Ablation of *ghrelin* improves the diabetic but not obese phenotype of *ob*/*ob* mice

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Summary

Ghrelin and leptin are suggested to regulate energy homeostasis as mutual antagonists on hypothalamic neurons that regulate feeding behavior. We employed reverse genetics to investigate the interplay between ghrelin and leptin. Leptin-deficient mice (*ob*/*ob*) are hyperphagic, obese, and hyperglycemic. Unexpectedly, ablation of *ghrelin* in *ob*/*ob* mice fails to rescue the obese hyperphagic phenotype, indicating that the *ob*/*ob* phenotype is not a consequence of ghrelin unopposed by leptin. Remarkably, deletion of *ghrelin* augments insulin secretion in response to glucose challenge and increases peripheral insulin sensitivity; indeed, the hyperglycemia exhibited by *ob*/*ob* mice is markedly reduced when *ob*/*ob* mice are bred onto the *ghrelin*$^{-/-}$ background. We further demonstrate that ablation of *ghrelin* reduces expression of *Ucp2* mRNA in the pancreas, which contributes toward enhanced glucose-induced insulin secretion. Hence, chronically, ghrelin controls glucose homeostasis by regulating pancreatic *Ucp2* expression and insulin sensitivity.

Introduction

The literature is replete with studies designed to establish the physiological role of ghrelin. However, these investigations involved pharmacological administration of ghrelin; therefore, the results, while valid, must be interpreted with caution. For example, each injection of exogenous ghrelin or ghrelin mimetic instantly causes the release of growth hormone releasing hormone, corticotrophin releasing hormone, neuropeptide Y (NPY), agouti-related protein (AGRP), adrenocorticotrophin, growth hormone, and glucocorticoids, as well as inhibiting the function and release of somatostatin (Cowley et al., 2003; Smith et al., 1997; Tschop et al., 2002; Tung et al., 2004). Plasma profiles of ghrelin suggest that ghrelin is normally released episodically with peak concentrations approximately double those of valleys. Ghrelin administered acutely interrupts and markedly alters the normal episodic pattern of ghrelin secretion, which confounds clear interpretation of the biological role of endogenous ghrelin. Accordingly, we employ genetic models to elucidate the physiological function of ghrelin, because even with the caveat of the possible influence of compensatory pathways, we believe that these models are more relevant for understanding ghrelin physiology.

Ghrelin and leptin act on feeding centers in the brain and regulate energy balance by counter-regulating the activity of NPY and AGRP hypothalamic neurons (Bagnasco et al., 2002; Cowley et al., 2003). Ghrelin is an octanoylated 28 amino acid orexigenic peptide produced mainly by the stomach (Kojima et al., 1999), which when administered acutely, increases appetite; however, mice lacking ghrelin have normal appetites (Sun et al., 2003; Wortley et al., 2004). The protein leptin is produced by adipocytes and inhibits food intake. Mice with a mutation in the leptin gene (*ob*/*ob*) are characterized by severe obesity, hyperglycemia, hyperinsulinemia, glucose intolerance, and hypothermia (Meinders et al., 1996; Muzzin et al., 1996). To investigate the interrelated physiological roles of ghrelin and leptin, we generated ghrelin- and leptin-deficient mutant mice (*ghrelin*(−/−).*ob*/*ob*).

Results

General characterization of ghrelin-deficient *ob*/*ob* mice

We backcrossed *ghrelin*$^{-/-}$ mice for ten generations to C57BL/6J mice and then bred them onto the *ob*/*ob* C57BL/6J background. We monitored body weight changes and food intake of wild-type (WT), *ghrelin*$^{-/-}$, *ob*/*ob,* and *ghrelin*(−/−).*ob*/*ob* mice. In agreement with our previous results, there were no differences in body weights or food intake of WT compared to *ghrelin*$^{-/-}$ mice. Compared to WT and *ghrelin*$^{-/-}$ mice, body weights in *ob*/*ob* and *ghrelin*(−/−).*ob*/*ob* mice were higher as early as 2 weeks of age; marked differences were apparent at 6 weeks of age. Surprisingly, ablation of *ghrelin* in *ob*/*ob* mice did not significantly reduce either body weight (Figure 1A) or food intake (data not shown), and fat content of *ob*/*ob* and *ghrelin*(−/−).*ob*/*ob* was identical (Figure 1B). We conclude that in the leptin deficient *ob*/*ob* mouse, ghrelin does not play a dominant role in either appetite regulation or fat deposition.

The *ob*/*ob* mice have a paucity of brown fat and impaired capacity for nonshivering thermogenesis and are unable to maintain body temperature under cold conditions (Arvaniti et al., 1998). Ghrelin suppresses sympathetic nerve activity in brown adipose tissue (Yasuda et al., 2003), suggesting that ablating *ghrelin* in *ob*/*ob* mice might restore the capacity for cold-induced thermogenesis. To determine whether *ghrelin* deletion improved thermoregulation, mice were exposed to 4ºC ambient temperature for 90 min, and core body temperature was measured at 15 min intervals. However, like *ob*/*ob* mice, the core body temperature of *ghrelin*(−/−).*ob*/*ob* mice dropped

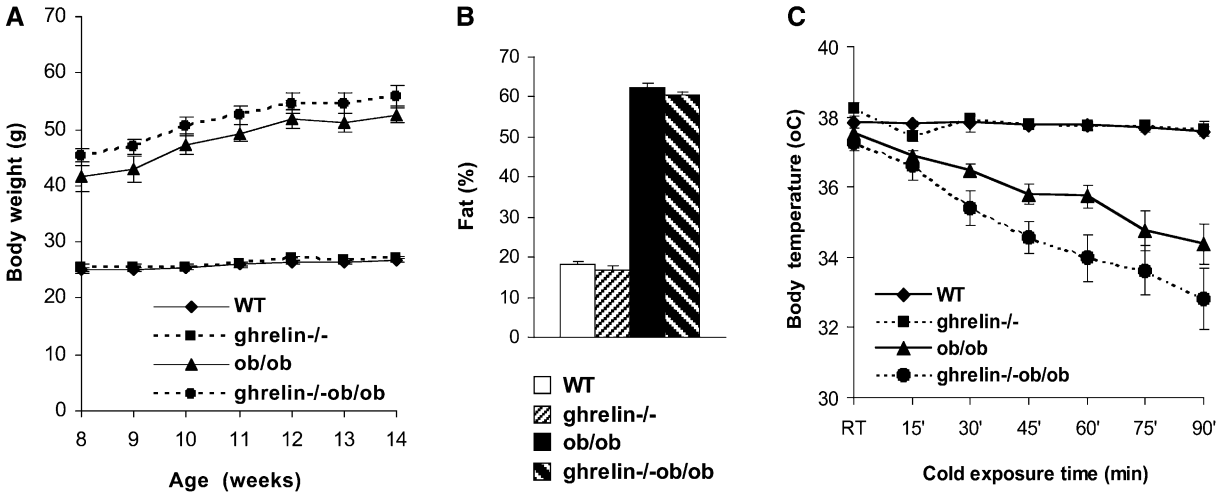

Figure 1. Growth curve, body composition, and thermoregulation

Experiments were performed with five littermate male mice. The results are presented as mean ± SE.

A) Body weights from 8–14 weeks of age, $p < 0.001$, obese mice (*ob/ob* and *ghrelin*(−/−).*ob/ob*) versus lean mice (WT and *ghrelin*$^{-/-}$); $p > 0.05$, *ob/ob* vs. *ghrelin*(−/−).*ob/ob*.

B) Whole-body fat content. $p < 0.001$, obese mice versus lean mice; $p > 0.05$, *ob/ob* versus *ghrelin*(−/−).*ob/ob*.

C) The rectal temperature of the mice exposed to 4°C temperature. $p < 0.05$ at 15 min; from 30 min on, $p < 0.001$, obese mice versus lean mice. The temperature drop in *ghrelin*(−/−).*ob/ob* was similar compared to *ob/ob*.

precipitously; by contrast, WT and *ghrelin*$^{-/-}$ maintained core body temperature (Figure 1C). Hence, in *ob/ob* mice, ablation of *ghrelin* does not restore the ability to maintain body temperature in a cold environment.

Deletion of *ghrelin* enhances glucose-dependent insulin release and increases insulin sensitivity

Glucose and insulin levels in *ob/ob* mice undergo dynamic changes, and the mice display their most severe diabetes at 12 weeks of age (Menahan, 1983). Ghrelin and its receptor (growth hormone secretagogue receptor, GHS-R) are expressed in pancreatic islets (Howard et al., 1996; Prado et al., 2004; Wierup et al., 2002, 2004). To investigate how ghrelin might influence glucose metabolism, we monitored glucose levels in lean mice (WT and *ghrelin*$^{-/-}$) and obese mice (*ob/ob* and *ghrelin(−/−).ob/ob*) at different ages. The lean mice were euglycemic; as expected, glucose and insulin levels were elevated in *ob/ob* and *ghrelin*(−/−).*ob/ob* mice (Figures 2A–2C). Blood glucose was elevated at age 4 weeks in *ob/ob* mice and at 6 weeks in *ghrelin*(−/−).*ob/ob* mice. Surprisingly, although obesity was as severe as in *ob/ob* mice, *ghrelin(−/−). ob/ob* exhibited lower glucose levels and their blood glucose normalized upon fasting (Figures 2A and 2B). Hence, ablation of *ghrelin* markedly improved glucose homeostasis in *ob/ob* mice.

The improvement in glucose homeostasis in *ghrelin(−/−). ob/ob* mice was accompanied by increased serum insulin levels (Figure 2C). C-peptide levels were measured and found to be significantly elevated in *ghrelin(−/−).ob/ob* mice compared to *ob/ob* mice (Figure 2D) indicating that the increase in serum insulin was due to increased insulin secretion. The data suggest that deletion of *ghrelin* improves pancreatic β cell function and that changes in ghrelin concentrations modulate glucose sensitivity of the β cells.

We next performed intraperitoneal glucose tolerance tests (IP-GTT). A low-glucose dose (0.625g/kg) was selected because *ob/ob* mice are glucose-intolerant. Remarkably, compared to *ob/ob* mice, *ghrelin*(−/−).*ob/ob* mice displayed reduced blood glucose concentrations 15, 30- and 60 min following glucose injection (Figure 2E), which was accompanied by increased insulin secretion (Figure 2F). Restoration of the first-phase of insulin secretion in *ob/ob* mice by *ghrelin* ablation has clinical relevance, because in humans the loss of first-phase insulin secretion is predictive of the development of Type 2 diabetes (Poitout and Robertson, 1996); therefore, in subjects at risk for Type 2 diabetes, treatment with a ghrelin antagonist may prove beneficial.

To investigate further the consequences of *ghrelin* ablation on insulin secretion and insulin sensitivity, we performed IP-GTT in *ghrelin*$^{-/-}$ mice using a higher glucose dose (2.5 g/kg). Compared to WT, *ghrelin*$^{-/-}$ mice exhibited significantly lower glucose levels at 30, 60 and 120 min following glucose challenge (Figure 3A) and correspondingly higher levels of insulin (Figure 3B). Notably, like *ghrelin*(−/−).*ob/ob* mice, the initial insulin response at 15 min was significantly ($p = 0.002$) higher in the *ghrelin*$^{-/-}$ compared to WT mice. To investigate the acute effect of ghrelin on glucose-induced insulin secretion, 18h fasted *ghrelin*$^{-/-}$ mice were subjected to IPGTT using vehicle + glucose, and ghrelin + glucose. The latter treatment produced higher blood glucose (Figure 3C) and markedly lower insulin levels (Figure 3D), showing that ghrelin acutely suppresses insulin release. These results further support our conclusion that improved glucose tolerance observed in *ghrelin(−/−).ob/ob* mice during IP-GTT is a consequence of *ghrelin* ablation, and *ghrelin* ablation improves pancreatic β cell function by increasing insulin secretion in response to a glucose challenge.

Improved glucose tolerance would also be facilitated if *ghrelin* ablation increased insulin sensitivity. To test this possibility, an insulin tolerance test (ITT) was performed. A greater reduction in glucose levels were observed 30 min following insulin dosing in *ghrelin* ablated mice, suggesting increased insulin sensitivity (Figure 3E). To more thoroughly address whether ablation of *ghrelin* improved insulin sensitivity, WT and *ghrelin*$^{-/-}$ mice were subjected to euglycemic hyperinsulinemic clamp studies. Glucose production rate was measured using [3-^{3}H] glucose

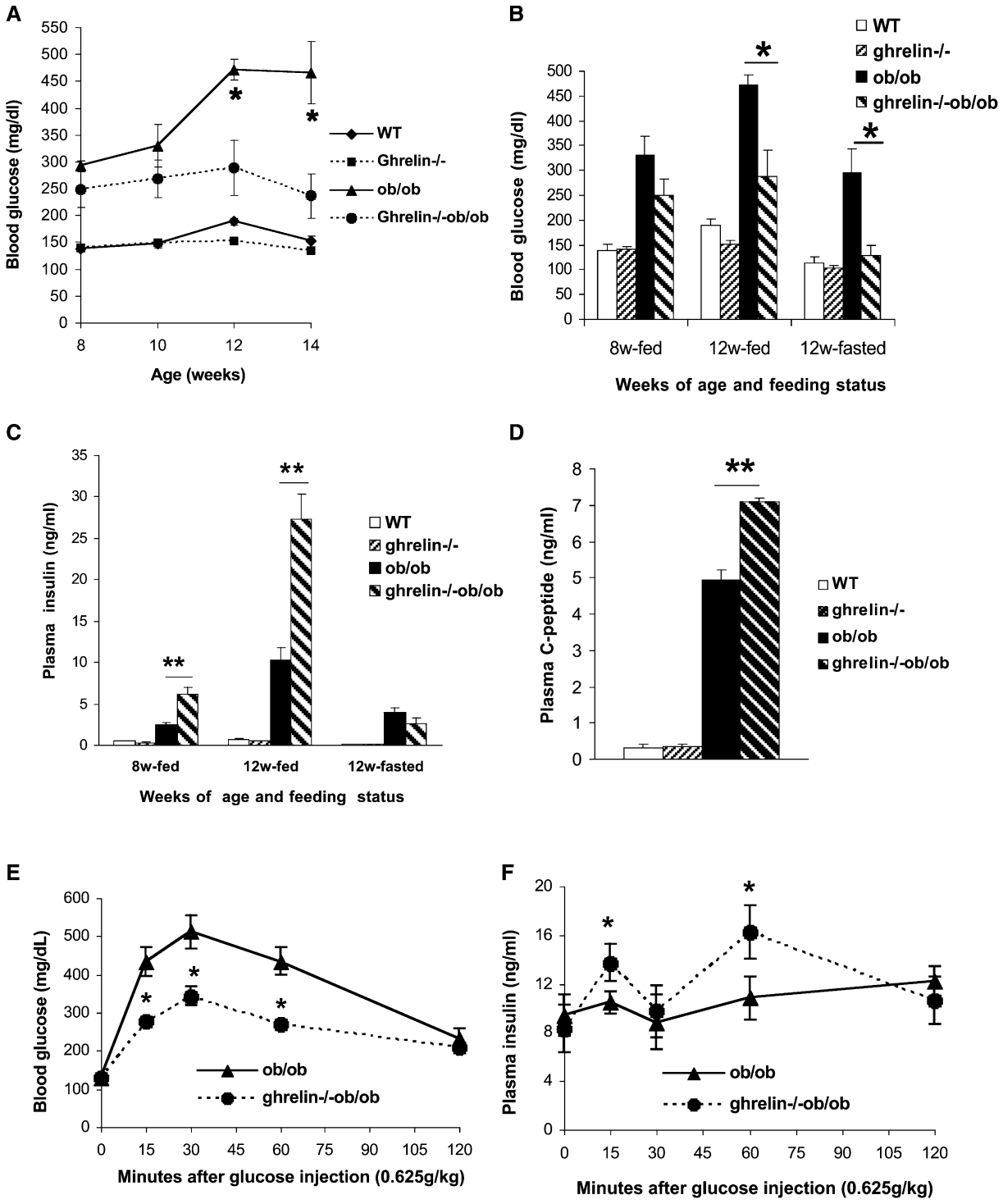

Figure 2. Glucose, insulin, C-peptide, and IP-GTT

The results are presented as mean ± SE. n = 5. *p < 0.05, **p < 0.001, *ob/ob* versus *ghrelin*(−/−).*ob/ob*.

A) Fed blood glucose.

B and C) Fed/fasted (24 hr) blood glucose and plasma insulin. Hyperglycemia and hyperinsulinemia were present in obese mice but not in lean mice.

D) Fasted (24 hr) plasma C-peptide.

E and F) IP-GTT (0.625 g/kg): blood glucose (**E**) and plasma insulin (**F**) values were assessed. *p < 0.05, at 15, 30, and 60 min for glucose, and 15 and 60 min for insulin.

infusion to allow estimation of whole body glucose disposal (Saha et al., 2004). Basal hepatic glucose production rate was the same in both genotypes (Figure 3F). However, during the low-dose insulin clamp, suppression of glucose production was higher in *ghrelin*$^{-/-}$ mice (Figure 3F, $p < 0.05$), suggesting that the liver of *ghrelin*$^{-/-}$ mice was more sensitive to insulin. Furthermore, a 33% increase ($p = 0.025$) in glucose infusion rate (GIR) and a 20% increase ($p = 0.036$) in glucose disposal

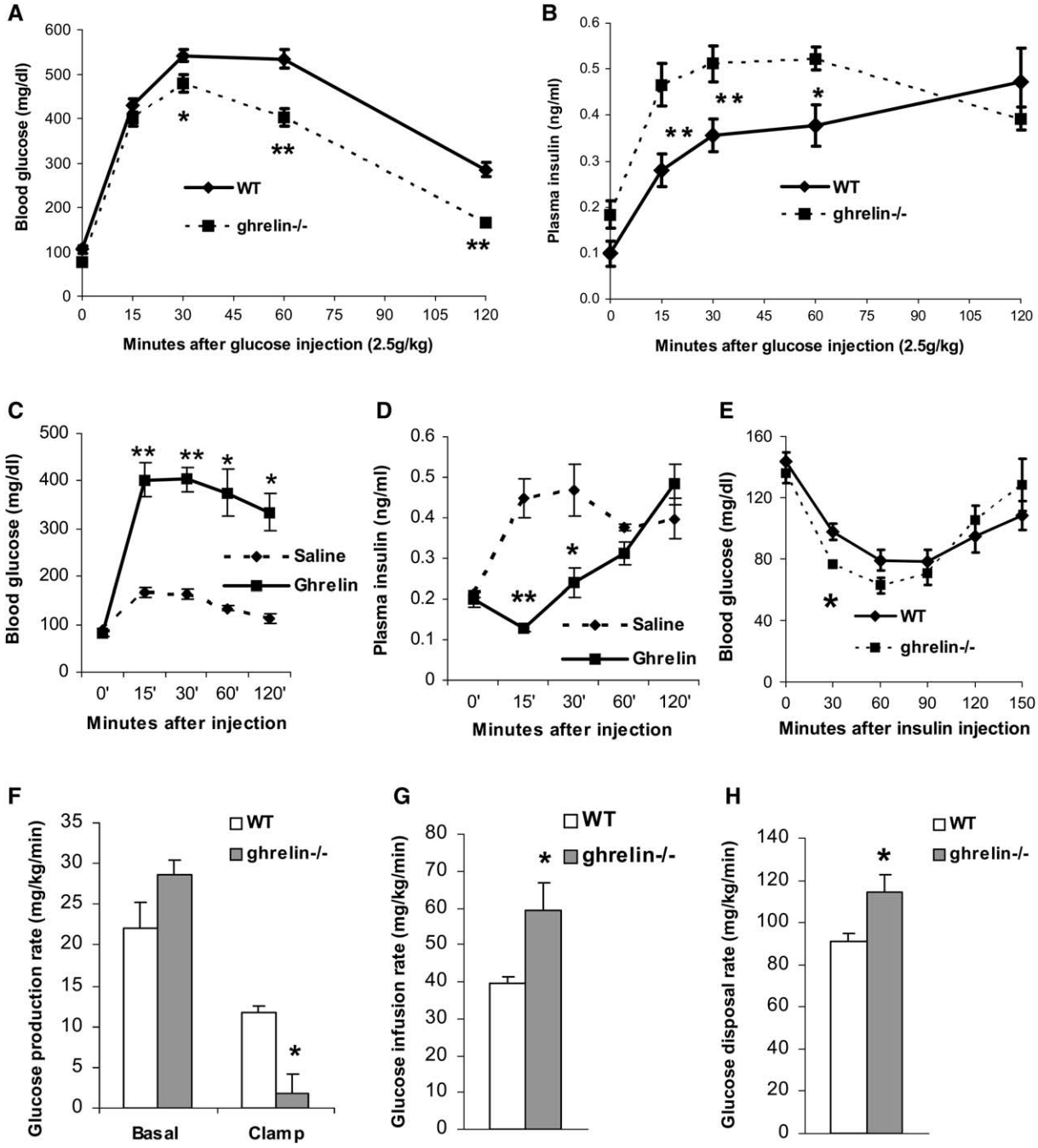

Figure 3. IP-GTT, ITT, and low-dose hyperinsulinemic-euglycemic clamp in *ghrelin*$^{-/-}$ mice

The results are presented as mean ± SE. n = 6–10. *p < 0.05, **p < 0.001. p value was evaluated between WT and *ghrelin*$^{-/-}$ in (**A**, **B**, and **E–H**).

A and B) IP-GTT (glucose 2.5 g/kg): 8-week-old conscious male mice were used. Glucose (**A**) and plasma insulin (**B**) values were assessed.

C and D) The effect of exogenous ghrelin on glucose-induced insulin secretion in *ghrelin*$^{-/-}$ mice. 2.5 g/kg glucose with or without 150 nmol/kg ghrelin were ip injected into 18 hr fasted *ghrelin*$^{-/-}$ mice, *p < 0.05, **p < 0.001 saline versus ghrelin treatment. Glucose (**C**) and insulin (**D**) responses were evaluated.

E) ITT (insulin 0.75 U/kg): Glucose responses of 8-week-old conscious male mice. Glucose responses: *p < 0.05 at time 30 min only.

F, G, and H) Hyperinsulinemic-euglycemic clamp studies: (**F**) glucose production rate (GPR) at basal and low-dose insulin clamp conditions; (**G**) glucose infusion rate (GIR); (**H**) glucose disposal rate (GDR).

rate (GDR) were detected (Figures 3G and 3H), indicating that besides augmenting glucose-induced insulin secretion, *ghrelin* ablation increased peripheral insulin sensitivity. By inference, ghrelin apparently modulates glucose homeostasis by regulating glucose-stimulated insulin secretion and insulin sensitivity.

Mechanism of ghrelin regulation of insulin secretion

Low plasma levels of leptin and high plasma levels of resistin are associated with obesity, insulin resistance and diabetes (Banerjee et al., 2004). To determine whether leptin or resistin explained the metabolic adaptation associated with ablation of

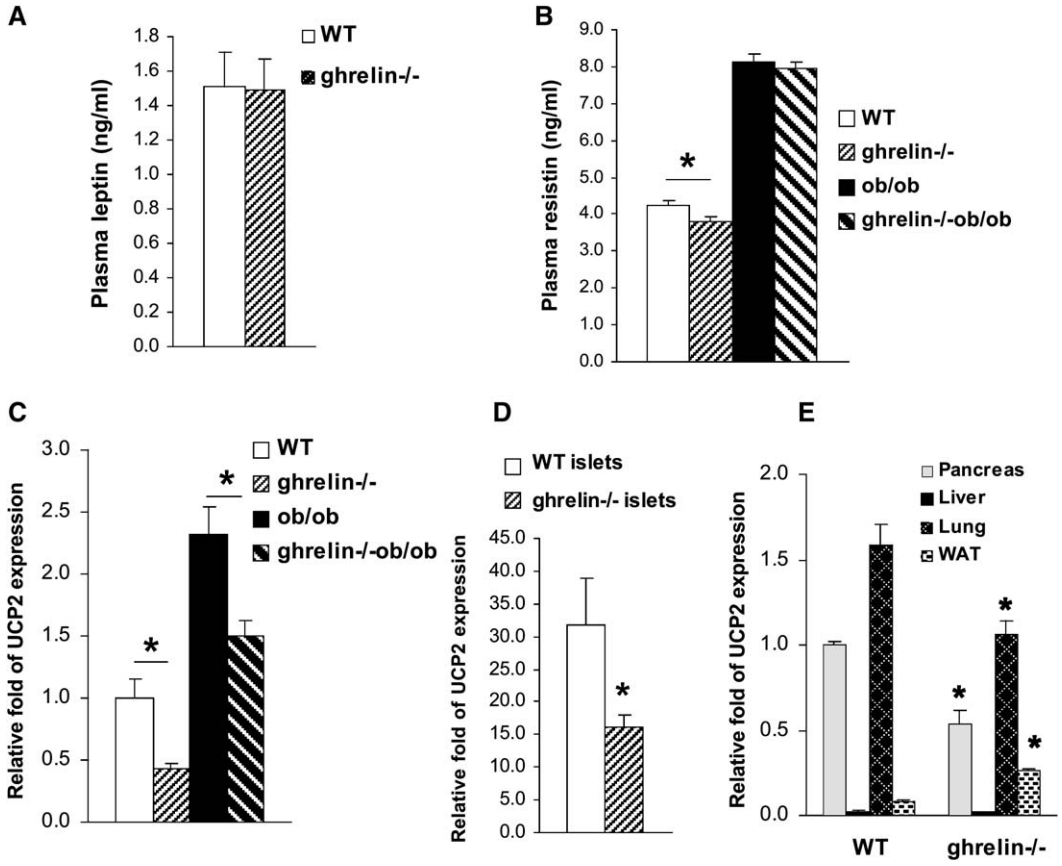

Figure 4. Plasma leptin and resistin levels and *Ucp2* mRNA expression

The results are presented as mean ± SE. n = 3-6.

A and B) Leptin (**A**) and resistin (**B**) plasma concentrations were measured in 8-month-old male mice after 24 hr fasting. * $p < 0.05$, WT versus *ghrelin*$^{-/-}$.

C and D) Taqman relative quantification of *Ucp2* mRNA expression in whole pancreas (**C**) and pancreatic islets (**D**) was measured in 5-month-old male mice. (**C**) The level of expression in WT was defined as 1, the rest of samples were expressed as relative fold of expression compared to WT. *$p < 0.05$, WT versus *ghrelin*$^{-/-}$, *ob/ob* versus *ghrelin(−/−).ob/ob*. The results in (**D**) are normalized to WT data in (**C**). *p< 0.05, WT islets versus *ghrelin*-/- islets.

E) UCP2 mRNA expression in liver, lung, and WAT of 8-week-old male littermate mice. *$p < 0.05$, WT versus *ghrelin*$^{-/-}$ for pancreas, lung, and WAT; $p = 0.058$ for liver.

ghrelin, plasma leptin and resistin were measured. Consistent with our previous findings, leptin levels were not significantly different in WT and *ghrelin*$^{-/-}$ mice (Sun et al., 2003; Figure 4A). Resistin levels were higher in obese than in lean mice and lower in *ghrelin*$^{-/-}$ than in WT mice. However, resistin levels were similar in *ob/ob* and *ghrelin(−/−).ob/ob* mice (Figure 4B); therefore, resistin does not play a role in the improved glucose homeostasis associated with *ghrelin* ablation.

Mitochondrial uncoupling protein-2 (UCP2) regulates ATP production. In the pancreas, an increase in the ATP/ADP ratio depolarizes the membrane of pancreatic β cells causing Ca^{2+} influx and insulin release. Accordingly, we measured *Ucp2* mRNA in the pancreas of WT, *ghrelin*$^{-/-}$, *ob/ob,* and *ghrelin*(−/−).*ob/ob* mice by real time RT-PCR. Obese mice had the highest levels of *Ucp2* expression, which was likely due to increased β cell mass resulting from leptin inactivation (Bock et al., 2003). Strikingly, ablation of *ghrelin* in both obese and lean mice reduces pancreatic *Ucp2* mRNA expression (Figure 4C). The same magnitude of attenuation was evident in pancreatic islets, where the concentration of *Ucp2* mRNA was 30-fold higher than in pancreas (Figure 4D). Therefore, chronic deprivation of ghrelin increases glucose sensitivity of pancreatic β cells by reducing *Ucp2* expression. *Ucp2* expression is also reduced in the liver ($p = 0.058$) and lung ($p < 0.05$) of *ghrelin*$^{-/-}$ mice (Figure 4E), but increased in WAT ($p < 0.05$). In skeletal muscle, Ucp2 mRNA was barely detectable in both WT and *ghrelin*$^{-/-}$ mice (data not shown). This relative tissue distribution and expression levels in WT mice are consistent with those described previously (Fleury and Sanchis, 1999).

Discussion

While pharmacologically, ghrelin and leptin have opposite effects on food intake, our results show that deletion of ghrelin in *ob/ob* mice does not rescue the obese phenotype (Figures 1A and 1B). Hence, unopposed ghrelin action in leptin deficient *ob/ob* mice is not the underlying cause of obesity. A study in obesity-prone juvenile rats also concluded that ghrelin was not a proximate cause of obesity (Levin et al., 2003). However, we did observe modest, but consistently higher body weight in *ghrelin(−/−).ob/ob* mice compared to *ob/ob* mice, which could be due to higher insulin levels. In brown adipose tissue, ghrelin suppresses sympathetic nerve activity (Yasuda et al., 2003); however, we showed that *ghrelin* ablation in *ob/ob* mice failed to improve cold exposure-induced thermogenesis (Figure 1C),

which is likely explained by the lack of brown fat associated with leptin deficiency.

By comparing the results of IP-GTT, ITT on WT and *ob/ob* mice with those from *ghrelin*$^{-/-}$ and *ghrelin*(−/−).*ob/ob* mice, we showed that *ghrelin* ablation improves glucose tolerance by amplifying glucose stimulated insulin secretion (Figures 2 and 3). We also showed that acute ghrelin administration to *ghrelin*$^{-/-}$ mice during an IP-GTT reverses the improved glucose tolerance of *ghrelin*$^{-/-}$ mice by markedly inhibiting insulin secretion (Figures 3C and 3D). These results indicate that ghrelin regulates β cell function. Furthermore, euglycemic hyperinsulinemic clamp studies showed that *ghrelin* ablation increases peripheral insulin sensitivity (Figures 3F–3H).

Dezaki *et al* reported that acute ghrelin administration negatively regulates glucose-stimulated insulin secretion in rats, which was prevented by a ghrelin antagonist (Dezaki et al., 2004). These investigators found that acute treatment with ghrelin and ghrelin receptor antagonists had no effect on insulin sensitivity. However, we found using euglycemic hyperinsulinemic clamps on mice that lacked endogenous ghrelin that *ghrelin* ablation improves insulin sensitivity as well β cell function. However, because insulin release is markedly elevated by *ghrelin* ablation, improved β cell function may play the dominant role in ghrelin regulation of glucose homeostasis.

During investigations to determine why *ghrelin* deletion causes increased insulin secretion in response to glucose, we found that *Ucp2* expression was lower in the pancreas of *ghrelin* ablated mice. This is significant because Ucp2 regulates ATP production and the membrane potential of β cells in pancreatic islets is regulated by the ATP-sensitive K^+ channel (K_{ATP}). Lowering Ucp2 increases the ATP/ADP ratio reducing opening of K_{ATP} which depolarizes the β cell and opens L-type Ca^{2+} channels, thereby increasing intracellular Ca^{2+} and insulin release. Hence, increased glucose sensitivity of the β cell in *ghrelin* ablated mice can be explained by increased depolarization of β cells allowing increased influx of Ca^{2+}. This explanation is consistent with observations made in *Ucp2* +/− mice, which express 50% lower levels of *Ucp2* mRNA than WT mice and their response to IPGTT closely resembles that of *ghrelin*$^{-/-}$ mice (Zhang et al., 2001). Furthermore, *Ucp2(−/−).ob/ob* mice, like *ghrelin*(−/−).*ob/ob* mice, also manifest improved glucose tolerance. Hence, chronically, ghrelin regulates the glucose sensitivity of pancreatic β cells by modulating *Ucp2* expression.

Altered *Ucp2* expression is also linked to another regulator of metabolic function, the silent information regulator 2 (Sir2) protein family (Blander and Guarente, 2004). Transgenic mice engineered to overexpress *Sirt1* (the mammalian version of *Sir2*) in pancreatic β cells (BESTO mice) show improved glucose tolerance and enhanced secretion of insulin in response to IPGTT (Moynihan et al., 2005). Furthermore, overexpression of *Sirt1,* like *ghrelin* ablation attenuates expression of *Ucp2.* Subsequent studies will investigate whether ghrelin regulates β cell function by repressing *Sirt1* expression.

Reduced *Ucp2* expression is consistent with increased sensitivity of pancreatic β cells to glucose, but this does not explain the acute inhibition of insulin secretion in *ghrelin*$^{-/-}$ mice following ip ghrelin injection (Figures 3C and 3D). Besides the K_{ATP} channel, inhibition of voltage-dependent tetraethylammonium (TEA) sensitive K^+ currents also depolarizes the β cell and augments Ca^{2+} influx and glucose dependent increased insulin secretion (MacDonald et al., 2001). However, the acute inhibitory effect of ghrelin on insulin secretion is only partially diminished by TEA (Dezaki et al., 2004), suggesting that enhancement of TEA-sensitive outward K^+ currents cannot completely explain the acute effects of ghrelin. Although the critical signaling element for insulin secretion is depolarization-dependent increase in intracellular Ca^{2+}, a concomitant outflux of a cation or influx of an anion must also occur (Neye et al., 2006). Since insulin secretion and Ca^{2+} influx is augmented following knockout or complete blockade of voltage-dependent K^+ currents, other currents must provide counterions for Ca^{2+} influx (Neye et al., 2006). Under conditions where voltage-dependent K^+ currents are completely blocked and cause augmentation of Ca^{2+} influx into β cells, the addition of nonspecific inhibitors of anion currents immediately block Ca^{2+} influx (Best, 1997; Neye et al., 2006). Since the immediate action of the anion current inhibitors is similar to that observed with ghrelin, we speculate that acute administration of ghrelin inhibits insulin release by inhibiting anion currents, perhaps in combination with enhancing TEA-sensitive outward K^+ currents.

Based on genetic models, we have made four significant conclusions regarding the physiological function of ghrelin. First, although pharmacologically, ghrelin and leptin have opposite effects on feeding centers in the hypothalamus, ghrelin unopposed by leptin does not explain the hyperphagic phenotype. Second, unopposed ghrelin is not the cause of increased fat deposition in the leptin deficient *ob/ob* mice. Third, ablation of ghrelin augments glucose dependent insulin secretion from the pancreatic β cell by reducing *Ucp2* expression. Fourth, ablation of ghrelin improves peripheral insulin sensitivity. Collectively, our data are consistent with the notion that ghrelin is a neuromodulatory sensor that controls whole body metabolism according to nutrient intake and body composition by linking the GH/IGF-1 axis, with glucose sensing, β cell function and fat mobilization.

Experimental procedures

Generation of *ghrelin*(−/−).*ob/ob* mice

The generation of *ghrelin*$^{-/-}$ was described by us previously (Sun et al., 2003). C57BL/6 *ob*/+ mice were purchased from The Jackson Laboratory. To generate *ghrelin(−/−).ob/ob*, *ghrelin*$^{-/-}$ mice were bred to *ob*/+ mice creating compound heterozygotes, *ghrelin*$^{(+/-)}$·*ob/OB*. In the second cross, compound heterozygotes were bred to each other, and *ghrelin*$^{-/-}$·*ob/OB* were identified and mated to produce *ghrelin*$^{-/-}$·*ob/ob* (ghrelin-deficient *ob/ob*) mice and *ghrelin*$^{-/-}$·*OB/OB (ghrelin*$^{-/-}$*)* mice. In parallel, *ghrelin*$^{(+/+)}$·*ob/OB* mice were bred to each other to produce *ghrelin*$^{(+/+)}$·*ob/ob (ob/ob)* as well as *ghrelin*(+/+).*OB/OB* (WT) mice. To generate the most reliable data, only littermate pairs were used. All results are presented as mean ± standard error (SE). For all measurements, statistical analysis was performed using ANOVA; $p < 0.05$ was defined as statistically significant.

Body weight, food intake, and blood glucose

Mice were individually housed and maintained from weaning on regular chow. Body weights and food intake were measured weekly, and blood glucose was measured biweekly at the same time of the day, from 8–14 weeks of age. Blood glucose values were determined by One-Touch Ultra glucometer (Lifescan, Milpitas, CA).

Body composition and bone density analysis

Body composition and bone density were analyzed by PIXImus densitometer (Lunar Corp.). The report includes fat and muscle (lean) contents, bone mineral density (BMD) and bone mineral content (BMC). Mice were anesthetized during the procedure.

Cold exposure and metabolic measurements

To determine thermoregulatory ability, mice were individually caged and placed in a 4ºC cold room for 90 min. The rectal core body temperature was measured at 15 min intervals using a Temperature Monitoring System from Indus Instruments (Houston, TX). Plasma insulin, C-peptide, leptin, and resistin were analyzed by the Mouse Metabolic Phenotyping Center at Vanderbilt University Medical Center.

Glucose tolerance test, insulin tolerance test, and low-dose hyperinsulinemic clamp

An intraperitoneal glucose tolerance test (IP-GTT) was performed on conscious mice, following an 18 hr fast, by ip injection of D-glucose (0.625 g/kg for obese mice and 2.5 g/kg for lean mice). Blood glucose was measured by tail bleeds at 0, 15, 30, 60, and 120 min, post glucose dose. The insulin tolerance test (ITT) was done similarly, except the mice were fasted for only 8 hr, and Humulin (Eli Lilly and Company, Indianapolis, IN) was administered by ip injection. 2 U/kg of Humulin was used for obese mice, and 0.75 U/kg was used for lean mice. A low-dose hyperinsulinemic clamp was performed, and calculated as described in our previous publication (Saha et al., 2004).

RNA Isolation and real-time RT-PCR

Mouse pancreases were first soaked in RNAlater (Ambion), subsequently homogenized in TRIzol (Invitrogen), then extracted using guanidinium thiocyanate method, as described (Chomczynski and Sacchi, 1987). RNA was subsequently treated with DNase I (Ambion). Mouse *Ucp2* gene-specific primers and TaqMan probes were designed with Primer Express 2.0 (Applied Biosystems) using default settings: forward primer, 5′-TCACTGTGCCCTTAC CATGCT; reverse primer, 5′-AGGCATGAACCCCTTGTAGAAG (transversing an exon boundary); and probe, 5′-6FAM-AAGGAGGGACCCCGC-MGB-3′. Assays were mixed at final concentrations of 900 nM primer and 250 nM probe. Reverse transcription was performed on 1 μg of total RNA with random primers, utilizing TaqMan reverse transcription reagents (Applied Biosystems) as recommended by the manufacturer. One tenth of the reverse transcription reaction was used as a template for the subsequent PCR reaction, which was carried out in triplicate. Thermal cycling was carried out with an ABI prism 7000 sequence detection system (Applied Biosystems) under factory defaults (50ºC, 2 min; 95ºC, 10 min; and 40 cycles at 95ºC, 15 s; 60ºC, 1 min). Threshold cycle number (Ct) was defined as fluorescence values exceeding baseline; it was further normalized to the Ct of internal control 18 S ribosomal RNA, and the relative quantification was calculated using ΔΔCt formula. Relative *Ucp2* mRNA expression was shown as fold expression over WT.

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