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Characterization of Adult Ghrelin and Ghrelin Receptor Knockout Mice under Positive and Negative Energy Balance

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Ghrelin and the ghrelin receptor (GH secretagogue receptor, GHS-R), are believed to have important roles in energy homeostasis. We describe results from the first studies to be conducted in congenic (N10) adult *ghrelin*$^{-/-}$ and *Ghsr*$^{-/-}$ mice under conditions of both positive (high-fat diet) and negative (caloric restriction) energy balance. In contrast to results from young N2 mutant mice, changes in body weight and energy expenditure are not clearly distinguishable across genotypes. Although respiratory quotient was lower in mice fed a high-fat diet, no differences were evident between littermate wild-type and null genotypes. With normal chow, a modest decrease trend in respiratory quotient was detected in *ghrelin*$^{-/-}$ mice but not in *Ghsr*$^{-/-}$ mice. Under caloric restriction, the weight loss of *ghrelin*$^{-/-}$ and *Ghsr*$^{-/-}$ mice was identical to wild-type littermates, but blood glucose levels were significantly lower. We conclude that adult congenic *ghrelin*$^{-/-}$ and *Ghsr*$^{-/-}$ mice are not resistant to diet-induced obesity but under conditions of negative energy balance show impairment in maintaining glucose homeostasis. These results support our hypothesis that the primary metabolic function of ghrelin in adult mice is to modulate glucose sensing and insulin sensitivity, rather than directly regulate energy intake and energy expenditure. (*Endocrinology* 149: 843–850, 2008)

GHRELIN HAS BEEN considered one of the most promising antiobesity targets; however, its physiological role in energy homeostasis is controversial. Pharmacological treatment of rats and mice with ghrelin stimulates food intake, increases body weight, and induces fat deposition (1–3). Circulating ghrelin is highest before feeding and declines immediately after nutrient ingestion, but levels are paradoxically low in obese humans and rodents (4, 5). In humans, a link between ghrelin and obesity was made after the observation that in obese subjects who underwent gastric bypass surgery, ghrelin production declined in parallel with sustained weight loss and reduced appetite (6, 7). However, gastric bypass was also reported to have no effect on ghrelin levels, but an inverse relationship between ghrelin and insulin was observed. This suggests that changes in ghrelin production reflect a new state of energy balance (8).

We have shown that *Ghsr*$^{-/-}$ mice are refractory to the stimulatory effects of ghrelin on GH release and appetite, confirming that the GH secretagogue receptor (GHS-R) is a physiologically relevant ghrelin receptor (9). Despite this, *ghrelin*$^{-/-}$ mice and *Ghsr*$^{-/-}$ mice have normal growth rates and normal appetites under conditions of standard laboratory housing (9, 10). Wortley *et al.* (11) made similar observations in their independently generated *ghrelin*$^{-/-}$ mice.

To appropriately interpret results derived from metabolic studies in gene-ablated mice, genetic background must be carefully considered. Clear differences have been observed in feeding and body weight in 129Sv and C57BL/6 mice (12, 13); indeed, the 129Sv mouse is far more resistant to diet-induced obesity (DIO) than the C57BL/6 mouse (14, 15). These differences are important considerations because conventionally, embryonic stem cells derived from 129Sv mice are targeted for gene ablation, and the modified embryonic stem cells are injected into the blastocyst of C57BL/6 mice to produce chimeric mice. When the chimeras are bred to produce wild-type (WT) (+/+) or null (−/−) mice, the genetic background is unevenly distributed so that the null genotype has more traits of the 129Sv (*e.g.* leanness) than the WT littermates; therefore, to make realistic comparisons, it is essential to backcross the mice to establish a pure background.

To minimize selective genetic traits, we backcrossed the *ghrelin*$^{-/-}$ and *Ghsr*$^{-/-}$ mice for 10 generations with C57BL/6J mice, and the phenotypes of adult congenic mutant mice were compared with WT mice under conditions of positive and negative energy balance. Positive energy balance was induced by 35% high-fat (HF) diet feeding, and negative energy balance was induced by 50% caloric restriction (CR). Ablation of ghrelin signaling also did not prevent DIO in these congenic adult mice. We did detect a modest decrease in respiratory quotient (RQ) in these adult *ghrelin*$^{-/-}$ mice compared with WT mice fed a normal diet, but no differences were observed when adult mice were fed a HF diet.

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Abbreviations: CR, Caloric restriction; DIO, diet-induced obesity; GHS-R, GH secretagogue receptor; HF, high fat; PI, phosphoinositide; RD, regular diet; RQ, respiratory quotient; VCO_2, carbon dioxide production; VO_2, oxygen consumption; WT, wild type.

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Materials and Methods

Animals and data analysis

All experiments were conducted on adult male WT, *ghrelin*$^{-/-}$, and *Ghsr*$^{-/-}$ mice. Mice were kept in a standard housing facility and singly housed 1 wk before and during the experiments. All procedures used in animal experiments were approved by the Institutional Animal Care and Use Committee at Baylor College of Medicine. Data are presented in the figures as mean ± SEM, except in Fig. 2, where to simplify the figures, only the mean value is displayed. The number of mice per group is indicated by n. Significant differences between the groups were evaluated by ANOVA tests (two-way ANOVA or repeated-measures ANOVA) using SigmaStat 3.0 software. Statistical significance was considered as $P < 0.05$.

Hormone assays

Blood was collected from the tail vein. Mice were fasted for 24 h, and blood was drawn from the tail, collected in EDTA-containing tubes, and kept at 4 C during processing. Samples were centrifuged at 3000 rpm for 30 min, and hydrochloric acid and phenylmethylsulfonyl fluoride were immediately added to the plasma. Samples were aliquoted into polypropylene vials and stored at −80 C until assayed. Total plasma ghrelin levels were measured by RIA using kits purchased from Linco Research (St. Charles, MO). This RIA kit uses a polyclonal antibody raised in rabbits and applies it against both octanoylated and des-octanoylated ghrelin and [^{125}I]ghrelin as the tracer. The lower limit of detection was 80 pg/ml, and the intraassay coefficient of variation was 4%. Active ghrelin levels were measured by another commercially available RIA kit from Linco Research. This RIA kit uses an antibody raised in guinea pigs and applies it against octanoylated ghrelin and ^{125}I-octanoylated ghrelin as the tracer. This assay has been found to be highly specific for active ghrelin, with less than 0.1% cross-reactivity for des-octanoyl ghrelin, and no cross-reactivity with ghrelin 14–28, motilin-related peptide, leptin, insulin, glucagon, or glucagon-like peptide 7–36. The lower detection limit was 10 pg/ml. The intraassay coefficient of variation was 5.3%. Blood glucose values were determined by One-Touch Ultra glucometer (Lifescan, Milpitas, CA). Plasma assays were carried out using the manufacturer's protocols: insulin, using rat insulin RIA kit (Linco Research), and IGF-I, using rat IGF-I RIA kit (Diagnostic Systems Laboratories, Inc., Webster, TX).

Body weight and food intake under different diets

The experimental mice were individually caged, provided with *ad libitum* access to water, and fed with either regular diet (RD) from PicoLab Rodent Diet (Oakville, Ontario, Canada) or HF diets from Harlan Teklad (Madison, WI). The percentage of macronutrients provided is based on weight, and the calorie equivalents are listed in parentheses. RD, diet 5053, has 9% fat (12% by calories), 20% protein, and 40% carbohydrate. The 35% HF diet, TD 0217, has 35.1% fat (62.2% by calories), 22.9% protein, and 24.2% carbohydrate. The 36% HF diet, TD12331, has 35.8% fat (58% by calories), 23% protein, and 35.5% carbohydrate. The 75% high-carbohydrate diet is TD 00199.

Indirect calorimetry

Metabolic parameters were obtained by using an Oxymax (Columbus Instruments, Columbus, OH) open-circuit indirect calorimetry system. Briefly, oxygen consumption (VO_2) (milliliters per kilogram per hour) and carbon dioxide production (VCO_2) ((milliliters per kilogram per hour) by each animal were measured for a 48-h period. VO_2 and RQ (ratio of VCO_2/VO_2) were then calculated. Energy expenditure (or heat) was calculated as the product of the calorific value of oxygen ($3.815 + 1.232 \times RQ$) and the volume of O_2 consumed. The light cycle was 0600–1800 h.

Evaluation of body weight and food intake during fasting and refeeding

The mice were weighed, and chow was removed. Forty-eight hours later, the animals were weighed again and then given a weighed amount of chow. Body weights were measured at 24 and 48 h after the start of the fast and 1, 2, 3, and 5 d after refeeding. The food intake was measured at 1, 2, 4, and 6 h and 1, 2, 3, and 5 d after refeeding.

CR

RD (diet 5053) was used during the CR protocol. All experimental mice were individually caged for 12 d before the start of CR, and average daily food consumption during these 12 d was calculated for each group. To determine whether the ghrelin$^{-/-}$ and Ghsr$^{-/-}$ mice could maintain blood glucose levels in an environment of chronic negative energy balance, mice were provided 50% of the average daily amount of food consumed *ad libitum* and fed daily at 1500 h. Body weight and blood glucose were measured before feeding, at 2- or 4-d intervals.

Results

Genetic background of ghrelin- and Ghsr- null mice

The null mice were initially generated on a 129Sv and C57BL/6J background. To reduce the impact of genetic heterogeneity on metabolic phenotype, the *ghrelin*$^{-/-}$ and *Ghsr*$^{-/-}$ mice were backcrossed with C57BL/6J mice for 10 generations. To determine whether both N10 null mice were congenic, the mice were analyzed for 110 microsatellite markers (Charles River Laboratory, Wilmington, MA). Besides the particular markers associated with gene deletion, all other markers were 100% identical to those characteristics of C57BL/6J mice, indicating that the N10 null mice are congenic (99.9% identical to C57BL/6J).

Phenotype of ghrelin- and Ghsr-null mice under positive energy balance

To investigate whether ghrelin and/or GHS-R might play a role in DIO, 16-wk-old adult male WT, *ghrelin*$^{-/-}$, and *Ghsr*$^{-/-}$ mice were fed a 35% HF diet or RD for 10 wk; body weight and food intake were monitored biweekly (Fig. 1, A and B). Body weights of mice fed the HF diet were significantly higher than those fed RD ($P < 0.05$), although the body weights of *Ghsr*$^{-/-}$ mice were slightly lower than those of WT, relative weight gain was not significantly different between WT, *ghrelin*$^{-/-}$, and *Ghsr*$^{-/-}$ mice. Consistent with our previous reports (9, 10), regardless of diet, the body weights of *ghrelin*$^{-/-}$ mice were the same as their WT littermates, whereas the body weights of *Ghsr*$^{-/-}$ mice remained slightly lower ($P < 0.05$). Irrespective of genotype, biweekly food intake was greater in mice fed RD and less in mice fed the HF diet ($P < 0.05$, Fig. 1, C and D). We also analyzed whole-body composition by PXImus densitometer and found no significant differences between WT and null mice fed any of the test diets (data not shown). Collectively, these data suggest that ablation of *ghrelin* or the *Ghsr* fails to prevent DIO in adult mice of C57BL/6J background.

Metabolic profile of mice fed HF diet and RD

The core body temperature of WT, *ghrelin*$^{-/-}$, and *Ghsr*$^{-/-}$ mice was identical under *ad libitum* fed or 24- to 48-h fasted conditions (data not shown), suggesting that ghrelin/GHS-R signaling had no major effect on metabolic rate. Tschop *et al.* (3) reported that peripheral daily administration of ghrelin caused weight gain by reduced fat utilization as manifested by an increase in RQ. Wortley *et al.* (11) reported a reduced RQ in their mixed background N2 *ghrelin*$^{-/-}$ mice fed a 45% HF diet. To determine whether genetic background might influence met-

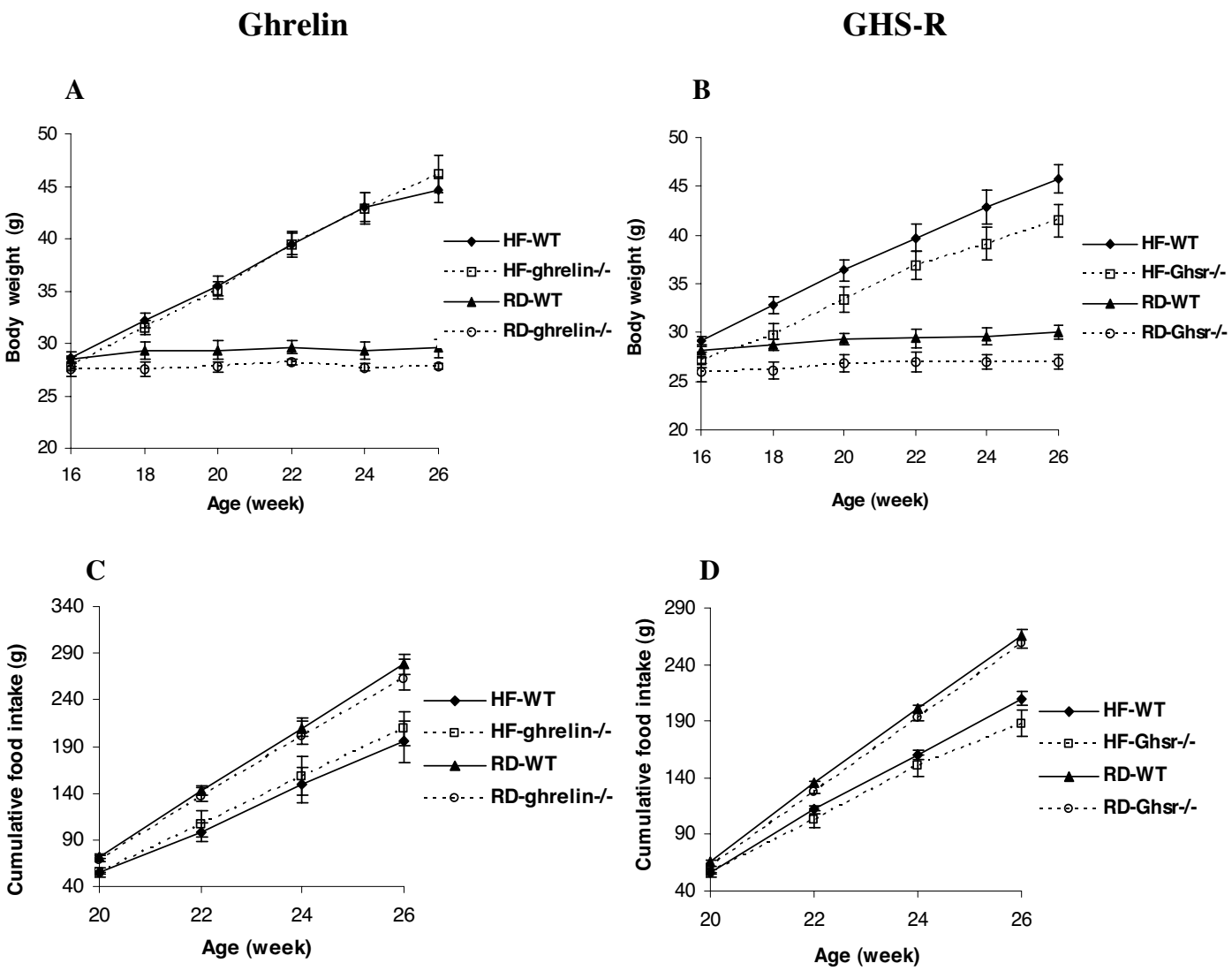

FIG. 1. Body weight (A and B) and cumulative food intake (C and D) of adult *ghrelin*- and $Ghsr^{-/-}$ mice fed 35% HF diet and RD. Body weight and food intake were measured biweekly from 16 to 26 wk of age in $ghrelin^{-/-}$ and $Ghsr^{-/-}$ mice and their WT littermates (n = 8). The data are presented as mean ± SEM. Body weight, $P < 0.05$ WT *vs.* $Ghsr^{-/-}$ with both HF and RD.

abolic fuel preference, we examined the metabolic characteristics of adult congenic $ghrelin^{-/-}$ and $Ghsr^{-/-}$ mice. Sixteen-week-old male mice were fed a 35% HF diet or RD for 10 wk, and then metabolic parameters were measured. We found no significant differences in energy expenditure (Fig. 2, A and B) between $ghrelin^{-/-}$ and $Ghsr^{-/-}$ mice compared with their WT littermates. RQ was significantly reduced in mice fed the HF diet, but the effects were indistinguishable between null mice and their WT littermates (Fig. 2, C and D). Interestingly, there was a modest but clearly decreased trend in the RQ of $ghrelin^{-/-}$ mice fed a RD. Indeed, the difference was statistically significant between WT and $ghrelin^{-/-}$ mice at a number of time points (as indicated by $P < 0.05$ from repeated-measures ANOVA, Fig. 2C); however, such a trend was absent in $Ghsr^{-/-}$ mice fed the same diet.

Total and active ghrelin levels in mice fed different diets

HF and high-carbohydrate diets influence total ghrelin concentrations differently (16). Active ghrelin (the octanoylated peptide) represents less than 10% of the total ghrelin peptide. To address whether composition of the diet plays a role in regulating active ghrelin concentrations, plasma levels of total ghrelin and active ghrelin were measured in WT mice fed different diets. Compared with mice fed normal and high-carbohydrate diets, total ghrelin was significantly lower in mice fed the HF diet (Fig. 3A, $P < 0.05$). Active ghrelin was also slightly reduced with HF feeding, but the difference did not reach statistical significance (Fig. 3B, $P = 0.057$).

Does deletion of Ghsr in DIO mice prevent weight gain after weight loss?

To address the question of whether the GHS-R has a role in adaptive response to weight loss, $Ghsr^{-/-}$ and WT control male mice were fed a HF diet for 10 wk, fasted for 48 h, then re-fed with HF diet. Body weight decreased and increased identically in both genotypes during the fasting and refeeding phases, respectively (Fig. 4A). This suggests that *Ghsr*

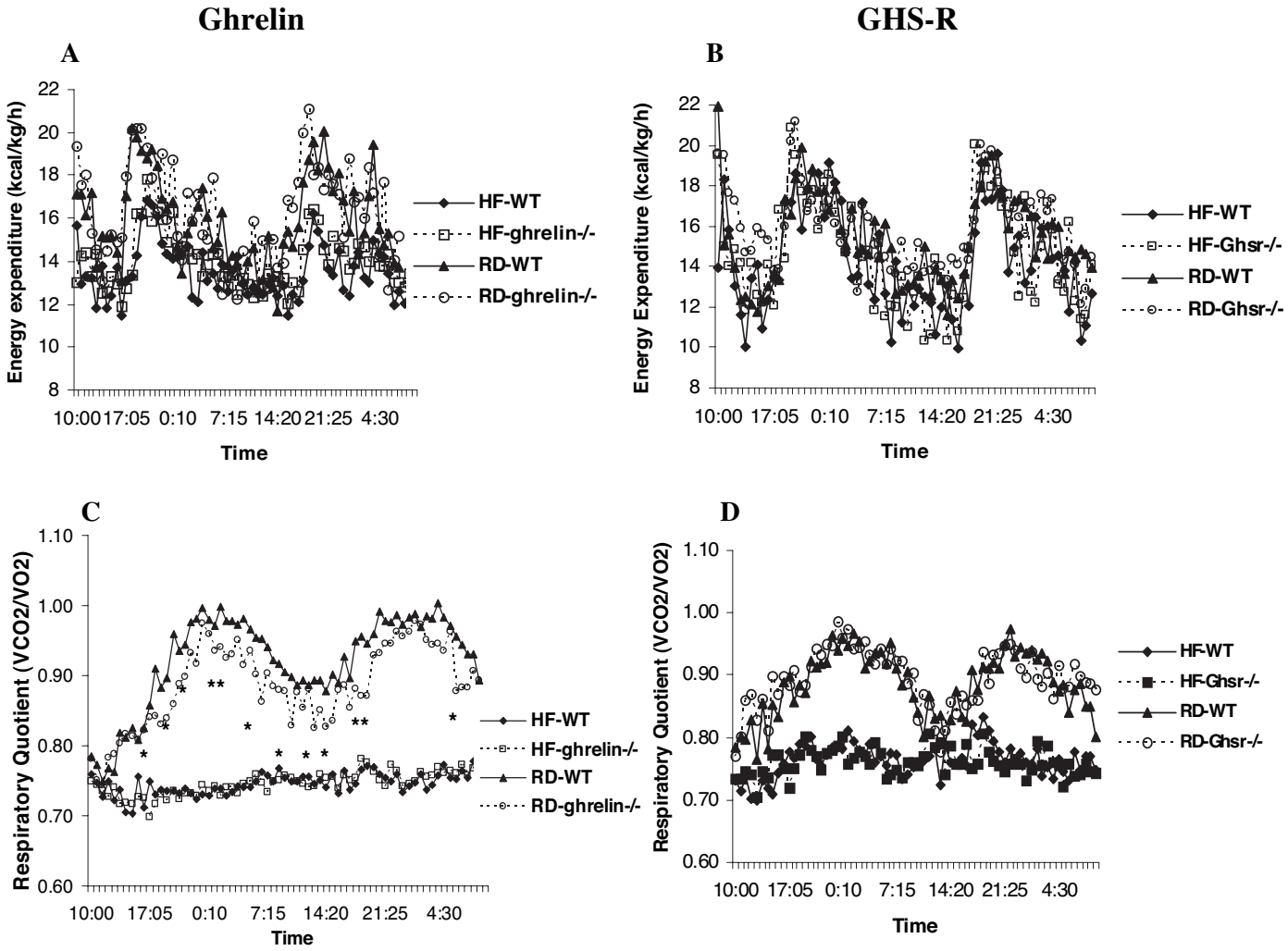

FIG. 2. Calorimetry parameters of adult *ghrelin*$^{-/-}$ and *Ghsr*$^{-/-}$ mice fed RD and HF diets. The 16-wk-old WT and *ghrelin*$^{-/-}$ and *Ghsr*$^{-/-}$ male mice were fed regular chow or 35% HF diet for 10 wk, and then the calorimetry studies were performed. The results are represented as the mean values of eight mice. *, Time points where $P < 0.05$ comparing RD-fed WT *vs.* *ghrelin*$^{-/-}$ mice. A and C, Energy expenditure (A) and RQ (C) in *ghrelin*$^{-/-}$ mice; B and D, energy expenditure (B) and RQ (D) in *Ghsr*$^{-/-}$ mice. The *lines* on the abscissas represent time points where data were collected. The light cycle was 0600–1800 h.

ablation neither inhibits weight loss in obese mice (fasting phase) nor compromises weight gain after weight loss (refeeding phase). Food intake during the refeeding phase was also identical in the WT and null genotypes (Fig. 4B). Hence, the absence of the *Ghsr* in obese mice does not prevent weight gain after weight loss or fasting-induced hyperphagia.

Glucose and insulin levels in Ghsr$^{-/-}$ *mice fed regular and HF diets*

An inverse association between ghrelin and insulin has been reported in humans (8). To address whether the ghrelin/GHS-R pathway is an essential regulator of glucose homeostasis according to diet, we compared the effects of HF feeding on glucose regulation in WT and *Ghsr*$^{-/-}$ mice. Although glucose levels were unaffected by HF feeding (Fig. 5A), insulin levels were increased in both genotypes (Fig. 5B). In WT and *Ghsr*$^{-/-}$ mice fed HF diet, fasting (18 h) had no effect on glucose but reduced plasma insulin levels identically in WT and *Ghsr*$^{-/-}$ mice. In contrast, in mice fed RD, fasting produced a marked reduction in blood glucose and plasma insulin in *Ghsr*$^{-/-}$ mice but not WT mice (Fig. 5B, $P < 0.05$). Plasma leptin levels were also compared. Leptin was 4-fold higher in mice fed the HF diet compared with those on RD irrespective of whether mice were of WT or mutant genotype (data not shown).

Ghrelin- and Ghsr-null mice under conditions of negative energy balance

To study ghrelin's role under conditions of 50% CR, body weight changes and blood glucose concentrations of individually caged 10-wk-old male WT, *ghrelin*$^{-/-}$, and *Ghsr*$^{-/-}$ mice were compared (Fig. 6). Average daily food intake was measured for 12 d, and the mice were calorie restricted by feeding them 50% of their average daily intake for 40 d. Weight loss during CR was identical in WT and null mutant genotypes. Glucose levels fell in WT and in both null genotypes, but the drop in blood glucose concentrations was significantly greater in *ghrelin*$^{-/-}$ and *Ghsr*$^{-/-}$ mice than in

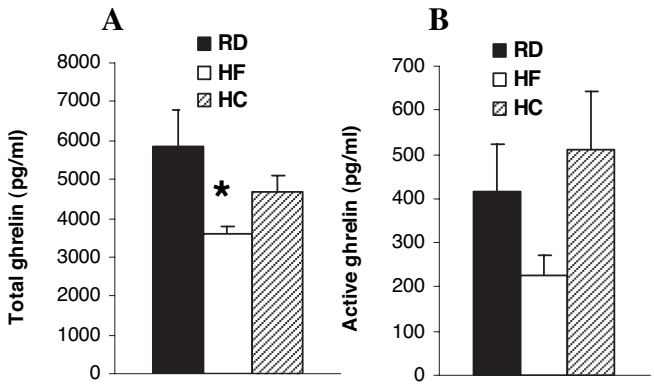

FIG. 3. Total (A) and active (B) plasma ghrelin concentrations in 24-h-fasted WT male mice. The 16-wk-old mice were fed RD, 35% HF diet, or 75% high-carbohydrate (HC) diet for 10 wk. Plasma was collected at 26 wk of age (n = 8). *, $P < 0.05$.

their WT littermates ($P < 0.05$), reaching a nadir after 16 d. Interestingly, glucose levels in all genotypes were identical after 28 d of CR. Upon restoring *ad libitum* feeding on d 40, glucose levels returned to pre-CR levels, and body weight was restored.

Discussion

When WT and null mice were fed either RD or HF diet, body weights of *ghrelin*$^{-/-}$ mice were no different from that of their WT littermates (Fig. 1A); however, body weights of *Ghsr*$^{-/-}$ mice were always modestly lower than WT littermates (Fig. 1B), consistent with their reduced IGF-I levels (9). IGF-I levels are not significantly lower in *ghrelin*$^{-/-}$ mice, as shown in our unpublished data and as reported by Wortley *et al.* (17). The lower IGF-I level in *Ghsr*$^{-/-}$ mice suggests that reduced body weight is likely due to the lack of GHS-R regulation of the GH/IGF-I pathway (18, 19).

Because the DIO-prone C57BL/6 mice generally exhibit increased body weight and adiposity after 3 months of age (12), we subjected young adult (16-wk-old) mice to HF diet and measured metabolic parameters at 26 wk. Our results of mature N10 congenic *ghrelin*$^{-/-}$ and *Ghsr*$^{-/-}$ mice show that ablation of the ghrelin/GHS-R signal does not prevent DIO (Fig. 1). Wortley *et al.* (17) and Zigman *et al.* (20) showed that mixed-background N2 *ghrelin*$^{-/-}$ and *Ghsr*$^{-/-}$ mice are resistant to DIO when HF diets were fed immediately after weaning. The discrepancy could be due to the differences in genetic background of the mice used. The mice we used had been backcrossed with C57BL/6J mice for 10 generations to reach a congenic state, 99.9% identical to C57BL/6J, whereas Wortley *et al.* (17) and Zigman *et al.* (20) used N2 mice that are mixed background of C57BL/6 and 129Sv (75% C57BL/6 and 25% 129Sv). Because the 129Sv mouse strain has a lean phenotype, whereas C57BL/6 is highly susceptible to DIO, it is possible that the lean trait of the 129Sv background present in their null mice contributed to the DIO-resistant phenotype.

Besides genetic background, the other difference between our studies and those of Wortley *et al.* (17) and Zigman *et al.* (20) is that they fed their mice HF diet immediately after weaning, whereas our mice were raised on RD and then fed

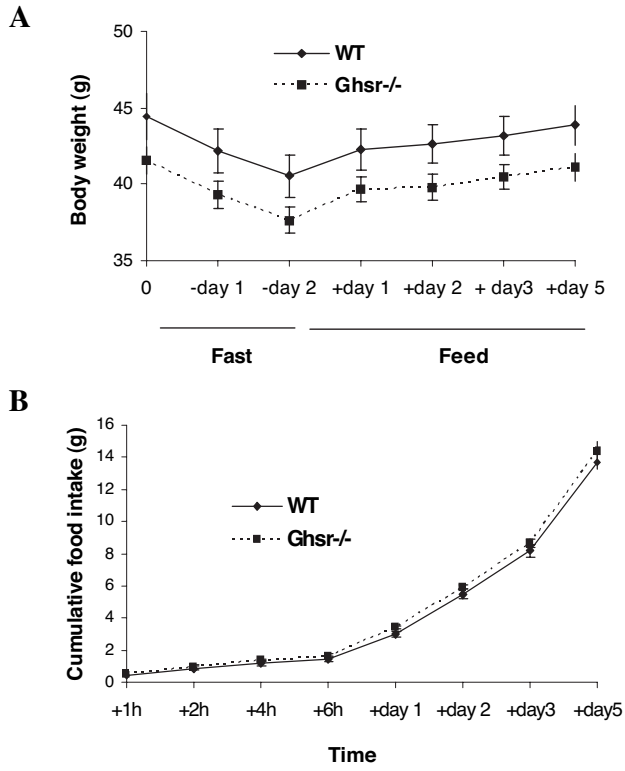

FIG. 4. Changes in body weight and food intake during fasting and refeeding. The 14-wk-old *Ghsr*$^{-/-}$ (N10) male mice were fed a 36% HF diet for 10 wk. At 24 wk of age, the mice were fasted for 48 h and then allowed free access to food. Body weight (A) and food intake (B) at different intervals were measured (n = 9). $P > 0.05$, WT *vs.* *Ghsr*$^{-/-}$ mice, for body weights and food intake.

HF diet as adults. Perhaps during the early postweaning period, ghrelin modifies the development of neural circuitry in hypothalamic areas that control food intake. Hence, the lack of a ghrelin signal in the developing orexigenic circuits might cause immature animals to be resistant to the HF diet. As the animals age, the central circuits may develop compensatory pathways so that adult mutant genotypes become responsive to HF feeding (21). It is possible that as mice reach adulthood, they develop compensatory pathways to adjust for the loss of a ghrelin/GHS-R signal. There is other evidence supporting the idea that ghrelin's effect on energy homeostasis is age dependent. Acute ghrelin injection increases feeding in fast-growing young (130 g) but not adult (370 g) rats (22). Vaccination to neutralize ghrelin in rats initially reduced weight gain (23), but the effect on appetite was transient and failed to show a long-term reduction on body weight (24). These results suggest that young, but not adult animals are responsive to regulation of ghrelin signaling. These collective results indicate that the metabolic effects of ghrelin are dependent upon age and genetic background.

Even though overwhelming pharmacological evidence suggests that ghrelin is an orexigenic factor (25, 26), direct evidence supporting a role for ghrelin as an obesity hormone in mammals is highly debatable. In rats selectively bred to develop DIO, both ghrelin and GHS-R expression are reduced in the hypothalamus (27). Furthermore, when ghrelin and GHS-R expression were studied in seasonal mammals

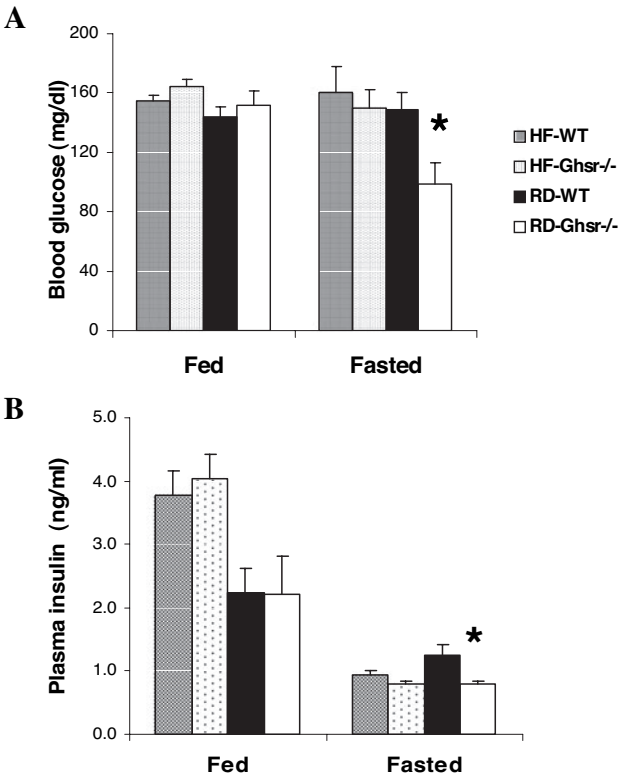

FIG. 5. Fed and fasted (18 h) blood glucose (A) and plasma insulin (B) of mice after 10 wk of 36% HF diet or RD feeding. Mice were 24 wk old when blood samples were collected (n = 9). *, $P < 0.05$ WT *vs.* $Ghsr^{-/-}$ for fasted glucose and insulin of mice fed RD.

(*e.g.* bears) that experience marked changes in body weight and fat mass, the seasonal changes are not accompanied by changes in either ghrelin or GHS-R (28). We considered that perhaps an intricate balance between ghrelin and leptin regulates appetite and body composition. However, we found that the leptin-deficient *ob/ob* mouse bred onto the $ghrelin^{-/-}$ background has the same hyperphagic and obese phenotype as the *ob/ob* mouse, indicating that ghrelin unopposed by leptin does not play a dominant role in orexigenic regulation and fat deposition (29). Together, these data suggest that the ghrelin/GHS-R signaling pathway is a modulator rather than a dominant regulator of energy homeostasis.

Studies comparing RQ and energy expenditure in WT, $ghrelin^{-/-}$, and $Ghsr^{-/-}$ mice have produced results that are at variance. Comparing with RD feeding, a marked reduction in RQ was observed in all genotypes (WT, $ghrelin^{-/-}$, and $Ghsr^{-/-}$ mice) that were fed a HF diet; however, no differences were observed between genotypes. In comparing adult WT and congenic $ghrelin^{-/-}$ fed RD, the latter exhibit a modest decrease in RQ (Fig. 2C), which is consistent with the report that under RD, exogenous ghrelin increases RQ (3). However, the lack of reduction in RQ with the HF diet contrasts with the report generated from 14- to 16-wk-old mixed background (N2) $ghrelin^{-/-}$ mice, where HF feeding reduced RQ greater in $ghrelin^{-/-}$ mice than in WT control mice without affecting energy expenditure (11). It is noteworthy that when the HF feeding was initiated immediately after weaning, the authors failed to detect a difference in RQ between their $ghrelin^{-/-}$ mice and WT controls, but instead they detected increased energy expenditure in $ghrelin^{-/-}$ mice (17). When $Ghsr^{-/-}$ mice of mixed background (N2) were fed HF diet immediately after weaning, Zigman *et al.* (20) reported a reduced RQ in $Ghsr^{-/-}$ mice compared with WT mice with no difference in energy expenditure. The discrepancy of RQ data between our studies and the reports by other investigators could be due to the differences in genetic background and/or age of the mice.

It has been reported that macronutrient type regulates total ghrelin levels. Administration of fat suppresses circulating ghrelin levels much less than carbohydrates or proteins (16). Our data show that in contrast to a normal or high-carbohydrate diet, a HF diet reduced plasma ghrelin levels, and HF feeding proportionately reduced the concentrations of ghrelin and desacyl-ghrelin (Fig. 3). Because total ghrelin concentrations are lower in obese humans and rodents (30, 31), we postulate that the decrease in ghrelin levels induced by the HF diet is related to DIO. It was reported that octanoate attenuates adipogenesis in 3T3-L1 preadipocytes (32), which suggests that the octanoyl group of ghrelin plays a role in diet-induced adipogenesis. However, our data showed that the ratio of ghrelin and desacyl ghrelin were comparable regardless of the diets.

Studies in patients subjected to stomach bypass surgery showed a correlation between low ghrelin level and sustained weight loss (6, 7); therefore, it was speculated that ghrelin ablation prevents weight gain after weight loss. However, our results show that deletion of *Ghsr* does not prevent DIO and does not prevent weight gain after fasting-induced weight loss or fasting-induced hyperphagia (Fig. 4). Our data suggest that sustained weight loss after stomach bypass surgery may not be regulated by ghrelin.

Under fasting conditions, RD-fed $Ghsr^{-/-}$ mice exhibit lower glucose and insulin levels than RD-fed WT mice, suggesting that GHS-R pays a role in glucose homeostasis and *Ghsr* deletion increases insulin sensitivity (Fig. 5). However, these differences are obscured in HF-fed mice, suggesting that the effects of GHS-R on glucose homeostasis are associated with the energy state of the animals.

How does ablation of ghrelin signaling increase insulin sensitivity? Phosphoinositide (PI) 3-kinase is involved in the insulin-mediated effects of glucose uptake, lipid deposition, and adiponectin secretion in adipocytes and genetic disruption of the PI 3-kinase subunit p85α increases insulin sensitivity. Adipose tissue plays a critical role in the antagonistic effects of GH on insulin actions on carbohydrate and lipid metabolism. GH regulates p85α expression and PI 3-kinase activity in white adipose tissue (33). Ghrelin activation of the GHS-R stimulates GH release (9); therefore, the amplitude of episodic GH is expected to be lower in $Ghsr^{-/-}$ mice. Because GH secretion is episodic with peaks approximately every 3 h, direct GH measurement requires sequential blood sampling and fluid replacement at 10-min intervals for up to 12 h, which is impractical in mice. However, consistent with reduced amplitude of GH release, serum IGF-I is lower in $Ghsr^{-/-}$ than in WT controls. GH, as a lipolytic hormone, is known to increase insulin resistance (34); therefore, increased insulin sensitivity exhibited by $Ghsr^{-/-}$ mice might be a consequence of lower GH levels.

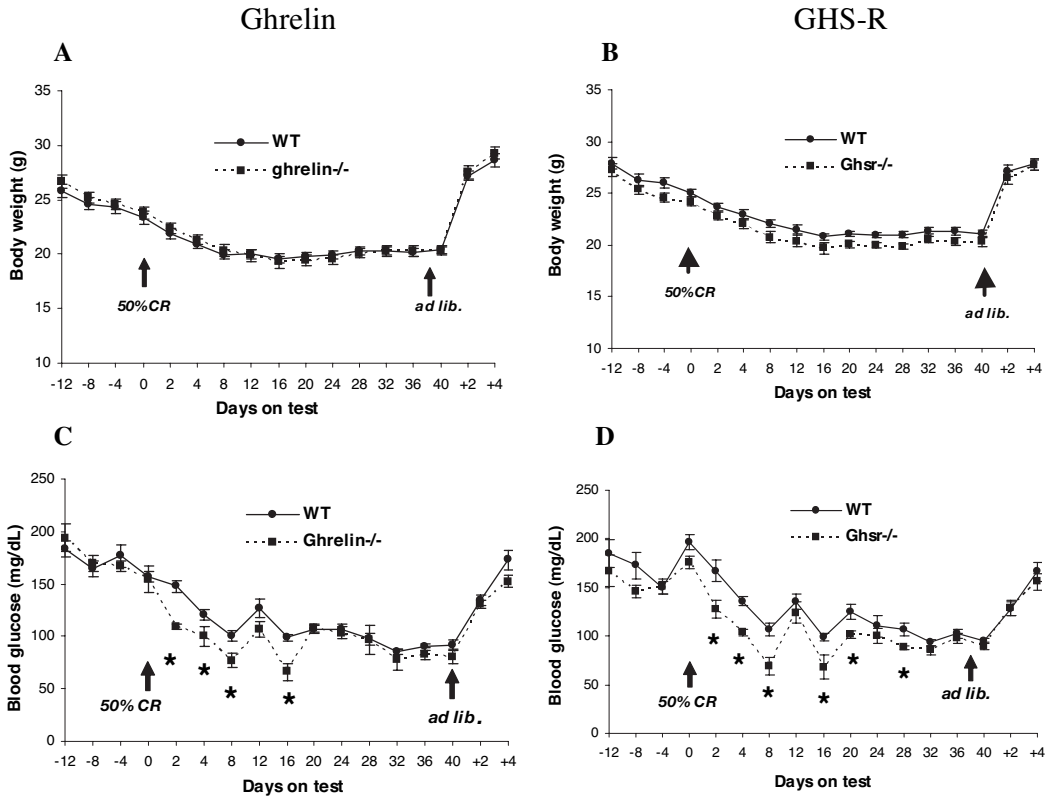

FIG. 6. Body weight (A) and blood glucose (B) of 10-wk-old *ghrelin*$^{-/-}$ and *Ghsr*$^{-/-}$ mice and their littermate controls (WT) during 50% CR. The experimental mice were individually caged, and food intake was measured from −12 d to −4 d, and average food consumption was calculated. Beginning on d 0, a calculated amount of food representing 50% CR was given to the mice daily at 1500 h for 40 d; then the mice were switched back to *ad libitum* feeding. For *ghrelin*$^{-/-}$ and WT, n = 9; for *Ghsr*$^{-/-}$ and WT, n = 7. Blood glucose was measured by One Touch Ultra glucometer. *, $P < 0.05$, WT *vs.* *ghrelin*$^{-/-}$ and *Ghsr*$^{-/-}$.

The *ghrelin*$^{-/-}$ and *Ghsr*$^{-/-}$ mice had lower blood glucose levels than their WT littermates when they were subjected to 50% CR (Fig. 6). Hence, ghrelin and the GHS-R appear to be involved in providing a counterregulatory glucose response during negative energy balance. Plasma ghrelin concentration is inversely correlated with body weight and body fat; CR is associated with increased plasma ghrelin concentration (35). Under CR conditions, fatty acids are mobilized and oxidized to potentially produce octanoic acid resulting in octanoylation of desacyl-ghrelin. The greater glucose levels of WT mice during the early phases of the CR may be driven by this increase in ghrelin. The fact that the difference in glucose is less pronounced during prolonged CR may be explained by depletion of the supply of free fatty acids and induction of ketosis.

Using glucose tolerance tests and hyperinsulinemic-euglycemic clamp studies, we demonstrated that *ghrelin*$^{-/-}$ mice have improved glucose tolerance and increased insulin sensitivity (29). In agreement with Dezaki *et al.* (36), we showed that ghrelin inhibits glucose-stimulated insulin release (29). Therefore, we speculate that the inhibitory effect of ghrelin on insulin release provides tonic regulation of pancreatic β-cells and restrains insulin secretory activity during food deprivation (37), which is consistent with the greater reductions in blood glucose levels observed during CR in *ghrelin*$^{-/-}$ and *Ghsr*$^{-/-}$ mice (Fig. 6).

In conclusion, ghrelin and the GHS-R appear to be nonessential regulators of appetite in adult mice. Deletion of *ghrelin* or the *Ghsr* in these adult C57BL/6J background mice does not prevent DIO or prevent weight gain after weight loss. The collective data suggest that ghrelin's effects on metabolic fuel preference are transient and may not have a significant effect throughout the lifespan. Perhaps adult C57BL/6J *ghrelin*$^{-/-}$ and *Ghsr*$^{-/-}$ mice are subject to metabolic adaptations especially in regard to energy intake and expenditure. The degree and type of adaptation may be age and background dependent. Nevertheless, the ghrelin/GHS-R pathway, at least in highly selective backcross bred male mice, appears to play an important role in glucose homeostasis by regulating insulin sensitivity and glucose sensing, particularly under conditions of negative energy balance.

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