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Ghrelin Contributes to Derangements of Glucose Metabolism Induced by Rapamycin in Mice

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Abstract

Aims/hypothesis—Rapamycin impaired glucose tolerance and insulin sensitivity. Our previous study demonstrated that rapamycin significantly increases the expression of gastric ghrelin, which is critical in the regulation of glucose metabolism. Here, we investigated whether ghrelin contributes to derangements of glucose metabolism induced by rapamycin.

Methods—The effects of rapamycin on glucose metabolism were examined in mice receiving ghrelin receptor antagonist or with ghrelin receptor gene deletion. Changes in Glut4, JNK, and pS6 were investigated by immunofluorescent staining or Western. Related hormones were detected by radioimmuno-assay kits.

Results—Rapamycin impaired glucose metabolism and insulin sensitivity not only in normal C57BL/6J mice but also in both obese mice induced by high fat diet and db/db mice. This was accompanied by elevation of plasma acylated ghrelin. Rapamycin significantly increased the levels of plasma acylated ghrelin in normal C57BL/6J mice, high fat diet induced obese mice, and db/db mice. Elevation in plasma acylated ghrelin and derangements of glucose metabolism upon administration of rapamycin was significantly correlated. The deterioration in glucose homeostasis induced by rapamycin was blocked by D-Lys3-GHRP-6, a ghrelin receptor antagonist, or by deletion of ghrelin receptor gene. Ghrelin receptor antagonism and ghrelin receptor gene deletion blocked the up-regulation of JNK activity, and GLUT4 expression and translocation in the gastrocnemius muscle induced by rapamycin.

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Contribution statement

Geyang Xu, Ziru Li and Hong Tang performed the experiments and analysed data; All authors contributed to the design of the experiments, interpretation of data and the writing of the manuscript. Geyang Xu, Ziru Li, Michael Mulholland and Weizhen Zhang revised the manuscript. All the authors approved the final version of the paper.

Duality of interest

The authors declare that there is no duality of interest associated with this manuscript.

Conclusions—The current study demonstrates that ghrelin contributes to derangements of glucose metabolism induced by rapamycin via altering the expression and translocation of GLUT4 in muscles.

Keywords

Ghrelin; glucose metabolism; rapamycin

Introduction

Ghrelin, a gastric 28 amino acid peptide hormone, is the endogenous ligand for the growth hormone secretagogue receptor (GHSR)[1]. Ghrelin is the only currently identified circulating hormone that is able to initiate food intake[2]. A variety of other physiologic processes are known to be regulated by ghrelin in addition to ingestive behavior, including effects on glucose metabolism[3]. Systemic administration of exogenous ghrelin has been reported to elevate blood glucose levels in humans[4] and rodents[5]. This effect is blocked by the ghrelin receptor antagonist, D-Lys3-GH-releasing peptide-6 (D-Lys3-GHRP-6)[5]. Deletion of the ghrelin gene in mice results in a phenotype with lower levels of circulating glucose and altered glucose tolerance upon exposure to high fat diet[6]. GHSR null mice fed a high fat diet demonstrate increased insulin sensitivity[7]. While both ghrelin and its receptor are expressed in human[8] and rat pancreatic islets[9], the mechanisms by which ghrelin produces these effects are unknown.

Rapamycin is a macrolide fungicide, first used as an immunosuppressant[10], which is also employed to explore the role of the mammalian target of rapamycin (mTOR) system in control of metabolic processes[11]. The mTOR signaling system is widely recognized to be operant in the hypothalamus where it is crucial to the central control of food intake[11–13]. Effects of rapamycin on blood glucose, serum triglycerides, free fatty acids and ketone bodies have also been reported both in animals and humans[14–16]. Chronic application of rapamycin has been associated with new onset diabetes in patients with organ transplantation[17]. Impaired glucose tolerance and new-onset diabetes are among the most serious metabolic complications of solid organ transplantation[18, 19]. Increased risk of diabetes in transplant recipients is largely due to the immunosuppressive agents used to treat transplant recipients[20]. There exist two mTOR complexes: mTORC1 and mTORC2. mTORC1 complex is sensitive to rapamycin and includes mTOR, Raptor, mLST8/GβL, PRAS40 and DEPTOR. mTORC1 is involved in the anabolic effect of insulin through activation of S6K1/2 and inhibition of 4E-BP1 and 4E-BP2[21]. In obesity and diabetes, mTORC1/S6K signaling is chronically activated[22]. This chronic activation promotes insulin resistance by a negative feedback mechanism involving serine phosphorylation and degradation of IRS1 as well as inhibition of IRS1 transcription[23,24]. Several genetically modified mice have been engineered to study the implication of mTORC1 in the regulation of insulin sensitivity. S6K1 knockout mice are protected against obesity and insulin resistance due to elevated energy expenditure and the loss of IRS1 serine phosphorylation by S6K1[25,26]. Global invalidation of 4E-BP1 and 4E-BP2 leads to the opposite phenotype[27]. Mice with adipose-specific raptor invalidation mainly recapitulate the phenotype of global S6K1 knockout mice[28]. These observations suggest that the mTOR signaling system may be an important factor in the regulation of glucose metabolism. While previous studies of mTOR signaling have extensively focused on insulin secretion by islet cells and its signaling pathways in the insulin targeted tissues, little has been known on its effect on hormones critical for energy metabolism. We have reported that mTOR signaling system regulates gastric mucosal expression of ghrelin[29, 30]. Systemic administration of the mTOR inhibitor, rapamycin, stimulated the transcription and translation of gastric ghrelin, increased circulating levels of ghrelin and stimulated feeding behavior[30]. In the

present study, we test the hypothesis that ghrelin plays a role in disordered glucose metabolism induced by rapamycin in normal and obese mice.

Methods

Materials

Rapamycin, mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody and goat anti-mouse Texas Red-conjugated IgG were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Dimethylsulfoxide (DMSO) was from Sigma Chemical Co. (St. Louis, MO). D-Lys3-GH-releasing peptide-6 (D-Lys3-GHRP-6) was from Phoenix Pharmaceuticals, Inc. (Burlingame, CA). Aprotinin was from Amersham Biosciences (Pittsburgh, PA). Phospho-S6 (ser235/236) rabbit antibody, S6 ribosomal mouse antibody, mouse anti-phospho-SAPK/JNK (Thr183/Tyr185) and mouse anti-GLUT4 were from Cell Signaling Technology (Beverly, MA). IRDye-conjugated affinity purified anti-rabbit and anti-mouse IgGs were purchased from Rockland (Gilbertsville, PA). Trizol reagent and the reverse transcription (RT) system were from Invitrogen Inc. (Carlsbad, CA). Acylated ghrelin and insulin radioimmunoassay kits were from Linco Bioscience Institute (St. Charles, MO).

Animals and treatments

Animals—Twelve-week-old male C57BL/6J mice, high fat diet induced obese mice (DIO), ghrelin receptor knockout mice and 12- to 16-week old male db/db mice were used in the present study. GHSR1a gene knockout mice in which exon 1 and exon 2 were deleted were obtained from the Shanghai Research Center for Biomodel Organism[31]. Deletion of GHSR1a gene fragment was confirmed by the absence of relative gene products examined by RT-PCR in hypothalamus and skeletal muscle in which GHSR1a are expressed (figure 5). Mice were housed in standard plastic rodent cages and maintained at a regulated environment (24 °C, 12-h light, 12-h dark cycle with lights on at 07:00 h). Regular chow and water were available *ad libitum* unless specified otherwise. In some experiments, rapamycin (1mg/kg) or D-Lys3-GHRP-6 (10 μmol/kg) was injected intraperitoneally for 6 days. Control animals received normal saline with same concentration of DMSO (0.1%).

Diets—Where indicated, 4-week-old mice were assigned to receive standard laboratory chow, or a high-fat diet (45% fat, D12451; Research Diets, New Brunswick, NJ) for 8 weeks. All animal protocols were approved by the Animal Care and Use Committee of Peking University.

Glucose tolerance test and insulin tolerance test

For the oral glucose tolerance tests, mice were fasted for 12 h before the gastric administration of glucose (3g/kg body weight) by gavage. For insulin tolerance tests, mice were fasted for 4 h, followed by intraperitoneal injection of insulin at a dose of 1 U/kg. Blood was drawn from a cut at the tip of the tail at 0, 30, 60, 90, and 120 min, and glucose concentrations were detected immediately with Glucotrend from Roche Diagnostics (Mannheim, Germany) according to the manufacturer's instruction.

Tissue sample preparations and immunofluorescent analysis

C57BL/6J mice were deeply anesthetized using pentobarbital, perfused transcardially with 20 ml 0.1 mol/l PBS (pH 7.4), followed by 20 ml 4% paraformaldehyde in PBS. Gastrocnemius muscle and pancreas were quickly removed and rinsed thoroughly with PBS. The tissues were postfixed in 4% paraformaldehyde, dehydrated, embedded in wax, and sectioned at 6 μm. Paraffin-embedded sections were de-waxed, rehydrated, and rinsed in

PBS. After boiling for 10 min in 0.01 mol/l sodium citrate buffer (pH 6.0), tissue sections were blocked in 5% goat pre-immune serum or 1% BSA in PBS for 1 h at room temperature, then incubated overnight with mouse monoclonal antibody to GLUT4 (1:100) or mouse anti-phospho SAPK/JNK (Thr183/Tyr185) (1:400) (for Gastrocnemius muscle) and goat anti-insulin (1:100) (for pancreas) antibodies. Tissue sections were then incubated at room temperature for 1 h with the following secondary antibodies: goat anti-mouse Texas Red-conjugated IgG or donkey anti-goat Texas Redconjugated IgG (1:100). Controls included substituting primary antibodies with mouse or goat IgG. Computerized image analysis (Model Leica Q550CW, Leica Qwin, Germany) was performed to quantify the immunostaining signals of GLUT4 and phospho-SAPK/JNK (Thr183/Tyr185) from mouse gastrocnemius muscle or insulin from pancreas.

Measurements of plasma acyl-ghrelin and insulin

Blood samples were immediately transferred to chilled polypropylene tubes containing EDTA-2Na (1 mg/ml) and aprotinin (1000 U/ml) and centrifuged at 4 °C. The plasma was separated and stored at -70 °C before use. Acylated ghrelin and insulin were measured using radioimmuno-assay kits according to the manufacturer's instruction. Concentrated HCl was added to the plasma at a final concentration of 0.1N and the acidified plasma was diluted with an equal volume of 0.9% NaCl solution. The anti-acylate ghrelin antiserum or anti-insulin antibody was used at final dilutions of 1/100,000. All assays were performed in duplicate.

Cell culture, transfection, treatment and immunofluorescent staining

C2C12 cells were maintained in DMEM containing 4.5 g/L glucose supplemented with 10% FBS. Cells were then transfected with HA-GLUT4 (4μg) or GFP (4μg) using Lipofectamine reagent according to the manufacturer's instructions. After transfection, cells were switched to differentiation medium containing DMEM supplemented with 2% fetal calf serum, 1% penicillin-streptomycin, 1% sodium pyruvate, and 1% glutamine. After 6 days of culture, cells were treated with ghrelin (10nmol/l for 30min), D-Lys3-GHRP-6 (1μmol/l for 30 min), insulin (10nmol/l for 10min) or D-Lys3-GHRP-6 added 5 min before ghrelin and/or insulin stimulation.

After treatment, cells were washed with PBS, fixed with 3.7% paraformaldehyde for 10 min at room temperature, then treated with 0.1% Triton X-100 in PBS for 5 min. After thorough washing, cells were blocked with 1% BSA in PBS for 30 min. Cells were next incubated with the mouse anti-HA Tag primary antibody (1:1000) overnight at 4°C, washed with PBS, then incubated with goat-anti-mouse Texas Red-conjugated IgG (1:100) for 1.5 h in the dark. Nuclei were visualized by staining with Hoechst 33254 for 15 min. Cells were mounted with 90% glycerol-PBS media and examined by confocal laser scanning microscopy (Leica). Computerized image analysis (Leica Q550CW) was performed to quantify the immunostaining signals of HA-GLUT4.

Western blot analysis

Gastric fundus and gastrocnemius muscle were isolated and homogenized in lysis buffer. Proteins were subjected to SDS-PAGE with a 10% running gel, then transferred to a polyvinylidene fluoride membrane. Membranes were incubated for 1 h at room temperature with 5% fat-free milk in Tris buffered saline containing Tween 20, followed by incubation overnight at 4 °C with primary antibodies. Specific reaction was detected using IRDye-conjugated second antibody and visualized using the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE).

RT-PCR

Total RNA was isolated from mouse hypothalamus, gastrocnemius muscle using the Trizol reagent. RT was performed using the RT system according to the manufacturer's instruction. The PCR program was as follows: hold at 95 °C for 5 min, then 95 °C 30 sec, 60 °C 30 sec, and 72 °C 1 min for 35 cycles. The nucleotide sequences of sense and antisense primers used were listed in the supplemental table.

20 µl of the RT-PCR samples were loaded onto 1.5% agarose gel. For negative controls, PCR reactions were performed for the primer pairs in the absence of transcript.

Statistical analysis

Data were expressed as mean $\pm$ SEM and analyzed by repeated-measures analysis of variance, one-way ANOVA, Student-Newman-Keul's test (comparisons between multiple groups), or unpaired Student's *t* test (between two groups) as appropriate, using GraphPad Prism software. $P < 0.05$ was considered significant. Pearson's correlation analysis was performed to determine the strength of the linear relationship between plasma concentration of acylated ghrelin and derangements of glucose metabolism or insulin resistance induced by rapamycin expressed as total areas under curves.

Results

Hyperglycemia, derangements of glucose metabolism and hyperghrelinemia induced by rapamycin

Because we have previously demonstrated that the mTOR signaling pathway is important in ghrelin expression and secretion[30], studies were performed using the mTOR pathway inhibitor, rapamycin. Mice treated with rapamycin demonstrated hyperglycemia, impaired glucose metabolism and glucose disposal curves typifying insulin resistance. These alterations were observed in normal C57BL/6J mice, as well as in high fat diet-induced obesity (DIO) mice and db/db obese mice (Fig. 1a–f). Disturbances of glucose homeostasis were accompanied by elevations in plasma acyl ghrelin. Significant increases of plasma acyl ghrelin in response to rapamycin administration were observed in normal C57BL/6J mice (27.6 ± 2.7 to 42.1 ± 5.8 pmol/l, $p < 0.05$), in high fat-induced obese mice (15.0 ± 1.4 to 22.0 ± 1.3 pmol/l, $p < 0.01$), and in db/db mice (20.0 ± 1.5 to 27.8 ± 2.3 pmol/l, $p < 0.05$) (Fig. 1g–i). Pearson's analysis demonstrated positive correlations between plasma acyl ghrelin and rapamycin-induced derangements of glucose metabolism and insulin sensitivity in normal C57BL/6J mice ($r = 0.922$ and 0.925 respectively, $p < 0.01$), DIO obese mice ($r = 0.943$ and 0.960 respectively, $p < 0.01$), and db/db obese mice ($r = 0.857$ and 0.907 respectively, $p < 0.01$). Systemic administration of rapamycin significantly decreased the phosphorylation of S6 protein, a down-stream target of mTOR signaling, in gastric fundus (Fig. 1j–l).

Effect of ghrelin receptor antagonism on rapamycin-induced impairment of glucose metabolism

If elevation in plasma acyl ghrelin is mechanistically linked to derangements of glucose metabolism induced by rapamycin, blockade of acyl ghrelin activity would be predicted to reverse the effects of rapamycin. The effects of D-Lys3-GHRP-6, a GHSR1a antagonist, were examined in C57BL/6J mice. Twelve-week-old male C57BL/6J mice were divided into four groups and received the following treatments for six days: control (DMSO), rapamycin (1 mg/kg ip), D-Lys3-GHRP-6 (10 µmol/kg ip) or D-Lys3-GHRP-6, administered 2 min before rapamycin. As shown in (Fig. 2a and b), D-Lys3-GHRP-6 blocked the effects of rapamycin on oral glucose tolerance and on insulin sensitivity even though plasma acyl ghrelin levels were significantly higher relative to controls (Fig. 2g).

Effect of ghrelin receptor gene deletion on rapamycin-induced alterations in glucose metabolism

To further confirm that ghrelin contributes to disturbance of glucose metabolism induced by rapamycin, a ghrelin receptor gene deletion mouse model was used. When fed standard chow, rapamycin caused significant shifts in oral glucose tolerance and insulin sensitivity curves in wild type littermates, but not in ghrelin receptor gene knockout mice (Fig. 2c and d). These changes were proportionately greater in animals maintained on a high fat diet (Fig. 2e and f). Rapamycin-induced derangements of glucose metabolism and insulin sensitivity were significantly less severe in ghrelin receptor gene knockout mice relative to wild type littermates (Fig. 2e and f) although similar trends in change were shown in ghrelin levels after glucose administration (Supplementary Fig. 1a).

Consistent with these observations, rapamycin stimulated food intake only in wild type littermates but not in GHSR1a null mice (Supplementary Fig. 2a–c) despite similar elevations in plasma acylated ghrelin for both GHSR1a null mice (34.7 ± 2.5 pg/ml for DMSO vs. 43.3 ± 2.1 pmol/l for rapamycin, $p < 0.05$) and wildtype littermates (27.3 ± 5.7 pg/ml for DMSO control vs. 43.8 ± 1.6 pmol/l for rapamycin, $p < 0.05$) (Fig. 2h). Rapamycin abolished S6 phosphorylation despite concurrent administration of D-Lys3-GHRP-6 or GHSR1a gene deletion (Fig. 2i–k).

Effects on insulin secretion and islet cells

No significant difference in plasma insulin was observed in mice treated with rapamycin in either control mice (70.42 ± 7.744 vs. 67.43 ± 8.07 pmol/l, $p = 0.8$) or D-Lys3-GHRP-6-treated mice (161.3 ± 37.44 vs. 142.4 ± 32.3 pmol/l, $p = 0.7$) (Supplementary Fig. 3a). Similarly, no significant difference in plasma insulin was observed in mice treated with rapamycin relative to control in either wildtype littermates (91.78 ± 11.05 vs. 122.9 ± 17.83 pmol/l, $p = 0.2$) or GHSR gene knockout mice (226.2 ± 49.15 vs. 151.7 ± 24.86 pmol/l, $p = 0.2$) (Supplementary Fig. 3b). Furthermore, similar trends in change were shown in insulin levels after glucose administration (Supplementary Fig. 1b). Analysis of pancreatic islets by HE staining and immunofluorescent staining showed no significant difference in islets morphology (Supplementary Fig. 4) and islets insulin immunoreactivity (Supplementary Fig. 5) between mice treated with or without rapamycin. This observation suggests that islets morphology and function was not significantly altered by rapamycin.

Ghrelin receptor and JNK phosphorylation

Since the stress-responsive c-Jun NH₂-terminal kinase pathway in muscle may account for insulin resistance induced by rapamycin[18], we next examined the effect of ghrelin receptor antagonism on JNK phosphorylation in gastrocnemius muscle. As shown in (Fig. 3a), rapamycin significantly increased levels of phosphorylated JNK. Pretreatment of mice with D-Lys3-GHRP-6 to block the ghrelin receptor markedly attenuated up-regulation of JNK induced by rapamycin. The role of ghrelin in regulation of JNK phosphorylation induced by rapamycin was further examined in GHSR null mice. As shown in (Fig. 3c), rapamycin failed to increase phosphorylation of JNK in gastrocnemius muscle of GHSR null mice, while wildtype littermates remained sensitive to the effects of this agent.

Effect of ghrelin receptor antagonism and ghrelin receptor gene deletion on glucose transport in muscle

Glucose transport into most tissues occurs through the action of members of a family of facilitative diffusion glucose transport proteins designated as GLUT1–5 and 7. GLUT1 is ubiquitously distributed and has been proposed to act as a constitutive transport protein. In contrast, the GLUT4 isoform is expressed almost exclusively in adipose cells and

gastrocnemius muscle and is responsible for insulin-stimulated glucose transport[32–34]. As shown in (Fig. 4a), rapamycin inhibited gastrocnemius muscle GLUT4 membrane translocation, both in the basal status and after insulin injection. Ghrelin antagonism rescued the suppression of GLUT4 translocation induced by rapamycin. As shown in (Fig. 4c), experiments using ghrelin receptor gene deletion mice confirmed that ghrelin suppresses GLUT4 translocation induced by rapamycin. Average area and intensity of GLUT4 staining signals were nearly unaffected by rapamycin in GHSR1a null mice, while significant reductions in these parameters were observed in wild type mice. Similar results were also observed in C2C12 myocytes transfected with HA-GLUT4 (Supplementary Fig. 6).

Expression of ghrelin receptor in muscle

Des-acyl-ghrelin failed to significantly alter blood glucose levels indicate that ghrelin increases blood glucose via specific interaction with GHSR1a[35]. Thus the relative tissue distribution of GHSR1a mRNA and protein were examined. High level of GHSR1a mRNA was detected in hypothalamus and gastrocnemius muscle in wild type mice, but not in GHSR1a null mice (Fig. 5).

Discussion

The major finding of this study is that increased concentration of ghrelin contributes to derangements of glucose metabolism induced by rapamycin. These effects were induced by exposure to rapamycin, indicating a critical role of the mTOR signaling system in ghrelin secretion and glucose homeostasis. This general conclusion is supported by the following observations: 1) there exists a positive correlation between circulating plasma acyl ghrelin levels and derangements of glucose metabolism induced by rapamycin; 2) either ghrelin receptor antagonism or ghrelin receptor gene deletion improves derangements of glucose metabolism induced by rapamycin; 3) up regulation of JNK phosphorylation induced by rapamycin is blocked by ghrelin receptor antagonism or gene deletion; 4) ghrelin receptor antagonism or gene deletion stimulates GLUT4 expression and translocation, and blocks the suppression of GLUT4 induced by rapamycin; 5) ghrelin receptor antagonism and ghrelin receptor deletion inhibits gastrocnemius muscle JNK activity; 6) ghrelin decreases the membrane GLUT4 in the differentiated C2C12 cells, while ghrelin receptor antagonism significantly increases the amount of GLUT4 under either basal condition or insulin stimulation.

mTOR signaling system is regulated independently by insulin, amino acids, and energy sufficiency[36]. It integrates signals from growth factors, hormones, nutrients, and cellular energy levels to regulate processes as diverse as protein translation, cell growth, proliferation, and survival[36]. mTOR is therefore a critical molecule linking many cellular activities with overall energy status at the organism level[13]. Several lines of evidences suggest that dysregulated mTOR signaling may link nutrient excess with obesity and insulin resistance. Aberrant mTOR activity has been reported during the development of obesity and diabetes[37]. Significant elevation of mTOR signaling has been observed in liver and skeletal muscle of insulin-resistant obese rats maintained on a high-fat diet[22]. In vitro experiments have demonstrated that mTOR signaling may operate a negative feedback loop toward PI3-kinase/Akt by increasing inhibitory serine phosphorylation of insulin receptor substrate-1[38]. Studies by Bell *et al* suggest that rapamycin has deleterious effects on min-6 cells, and rat or human islets[17]. Our study confirms that systemic rapamycin causes hyperglycemia in normal mice and worsens hyperglycemia in both high-fat diet obese mice and db/db obese mice.

The mechanism underlying rapamycin-induced hyperglycemia is complicated and may involve both insulin secretion from pancreatic islet cells and insulin sensitivity in the liver,

muscle or adipose tissue[6]. While rapamycin has been associated with an increase in apoptosis of pancreatic beta-cells and reduction in beta-cell mass, insulin secretion has also been reported to be unchanged in rats or in cultured islet cells[18]. Our study shows that rapamycin does not alter levels of serum insulin, the islet morphology and islet insulin immunoreactivity, suggesting that hyperglycemic effects may rather involve impaired glucose metabolism in insulin-sensitive tissues. Skeletal muscle is a major site of glucose uptake after insulin administration. Insulin stimulation leads to an uptake of glucose through Akt-mediated translocation of the glucose transporter-4 to the cell surface[39]. Our study provides evidence that skeletal muscle is one of the main targets of rapamycin and contributes to the derangements of glucose metabolism induced by this agent. Upon treatment with rapamycin, skeletal muscle showed a mark increase in JNK phosphorylation, and a significant reduction in GLUT4 membrane expression.

The evidence whether rapamycin exerts its effect on skeletal muscle by a direct or indirect mechanism is conflicting. Studies by Tremblay and Marette show that rapamycin acts directly on cultured skeletal muscle to prevent the insulin-resistant effects of nutrient excess on insulin-mediated glucose transport[38]. The current study reveals a novel mechanism for rapamycin-induced derangements of glucose metabolism. This study demonstrates that ghrelin contributes to the development of derangements of glucose metabolism induced by rapamycin. Hyperglycemia induced by rapamycin was highly correlated with levels of plasma acylated ghrelin in both lean mice and obese animals. Rapamycin-induced derangements of glucose metabolism were blocked by ghrelin receptor antagonism or ghrelin receptor gene deletion. Increase of JNK phosphorylation induced by rapamycin in skeletal muscle was significantly attenuated by ghrelin receptor antagonism or ghrelin receptor gene deletion. In addition, ghrelin receptor antagonism or GHSR1a gene deletion reversed down-regulation of GLUT4 induced by rapamycin in skeletal muscle. Together with our previous report[30] that rapamycin inhibits mTOR signaling in gastric mucosa and stimulates transcription and translation of ghrelin, this study indicates that rapamycin may act on the stomach to regulate the production of acylated ghrelin, which in turn causes impairment of glucose transport in skeletal muscle. While direct evidence is still absent and requires the conditional deletion of mTOR signaling molecules in gastric ghrelin secreting cells, a line of findings[30] support the direct effect of rapamycin on gastric ghrelin secreting cells. (1) mTOR signaling molecules are selectively expressed in gastric ghrelin secreting cells and mTOR activity is reciprocally related to energy levels; (2) Reciprocal relationship exists between gastric mTOR activity and the expression and secretion of ghrelin during changes in energy status; (3) Inhibition of gastric mTOR signaling leads to increased expression of ghrelin and circulating ghrelin, while activation of gastric mTOR signaling suppresses its expression and secretion; (4) Overexpression of mTOR decreases ghrelin promoter activity, while blocking mTOR signaling by TSC1 or TSC2 increases its activity.

In addition to its orexigenic effect, ghrelin plays an important role in the regulation of glucose homeostasis[6]. Previous studies have demonstrated that ghrelin is able to suppress insulin secretion in several in vitro experimental models, including rodent islets[5], pancreatic cell lines[40], and perfused rat pancreas[41]. Ghrelin receptor antagonism or neutralization of endogenous ghrelin significantly increases insulin release from perfused pancreas[5]. Ghrelin deficiency in *ob/ob* mice causes enhanced insulin secretion and improved insulin sensitivity[42]. Blockade of pancreatic islet-derived ghrelin enhances insulin secretion to prevent glucose intolerance in mice fed high fat diet[7]. Ghrelin gene knockout mice demonstrate a significant increase in plasma insulin and a marked reduction of blood glucose levels[6]. In addition, the number of ghrelin-positive epsilon (ϵ)-cells is greater in transgenic mouse models lacking functional Nkx2-2, Pax6 or Pax4, with reciprocal beta-cell deficiency[43]. Taken together, these studies suggest that ghrelin may

alter glucose homeostasis by modulating the secretion of insulin. Our study demonstrates additionally that endogenous ghrelin may contribute to derangements of glucose metabolism by impairing glucose transport in the skeletal muscle. While ghrelin has been demonstrated to exercise its effect on energy metabolism via a central mechanism[3, 44], our study suggests that ghrelin may directly act through its receptor on the myocytes to regulate their glucose transportation. This data is consistent with previous reports demonstrating that ghrelin regulates the differentiation of myocytes[45] and that acylation is critical for the effect of ghrelin on myogenesis[46]. Other studies may limit the validity of this conclusion. Existence of a novel ghrelin receptor subtype has been reported[2]. While the involvement of a novel ghrelin receptor other than GHSR1a in the rapamycin-induced derangements of glucose metabolism cannot be completely excluded, following evidences indicate that ghrelin increases blood glucose via specific interaction with GHSR1a. The novel ghrelin receptor, if exists, is activated by des-acyl-ghrelin which demonstrates no effect on blood glucose levels[35]. In addition, both circulating and duodenal lipids have been shown to regulate glucose homeostasis by activating neurons in the hypothalamus and/or midbrain which subsequently inhibit glucose production in the liver[47]. These effects are likely mediated by the protein kinase C[48,49]. Since ghrelin has been reported to stimulate food intake by activation of its receptor in the hypothalamus, the possibility that ghrelin acts through a central neuronal mechanism to regulate the hepatic glucose production and therefore mediates the effect of rapamycin-induced glucose dysfunction cannot be excluded.

In summary, our study demonstrates that direct modulation of acylated ghrelin secretion is a crucial mechanism accounting for the derangements of glucose metabolism induced by rapamycin. Antagonism of ghrelin may therefore provide a potential therapeutic strategy for new-onset diabetes mellitus associated with rapamycin treatment after transplantation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

GHSR	growth hormone secretagogue receptor
mTOR	mammalian target of rapamycin
Rapa	rapamycin
S6	ribosomal protein S6

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FIG. 1. Hyperglycemia, derangements of glucose metabolism and hyperghrelinemia induced by rapamycin

Oral glucose tolerance tests (a, b and c) for normal C57BL/6J mice, high fat-induced obesity (DIO) mice and db/db mice. Insulin tolerance tests (d, e and f) for normal, DIO and db/db mice. Plasma acyl-ghrelin levels (g, h and i) for normal, DIO and db/db mice. (j, k and l) Depict western blot analysis of gastric S6 and phospho-S6 levels in normal, DIO and db/db mice. Data expressed as mean $\pm$ SEM; Six mice were examined for each condition. *P < 0.05 vs. control mice.

FIG. 2. Effect of ghrelin receptor antagonism or ghrelin receptor gene deletion on rapamycin-induced impairment of glucose metabolism

(a) and (b) depict results of oral glucose tolerance tests (a) and insulin tolerance tests (b) in male C57BL/6J mice receiving DMSO (black line), rapamycin (Rapa; 1 mg/kg, red line), D-Lys3-GHRP-6 (10 μ mol/kg, green line) or D-Lys3-GHRP-6 plus rapamycin (blue line). (g) Plasma acyl-ghrelin levels in C57BL/6J mice receiving DMSO, rapamycin, D-Lys3-GHRP-6 or D-Lys3-GHRP-6 plus rapamycin. (i) Depicts western blot results for gastric pS6 and gastric total S6 in the presence of various treatments. Results are expressed as mean $\pm$ SEM. Six mice were examined for each condition. * $P < 0.05$ vs. control, # $P < 0.05$ vs. D-Lys3-GHRP-6 treatment. (c) and (d) Illustrate oral glucose tolerance tests and insulin tolerance tests in normal and GHSR1a null mice. All animals were fed a normal chow diet.

(c) Rapamycin administration (Rapa; 1 mg/kg ip, red line) induced derangements of glucose metabolism in wild type mice but not in ghrelin receptor knock out littermates. (d) Similarly, rapamycin treatment caused abnormal insulin tolerance tests in normal mice but not in ghrelin receptor gene knockout mice.

Oral glucose tolerance tests and insulin tolerance tests in normal (WT) and GHSR1a null mice (GHSR^{-/-}) fed a high fat diet for 8 weeks were shown in (e, f). As shown in (e, f), oral glucose tolerance is significantly worsened and insulin sensitivity is decreased in mice fed a high fat diet following exposure to rapamycin (Rapa; 1 mg/kg ip). Rapamycin failed to induce alterations in glucose tolerance in ghrelin receptor knockout mice fed a high fat diet and effects on insulin sensitivity were significantly less pronounced. (h) Plasma acyl-ghrelin levels in WT and GHSR^{-/-} mice receiving DMSO or rapamycin. Western blot for gastric pS6 and gastric S6 for WT and GHSR^{-/-} mice receiving DMSO or rapamycin in the presence of normal chow diet (NCD) (j) or high fat diet (HFD) (k). Results expressed as mean±SEM. Six mice were examined for each condition. * denotes P <0.05 vs. control mice, # denotes P <0.05 vs. GHSR^{-/-} mice without rapamycin administration.

Total areas under curves (AUC) are calculated with Y values set as zero. Results are expressed as mean±SEM. * denotes P<0.05 vs control (DMSO) mice.

FIG. 3. Effect of ghrelin receptor antagonism or ghrelin receptor gene deletion on JNK activity in skeletal muscle

(a) Illustrates immunofluorescent staining for phospho-JNK (red) in gastrocnemius muscle derived from mice treated with DMSO, rapamycin (1mg/kg), D-Lys3-GHRP-6 or D-Lys3-GHRP-6 plus rapamycin for six days. Nuclei were stained with Hoechst dye. Controls include substituting primary antibodies with mouse IgG. Shown are representative results from six individual experiments.

(c) Depicts immunofluorescent staining for phospho-JNK (red) in gastrocnemius muscle derived from ghrelin receptor gene null mice or wild type littermates treated with DMSO or rapamycin (1mg/kg). The middle panels are results from mice treated with insulin (2 IU/mouse, ip) 10 min before harvest of tissues. Controls include substituting primary antibodies with mouse IgG.

(b, d) Fluorescent area and intensity of JNK staining were measured to quantify changes in phospho-JNK and expressed as mean $\pm$ SEM. Shown were representative results from six individual experiments.

FIG. 4. Effect of ghrelin receptor antagonism or ghrelin receptor gene deletion on glucose transport in skeletal muscle
 (a) Depicts immunofluorescent staining for GLUT4 (red) in gastrocnemius muscle derived from mice treated with DMSO, rapamycin (1mg/kg), D-Lys3-GHRP-6 or D-Lys3-GHRP-6 plus rapamycin for six days. Nuclei were stained with Hoechst dye. Controls include substituting primary antibodies with mouse IgG. Shown were representative results from six individual experiments.
 (c) Images depict immunofluorescent staining for GLUT4 (red) in gastrocnemius muscle derived from ghrelin receptor gene null mice or wild type littermates treated with DMSO or rapamycin (1mg/kg). The middle panels are results from mice treated with insulin (2 IU/

mouse, ip) 10 min before harvest of tissues. Controls include substituting primary antibodies with mouse IgG.

(b, d) Fluorescent area and intensity of GLUT4 staining in gastrocnemius muscle membrane were measured to quantify GLUT4 and expressed as mean $\pm$ SEM. Shown were representative results from six individual experiments.

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FIG. 5. Expression of GHSR1a mRNA
GHSR1a mRNAs were detected by RT-PCR using total RNA extracted from various mouse tissues as described in the Experimental Procedures. GAPDH mRNA was used as an internal control. Negative control used reaction product without reverse transcription. Shown was the result of four individual experiments.