In vivo estimation of gluconeogenesis

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Chapter 6

Effect of ghrelin on glucose regulation in mice

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Abstract

Objective: To determine whether ghrelin has an impact on gluconeogenesis, glycogenolysis and insulin sensitivity using a mice model.

Research Design and Methods: Rate of appearance of glucose, gluconeogenesis and glycogenolysis were measured in a set of five animals in each group of wild type (WT), ghrelin knockout (ghrelin$^{-/-}$) and growth hormone secretagogue receptor knockout (Ghsr$^{-/-}$) mice following a short term (8h) and prolonged fast (18h). Concentrations of glucose and insulin were measured, and insulin resistance and hepatic insulin sensitivity were calculated.

Results: Glucose concentrations were not different among the groups either following the 8 or 18h fast; however, they were lower after the 18h fast. Plasma insulin concentrations were lower in the ghrelin$^{-/-}$ and Ghsr$^{-/-}$ than WT animals following the 8h fast, but were not different after the 18h fast. The rates of gluconeogenesis, glycogenolysis and glucose clearance, and indices of insulin sensitivity were higher in the ghrelin$^{-/-}$ and Ghsr$^{-/-}$ than WT animals after the 8h fast, but were not different between the ghrelin$^{-/-}$ and Ghsr$^{-/-}$ groups. Following an 18h fast, gluconeogenic rates were lower in the ghrelin$^{-/-}$ and Ghsr$^{-/-}$ than WT animals and gluconeogenesis accounted for nearly all glucose production in all groups.

Conclusions: This study demonstrates that gluconeogenesis and glycogenolysis are increased and insulin sensitivity is improved by the ablation of ghrelin or growth hormone secretagogue receptor in mice.

Introduction

The improvement in insulin sensitivity and in many cases reversal of Type-2 diabetes after bariatric surgery procedures are well established (1-5). However, the underlying reason(s) for this improvement is not clear. Improved glycemic control achieved immediately after surgery but before weight loss suggests a hormonal mechanism as the explanation for improved glucose homeostasis (6). The plasma ghrelin concentration was decreased by ~ 70 % following gastric bypass surgery as compared to matched obese and normal-weight controls (6). The relationship between the improvement of glucose metabolism and reduced ghrelin concentration remains to be determined.

The stomach is the major source of circulating ghrelin (7). Further, ghrelin-containing cells are abundant in the fundus region of the stomach (7-10). Sleeve gastrectomy, which involves the complete resection of the gastric fundus, resulted in better glycemic control when compared to other forms of bariatric surgery (3).

Ghrelin stimulates the release of growth hormone via the growth hormone secretagogue receptor (11-13). Consequently, studying the effects of ghrelin on glucose metabolism using ghrelin infusion can potentially be confounded by the effects of growth hormone. Moreover, ghrelin is produced by cells scattered in various tissues (14-17). Therefore, studies of glucose kinetics in the absence of ghrelin or its receptor using transgenic mice models (13; 18-20) provide an opportunity to investigate the effects of ghrelin on glucose metabolism independent of any action of growth hormone.

Ghrelin infusion in growth hormone deficient and normal mice (21) and in humans (22) indicated that the effects of ghrelin on glucose metabolism also occur in the absence

of growth hormone. Another adipocytokine, leptin, also influences glucose metabolism. Intracerebral infusion of leptin stimulated gene expression of hepatic glucose-6-phosphatase and PEPCK, and increased gluconeogenesis (23). Similarly infusion of leptin increased both hepatic and peripheral insulin sensitivity (24-26) and inhibited insulin secretion (27; 28). Antagonizing effects of ghrelin on leptin demonstrated by co-administration of leptin and ghrelin (28) indicate that leptin's influence on gluconeogenesis and insulin secretion may be counteracted by the presence of ghrelin. Insulin has been shown to decrease glycogenolysis in a dose dependent manner (29; 30).

The present study was designed to address the following hypotheses; 1) gluconeogenesis and insulin sensitivity are higher in the absence of ghrelin, 2) glycogenolysis is higher during post absorptive conditions due to a lower insulin concentration in the absence of ghrelin. To test these hypotheses, we compared glucose kinetics among wild type (WT), ghrelin (ghrelin$^{-/-}$) and growth hormone secretagogue receptor (Ghsr $^{-/-}$) knockout mice.

Materials and Methods

Animals and housing: Four to five months old adult male WT, ghrelin$^{-/-}$, and Ghsr $^{-/-}$ mice were used for all the experiments. Mice were kept in a standard housing facility and had access to standard chow diet (Harlan teklad rodent diet 2920x) with ad-lib access to autoclaved reverse osmosis water. Mice were maintained under a 12 h light cycle (600-1800h) and constant temperature (75 ± 2ºF). Glucose kinetic measurements were performed in a set of five animals (n=5) in each group. Body composition was measured in another set of animals; WT (n=15), ghrelin$^{-/-}$ (n=9), and Ghsr $^{-/-}$ mice (n=7). Glucose

and insulin concentrations were measured in identically fasted WT (n=10), ghrelin$^{-/-}$ (n=5), and Ghsr $^{-/-}$ mice (n=5). All procedures used in the animal experiments were approved by the Baylor College of Medicine Institutional Animal Care and Use Committee.

Body Composition: Body composition parameters such as total body fat and total lean body mass were measured by magnetic resonance imaging (MRI) using EchoMRI-100 (QNMR systems, Houston, TX).

Stable isotopes: Sterile and pyrogen free deuterium oxide (^2H_2O), 99 atom percent ^2H and [6,6-$^2H_2$]glucose, 99 atom% ^2H were purchased from Cambridge Isotopes Laboratories (Andover, MA). [6,6-$^2H_2$]glucose was dissolved in 0.5% ^2H_2O (made isotonic by addition of sodium chloride), filtered and prepared for intravenous infusion.

Study design: Rate of appearance of glucose and gluconeogenesis were measured in each group at the end of a short term fast of 8 h and a prolonged fast of 18 h, respectively. On the day of the infusion, all mice were weighed and received an IP dose of 99% ^2H_2O (4mg/g body weight) resulting in a deuterium enrichment of (~) 0.5% in body water. After this ^2H_2O dose, the animals were given ad libitum access to water (0.5% ^2H_2O) for the rest of the study to maintain the body water deuterium enrichment at ~0.5%. The mice were then restrained in an infusion box and a tail vein catheter was inserted as described previously (31). Two hours following the ^2H_2O dose, a primed constant rate infusion of [6,6-$^2H_2$]glucose at (~) 0.75 mg/kg·min (150 µL/hour) was started and continued for 4 h.

After 4 h of infusion, blood was drawn from the submaxibular bundle and centrifuged for 15 min at 4º C. Plasma was separated and kept frozen at -80º C until

analyzed. Blood samples from five mice were collected by lateral vein bleeding before start of the tracer infusion to determine baseline enrichments. A pilot experiment in 3 animals was initially performed to ascertain that the isotopic enrichment had reached steady state between 3 and 4 hrs. During the pilot study, one sample was collected before the start of the infusion from the lateral tail vein and two samples at 3h, and 4h (60µl/sample) by submaxibular bleeding technique.

Analyses: The Isotopic enrichment of [6,6-$^{2}H_2$]glucose was measured by gas chromatography – mass spectrometry (GCMS) (6890/5973 Agilent Technologies, Wilmington, DE) using the pentaacetate derivative. The incorporation of deuterium in glucose was determined using the average deuterium enrichment in glucose carbons 1,3,4,5 and 6 as previously described (32; 33).

Briefly, this method (32; 33) involves preparation of the pentaacetate derivative of glucose, followed by sample analysis using GCMS in the positive chemical ionization mode. Selective ion monitoring of m/z 170/169 is performed to determine the M+1 enrichment of deuterium in the circulating glucose carbons (C-1,3,4,5,6,6) (M is the base mass, 169, representing unlabeled glucose). After subtracting the enrichment of M+1 resulting from the natural abundance, the average enrichment of deuterium on a gluconeogenic carbon is calculated from these M+1 data (32; 33). Deuterium enrichment in plasma water is determined by Isotope Ratio Mass Spectrometry (Delta^{+}XL IRMS Thermo Finnigan, Bremen, Germany).

Insulin concentrations were determined by radioimmunoassay (Millipore, Billerica, MA) and plasma glucose concentrations using the Precision Xtra blood glucose monitoring system (Abbott Inc, Alameda, CA).

Calculations: All kinetic measurements were performed under steady state conditions. Total plasma glucose appearance rate (glucose Ra) was calculated from the M+2 enrichment of [6,6-$^2H_2$]glucose in plasma using established isotope dilution equations (34).

Rate of glucose production (mg/kg·min) *(GPR) = glucose Ra – exogenous glucose* (i.e only the tracer since the animals were fasting).

Fractional gluconeogenesis (i.e. gluconeogenesis as a fraction of glucose Ra) was calculated according to Chacko et. al (32; 33) as follows:

Fractional gluconeogenesis (GNG % Ra) = [(M+1) (^2H) $_{(m/z\ 170/169)}$ /6] / E ^2H_2O

where (M+1)(^{2}H) $_{(m/z\ 170/169)}$ is the M+1 enrichment of deuterium in glucose measured using m/z 170/169 and '6' is the number of ^{2}H labeling sites on the m/z 170/169 fragment of glucose (i.e the average M+1 enrichment derived from deuterated water) and *E ^2H_2O* is the deuterium enrichment in plasma water.

Rate of gluconeogenesis was calculated as the product of total glucose appearance rate and fractional gluconeogenesis.

Rate of Gluconeogenesis (mg/kg·min) *(GNG rate) = gluc Ra × GNG % Ra*

Glycogenolysis was calculated by subtracting the rate of gluconeogenesis from the glucose production rate.

Rate of glycogenolysis (mg/kg·min) = *GPR - GNG rate*

Glucose Clearance (ml/kg·min) = *gluc Ra / C*

where gluc Ra is the rate of appearance, which equals the rate of disappearance of glucose (mg/kg·min) under steady state conditions and C is the plasma glucose concentration in mg/mL.

Insulin resistance was calculated by the homeostasis model assessment, HOMA-IR (fasting insulin μU/ml x fasting glucose mM /22.5) (35; 36).

Hepatic insulin sensitivity was calculated in the fasting state by the hepatic insulin sensitivity index (HISI): 1000/ (GPR [μmol/kg · min) x fasting plasma insulin (μU/mL)], where 1000 is a constant that results in numbers between 1 and 10, as described by Matsuda et al. (37; 38).

Statistical analyses: ANOVA was used to test significance among groups. ANOVA was followed by unpaired t-test to compare significant differences between groups. A p value <0.05 was used to define significance. All results are provided as mean ± SE.

Results

Body Composition (Table 1): Lean body mass was similar in all groups, however, total body fat was lower in ghrelin$^{-/-}$, and Ghsr $^{-/-}$ as compared to WT mice.

Table 1. Body composition measurements in WT, ghrelin$^{-/-}$ and Ghsr $^{-/-}$ mice.

	WT	ghrelin$^{-/-}$	Ghsr $^{-/-}$	p Value WT vs. ghrelin$^{-/-}$	p Value WT vs. Ghsr $^{-/-}$	p Value ghrelin$^{-/-}$ vs. Ghsr $^{-/-}$
Lean body mass (g)	23.0 ± 0.3	22.0 ± 0.6	22.4 ± 0.4	NS	NS	NS
Total body fat (g)	6.7 ± 0.7	4.0 ± 0.3	3.1 ± 0.5	0.006	0.002	NS
Body fat (%)	19.8 ± 1.5	13.7 ± 0.9	10.5 ± 1.4	0.007	0.001	NS

Measurements following a short term fast (8 h): Glucose concentrations were similar among the groups (Table 2). Plasma insulin concentrations were significantly lower in ghrelin$^{-/-}$ and Ghsr $^{-/-}$ mice than WT mice (p=0.012 and 0.009, respectively), however,

were not different between ghrelin $^{-/-}$ and Ghsr $^{-/-}$ mice (Table 2). The glucose production rates in ghrelin$^{-/-}$ and Ghsr $^{-/-}$ mice were nearly 60% higher when compared to the WT group, p=0.008 and 0.0004, respectively (Table 3). The rates were similar (NS) in ghrelin$^{-/-}$ and Ghsr $^{-/-}$ animals. The rates of gluconeogenesis were higher in ghrelin$^{-/-}$, and Ghsr $^{-/-}$ mice than WT (p=0.014 and 0.002, respectively), however, no difference was observed between the ghrelin$^{-/-}$ and Ghsr $^{-/-}$ groups (Fig 1). Gluconeogenesis accounted for ~ 70% of glucose production in all three groups. Rates of glycogenolysis (Fig 2) were higher in ghrelin$^{-/-}$ and Ghsr $^{-/-}$ mice than in WT mice (p=0.017 and 0.003, respectively), but no difference was observed between the ghrelin$^{-/-}$ and Ghsr $^{-/-}$ groups. Glucose clearance rate was significantly higher in ghrelin$^{-/-}$, and Ghsr $^{-/-}$ mice as compared to WT (p=0.002 and 0.0003, respectively) and no difference was observed between the ghrelin$^{-/-}$ and Ghsr $^{-/-}$ groups (Table 3). HOMA-IR was lower in ghrelin$^{-/-}$ and Ghsr $^{-/-}$ mice than WT (p=0.02 and p=0.02, respectively) (Table 2) and HISI was higher in ghrelin$^{-/-}$ and Ghsr $^{-/-}$ mice than WT (p=0.005 and p=0.0002, respectively).

Table 2. Concentrations of glucose and insulin in WT, ghrelin$^{-/-}$ and Ghsr $^{-/-}$ mice at the end of 8 h and 18 h fast.

	8 h Fast			18 h Fast		
	WT	ghrelin$^{-/-}$	Ghsr $^{-/-}$	WT	ghrelin$^{-/-}$	Ghsr $^{-/-}$
Glucose (mM)	14.0 ± 0.6	12.0 ± 0.9	13.7 ± 0.4	8.2 ± 0.4	7.3 ± 0.6	8.6 ± 0.7
Insulin (ng/mL)	2.07 ± 0.25	0.98 ± 0.14*	0.88 ± 0.21*	0.86 ± 0.18	0.51 ± 0.10	0.53 ± 0.13

Data expressed Mean ± SE and * denotes p<0.05 ghrelin$^{-/-}$ vs. WT group and Ghsr $^{-/-}$ vs. WT group.

Table 3. Rates of glucose appearance (Ra), glucose production (GPR) and glucose clearance, and Indices of HOMA-Insulin resistance (HOMA-IR) and Hepatic insulin sensitivity (HISI) in WT, ghrelin$^{-/-}$ and Ghsr $^{-/-}$ mice at the end of 8 h and 18 h fast, respectively.

	8 h Fast			18 h Fast		
	WT	ghrelin$^{-/-}$	Ghsr $^{-/-}$	WT	ghrelin$^{-/-}$	Ghsr $^{-/-}$
Glucose Ra (mg/Kg·min)	12.89 ± 0.82	20.12 ± 1.89*	19.66 ± 0.82*	13.83 ± 0.51	9.15 ± 0.67*	11.83 ± 1.34
GPR (mg/Kg·min)	12.17 ± 0.81	19.34 ± 1.89*	18.93 ± 0.81*	12.33 ± 0.50	8.32 ± 0.65*	11.03 ± 1.34
Glucose clearance rate (ml/kg·min)	5.13 ± 0.33	9.33 ± 0.88*	7.98 ± 0.33*	9.41 ± 0.35	6.94 ± 0.51*	7.68 ± 0.87
HOMA-IR	32.3 ± 6.6	13.1 ± 2.6*	13.1 ± 3.5*	8.3 ± 3.3	4.0 ± 0.7	5.0 ± 1.3
HISI	0.29 ± 0.01	0.40 ± 0.04*	0.44 ± 0.02*	0.77 ± 0.10	1.76 ± 0.17*	1.21 ± 0.13*

Data expressed Mean ± SE and * denotes $p<0.05$ ghrelin$^{-/-}$ vs. WT group and Ghsr $^{-/-}$ vs. WT group.

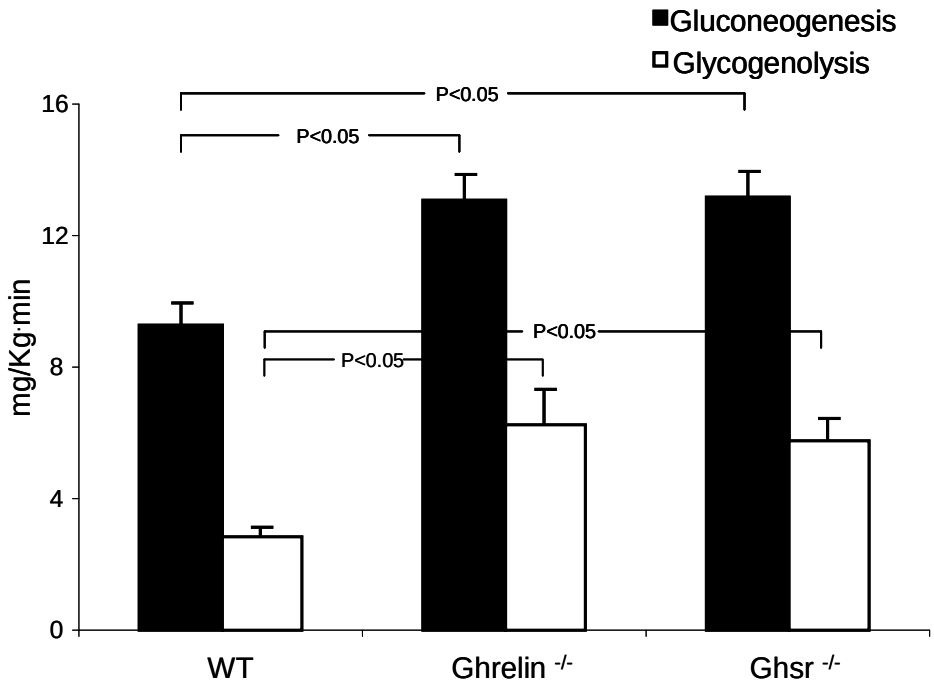

Figure 1. Rate of gluconeogenesis and glycogenolysis was significantly higher in ghrelin$^{-/-}$ and Ghsr $^{-/-}$ mice at the end of the 8 h fast as compared to those in WT group.

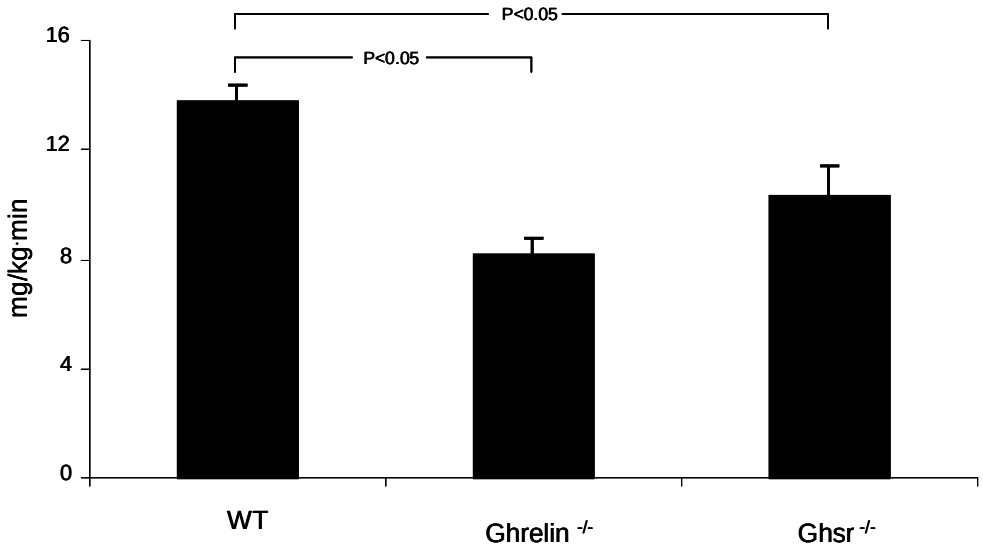

Figure 2. Rate of gluconeogenesis was significantly lower in ghrelin$^{-/-}$ and Ghsr $^{-/-}$ mice at the end of the 8 h of fast as compared to that in WT group.

Measurements following a prolonged fast (18 h): Both glucose and insulin concentrations were not different among the three groups (Table 2). The glucose production rates were lower in ghrelin$^{-/-}$ when compared to the WT group, (p=0.001) (Table 3), however, the differences between the Ghsr $^{-/-}$ and WT groups were not significant. The rates of gluconeogenesis (Fig 2) were lower in the ghrelin$^{-/-}$ and Ghsr $^{-/-}$ as compared to the WT group, (p=0.0001 and 0.024, respectively), but were not different between the ghrelin$^{-/-}$ and Ghsr $^{-/-}$groups. At the end of the 18 h fast, glycogenolysis was essentially zero in all groups and gluconeogenesis accounted for nearly all glucose production in all the groups. The glucose clearance rate was lower in the ghrelin$^{-/-}$ knockout group than WT, (p=0.004), but the difference did not reach significance between WT and the Ghsr $^{-/-}$ mice. Following the prolonged fast, HOMA-IR was not different among groups (Table 2) while HISI was higher in ghrelin$^{-/-}$ and Ghsr $^{-/-}$ mice as compared to WT (p=0.0002 and p=0.026, respectively).

Glucose concentrations were significantly lower after the 18 h as compared to 8 h fast in all groups ($p<0.004$ in all groups). Glucose production rates were lower in both ghrelin$^{-/-}$ and Ghsr $^{-/-}$ groups following the18 h as compared to the 8 h fast, while no difference was observed in the WT group.

Discussion

Changes in gut hormones involved in the regulation of glucose metabolism are well established (39; 40). However, the effect of ghrelin, an endogenous ligand for the growth hormone secretagogue receptor (13; 15), on endogenous glucose synthesis remains unclear. In the present study, we demonstrated that ablation of ghrelin or its receptor in 8h fasted mice resulted in increase of gluconeogenesis and glycogenolysis (Fig 1).

Interestingly, rates of gluconeogenesis and glycogenolysis were not different between ghrelin$^{-/-}$ and Ghsr $^{-/-}$ mice. These data demonstrate that ghrelin plays a role in the regulation of gluconeogenesis and glycogenolysis, and that at least part (if not all) of those effects occurs via the growth hormone secretagogue receptor.

A potential mechanism for increased gluconeogenesis and glycogenolysis could be secondary to the opposing effects of ghrelin on leptin action (28). Intracerebral infusion of leptin stimulated gene expression of the hepatic enzymes glucose-6-phosphatase and PEPCK with subsequent increase in gluconeogenesis (23). At the end of the 8 h fast in our study, in the absence of ghrelin (ghrelin$^{-/-}$) or its mediation through its purported receptor (Ghsr $^{-/-}$), gluconeogenesis was higher than in WT mice. The increased rate of gluconeogenesis might be because of the absence of the inhibiting effect of ghrelin on leptin since we previously reported that the plasma leptin concentrations were similar in ghrelin$^{-/-}$, Ghsr $^{-/-}$ and WT mice (13; 41).

It has also been reported that leptin inhibits insulin secretion and ghrelin reverses leptin's inhibiting effect on insulin secretion (27; 28). This suggests reduced insulin concentration in the absence of ghrelin. Consistent with this we observed that insulin concentrations were lower in both the ghrelin$^{-/-}$ and Ghsr $^{-/-}$ mice as compared to WT mice following the 8 h fast (Table 1). Previous studies have shown that insulin decreases glycogenolysis in a dose dependent manner (29; 30). In the present study, in response to lower insulin concentration in the knockout groups than in the WT, glycogenolysis was significantly higher following 8 h of fasting (Fig 1).

The significantly higher hepatic insulin sensitivity index and improved insulin resistance in ghrelin$^{-/-}$ and Ghsr $^{-/-}$ as compared to WT mice demonstrate increased insulin

sensitivity in the absence of ghrelin action on the purported ghrelin receptor. Ghrelin infusion has been reported to induce insulin resistance and stimulate lipolysis (22; 42; 43), whereas, leptin increased both hepatic and peripheral insulin sensitivity (24-26). This indicates that leptin unopposed by ghrelin action might be the reason for this increased insulin sensitivity. Both ghrelin $^{-/-}$ and Ghsr $^{-/-}$ knockout mice are more insulin sensitive than WT mice (41; 44-46). Sun et al. reported that glucose production was more suppressed in ghrelin$^{-/-}$ than WT mice during a low-dose insulin clamp suggesting increased hepatic insulin sensitivity in the absence of ghrelin (41).

Enhanced hepatic insulin sensitivity can potentially stimulate glycogen synthesis during glucose availability resulting in increased glycogen stores. This might explain our observation of significantly higher rates of glycogenolysis in the ghrelin$^{-/-}$ and Ghsr $^{-/-}$ as compared to WT mice at the end of the 8 h fast. A previous report that ghrelin down-regulates markers of glycogen synthesis (47) is consistent with this observation.

Blood glucose concentrations were similar in all groups of animals at the end of the 8 h fast despite significantly higher rates of gluconeogenesis and glycogenolysis in ghrelin$^{-/-}$ and Ghsr $^{-/-}$ mice indicating increased glucose uptake in the absence of ghrelin or its receptor. Higher glucose clearance observed at lower insulin concentration during short term fast in ghrelin$^{-/-}$ and Ghsr $^{-/-}$ as compared to WT mice in our study might suggest that less insulin is required for peripheral glucose uptake in the absence of ghrelin or its receptor under normal physiologic conditions. This is in line with the improved peripheral insulin sensitivity reported in these knockout mice (41; 44-46). Consistent with our observation, continuous ghrelin infusion was demonstrated to induce insulin resistance in muscle and to stimulate lipolysis (22; 42; 43). Reduction of plasma ghrelin

below physiological levels by insulin infusion during glucose clamp resulted in a sharp increase of insulin sensitivity in humans (48).

In agreement with other reports (45; 49; 50), the measurements of body composition (table 2) revealed that both knockout groups had significantly smaller fat mass as compared to WT. Lower fat accretion stimulated by the absence of ghrelin or its receptor might be another reason for improved insulin sensitivity. In contrast, we found that the lean body mass was not different among the three groups.

During prolonged fasting (18 h), rates of glycogenolysis were essentially zero and insulin concentrations were appropriately decreased and similar in all groups as reported previously (19). In WT mice, gluconeogenesis was significantly increased. However, no increase in gluconeogenesis was observed in the knockout groups (Fig 2). The plasma glucose concentrations remained similar in all groups. We observed that glucose clearance was significantly lower in the ghrelin$^{-/-}$ and Ghsr $^{-/-}$ groups suggesting lower demand for glucose via gluconeogenesis in the knockout groups. Alternatively, smaller lipid stores in the knockout groups and thus, less mobilization of gluconeogenic substrates might be another reason for this lower rate of gluconeogenesis.

Thus, our study demonstrates that gluconeogenesis and glycogenolysis are increased, and insulin sensitivity is improved in mice by the absence of ghrelin action. This could be a potential explanation for the improvement of glucose metabolism observed in patients after bariatric surgery. We speculate that improved glucose metabolism in the absence of ghrelin in mice might be associated with leptin. A recent report demonstrated that antidiabetic actions of leptin are mediated via the central nervous system dependent mechanisms (51). Further studies are required to determine the

mechanisms underlying the association between leptin and ghrelin in the improvement of glucose metabolism. Our present data supports a potential prospect of improving insulin sensitivity in the diabetic condition by the use of ghrelin receptor antagonists.

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