first regards the doses of acyl-ghrelin that were used to demonstrate an effect on glucagon release. Although it is true that some of the *in vivo* experiments involved methods that achieved supraphysiological levels of ghrelin (chronic pharmacological delivery of ghrelin via osmotic minipumps, transgenic overexpression of ghrelin, and acute sc administration of 1 and 2 mg/kg BW ghrelin), other *in vivo* methods used did demonstrate raised plasma glucagon levels upon changes of ghrelin within the supposed physiological range. In particular, significant increases in plasma glucagon were observed within 30 min of sc injection of 0.1 mg/kg BW ghrelin. The corresponding level of plasma acyl-ghrelin that was achieved 30 min after this sc ghrelin injection (380.9 ± 115.0 pg/ml, Fig. 2) is very close to that achieved physiologically in wild-type mice upon a 16-h fast (343.7 ± 41.7 pg/ml, Fig. 4). Also,

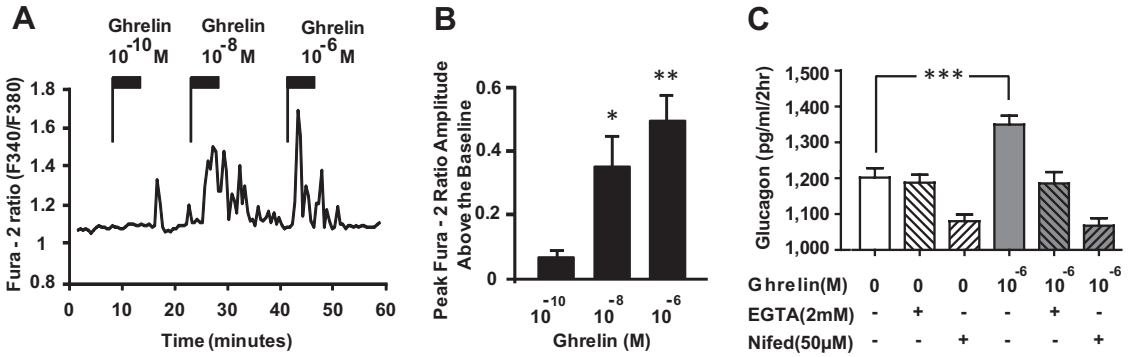

FIG. 6. Role of $[Ca^{2+}]_i$ in ghrelin-induced glucagon release. A, Tracing of ghrelin-induced increases in ($[Ca^{2+}]_i$ within a representative αTC1 cell, as indicated by assessing ratios of Fura-2 emission at 340 nm and 380 nm. Ghrelin at 10^{-8} M and 10^{-6} M increased $[Ca^{2+}]_i$, whereas incubation at 10^{-10} M was with little effect on this cell. B, Addition of different concentrations of ghrelin to αTC1 cell cultures increased peak Fura-2 ratio amplitudes above the baseline, which positively correlates with increased $[Ca^{2+}]_i$, as analyzed by one-way ANOVA followed by Dunnett's *post hoc* test (n = 8 cells per each ghrelin concentration). C, Interference with entry of calcium into αTC1 cells by addition of EGTA or nifedipine blocked ghrelin-induced glucagon release, as analyzed by two-way ANOVA followed by Bonferroni *post hoc* analysis (n = 8 wells containing 5×10^5 αTC1 cells per well, per each condition; *, $P < 0.05$; **, $P < 0.01$). C, ***, $P < 0.001$.

the corresponding level of plasma acyl-ghrelin achieved 30 min after the sc injection of 0.25 mg/kg BW ghrelin (1322.8 ± 726.3 pg/ml) is very close to that previously observed in mice upon a 24-h fast [1169.9 ± 27.3 pg/ml, (32)]. Furthermore, as mentioned, glucagon and blood glucose levels in fasted GHSR-null mice, which are presumably unable to respond to fasting-induced elevations in acyl-ghrelin (due to the genetic deletion of GHSR), were lower than those in fasted wild-type mice. The plasma acyl-ghrelin levels achieved with the 0.1 mg/kg BW and 0.25 mg/kg BW sc ghrelin doses also approach that observed upon exposure of mice to a 10-d chronic social defeat stress protocol (592 ± 106 pg/ml, Chuang, J.-C., and J.M. Zigman, unpublished observations). These examples suggest that we have demonstrated statistically significant rises in glucagon following maneuvers that raise acyl-ghrelin within the physiological range, *in vivo*. For our *in vitro* models, we did need to use slightly higher levels of ghrelin than those achieved in the plasma after a fast to demonstrate a statistically significant effect on glucagon secretion. In particular, cultured α-cells and islets exposed to 1×10^{-9} M acyl-ghrelin resulted in statistically significant release of glucagon into the incubation media (Fig. 5). This 10×10^{-10} M concentration of acyl-ghrelin is only slightly higher than the plasma ghrelin level achieved physiologically upon a 24-h fast [1169.9 ± 27.3 pg/ml, which is the equivalent of 3.5×10^{-10} M (32)]. Of note, the levels of glucagon release observed from the cultured α-cells and islets upon incubation with the more physiologically-relevant 10^{-10} M concentration of ghrelin were intermediate to those achieved with 10^{-11} M and 10^{-9} M concentrations, but the statistical power was not achieved with the 10^{-10} M dose to demonstrate a difference from levels observed when no ghrelin was added (Fig. 5).

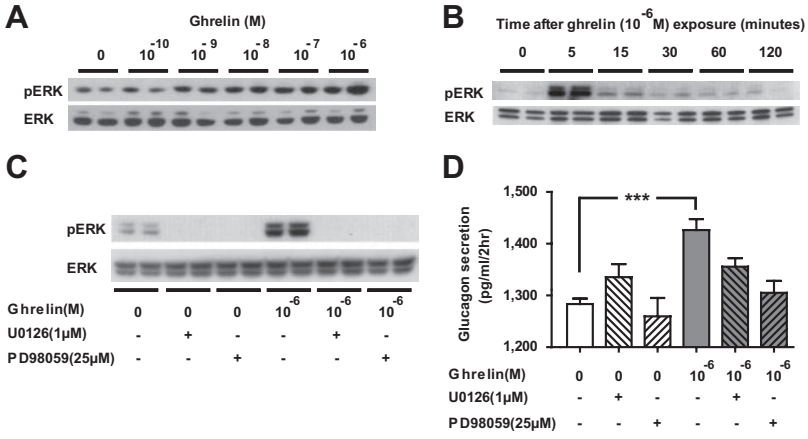

FIG. 7. ERK is activated by ghrelin in αTC1 cells and is required for ghrelin-induced glucagon secretion. A, Ghrelin induces phosphorylation of ERK in a dose-dependent manner (as assessed 5 min after addition of ghrelin). B, Ghrelin-induced ERK phosphorylation occurs rapidly and is most evident at 5 min after treatment. Ghrelin-induced ERK activation in αTC1 cells is blocked by U0126 and by PD98059, selective inhibitors of ERK phosphorylation (panel C), as is ghrelin-induced glucagon secretion from αTC1 cells (panel D). D, n = 8 wells containing 5×10^5 αTC1 cells per well, per each condition. Two-way ANOVA followed by Bonferroni *post hoc* analysis was used to analyze these results; ***, $P < 0.001$.

It has been suggested that ghrelin's role in mediating whole-body glucose handling is of critical importance for survival in the setting of chronic, severe caloric restriction. In fact, a precipitous drop in blood glucose and the development of a moribund state developed in mice lacking GOAT but not wild-type mice after only 7 d on a 60% calorie restriction protocol (15). Thus, any new insight into the mechanism by which ghrelin defends against the development of this extreme hypoglycemia is of importance. Of interest, in the aforementioned calorie restriction study, the investigators demonstrated that such falls in blood glu-

cose within GOAT-deficient mice were associated with a blunted GH-secretory response and were preventable upon GH infusion (15). However, both wild-type and GOAT-deficient mice were noted to have similar levels of both insulin and glucagon after the 7-d calorie restriction protocol despite significantly lower blood glucose levels in the GOAT-deficient mice (15). The exact reason why the severely calorie-restricted GOAT-deficient mice (15) did not exhibit an altered glucagon response as observed here in our overnight-fasted GHSR-null mice, is unclear. However, differences in severity and length of the calorie restriction protocols, timing of the assays, mouse genetic background, and effects of GOAT *vs.* GHSR deletion could all contribute to the differing results. Further studies will be needed to more comprehensively determine the role of ghrelin-mediated glucagon secretion during the setting of chronic, severe caloric restriction.

The current study demonstrates the importance and requirement of intracellular calcium fluxes and phosphorylation of ERK in ghrelin-mediated glucagon release. Neither of these findings was unexpected, because $[Ca^{2+}]_i$ and/or pERK had been shown in various primary or immortalized cells with endogenous GHSR expression to be activated by ghrelin (1, 4, 34–38, 41). It is also known that both pERK and $[Ca^{2+}]_i$ are involved in exocytosis in endocrine cells (42, 43), and specifically, increases in $[Ca^{2+}]_i$ have previously been linked to palmitic acid stimulation of glucagon secretion (44). The current study is the first to demonstrate that both ERK phosphorylation and increases in $[Ca^{2+}]_i$ are induced by ghrelin in an endocrine cell line with endogenous GHSR. Thus, the α-cell lines used here seem to be ideal models to investigate intracellular signaling cascades initiated by ghrelin, and future studies likely should include those in which a connection between ghrelin-mediated changes in Ca^{2+} flux and ERK phosphorylation, as has been accomplished in other systems (45), can be established.

Of mention, the reported demonstration of ghrelin-induced glucagon release from α-cell lines and confirmation of GHSR expression within α-cells does not exclude effects on glucagon secretion by acyl-ghrelin acting at other sites. For instance, the hypothalamus, which normally expresses GHSR abundantly in several nuclei, mediates glucose homeostasis (46). Ghrelin's actions on glucagon release might also be mediated indirectly via ghrelin-mediated reductions in insulin (3, 4) and/or via changed autonomic outflow (47). Alternatively, the effects of ghrelin observed potentially could be mediated, at least in part, by desacyl-ghrelin, presumably acting via a non-GHSR mechanism, because some studies have demonstrated anorexigenic and other actions for desacyl-ghrelin (48). The findings here, however, suggest that direct effects on α-cell GHSR do have the capacity to contribute to ghrelin's overall effects on glucagon levels.

Although not a central focus of the current study, we also did not observe differences in plasma insulin levels in mice acutely injected or chronically infused with ghrelin, transgenic mice expressing supraphysiological levels of ghrelin, or in mice with blocked GHSR expression. This is unlike what we may have expected from the majority of papers that demonstrate ghrelin's ability to restrict insulin release through direct effects on pancreatic β-cells. Nonetheless, we previously reported that GHSR deletion led to lower blood glucose levels in male mice maintained on a standard chow diet, whereas corresponding plasma insulin for these GHSR-null mice was not increased, and in fact was statistically lower in the early a.m (8). Thus, we proposed that GHSR deletion was associated with enhanced insulin sensitivity, and such has also been supported by several other studies, as discussed above (9–13). We did, however, here observe a ghrelin-mediated reduction of insulin secretion from isolated islets, as had been demonstrated previously (3, 7). The contributions of ghrelin-mediated changes in glucagon secretion, insulin release, insulin sensitivity, and GH secretion will need to be investigated in an integrated fashion in future studies to best understand how these mechanisms work together in various clinical settings to influence glycemia.

Materials and Methods

Animals

Tissues for qPCR were from 3-month-old male A129/SvJ mice housed with *ad libitum* access to water and standard rodent diet (diet 7001; Harlan Teklad, Madison, WI) in a light- and temperature-controlled facility. GHSR-null mice and wild-type littermates were generated by breeding heterozygous mice, obtained after more than 10 generation backcrossing onto a C57BL6/J genetic background (8). Mice with ghrelinomas were generated as reported (32), and blood samples were collected and analyzed at 20 wk of age, a time point before the manifestation of visible tumors. As reported, BW of GHSR-null or ghrelinoma-containing mice did not differ from their wild-type littermates (8, 32). All studies were approved by the Institutional Animal Care and Use Committee of University of Texas Southwestern Medical Center (UTSW).

Islet studies

To determine the effect of glucose on islet GHSR mRNA levels, mouse islets were isolated using standard methods (49). Briefly, whole pancreata were perfused and digested with liberase R1 (Roche, Indianapolis, IN). Islets were isolated by Ficoll gradient centrifugation and hand selected under a stereomicroscope before transfer to RPMI 1640 containing 11.1 mM glucose and supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (FBS), 100 IU/ml penicillin, and 100 μg/ml streptomycin. Isolated islets were incubated overnight (37 C, 5% CO_2)

before experimentation. Next, islets (~200 islets per replicate) were incubated in 11 mM glucose for 8 h and then shifted to 5 mM (low glucose) or 17.5 mM (high glucose) for 16 h before RNA isolation.

To determine the effect of ghrelin on glucagon and insulin secretion from islets, we performed studies on islets freshly isolated from the splenic portion of mouse pancreata, using reported methods (27). Previously, acyl-ghrelin had been shown to stimulate glucagon release from mouse islets freshly collected from the splenic part of the pancreas, where glucagon cells are more numerous, but not from the duodenal part of the pancreas (27). Here, islets from the splenic portions of pancreata were isolated as above (pancreata were digested and centrifuged and islets were hand selected using a stereomicroscope), from male GHSR-null or wild-type littermates. Instead of an overnight incubation (as described above for the islets from which RNA was obtained), islets immediately were preincubated for 30 min at 37 C in secretion assay buffer (SAB, containing 0.114 M NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.16 mM $MgSO_4$, 12.75 mM $NaHCO_3$, 25 mM $CaCl_2$, 20 mM HEPES, and 0.2% BSA, pH 7.4) with 1 mM glucose. After preincubation, islets were transferred to a 24-well plate (10 size-matched islets per well) containing 500 μl SAB with 12 mM glucose and different concentrations of ghrelin (0–10^{-6} M). After a 60-min incubation at 37 C, aliquots of SAB were removed from the wells and stored at −80 C before insulin and glucagon assays. Islets from each of the wells were transferred to 500 μl cold PBS and then were sonicated before assays to determine total insulin and glucagon content. Glucagon and insulin secretion are reported as percent of content and determined by expressing the hormone concentration in the culture media divided by the sum of hormone in media and islets multiplied by 100. Note that the observation of ghrelin-stimulated glucagon secretion by mouse islets was dependent on the isolation and culture conditions, being observed only in freshly isolated islets [as has previously been reported (25–27)], and not in those that were incubated overnight before ghrelin exposure. This observation suggests that either hormones secreted into the culture media overnight, or cell-cell interactions of the incubated islet may affect ghrelin/GHSR/glucagon signaling.

Cell culture

The insulinoma cell line βTC6 [CRL-11506 (50)] and the glucagonoma cell line αTC1-clone 9 [CRL-2350 (51)] were obtained from American Type Tissue Culture. MIN6 (52) and InR1G9 cell (53) lines were provided by Drs. Melanie Cobb and Roger Unger (UTSW). βTC6 cells were cultured in DMEM (4.5 g/liter glucose, 4 mM L-glutamine) with 15% heat-inactivated FBS. MIN6 cells were cultured in DMEM (4.5 g/liter glucose) with 2 mM L-glutamine, 1 mM sodium pyruvate, and 10% heat-inactivated FBS. αTC1 cells were cultured in DMEM with 4 mM L-glutamine, 1.5 g/liter sodium bicarbonate, 3 g/liter glucose, 10% heat-inactivated FBS, 15 mM HEPES, 0.1 mM nonessential amino acids, and 0.02% BSA. InR1G9 cells were cultured in RPMI with 11.1 mM glucose.

To determine the effect of glucose on αTC1 GHSR mRNA levels, αTC1 cells were incubated in medium identical to their above-described standard medium with the exception of 11 mM glucose (instead of 3 g/liter glucose) for 8 h and then shifted to 5 mM (low glucose) or 17.5 mM (high glucose) for 16 h before RNA isolation.

RNA measurement

Total RNA was isolated from tissues or cultured cells using RNA STAT-60 (Tel-Test, Inc., Friendswood, TX). Total RNA (2 μg) was treated with ribonuclease-free deoxyribonuclease (Roche) and then reverse transcribed with random hexamers using SuperScript II (Invitrogen, Carlsbad, CA). qPCR was performed using an Applied Biosystem Prism 7900HT sequence detection system (Applied Systems, Foster City, CA) and SYBR-green chemistry. Gene-specific primers were designed using Primer Express Software (PerkinElmer Life Sciences, Boston, MA) and validated by analysis of template titration and dissociation curves. [Primer sequences: GHSR forward, 5′-ACCGTGATGGTATGGGTGTCG-3′; and reverse, 5′-CACAGTGAGGCAGAAGACCG-3′ (product spanning nucleotides 878–937, NM_177330)]. qPCR containing 25 ng reverse-transcribed RNA, each primer (150 nM) and 5 μl 2X SYBR Green PCR master mix (Applied Biosystems) were evaluated by the comparative C_T method using cyclophilin (Fig. 1A) or hypoxanthine-phophoribosyltransferase (Fig. 1, B and C) as the invariant control gene.

ISHH and IHC

Mice were deeply anesthetized with ip injection of chloral hydrate and perfused transcardially with diethylpyrocarbonate-treated 0.9% PBS followed by 10% neutral buffered formalin. Pancreata were removed, stored in the formalin for 4–6 h at 4 C, immersed in 20% sucrose in diethylpyrocarbonate-treated PBS, pH 7.0, at 4 C overnight, and cut on a cryostat at 15-μm intervals. The sections were mounted on SuperFrost slides (Fisher Scientific, Pittsburgh, PA), air dried, and stored at −80 C until further processing. ISHH for GHSR mRNA was performed as reported (47) using an ^{35}S-labeled mouse GHSR type 1a-specific riboprobe generated from a 916-bp fragment of cDNA amplified with GHSR-specific primers (mGHSR1047, 5′-GTGGTGTTTGCTTTCATCCTC-3′ and mGHSR1962, 5′-CATGCTCAAATTAAATGCATCC-3′). IHC for insulin and glucagon was performed on sections adjacent to those used for ISHH using previously described methods (47). Antisera included: guinea pig antiinsulin antiserum (1:300 in 0.1% Tween-20 in 1× PBS, DakoCytomation, Carpinteria, CA), rabbit antiglucagon antiserum (1:300 in 0.1% Tween-20 in 1× PBS, Millipore Corp., Billerica, MA), antiguinea pig IgG-Alexa 594 conjugate (1:250, Invitrogen) and antirabbit IgG-Alexa 488 conjugate (1:250, Invitrogen).

Acute acyl-ghrelin injections

C57BL6/J mice aged 10–11 wk were injected sc at 0011 h with a range (0–2 mg/kg BW) of rat acyl-ghrelin (Pi Proteomics, Huntsville, AL) doses. GHSR-null littermates were similarly treated with either 0 or 2 mg/kg BW acyl-ghrelin. Blood (200 μl) was collected for immediate blood glucose measurements [using a OneTouch Ultra (LifeScan, Milpitas, CA)] and subsequent glucagon and insulin assays 30 min after injection. During this 30-min period, access to food was removed. Another cohort of 10- to 11-wk-old C57BL6/J mice was similarly treated with sc ghrelin, without access to food, and blood was collected for circulating acyl-ghrelin measurements. To determine whether the lower acyl-ghrelin doses could initiate an established ghrelin-induced action, a separate cohort of mice was injected with a similar range of acyl-ghrelin doses in the presence of standard rodent diet, and subsequent food intake was monitored.

Osmotic pump chronic ghrelin delivery

Osmotic pumps (Alzet, Cupertino, CA; model 1002) prefilled with saline or 16.7 mg/ml rat acyl-ghrelin were inserted sc into the interscapular region of mice under anesthesia. Acyl-ghrelin was delivered at a rate of 0.25 μl/h to achieve a daily dose of 4 mg/kg BW. Acyl-ghrelin or saline administration for 2 wk increased BW by 9.7% or 3.5%, respectively (data not shown).

Measurement of plasma parameters

Mice were euthanized with chloral hydrate, after which blood was collected from the inferior vena cava in EDTA-coated tubes containing *p*-hydroxymercuribenzoic acid (final concentration, 1 mM). The plasma was separated immediately and stored at −80 C. Samples used for measurement of ghrelin were first acidified with 0.1 M HCl. Plasma glucose was measured using glucose oxidase techniques (Sigma Chemical Co., St. Louis, MO), except as noted. Insulin, glucagon, and C-peptide were measured by RIA (Millipore Corp.) using 20 μl, 100 μl (or 20 μl for transgenic mice with ghrelinomas), and 15 μl plasma, respectively. Acyl- and desacyl-ghrelin were measured by ELISA using 25 μl and 10 μl acid-stabilized plasma, respectively (Cayman Chemical, Ann Arbor, MI).

Glucagon secretion from pancreatic α-cell lines

αTC1 cells were split into polylysine-coated 24-well plates (5×10^5 cells per well) 2 d before experimentation. Cells were preincubated in SAB with 16.7 mM glucose for 1 h and next were stimulated with SAB (250 μl per well) containing 5 mM glucose and different concentrations of acyl-ghrelin for 2 h. InR1G9 cells were similarly treated except there were 2×10^5 cells per well and preincubation was performed in SAB with 11.1 mM glucose.

To block ERK activation, αTC1 cells were exposed to U0126 (Cell Signaling Technology, Danvers, MA) or PD98059 (Calbiochem, San Diego, CA) beginning 1 h before and continuing during the 2-h treatment with acyl-ghrelin or saline. Glucagon in the medium was measured as described above. To block calcium influx, EGTA (2 mM, Sigma) or nifedipine (50 μM, Tocris Bioscience, Bristol, UK) was applied to αTC1 cells during the 2-h incubation with either acyl-ghrelin or saline. Aliquots of media collected after the 2-h treatment periods were stored at −80 C until glucagon assay. These experiments were repeated to collect samples for Western blot analysis.

$[Ca^{2+}]_i$ measurement

Cells on glass-bottom dishes were incubated with 2 μM Fura-2 acetoxymethyl ester (Sigma) in 10 mM HEPES-buffered Krebs-Ringer bicarbonate buffer containing 5 mM glucose and 0.1% BSA for 30 min at 37 C. Next, the dishes were placed on the stage of a Zeiss Axiovert 200M microscope (Carl Zeiss, Thornwood, NY), and the cells were superfused with HEPES-buffered Krebs-Ringer bicarbonate buffer at a rate of 1 ml/min at 37 C. Cells were excited alternately at 340 and 380 nm every 10 sec, and emission signals at 510 nm were detected with a cooled charge-coupled device SensiCam QE camera (Cooke Corp., Auburn Hills, MI). The intensity of emission signals and ratio were calculated by SlideBook 4.2 (Intelligent Imaging Innovations, Denver, CO). The Fura-2 ratio amplitude was calculated by subtracting the basal ratio from the acyl-ghrelin-stimulated peak ratio.

Western blot analysis

Protein extracts were prepared by lysing cells in SDS sample buffer (2% SDS, 150 mM NaCl, 62 mM Tris-HCl, pH 6.8, 10% glycerol, 2 5% dithiothreitol, 0.01% Bromophenol blue) and boiling for 10 min. Proteins were size fractioned on an 8% denaturing SDS-polyacrylamide gel and transferred to nitrocellulose membranes. Rabbit anti-pERK antibody (1:1000, Cell Signaling Technology) and mouse anti-ERK antibody (1:1000, Cell Signaling Technology) were used with the species-appropriate horseradish peroxidase-conjugated secondary antibodies and the Supersignal West picochemiluminescent substrate (Thermo Scientific, Rockford, IL) to generate a signal by autoradiograph.

Statistical analyses

Data are expressed as mean ± SEM. GraphPad Prism 5.0 (GraphPad Software, Inc., San Diego, CA) was used for statistical analyses. If unequal variance among groups was detected by Bartlett's test, data were transformed before analysis, as required. Pair-wise comparisons were performed by two-tailed Student's *t* test. One-way ANOVA followed by Dunnett's *post hoc* test were used to assess the effects of administered ghrelin concentration on acute food intake, and on plasma acyl-ghrelin, glucagon, glucose, and insulin levels in wild-type mice, glucagon release from cultured α-cell lines, glucagon and insulin release from islets, and on Fura-2 ratio amplitude. Two-way ANOVA followed by Bonferroni *post hoc* analysis was used to assess the effects of acyl-ghrelin and calcium blockers or ERK inhibitors on glucagon secretion from α-cell lines (Figs. 6C and 7D).

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