Ablation of ghrelin receptor in leptin-deficient ob/ob mice has paradoxical effects on glucose homeostasis when compared with ablation of ghrelin in ob/ob mice

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Ma X, Lin Y, Lin L, Qin G, Pereira FA, Haymond MW, Butte NF, Sun Y. Ablation of ghrelin receptor in leptin-deficient ob/ob mice has paradoxical effects on glucose homeostasis when compared with ablation of ghrelin in ob/ob mice. Am J Physiol Endocrinol Metab 303: E422–E431, 2012. First published June 5, 2012; doi:10.1152/ajpendo.00576.2011.—The orexigenic hormone ghrelin is important in diabetes because it has an inhibitory effect on insulin secretion. Ghrelin ablation in leptin-deficient ob/ob (Ghrelin−/−:ob/ob) mice increases insulin secretion and improves hyperglycemia. The physiologically relevant ghrelin receptor is the growth hormone secretagogue receptor (GHS-R), and GHS-R antagonists are thought to be an effective strategy for treating diabetes. However, since some of ghrelin’s effects are independent of GHS-R, we have utilized genetic approaches to determine whether ghrelin’s effect on insulin secretion is mediated through GHS-R and whether GHS-R antagonism indeed inhibits insulin secretion. We investigated the effects of GHS-R on glucose homeostasis in Ghsr-ablated ob/ob mice (Ghsr−/−:ob/ob). Ghsr ablation did not rescue the hyperphagia, obesity, or insulin resistance of ob/ob mice. Surprisingly, Ghsr ablation worsened the hyperglycemia, decreased insulin, and impaired glucose tolerance. Consistently, Ghsr ablation in ob/ob mice upregulated negative β-cell regulators (such as UCP-2, SREBP-1c, ChREBP, and MIF-1) and downregulated positive β-cell regulators (such as HIF-1α, FGF-21, and PDX-1) in whole pancreas; this suggests that Ghsr ablation impairs pancreatic β-cell function in leptin deficiency. Of note, Ghsr ablation in ob/ob mice did not affect the islet size; the average islet size of Ghsr−/−:ob/ob mice is similar to that of ob/ob mice. In summary, because Ghsr ablation in leptin deficiency impairs insulin secretion and worsens hyperglycemia, this suggests that GHS-R antagonists may actually aggravate diabetes under certain conditions. The paradoxical effects of ghrelin ablation and Ghsr ablation in ob/ob mice highlight the complexity of the ghrelin-signaling pathway.

ghrelin ablation in ob/ob mice has paradoxical effects on glucose homeostasis.

Ghrelin ablation increases insulin secretion and improves hyperglycemia.

Ghsr ablation did not rescue hyperphagia, obesity, or insulin resistance.

Ghsr ablation worsened hyperglycemia, decreased insulin, and impaired glucose tolerance.

Ghsr ablation upregulated negative β-cell regulators and downregulated positive β-cell regulators.

Ghsr−/−:ob/ob mice have similar islet size compared to ob/ob mice.

Ghsr ablation in ob/ob mice highlights the complexity of ghrelin-signaling pathway.

GHS-R ablation impairs pancreatic β-cell function.

GHS-R antagonists may actually aggravate diabetes under certain conditions.

Paradoxical effects of ghrelin ablation and Ghsr ablation in ob/ob mice highlight the complexity of ghrelin-signaling pathway.

Obesity is one of the most alarming health concerns in Western and developing countries. Obesity causes insulin resistance, often leading to type 2 diabetes. Ghrelin is a multifaceted hormone best known for its orexigenic action; growth hormone secretagogue receptor (GHS-R) is recognized as a physiologically relevant receptor for ghrelin, mediating ghrelin’s effects on growth hormone release, food intake, and adiposity (6, 12, 46). Both ghrelin and GHS-R are expressed in pancreatic islets (51, 53). We demonstrated previously that whereas ghrelin gene ablation in wild-type mice has no effect on hyperphagia or obesity, ghrelin ablation in leptin-deficient ob/ob mice ameliorates the diabetic condition (44). Studies have demonstrated that exogenous ghrelin administration inhibits insulin secretion both in vivo and in vitro (4, 16, 41, 44), confirming ghrelin as a negative regulator of insulin secretion and implying that ghrelin has an important role in glucose homeostasis.

We reported previously that circulating ghrelin increases during fasting and that glucose concentrations decrease in calorie-restricted ghrelin−/− and Ghsr-null mice, which suggests that both ghrelin and GHS-R are involved in glucose sensing (45, 46). We have shown that ghrelin’s stimulatory effects on growth hormone (GH) release and feeding are mediated through GHS-R (46). However, it is unknown whether ghrelin’s effect on insulin secretion is mediated through GHS-R. A widely used GHS-R antagonist, [D-Lys3]-GHRP-6, has been shown to block ghrelin’s inhibitory effect on insulin secretion (16). It was thus inferred that ghrelin’s effect on insulin secretion is mediated through the GHS-R and that GHS-R antagonists would be a viable approach for treating diabetic hyperglycemia (1, 2, 16). However, a recent report showed that [D-Lys3]-GHRP-6 blocks receptors other than GHS-R; for example, the CCR5 and CXCR4 receptors, which are used by HIV to enter cells, can also be blocked by [D-Lys3]-GHRP-6 (34). In addition, GHS-R is known to have constitutive activity, which is capable of producing its biological response in the absence of a bound ligand (21). Some GHS-R-associated effects may be due to the constitutive activity of GHS-R rather than the activation of ghrelin. We have shown recently that ghrelin−/− and Ghsr-null mice have differential phenotypes in thermogenic regulation and sleep; others have shown that ghrelin directly stimulates liver glucose output and adipogenesis by mechanisms independent from GHS-R (18, 30, 47, 48, 50). The evidence collectively suggests that some ghrelin functions may be mediated by subtype receptor(s) other than GHS-R. It is unknown whether ghrelin’s effect on pancreatic β-cells is mediated exclusively by GHS-R or whether GHS-R antagonists can be used as antidiabetic agents.

The objectives of this study were to 1) assess the effect of GHS-R ablation on glucose homeostasis under obese and diabetic states, 2) determine whether ghrelin deletion and GHS-R deletion have similar effects on glycemic control in leptin-deficient mice,
and 3) shed light on whether ghrelin’s inhibitory effect on insulin secretion is mediated through GHS-R. To enable these studies, we bred our Ghsr−/− mice with leptin-deficient ob/ob mice to generate a mouse model lacking both GHS-R and leptin (Ghsr−/−: ob/ob).

**METHODS**

**Generation of Ghsr−/−: ob/ob mice.** Our studies were approved by the Institutional Animal Care and Use Committee at Baylor College of Medicine. The generation of ghrelin−/−, Ghsr−/−, and ghrelin−/−; ob/ob mice has been described by us previously (43, 44, 46). All mice used in the study were on the C57BL/6J background. To generate Ghsr−/−: ob/ob mice, N12 Ghsr−/− mice were bred to ob/+ mice from the Jackson Laboratory, creating compound heterozygotes, i.e., Ghsr−/−: ob/OB mice. In the second cross, these mice were interbred to generate Ghsr−/−: ob/ob mice and Ghsr−/−: OB/OB (Ghsr−/−) mice. In parallel, Ghsr−/+: ob/ob mice were bred to each other to produce Ghsr−/−: ob/ob (ob/ob) and Ghsr−/−: OB/OB (wild-type (WT)) mice. To minimize animal-to-animal variations, only littermate male mice were used. Mice were maintained under controlled temperature (≈75°F) and illumination (12:12-h light-dark cycle, 6 AM to 6 PM), with free access to water and regular chow.

**General phenotypical characterization.** Body weight and food intake were measured weekly, and blood glucose was measured biweekly. Measurements were taken at the same time each day (between 9 and 10 AM) from 8 to 16 wk of age. Blood glucose concentrations were determined by a One-Touch Ultra glucometer (Lifescan, Milpitas, CA). Plasma triglycerides, total cholesterol, HDL, LDL, and free fatty acids (FFA) were measured by the lipid core of Hormone Assay & Analytical Services at Vanderbilt University.

**Body composition and indirect calorimetry.** Whole body composition (fat and lean mass) of mice was measured by an Echo MRI-100 whole body composition analyzer (Echo Medical Systems, Houston, TX). Metabolic parameters were obtained using the Oxymax (Columbus Instruments, Columbus, OH) open-circuit indirect calorimetry system. To minimize the confounding effects of stress, mice were caged individually in metabolic chambers and given free access to regular chow and water for 1 wk. They were then placed in metabolic cages for 2–4 days before the indirect calorimetry testing. Indirect calorimetry studies were carried out for 72 h. The first 24 h were considered the acclimation phase, and average data of the final 48 h were analyzed. Oxygen consumption (VO2; ml·kg−1·h−1), carbon dioxide production (VCO2; ml·kg−1·h−1), and locomotor activity (beam break counts) were measured. Respiratory exchange ratio (RER) and energy expenditure (EE; or heat generation) were calculated. The characteristics of light and dark circadian rhythm were totally lost in mice on ob/ob background (Fig. 2, C and D). No differences were observed in total VO2, VCO2, or energy expenditure between ob/ob and Ghsr−/−: ob/ob mice whether normalized by either body weight or lean mass (Fig. 2C). The Ghsr−/−: ob/ob mice showed a lower RER compared with ob/ob mice, and the difference persisted throughout the light and dark phases (Fig. 2D). There were no differences in energy expenditure or RER between ob/ob and Ghrelin−/−: ob/ob mice (data not shown).

**Histological analysis.** Entire pancreases were fixed overnight in 10% formaldehyde solution at room temperature and then dehydrated and embedded in paraffin. Tissue blocks of whole pancreas (showing head, body, and tail of the pancreas) were then sectioned at 5 μm and stained with hematoxylin and eosin (H & E) for morphometric analysis. The H & E staining was carried out according to the standard protocols. The average area of pancreatic islets was measured using Axiohot microscope, and the image was analyzed using the Scion Image for Windows analysis software [National Institutes of Health (NIH), Bethesda, MD]. The sectional area of islets was measured at a magnification of ×10. All islets on each randomly selected section were counted, and ≥120 islets were counted for each mouse.

**Statistical analyses.** All data are expressed as means ± SE. We used a two-tailed Student t-test, or one- or two-way ANOVA, to determine significance of differences between genotypes or treatments. P < 0.05 is defined as statistical significance.

**RESULTS**

GHS-R deficiency affects neither the body weight nor food intake of ob/ob mice. No differences were observed in food intake or body weight between WT and Ghsr−/− mice (Fig. 1, A and B). Ghsr−/−: ob/ob mice were hyperphagic compared with the lean WT and Ghsr−/− mice, but food intake was similar to that of ob/ob mice (Fig. 1A). Similar to Ghrelin−/−: ob/ob mice, the body weight of Ghsr−/−: ob/ob mice was significantly higher than that of WT and Ghsr−/− mice but similar to that of ob/ob mice (Fig. 1B). These results suggest that ablation of GHS-R in ob/ob mice does not protect mice from hyperphagia or obesity resulting from leptin deficiency.

GHS-R ablation affects neither body composition nor lipid profile of ob/ob mice. The body fat contents of ob/ob and Ghsr−/−: ob/ob mice were significantly higher than that of WT and Ghsr−/− mice, but there was no difference between ob/ob and Ghsr−/−: ob/ob mice (Fig. 1C). Furthermore, compared with ob/ob mice, the Ghsr−/−: ob/ob mice showed similar plasma lipid profiles in triglyceride, cholesterol, HDL, and LDL (Fig. 1D) as well as FFA (Fig. 1E). Thus, GHS-R ablation affects neither body composition nor lipid profiles of ob/ob mice.

**Metabolic profile of Ghsr−/−: ob/ob mice during indirect calorimetry.** Under the ad libitum-fed condition, there was no difference in total food intake (Fig. 2A) or locomotor activity (Fig. 2B) between ob/ob and Ghsr−/−: ob/ob mice during calorimetry study. The characteristics of light and dark circadian rhythm were totally lost in mice on ob/ob background (Fig. 2, C and D). No differences were observed in total VO2, VCO2, or energy expenditure between ob/ob and Ghsr−/−: ob/ob mice whether normalized by either body weight or lean mass (Fig. 2C). The Ghsr−/−: ob/ob mice showed a lower RER compared with ob/ob mice, and the difference persisted throughout the light and dark phases (Fig. 2D). There were no differences in energy expenditure or RER between ob/ob and Ghrelin−/−: ob/ob mice (data not shown).

**GHS-R ablation aggravates hyperglycemia of ob/ob mice.** As expected, under the ad libitum-fed condition, the lean mice were euglycemic, and the ob/ob mice developed hyperglycemia when compared with lean mice. Surprisingly, in contrast to the improved glycemic condition observed in Ghrelin−/−: ob/ob mice, the Ghsr−/−: ob/ob mice exhibited worsened hyperglycemia (Fig. 3A) and reduced plasma insulin and C-peptide (Fig. 3B) compared with ob/ob mice. Whereas plasma glucagon levels of Ghsr−/− mice were significantly lower than those of WT
mice, the glucagon levels in Ghsr⁻/⁻:ob/ob mice showed a trend of decrease compared with that of ob/ob mice, but this trend failed to reach statistical significance (Fig. 3C). These results suggest that ablation of GHS-R further exacerbates hyperglycemia of ob/ob mice by inhibiting insulin secretion.

GHS-R ablation inhibits insulin secretion of ob/ob mice but has no effect on insulin sensitivity. A low-dose (0.625 g/kg) ip glucose tolerance test (GTT) was selected to study the glucose and insulin responses of Ghsr⁻/⁻:ob/ob mice, because mice on ob/ob background are glucose intolerant (44). Compared with ob/ob mice, Ghsr⁻/⁻:ob/ob mice displayed increased blood glucose excursions at 30, 60, and 120 min following an ip glucose bolus (Fig. 3D); first-phase (15 min) plasma insulin concentrations were decreased in Ghsr⁻/⁻:ob/ob mice compared with ob/ob mice (Fig. 3E). To elucidate whether the worsened hyperglycemia of Ghsr⁻/⁻:ob/ob mice was also attributable to insulin sensitivity, ITTs were performed. No statistically significant differences were observed in blood glucose concentrations between ob/ob and Ghsr⁻/⁻:ob/ob mice (Fig. 3F). These data suggest that GHS-R ablation in leptin-deficient mice further impairs ß-cell insulin secretory function but has no effect on insulin sensitivity.

Morphology of pancreatic islets in Ghsr⁻/⁻:ob/ob mice. Although obese mice (ob/ob, Ghrelin⁻/⁻:ob/ob, and Ghsr⁻/⁻:ob/ob) had significantly enlarged islets compared with lean WT mice (Fig. 4, A–D), there was no difference in islet size between ob/ob and Ghsr⁻/⁻:ob/ob mice. On the other hand, Ghrelin⁻/⁻:ob/ob mice appeared to have larger islets than ob/ob and Ghsr⁻/⁻:ob/ob mice, but the difference failed to reach statistical significance (P = 0.08) due to the wide variation in islet size (Fig. 4E).

We noted that ob/ob and Ghsr⁻/⁻:ob/ob mice appeared to have more islet vascularization than WT and Ghrelin⁻/⁻:ob/ob mice (Fig. 4, A–D). Ghsr⁻/⁻:ob/ob mice had pancreatic islet morphology similar to that of ob/ob mice but were different from that of Ghrelin⁻/⁻:ob/ob mice.

Expression profiles of ß-cell regulatory genes in whole pancreas of ob/ob, Ghrelin⁻/⁻:ob/ob, and Ghsr⁻/⁻:ob/ob mice. We studied the expression of regulators involved in insulin secretion and pancreatic ß-cell mass. We showed previously that pancreatic uncoupling protein 2 (UCP2) mRNA expression is decreased in whole pancreas of Ghrelin⁻/⁻:ob/ob mice (44). In contrast, the Ghsr⁻/⁻:ob/ob mice have increased UCP2 expression in whole pancreas compared with ob/ob mice (Fig. 5A). The sterol regulatory element-binding protein-1c (SREBP-1c) and peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) are positive transcriptional regulators of UCP2, targeting the E-box and TRE line with UCP2 levels shown in these mouse models; however, there is no difference in PGC-1α levels (Fig. 5C). Whereas UCP2 decreases ATP levels in ß-cells, hypoxia-inducible factor-1α (HIF-1α) exerts its effect on ß-cell function by stimulating ATP (9). Fibroblast growth factor-21 (FGF-21) has been shown to improve ß-cell function and survival by activation of extracellular signal-regulated kinase 1/2 and the Akt signaling pathway (52).

Remarkably, although we detected significant increases in the expression of both HIF-1α and FGF-21 in whole pancreases of Ghrelin⁻/⁻:ob/ob mice, we found much lower levels of HIF-1α...
and FGF-21 expression in those of Ghsr−/−:ob/ob mice (Fig. 5, D and E). Carbohydrate response element-binding protein (ChREBP) is a key regulator of glucose metabolism and fat storage (15). Pancreatic and duodenal homeobox-1 (PDX-1) is a transcription factor necessary for pancreatic development and β-cell maturation. It has been shown that PDX-1 is negatively regulated by ChREBP (13). Our results showed that ChREBP expression was decreased in whole pancreases of Ghsr−/−:ob/ob mice but increased in those of Ghrelin−/−:ob/ob mice (Fig. 5F).

In contrast, PDX-1 showed an opposite expression pattern in whole pancreas of both double-null mice compared with ChREBP (Fig. 5G). Deficiency of macrophage migration inhibitory factor-1 (MIF-1) has been shown to protect pancreatic islets from palmitic acid-induced apoptosis (40). Yet whereas we detected lower levels of MIF-1 in whole pancreases of Ghrelin−/−:ob/ob mice, we observed higher levels of ChREBP in Ghsr−/−:ob/ob mice (Fig. 5H). In addition, we have studied expression of forkhead box protein O1 (FOXO1). FOXO1 has been shown to protect pancreatic β-cells from fatty acid insult (31), but FOXO1 levels did not change in our mouse models (Fig. 5I). Collectively, these data exemplify the dramatic differences between the pancreatic gene expression profiles of Ghrelin−/−:ob/ob and Ghsr−/−:ob/ob mice, suggesting that whereas ablation of ghrelin in ob/ob mice improves β-cell function, ablation of Ghsr in ob/ob mice may worsen β-cell function.

DISCUSSION

The best-known functions of ghrelin are its roles in stimulating GH release and promoting appetite and adiposity. We and others have shown that ghrelin is a negative regulator of insulin secretion (16, 44, 45). We reported that ablation of ghrelin expression augments insulin secretion and improves insulin sensitivity, resulting in the improvement of hyperglycemia and glucose intolerance in diabetic ob/ob mice (44). Blockade of ghrelin has also been shown to increase insulin secretion and prevent glucose intolerance induced by a high-fat diet (16). These findings suggest that suppressing ghrelin signaling prevents both genetically and environmentally induced β-cell impairments. It has thus been speculated that
GHS-R antagonists may serve as antidiabetic agents. Although the effects of deletion and/or pharmacological blockade of GHS-R on glucose homeostasis have been examined in normal lean mice (11, 29, 60), the effect of GHS-R in obese and diabetic subjects is unknown. Most diabetic patients are obese, so it is important to understand the role of GHS-R on glucose homeostasis under an obese diabetic state.

Leptin and ghrelin have opposite effects on food intake and body weight regulation. It is thus important to understand the interplay between leptin signaling and ghrelin signaling. Both the leptin receptor and the ghrelin receptor are expressed within the same nuclei of the hypothalamic arcuate nucleus, which regulate appetite and satiety (35). One report showed that the effects of ghrelin on GH secretion and food intake are suppressed in leptin receptor-knockout db/db mice, suggesting that cross-talk exists between the ghrelin- and leptin-signaling pathways (24). Another report showed that ghrelin receptor deficiency does not impact the anorexigenic effect of leptin, suggesting that ghrelin and leptin may act on separate and distinct neuronal populations (35). In diet-induced obese mice, the circulating ghrelin level is lower, and the role of ghrelin on food intake is suppressed (5, 24). In obese leptin-resistant Zucker rats, GHRP-6-induced Fos response is increased, but central infusion of leptin suppresses this GHRP-6-induced Fos response (22). These studies suggest that ghrelin-regulatory circuits in the hypothalamus are dynamically regulated, and the regulation may vary based on nutritional states.

To study the role of GHS-R on glucose homeostasis under obese and diabetic conditions, we bred the Ghsr−/− mice with leptin-deficient ob/ob mice to generate Ghsr−/−:ob/ob mice. Similarly to Ghrelin−/−:ob/ob mice, we observed that GHS-R ablation in ob/ob mice failed to rescue the hyperphagic or obese phenotypes of ob/ob mice (Fig. 1, A–C). Plasma lipids, such as plasma levels of triglyceride, cholesterol, HDL, LDL, and FFA, play important roles in insulin resistance and glucose homeostasis. Here, we found that Ghsr−/−:ob/ob mice had lipid profiles similar to those of ob/ob mice (Fig. 1, D and E). These findings indicate that unopposed action of ghrelin or GHS-R in ob/ob mice is not the underlying cause of leptin-dependent obesity and that leptin has a dominant effect on energy homeostasis.

We reported recently that ghrelin ablation and Ghsr ablation have distinct effects on energy expenditure in older mice; older Ghsr−/− mice (but not older Ghrelin−/− mice) have an elevated energy expenditure (30) due to increased thermogenesis in brown adipose tissue (27). Characterization of the metabolic state of Ghsr−/−:ob/ob mice revealed no differences in energy intake or locomotor activity (Fig. 2, A and B). Regardless of normalizing by body weight or lean mass, the energy expen-
diture of Ghsr<sup>−/−</sup>:<sup>ob/ob</sup> mice was no different from that of <sup>ob/ob</sup> mice (Fig. 2C). It is known that <sup>ob/ob</sup> mice have almost no brown adipose tissue, which makes them severely thermo-genetically impaired (23). The lack of brown adipose tissue in Ghsr<sup>−/−</sup>:<sup>ob/ob</sup> may thus obscure the effect of the GHS-R on energy expenditure. Interestingly, Ghsr<sup>−/−</sup>:<sup>ob/ob</sup> mice had a significant reduction in RER during both the light and the dark periods (Fig. 2D), indicating that the Ghsr<sup>−/−</sup>:<sup>ob/ob</sup> mice preferentially utilize fat as an energy source under the leptin-deficient background. Our data are consistent with previous reports that Ghrelin<sup>−/−</sup>:<sup>ob/ob</sup> and Ghsr<sup>−/−</sup>:<sup>ob/ob</sup> mice have decreased RER in a diet-induced obese state (54, 60). However, the body composition analysis (Fig. 1C) showed that the preferential fat consumption associated with GHS-R ablation is insufficient to rescue the obesity of <sup>ob/ob</sup> mice. This again supports the conclusion that leptin plays a dominant role in regulating energy metabolism and body composition.

In surprising contrast to Ghrelin<sup>−/−</sup>:<sup>ob/ob</sup> mice, we found that Ghsr<sup>−/−</sup>:<sup>ob/ob</sup> mice exhibited higher basal glucose and lower insulin and C-peptide levels than <sup>ob/ob</sup> mice (Fig. 3, A and B). C-peptide, generated during proinsulin processing and secreted along with insulin, is a more accurate measurement for insulin secretion than plasma insulin itself, because plasma insulin concentrations can also be affected by degradation (55). The decreased C-peptide levels along with lower insulin concentrations confirm that insulin secretion is indeed reduced in Ghsr<sup>−/−</sup>:<sup>ob/ob</sup> mice. GHS-R is reported to be expressed in both α- and β-cells of pancreatic islets (14, 25). Chuang et al. (11) showed that ghrelin injections increase blood glucose and plasma glucagon in wild-type mice; consis-
tently, they showed that GHS-R knockout mice have lower glucagon and fasting blood glucose. This suggests that ghrelin’s regulation of blood glucose may also involve stimulation of glucagon secretion from \( \text{H9251} \)-cells. Similar to the observation made by Chuang et al. (11), we detected lower glucagon levels in \( \text{Ghsr}^{+/+} \)/\( \text{ob/ob} \) mice compared with those of WT mice (Fig. 3C).

As expected, both obese \( \text{ob/ob} \) and \( \text{Ghsr}^{-/-} \)/\( \text{ob/ob} \) mice were hyperglucagonemic compared with lean WT and \( \text{Ghsr}^{-/-} \)-mice. Although there was a trend toward decreasing glucagon in \( \text{Ghsr}^{-/-} \)/\( \text{ob/ob} \) mice compared with that of \( \text{ob/ob} \) mice, it did not reach statistical significance (Fig. 3C). The discrepancy between the glucagon phenotype observed in \( \text{Ghsr}^{-/-} \)/\( \text{ob/ob} \) mice vs. that of \( \text{Ghsr}^{+/+} \)/\( \text{ob/ob} \) mice might be due to the nutritional state of the double-null mice and/or the leptin-deficient background. Since we did not detect significant elevation of glucagon in \( \text{Ghsr}^{-/-} \)/\( \text{ob/ob} \) mice, the worsened hyperglycemia of the double-mutant mice cannot be explained by elevated glucagon secreted by \( \text{H9251} \)-cells.

Fig. 5. The mRNA expression of negative and positive \( \beta \)-cell regulatory genes in pancreata of obese mice. Uncoupling protein 2 (UCP2; A), sterol regulatory element-binding protein-1c (SREBP-1c; B), peroxisome proliferator-activated receptor-\( \gamma \) coactivator-1\( \alpha \) (PGC-1\( \alpha \); C), hypoxia-inducible factor-1\( \alpha \) (HIF-1\( \alpha \); D), fibroblast growth factor-21 (FGF21; E), carbohydrate response element-binding protein (ChREBP; F), pancreatic and duodenal homeobox-1 (PDX-1; G), macrophage migration inhibitory factor-1 (MIF-1; H), and forkhead box protein O1 (FOXO1; I). The data are presented as means \( \pm \) SE (\( n = 9–12 \)). *\( P < 0.05 \) and **\( P < 0.001 \), \( \text{ob/ob} \) vs. \( \text{Ghrelin}^{-/-} \)/\( \text{ob/ob} \) mice or \( \text{ob/ob} \) vs. \( \text{Ghsr}^{-/-} \)/\( \text{ob/ob} \) mice. #\( P < 0.05 \), \( \text{Ghrelin}^{-/-} \)/\( \text{ob/ob} \) mice or \( \text{Ghsr}^{-/-} \)/\( \text{ob/ob} \) mice.
Our hypothesis analysis data suggest that the Ghsr ablation in ob/ob mice decreases insulin secretion. In agreement, glucose tolerance tests revealed that Ghsr<sup>-/-</sup>:ob/ob mice have worsened glucose tolerance, showing increased glucose but reduced insulin when compared with ob/ob mice (Fig. 3, D and E). First-phase insulin secretion is the earliest detectable sign of prediabetes (36). In line with the attenuated glucose response in Ghsr<sup>-/-</sup>:ob/ob mice, first-phase (15 min) plasma insulin concentrations were decreased in Ghsr<sup>-/-</sup>:ob/ob mice compared with ob/ob mice, supporting that Ghsr ablation attenuates insulin secretion in the leptin-deficient background (Fig. 3E). GHS-R ablation in ob/ob mice does not have a significant impact on insulin sensitivity (Fig. 3F), suggesting that insulin sensitivity does not contribute to the worsened hyperglycemia and glucose intolerance of Ghsr<sup>-/-</sup>:ob/ob mice. Collectively, our data suggest that Ghsr ablation in ob/ob mice does not affect glucose counterregulation or insulin sensitivity but further diminishes insulin secretion and aggravates hyperglycemia. This surprising outcome reveals that ghrelin and GHS-R have distinct effects on insulin secretion in ob/ob mice.

Pancreatic function can be affected by islet function or islet cell mass. The ob/ob mice have increased islet cell mass, due primarily to islet hyperplasia and hypertrophy (26, 49). To exclude the possibility that the reduced pancreatic function of Ghsr<sup>-/-</sup>:ob/ob mice was due to reduced islet cell mass, we performed histological examinations by H & E staining to evaluate islet size. As expected, we found that islet size of obese (Ghrelin<sup>-/-</sup>:ob/ob, Ghsr<sup>-/-</sup>:ob/ob, and ob/ob) mice was significantly greater than that of lean WT mice, but there was no difference in islet size between ob/ob and Ghsr<sup>-/-</sup>:ob/ob mice (Fig. 4E). This suggests that the islet impairment in Ghsr<sup>-/-</sup>:ob/ob mice is likely due to the effect of GHS-R on β-cell function but not on islet cell mass. It is intriguing that the islet blood vessel distribution of Ghrelin<sup>-/-</sup>:ob/ob mice is similar to normal WT lean mice, whereas the islet vasculature morphology of Ghsr<sup>-/-</sup>:ob/ob mice closely resembles that of ob/ob mice (Fig. 4, A–D). The ob/ob mouse islets are more sensitive to sympathetic inhibition of catecholamines in the circulation (39) and have reduced capacity for blood flow (28). This vascular feature of ob/ob mice increases β-cell stress and causes more islet cell damage. These morphology data suggest that ghrelin ablation may improve islet vasculature of ob/ob mice, but Ghsr ablation does not. Again, this is in line with our observation that ghrelin<sup>-/-</sup>:ob/ob and Ghsr<sup>-/-</sup>:ob/ob mice have differential diabetic phenotypes. Ghsr ablation-associated pancreatic impairment is likely attributable to the effect of GHS-R on pancreatic function but not islet cell mass.

Mitochondrial UCP2 is a negative regulator of pancreatic β-cell function. UCP2 decreases ATP production and results in a reduced ATP/ADP ratio, thereby inhibiting insulin secretion in pancreatic β-cells (17, 56). Overexpression of UCP2 leads to reduced insulin secretion (7); in contrast, UCP2-deficient mice have increased insulin secretion (56). UCP2-ablated ob/ob mice have restored first-phase insulin secretion and improved glycemia (56). Previously, we showed that Ghrelin<sup>-/-</sup>:ob/ob mice have attenuated hyperglycemia and improved β-cell function, compared with that of ob/ob mice, resulting from down-regulation of UCP2 (44). Our current study shows that pancreatic UCP2 mRNA was increased significantly in whole pancreas of Ghsr<sup>-/-</sup>:ob/ob mice compared with that of ob/ob mice (Fig. 5A). Similarly, Ghsr ablation in ob/ob mice upregulated negative β-cell regulators (such as SREBP-1c, ChREBP, and MIF-1) and downregulated positive β-cell regulators (such as HIF-1α, FGF-21, and PDX-1) (Fig. 5, B–I) in whole pancreas. The differential gene expression profiles are in line with the worsened hyperglycemia in Ghsr<sup>-/-</sup>:ob/ob mice and the improved glycemic control in Ghsr<sup>-/-</sup>:ob/ob mice. This supports our conclusion that ghrelin ablation in ob/ob mice improves pancreatic β-cell function, whereas Ghsr ablation in ob/ob mice impairs pancreatic β-cell function. It is worth noting that the pancreatic phenotypes may not be a reflection of changes solely in β-cells, since the gene expression studies were carried out in whole pancreas. Pancreatic cell types other than β-cells may contribute to the pancreatic phenotypes via endocrine and/or exocrine mechanisms.

It is important to emphasize that the glycemic phenotypes of the double-null mice were observed on the leptin-deficient obese background, which are different from that observed in either ghrelin ablation or Ghsr ablation on the wild-type lean background. The glycemic phenotypes of the double-null mice likely result from the interplay between leptin signaling and ghrelin/GHS-R signaling. Our data showed that ghrelin and GHS-R in a leptin-deficient background have differential roles in glucose homeostasis. It is possible that ghrelin’s inhibitory effect on insulin secretion is mediated through subtype receptor(s) other than GHS-R. Culture studies of the direct effect of ghrelin in GHS-R knockout pancreatic β-cells or GHS-R-null islets may help to further address this question.

It is also noteworthy that three peptides, ghrelin (acylated ghrelin), des-acyl ghrelin, and obestatin, are derived from the preproghrelin gene, and all are expressed in the pancreas (8, 42). Whereas ghrelin is known to activate GHS-R, des-acyl ghrelin and obestatin do not, and their receptors are either unknown or debatable. Des-acyl ghrelin is shown to increase food intake and promote obesity (38, 48, 50, 58), whereas obestatin inhibits food intake and enhances energy expenditure (3, 57). More intriguingly, des-acyl ghrelin and obestatin have been shown to have opposing glucoregulatory effects compared with ghrelin; des-acyl ghrelin functions as a potent insulin secretagogue (19, 58), and des-acyl ghrelin and obestatin increase islet cell mass and prevent streptozocin-induced diabetes (10, 20, 37). In Ghrelin<sup>-/-</sup> mice, the signaling of ghrelin, des-acyl ghrelin, and obestatin are abolished. In Ghsr<sup>-/-</sup> mice, the signaling pathway of ghrelin is abolished, but the signaling pathways of des-acyl ghrelin and obestatin remain intact. The glycemic phenotype of Ghsr<sup>-/-</sup> mice may be a result of unopposed des-acyl ghrelin signaling and/or obestatin signaling.

Ghrelin O-acyltransferase (GOAT) is an acyltransferase that catalyzes ghrelin octanoylation. Ablation of GOAT in mice has no phenotype under a regular diet or high-fat diet. Intriguingly, at 60% calorie restriction, the GOAT-null mice show severe hypoglycemia and low circulating GH (59). This result indicates that ghrelin is essential for maintaining GH levels during severe calorie restriction to prevent hypoglycemia and death. In contrast, our studies were overnourished obese mice, wherein ablation of ghrelin in ob/ob mice appeared to be beneficial, but ablation of GHS-R in ob/ob mice appeared to be detrimental. The differential effects of ghrelin deficiency in leptin-deficient background mice vs. the GOAT-ablated mice may be due to the difference in nutritional states of the mice and/or the effects of des-acyl ghrelin and obestatin present in...
GOAT-null mice. Our findings in obese and diabetic leptin-deficient mice suggest that GHS-R antagonists may be harmful when used in treating diabetes in certain obese conditions. However, since most obese humans are leptin resistant rather than leptin deficient (having a metabolic state more like leptin receptor-deficient db/db mice), further studies of the phenotype of GHS-R ablation in db/db mice may provide additional insight.

In conclusion, our data show that ghrelin ablation and GHS-R ablation have opposite effects on glycemic control of leptin-deficient ob/ob mice; ghrelin ablation improves it, and GHS-R ablation worsens it. Ghrelin and Ghsr ablation have differential effects on glucose-induced pancreatic insulin secretion in the leptin-deficient background. In contrast to ghrelin neutralization, GHS-R antagonism may inhibit pancreatic insulin secretion and has deleterious effects on pancreatic function. Ghrelin’s inhibitory effect on insulin secretion may be mediated by receptor(s) other than GHS-R. Our new findings highlight the extreme complexity of the ghrelin-signaling pathway in the pancreas and demonstrate that it is critically important to distinguish the effects of ghrelin neutralization from that of GHS-R antagonism. Further studies are needed to fully understand the molecular mechanisms by which ghrelin and GHS-R regulate pancreatic β-cell function and to determine the proper therapeutic applications for ghrelin neutralization and GHS-R antagonists.

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