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Journal of Surgical Research 130, 1–7 (2006)
doi:10.1016/j.jss.2005.08.003

Postoperative Ghrelin Levels and Delayed Recovery from Body Weight Loss after Distal or Total Gastrectomy

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Submitted for publication November 16, 2004

Background. **Body weight loss is a common but one of the most serious sequelae after gastrectomy. Ghrelin, a novel gastric hormone that up-regulates body weight through appetite control and metabolic reaction, seems to be affected by gastric surgery.**

Patients and methods. **Early postoperative alteration of serum ghrelin was evaluated at days 3 and 7 after gastrectomy for 13 hospital patients. In 64 outpatients who had previously undergone total gastrectomy (TG: 26 patients) or distal gastrectomy (DG: 38 patients) 4.6 months to 136 months (average, 41 months) earlier, the association between their serum ghrelin and leptin levels and postoperative body weight was investigated.**

Results. **Serum ghrelin declined immediately and greatly after TG to 12% of the preoperative level (day 3 and day 7), whereas the decline was less significant after DG at 39% (day 3) and 56% (day 7). In outpatients, serum ghrelin after TG was very low compared with the control (18.6 fmol/mL *versus* 92.1 fmol/mL, $P < 0.0001$), irrespective of the period after surgery, whereas the level after DG recovered and was equivalent to the control (73.4 fmol/mL, $P = 0.355$). Body weight loss was more apparent in TG patients than in DG patients, showing postoperative reduction of body mass index (ΔBMI) −3.940 *versus* −1.949 ($P < 0.0001$). Serum leptin concentration, reflecting the systemic fat volume, significantly correlated with BMI in both TG and DG patients, and tended to be lower in TG patients than in DG patients (800 pg/mL *versus* 1158 pg/mL, $P = 0.236$).**

Conclusion. **Persistent decline of serum ghrelin and body weight was observed commonly after total gastrectomy. Further study is needed as to whether or not ghrelin administration can improve the body weight level for these patients.**

Key Words: **ghrelin; gastrectomy; gastric cancer; body weight loss; post-operative sequelae.**

INTRODUCTION

Body weight loss is the one of the most serious sequelae of gastrectomy. Complex mechanisms, including reduced food intake because of early satiety and appetite loss [1–3], decrease of gastric acid [4], perturbation of fat absorption caused by reduced pancreatic excretion [3, 5], reflux esophagitis [6], alteration of intestinal floral [7], increased peristalsis, and diarrhea [8] have been considered, but there has been no completely satisfactory explanation. Alteration of endocrine status such as reduced gastrin [5] or increased cholecystokinin [2, 5] also has been suggested to be involved in body weight loss after surgery. On the other hand, in the case of morbid obesity, body weight loss caused by gastric surgery can be one of the most powerful treatment strategies, offering important clues for elucidating the mechanism of body weight loss after gastrectomy. Gastroplasty, which reduces the volume of the stomach, generally is less effective for weight reduction because of compensation by less but frequent food intake, whereas gastric bypass is more effective by suppressing appetite for a long time [9, 10]. Recently, some studies have shown that the effectiveness of gastric bypass might be associated with the reduction of a novel gastric hormone, ghrelin [10, 11].

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0022-4804/06 $32.00

Ghrelin was first identified as an intrinsic ligand for the growth hormone secretagogue (GHS) receptor in 1999 [12]. However, recent studies have revealed its function of stimulating not only GH secretion from the pituitary gland but also the appetite signal in the hypothalamus, opposing leptin [13] and gastrointestinal activity through the vagal nerve [14]. These pleiotrophic effects of ghrelin result in body weight gain associated with reduced fat metabolism [15]. Secretion of ghrelin is increased by starvation and suppressed by foods [16], glucose [17], fat [18], insulin [19], and somatostatin [20]. Most of the ghrelin is produced in the stomach in A-like cells in the fundic gland, and the duodenum and the brain secrete far smaller amounts [21]. The mechanism for reduced ghrelin levels associated with gastric bypass surgery is not known. It is speculated that although short periods of starvation stimulate the production of ghrelin, loss of contact with ingested foods for a long time may result in marked reduction of ghrelin production [11].

Although a few studies have reported the reduction of plasma ghrelin after gastrectomy [10, 16, 22] to date, the association between body weight loss and ghrelin reduction after gastrectomy has not been studied in detail. Thus, in this study, we investigated this association in representative surgical treatment for gastric cancer, including total gastrectomy (TG) with Roux-Y reconstruction and distal gastrectomy (DG) with Billroth-I reconstruction.

PATIENTS AND METHODS

Patients

Two groups of gastric cancer patients who gave their informed consent were enrolled in this study. Thirteen hospital patients were investigated for the early phase effects of gastrectomy on ghrelin production, and 64 outpatients were studied for the late-phase effects of gastrectomy on ghrelin and leptin production and their implications for postoperative body weight. Curative resection with standard D2 lymph node resection as previously described [23] was conducted for all patients. Roux-Y reconstruction was used for TG and Billroth-I reconstruction for DG. No surgical complications, such as pneumonia, infection, anastomotic leakage, pancreatitis, and ileus, were observed for both groups of hospital patients and outpatients. Those patients who exhibited body weight loss of more than 3 kg before surgery as the result of advanced gastric cancer were excluded from this study. Thus, the average body weight loss of enrolled patients was 0.82 kg and not statistically significant. Postoperatively, TG and DG patients underwent total and peripheral parenteral nutrition, respectively, and patients in neither group were given enteral nutrition.

For the 13 hospital patients, who underwent TG (8 patients) and DG (5 patients) from January to June 2003, 10 mL of blood sample was collected before and on day 3 and day 7 after gastrectomy in the morning before breakfast. None of them started oral food intake until day 3 after surgery, but DG patients started it on day 4 or 5 after surgery. The 64 outpatients who had undergone TG (26 patients) and DG (38 patients) came to our hospital for periodic examination of tumor recurrence from January to June 2003. A blood sample was collected from each patient at around 9 to 10 a.m. before breakfast. Clinical data, including height and body weight before and after surgery, the results of routine blood examinations, and the pathological stage and treatment of gastric cancer, were obtained from the patient medical records. Body mass index (BMI) was calculated as body weight (kg)/height^2 (m^2). The interval from the surgery of the subjects ranged from 4.6 to 136 months (average, 41 months), and none of them displayed tumor recurrence by chest x-ray, physical examination, abdominal CT scan or sonography, and serum tumor markers. The control group consisted of 29 patients with early digestive cancers who did not show significant body weight change attributable to cancer. Blood samples were collected before surgery, when they were admitted for surgical treatment (hemicolectomy 10 cases, anterior resection 7 cases, esophagectomy 9 cases and pancreaticoduodenectomy 3 cases).

Measurement for Serum Ghrelin and Leptin

The serum was obtained from each blood sample and kept at −70°C until measurement of ghrelin and leptin. Both serum ghrelin and leptin were measured with sandwich-type enzyme immunoassay kits for ghrelin (SCETI Co., Ltd., Tokyo, Japan) and leptin (IBL Co., Ltd., Gunma, Japan) according to the manufacturer's instructions. Measurement was performed in duplicate for each sample and the mean value was used. Two different forms of ghrelin, active (acyl) and inactive (desacyl) forms [24], were measured with different kits from the same company. Because the amount of the active form of ghrelin was very small and unstable in serum, the active form could not be measured for most serum samples, being only at most 3% to 5% of the inactive form, even when measurable. Thus, the amount of the inactive form was used in this study as being representative of serum ghrelin.

Statistical Analysis

The difference of variables, including BMI, postoperative decrease of BMI, serum ghrelin and leptin concentration, and days after surgery were evaluated by Student's t test between two groups (DG and TG patients) or by Bonferroni/Dunn test for three groups (DG, TG, and control group). The correlation between two each of these parameters was investigated by linear regression analysis. Early-phase postoperative change of ghrelin in DG and TG was evaluated by a repeated-measures analysis of variance. A P value of less than 0.05 was considered to indicate statistical significance. All statistical analyses were performed with the software package StatView ver.5.0 (Abacus Concepts, Berkeley, CA).

RESULTS

Early Phase Effect of Gastrectomy on Serum Ghrelin Concentration

The presence of gastric cancer did not affect serum ghrelin before gastrectomy in comparison with that of the control group (95.7 fmol/mL *versus* 92.1 fmol/mL, $P = 0.8553$). Early postoperative changes of serum ghrelin concentration in TG and DG patients are shown in Fig. 1. In TG patients, serum ghrelin levels were markedly decreased to 12% of the preoperative concentration at day 3 (11.7 fmol/mL) and remained at this level to day 7 (12.1 fmol/mL). In contrast, serum ghrelin concentration decreased to 39% (36.5 fmol/mL) of the preoperative level at day 3 after DG and slightly recovered up to 56% (52.3 fmol/mL) by day 7. The postoperative ghrelin was significantly lower than the preoperative level in both TG and DG patients ($P =$

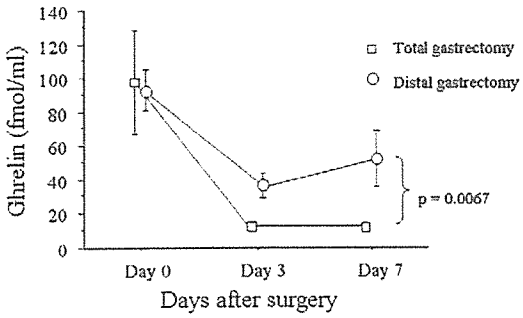

FIG. 1. Early phase effect of gastrectomy on serum ghrelin concentration. Serum ghrelin was measured before and on days 3 and day 7 after surgery. The averages of eight total (open squares) and five distal (open circles) gastrectomy patients are indicated with standard error bars. Significant reduction of serum ghrelin was observed at days 3 and day 7 for total gastrectomy patients and day 3 for distal gastrectomy patients.

0.0002 and $P = 0.0356$), and the decline was significantly less in the DG patients than in the TG patients ($P = 0.0067$).

Long-Term Effect of Gastrectomy on Serum Ghrelin and Leptin and Body Weight in Outpatients

The long-term effect of gastrectomy on serum ghrelin and leptin and body weight were evaluated for outpatients who had previously undergone surgery. Body weight loss after gastrectomy was ubiquitous in gastric cancer patients, and the reduction of BMI after surgery was more apparent in TG patients than in DG patients (–3.940 *versus* –1.949, $P < 0.0001$; Fig. 2A). The BMI after gastrectomy itself showed the same trend but was not statistically significant because of a large deviation of BMI both before and after surgery among patients. No significant difference was observed between TG and DG patients in their clinical background, including age, gender, BMI at surgery, duration after surgery, and stage of gastric cancer (Table 1). The average of serum ghrelin concentration was 18.6 fmol/mL in TG patients, 73.4 fmol/mL in DG patients, and 92.1 fmol/mL in the control group. Thus, there was little difference between DG patients and the control group ($P = 0.3851$), whereas TG patients displayed a significantly lower serum ghrelin level than the DG patients ($P = 0.0034$) or the control group ($P < 0.0004$; Fig. 2B). The average serum leptin concentration was 800 pg/mL in TG patients, 1158 pg/mL in DG patients, and 1507 pg/mL in the control group (Fig. 2C). This trend was consistent with their body weight, but the difference among them was not statistically significant. With respect to blood examinations, no differences were noted for serum albumin and lymphocytes, but hemoglobin was significantly lower in TG patients than in DG patients (Table 1).

The association between these weight control hormones and BMI is plotted in Fig. 3. Serum ghrelin concentration showed significant negative correlation with BMI in DG patients (Fig. 3C). However there was no correlation for TG patients (Fig. 3A), probably because ghrelin levels were consistently low. In contrast, serum leptin concentration correlated well with the BMI in both groups, showing the same regression line (Fig. 3B and 3D).

Instead of long-term follow up for each patient, the association of the duration after gastrectomy, ranging from 4.6 months to 11.2 years, with body weight and serum ghrelin was investigated, to speculate their time-dependent alteration (Fig. 4). BMI did not show correlation with postoperative time in either TG or DG patients (Fig. 4A and 4C). Ghrelin tended to increase with postoperative time in DG patients (Fig. 3D, $P = 0.1007$), however such trends were not seen in TG patients (Fig. 3C, $P = 0.2685$). Likewise, serum leptin tended to increase in DG but not in TG patients (data not shown).

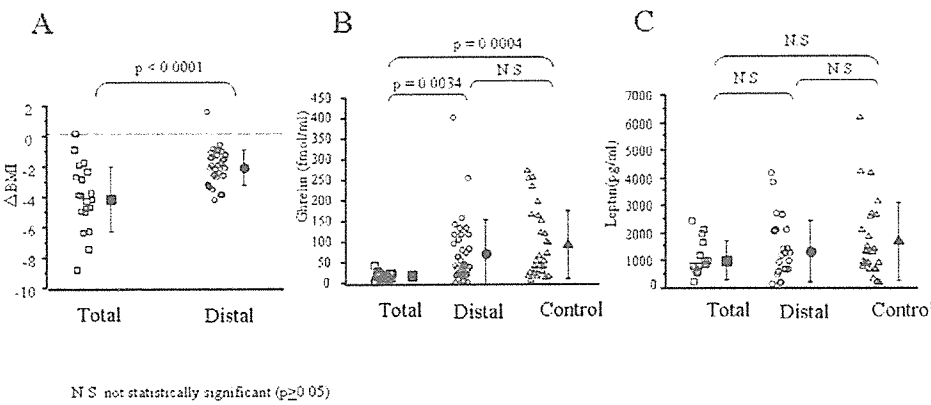

FIG. 2. Alteration of body weight and weight control hormones (ghrelin and leptin) after gastrectomy. Body weight loss (ΔBMI) (A), serum ghrelin (B), and serum leptin (C) of total gastrectomy patients (squares), distal gastrectomy patients (circles), and control individuals (triangles) were plotted (open) along with their averages (closed) and standard deviation bars. Differences between the two groups were evaluated by Student's *t* test and *P* values are indicated.

TABLE 1

Background of 64 Outpatients Who Underwent Gastrectomy

	Total gastrectomy (n = 26)	Distal gastrectomy (n = 38)
Age (y.o.) at surgery	65.0	60.1
Gender (M : F)	18 : 8	27 : 11
Duration from surgery (months)	41.5	42.9
BMI at surgery	23.7	22.6
Depth of invasion (T1 : T2 : T3)	10 : 9 : 7	20 : 12 : 6
Lymph node metastasis (absent : present)	10 : 16	20 : 18
Histological grade (G1,2 : G3,4)	10 : 16	23 : 15
Hemoglobin (g/dl) after surgery	11.5	13.5
Albumin (g/dl) after surgery	4.4	4.5
Lymphocytes (/mm^3) after surgery	2264	1908

Note. Average values are shown for age, duration from surgery, BMI at surgery, hemoglobin, albumin, and lymphocytes. TNM classification was used for depth of invasion and histological grade. Hemoglobin, albumin, and lymphocyte were measured along with ghrelin and leptin. Significant difference was present only for hemoglobin ($P < 0.0001$, Student's t test).

DISCUSSION

In this study, we found that gastric cancer patients after TG displayed significant body weight loss and immediate and remarkable decline of serum ghrelin, which did not recover even after a long postoperative period. This relationship raised the novel question of whether or not the decline of ghrelin causes body weight loss after TG. The most obvious reason for body weight loss after TG is reduction of oral food intake [1, 3]. Surgeons dealing with gastric cancers have tried to increase food intake by producing a gastric substitute, such as a jejunal pouch, but have not always been successful. However, surgeons dealing with morbid obesity have shown that the effect of gastric volume reduction for body weight control is limited because of a rebound action, i.e., small-but-frequent food intake [9, 10]. In addition, under a regulated program, most TG patients can eat as much as healthy subjects [1]. These phenomena suggest early satiety and loss of appetite are causes of reduced food intake. Appetite is regulated by the hypothalamus via many hormones. Although it is still controversial, some studies speculate appetite loss after gastrectomy to be associated with increased intestinal hormones such as cholecystokinin [2, 25, 26] and serotonin [26, 27]. The present study suggests that ghrelin may play a central role in regulating appetite by the digestive tract. It is of interest that intestinal hormones other than ghrelin generate appetite-suppressing signals but only ghrelin promotes a stimulating signal. Interference of fat digestion and accumulation are characteristic metabolic perturbations of post gastrectomy patients [3, 28]. This is partly caused by disorder of pancreatic excretion. Although

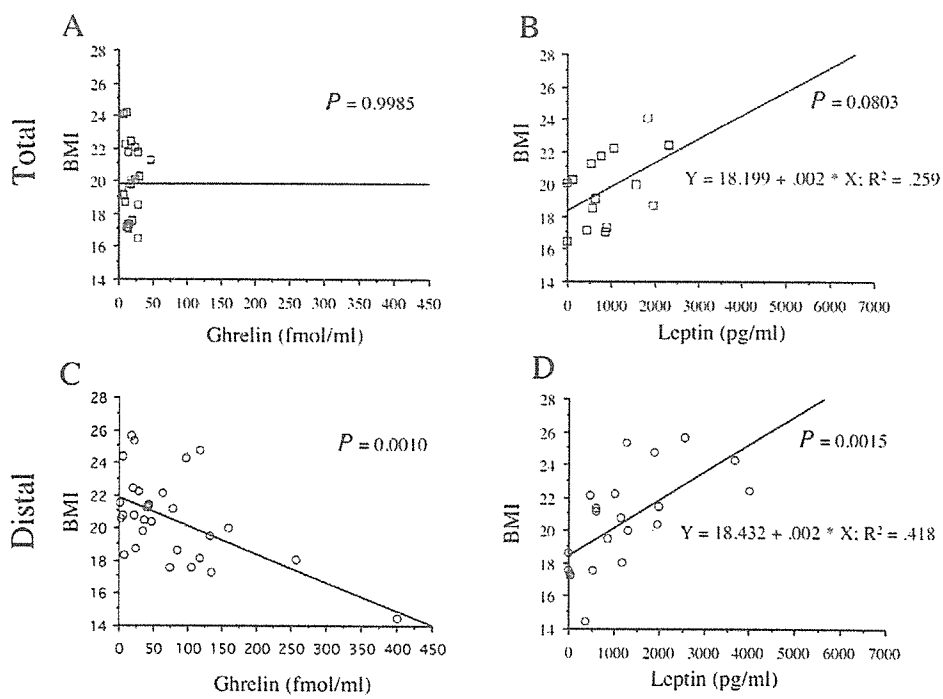

FIG. 3. Correlation between postoperative body weight and weight control hormones. Association of postoperative body weight with serum ghrelin (A, C), leptin (B, D) in total (A, B), and distal gastrectomy (C, D) patients are shown with each linear regression line and P value. The formula of the regression line is given in B and D.

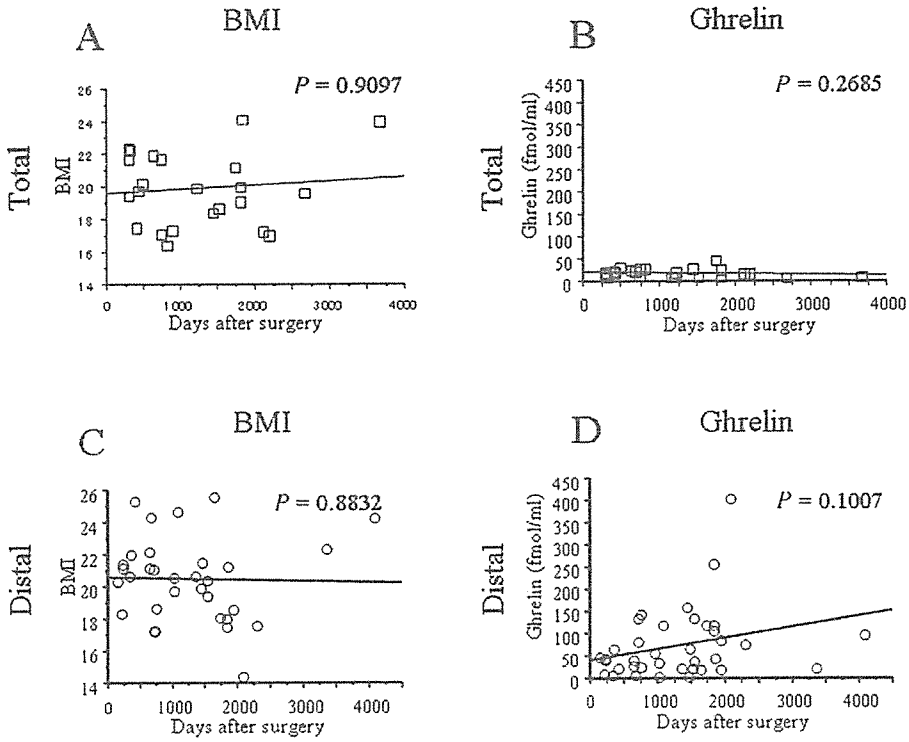

FIG. 4. Association of postoperative duration with body weight and serum ghrelin. The association of post-operative duration with body weight (A, C) and serum ghrelin (B, D) for total (A, B) and distal gastrectomy (C, D) patients is shown with each linear regression line and *P* value.

the influence of ghrelin on pancreatic excretion has not been fully elucidated [29, 30], alteration of the fat metabolism [15] is influenced by ghrelin administration. Endocrine of growth hormone (GH) is regulated mainly by GH-releasing hormone, ghrelin, and somatostatin [12]. Reduction of GH and prevention of body weight loss after gastrectomy by exogenous GH strongly [31] suggest the clinical usefulness of ghrelin supplementation after gastrectomy. However, our finding that TG patients displayed various degrees of postoperative body weight loss despite extreme and ubiquitous ghrelin decline may suggest the involvement of pathways other than ghrelin in post operative body weight control. Further work is needed to clarify this.

In DG patients, serum ghrelin declined by approximately 50% after surgery, but recovery was immediate, and no significant difference from the control was found after longer follow up, in agreement with a recent study [32]. In addition, a significant negative correlation was found between BMI and ghrelin for these patients. This means that as for nongastrectomized individuals [33, 34], ghrelin works in a negative feedback loop against body weight, i.e., ghrelin alteration is not a cause of, but a result of, postoperative body weight loss. The procedure of DG removes two-thirds of the stomach, including approximately half of the fundic gland lesion, which matches the serum ghrelin concentration after surgery. Ghrelin recovered to the normal level and some patients with longer survival periods after DG even showed a higher level than the control. Ghrelin production may be stimulated in the residual stomach, such as by means of hyperplasia or hypertrophy. This possibility could be investigated by immunohistochemistry and mRNA measurement of the residual stomach. Another possibility is that ghrelin reduction by gastric removal is compensated for by increased ghrelin production in the duodenum. It is commonly understood that reconstruction for gastrectomy with ingestion passing the duodenum is superior to that without it, with respect to postoperative body weight and nutritional status, for example, Billroth-I *versus* Billroth-II for distal gastrectomy [35] or jejunal interposition *versus* Roux-Y for total gastrectomy [36, 37]. The reason for this has also not been well understood. It should be interesting to investigate the amount of ghrelin in these groups.

Ghrelin is secreted as an active (acyl) form and immediately inactivated (des-acyl form) by elastase and protease in the blood [38]. We measured des-acyl ghrelin because most of the serum ghrelin is inactivated in the serum and the proportion of active/inactive ghrelin is relatively constant. However, recent study revealed an additional role of des-acyl ghrelin and the alteration of the active/inactive ghrelin ratio under some pathological conditions [38]. For precise

measurement of acylated ghrelin, the plasma sample must be preserved with protease inhibitors. Further studies are needed to clarify these issues.

Excess body weight loss after gastrectomy induces general fatigue and decline of social activity. In addition, it impairs the immune system [39], making the patient more susceptible to infectious disease and cancer recurrence. Antagonists of intestinal hormones, including cholecystokinin or serotonin, have been reported to increase body weight after gastrectomy in animal experiments [27, 40] and their clinical application has been considered. However, the side effects of such non-physiological compounds have not been evaluated. Ghrelin is an intrinsic hormone, and the safety of recombinant ghrelin and its clinical usefulness have been confirmed by clinical trials for patients with heart failure [41]. To prevent body weight loss after gastrectomy, the effect of ghrelin should be better than that of antagonists of intestinal hormones because of its pleiotrophic function, including increasing appetite, metabolism and GH release. The increase of early gastric cancers and development of fiberscopic treatment should urge surgeons to try to improve the quality of life after gastrectomy by choosing a suitable therapeutic modality. Ghrelin offers much promise for a new form of drug therapy for gastrectomy patients.

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Ghrelin Improves Renal Function in Mice with Ischemic Acute Renal Failure

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Growth hormone and IGF-1 have been suggested to have tissue-protective effects. Ghrelin is a stomach-derived growth hormone secretagogue. The effects of ghrelin on ischemia/reperfusion-induced renal failure in mice were examined. Ischemic acute renal failure was induced by bilateral renal artery clamping for 45 min and reperfusion for 24 h. Ghrelin (100 μg/kg mouse) or vehicle was injected subcutaneously six times before surgery and three times after surgery every 8 h. Twenty-four hours after reperfusion, the right kidney was isolated and perfused. Acetylcholine (ACh)- and adrenomedullin-induced endothelium-dependent vasorelaxation of renal vessels significantly improved in ghrelin-pretreated mice (%Δ renal perfusion pressure by 10^{-7} M ACh −63.5 ± 3.7 *versus* −41.2 ± 5.5%; $P < 0.05$). This change was associated with significant increases of nitric oxide release in the kidneys of ghrelin-treated mice (10^{-7} M ACh 35.5 ± 5.8 *versus* 16.9 ± 3.5 fmol/g kidney per min; $P < 0.05$). Serum concentration of urea nitrogen (53 ± 7 *versus* 87 ± 15 mg/dl; $P < 0.05$) and renal injury score were significantly lower in the ghrelin group (2.5 ± 0.8 *versus* 5.3 ± 1.5; $P < 0.01$). Tubular apoptotic index was significantly lower in the ghrelin group (5 ± 5 *versus* 28 ± 4; $P < 0.05$). Furthermore, the survival rate after the 60-min ischemic period was higher in the ghrelin group (80 *versus* 20%; $P < 0.05$). Ghrelin treatment significantly increased the serum level of IGF-1. However, such renal protective effects of ghrelin on ischemia/reperfusion injury were not observed in insulin receptor substrate-2 knockout mice. These results suggest that ghrelin may protect the kidneys from ischemia/reperfusion injury and that this effect is related to an improvement of endothelial function through an IGF-1–mediated pathway.

J Am Soc Nephrol 17: 113–121, 2006. doi: 10.1681/ASN.2004080626

Ghrelin has a growth hormone (GH)-releasing effect. It was first isolated from the stomach and is known as an endogenous ligand for GH secretagogue receptor (GHSR) (1). Ghrelin is a peptide of 28 amino acids with an *n*-octanoyl modification at serine-3, and this modification is closely related to its physiologic activity. Studies in healthy volunteers have shown that ghrelin increases the cardiac index and stroke volume and decreases the mean arterial pressure. These effects were associated with upregulation of GH and IGF-1 (2). Ghrelin also has beneficial effects on left ventricular systolic function and energy metabolism in severe heart failure and improves cardiac cachexia (3–5). GH and IGF-1 improve severe heart failure caused by dilated cardiomyopathy and ischemic heart disease (6–8). These findings suggest that the cardiac effect of ghrelin is exerted through an increase of GH release. There have also been reports on the renal protective effects of GH and IGF-1 against various types of renal damage (9), although several other reports did not confirm this effect (10). As for the renal protective mechanism, induction of nitric oxide (NO) and cGMP in the kidney by GH and IGF-1 were suggested to improve renal circulation (11,12).

It is still controversial whether ghrelin has a GH-independent effect on cardiovascular function. GHSR widely distributes and exists in the heart and vessels (13). Moreover, intra-arterial infusion of ghrelin in healthy individuals dose-dependently increased blood flow without changes in serum IGF-1 concentration (14), indicating the possibility of a direct cardiovascular action of ghrelin.

Despite the reports on the cardiac effects of ghrelin, there are no data on the protective effect of ghrelin in organs other than the heart. In this study, we investigated whether ghrelin improved ischemic acute renal failure (iARF) and whether ghrelin influenced vascular endothelial function in mice. Furthermore, to explore the role of IGF-1 in the effects of ghrelin, we studied the effects of ghrelin in insulin receptor substrate-2 (IRS-2) knockout (KO) mice.

Materials and Methods

Animals

All studies were performed in concordance with the university guidelines for animal experiments. Adult male BALB/C mice that weighed 30 to 35 g were obtained from Charles River Laboratories

Received August 1, 2004. Accepted September 29, 2005.

Published online ahead of print. Publication date available at www.jasn.org.

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ISSN: 1046-6673/1701-0113

(Yokohama, Japan). The IRS-$2^{-/-}$ mice were maintained on the original C57BL6/CBA hybrid background and were prepared by IRS-$2^{+/-}$ mouse intercrosses (15).

iARF

iARF was induced in 8- to 12-wk-old BALB/C and IRS-$2^{-/-}$ mice as described elsewhere (16). In brief, after anesthesia with pentobarbital (40 mg/kg, intraperitoneally), a middle abdominal incision was made and bilateral renal arteries were clamped for 45 min. After declamping, we confirmed the restoration of renal blood flow and closed the incision. Twenty-four hours after the start of reperfusion, 1.0 ml of blood was drawn to measure the serum level of blood urea nitrogen (BUN), creatinine, and IGF-1. Thereafter, the right kidney was used for isolated perfusion and the left kidney was used for histologic examination and analysis of renal tubular cells apoptosis.

Administration of Ghrelin

Rat ghrelin was obtained from the Peptide Institute (Osaka, Japan). Ghrelin (100 μg/kg mouse) was dissolved in 0.9% saline that contained BSA and was subcutaneously injected six times before ischemia every 8 h and three times after ischemia. An equal volume of the vehicle was injected into the control mice. To confirm the rationality of our protocol, we also examined ghrelin's effect after a single injection given just before ischemia and 8 h after reperfusion.

Isolated Perfused Kidney

Male BALB/C and IRS-$2^{-/-}$ mice that were treated with vehicle or ghrelin were anesthetized with pentobarbital (40 mg/kg, intraperitoneally), then the right kidney was isolated and perfused as described previously (17). In brief, after an abdominal incision, a 24-G needle was inserted into the right renal artery and then renal perfusion was started with Krebs-Henseleit buffer. The buffer was saturated with 95% O_2/5% CO_2 and contained 10^{-6} mol/L angiotensin II and 10^{-5} mol/L indomethacin to maintain the renal perfusion pressure (RPP) at approximately 100 mmHg. After a 60-min equilibrium period, graded doses of acetylcholine (ACh; 10^{-8} to 10^{-7} M) and adrenomedullin (AM; 10^{-10} to 10^{-7} M) were added to the buffer at 10-min intervals, and RPP was monitored through a pressure transducer (Datex-Ohmeda K.K., Tokyo, Japan). The renal vein was also cannulated to drain the perfusate into the NO assay system.

Measurement of NO Released from Kidney

We measured NO concentration in the perfusate from the renal vein using a chemiluminescence assay as described previously (17–19). The venous effluent was introduced into a rotatory mixer with a chemiluminescence assay probe of 10 mmol/L H_2O_2, 18 mmol/L recrystallized luminol, 2 mmol/L potassium carbonate, and 150 mmol/L desferrioxamine. The mixture of the perfusate and probe then entered a chemiluminescence detector. The chemiluminescent signal was measured continuously and was recorded using a pen recorder. The NO signal was calibrated using an NO solution. NO release was normalized by kidney weight and expressed as femtomoles per minute per gram of renal tissue.

Measurement of cGMP Level in the Mouse Kidney

After the NO measurement, we perfused the kidney for 15 min with 10^{-8} M AM through the renal artery. Then the kidneys were homogenized in 4% TCA (pH 4.0) on ice. After centrifugation, the supernatant was extracted four times with water-saturated ether and then evaporated. The pellets were redissolved in a buffer solution. The cGMP content was assayed using an ELISA kit according to the manufacturer's recommendation (Amersham Biosciences Corp., Piscataway, NJ) (20).

Histologic Studies

Tissue samples were fixed in 4% paraformaldehyde and embedded in paraffin. We obtained 5-mm sections and stained them with the periodic acid-Schiff reagent. We conducted a semiquantitative histologic analysis. Twenty tubules or glomeruli in each kidney were randomly selected at a ×400 magnification, and the degree of renal damage was scored using the scoring system for renal injury reported by Solez *et al.* (21). We calculated the mean renal injury score in each mouse and then averaged the scores for each group. The sections were examined by a pathologist in a blinded manner. We examined the tissues for the presence of expansion of Bowman's space, interstitial edema, epithelial detachment, and tubular cells casts. Renal morphologic changes were graded on a scale of 0 to 3+: 0, normal; 1+, slight; 2+, moderate; and 3+, severe.

Detection of Apoptotic Cells

To examine the antiapoptotic effect of ghrelin, we performed terminal deoxynucleotidyl transferase mediated dUTP nick end-labeling (TUNEL) staining of renal tubular cells. Nuclei were also counterstained with propidium iodine and mounted with ProLong Antifade Kit (Molecular Probes, Eugene, OR). The sections were observed using a confocal microscope (FLUOVIEW FV300, Olympus, Tokyo). The apoptotic index was calculated as the number of TUNEL-positive nuclei per high-power field (×400).

Survival Rate of Mice with iARF

To examine the effect of ghrelin on the survival of mice with ARF, we prolonged the duration of renal arterial clamping from 45 to 60 min. After removal of the clamp, we closely observed the mice during a 36-h reperfusion period.

Statistical Analyses

All data are expressed as the mean value ± SEM. Statistical comparisons were made by ANOVA followed by the Student-Neumann-Keuls test. To compare renal injury scores, we used the nonparametric Kruskal-Wallis test. The survival rate of mice after 60 min of ischemia and 36 h of reperfusion was estimated with the Kaplan-Meier method. $P < 0.05$ was considered statistically significant.

Results

Effects of Ghrelin on Renal Vascular Endothelial Function

Body weight, kidney weight, and RPP of the four groups of mice are summarized in Table 1. Bilateral kidneys from BALB/C mice were macroscopically normal. The kidney weight was significantly greater in iARF mice than in sham-operated mice. Baseline RPP in the iARF group was higher than in the sham-operated group. Vehicle-treated mice with iARF showed significantly higher RPP than ghrelin-treated mice with iARF (Table 1).

The effect of ACh and AM on RPP and NO release in the four groups are shown in Figure 1. They lowered RPP of kidneys in all groups in a dose-dependent manner. The endothelium-dependent vasodilatory effect of them was significantly greater in the sham-operated mice than in the iARF mice. In sham-operated mice, ghrelin did not modify the renal vascular response. However, in iARF mice, treatment with ghrelin significantly increased ACh- and AM-induced vasodilation. The ACh- and AM-induced NO release from the kidney was greater in the ghrelin group of iARF mice than in the vehicle group (Figure 1).

Table 1. Baseline characteristics of mice that had iARF and were treated with vehicle or ghrelin[a]

	n	BW (g)	KW (g)	KW/BW (%)	Baseline RPP (mmHg)
BALB/C					
sham + vehicle	8	27.6 ± 2.6	0.137 ± 0.042	0.496 ± 0.125	79.6 ± 5.5
sham + ghrelin	8	28.6 ± 2.1	0.132 ± 0.058	0.462 ± 0.117	87.6 ± 4.2
iARF + vehicle	8	25.9 ± 3.5	0.173 ± 0.079[b]	0.668 ± 0.098[b]	110.3 ± 10.9[c]
iARF + ghrelin	8	27.8 ± 2.2	0.159 ± 0.059[d]	0.572 ± 0.143[e]	95.3 ± 7.5[e,f]
IRS-2 KO					
iARF + vehicle	4	29.5 ± 2.1	0.158 ± 0.009	0.54 ± 0.04	110.6 ± 5.4
iARF + ghrelin	4	28.8 ± 0.8	0.165 ± 0.006	0.57 ± 0.03	111.3 ± 7.5

[a]Values are means ± SEM. BW, body weight; KW, kidney weight; RPP, renal perfusion pressure; KO, knockout.
[b]$P < 0.05$ *versus* sham + vehicle.
[c]$P < 0.01$ *versus* sham + vehicle.
[d]$P < 0.05$ *versus* sham + ghrelin.
[e]$P < 0.05$ *versus* iARF + vehicle.
[f]$P < 0.01$ *versus* sham + ghrelin.

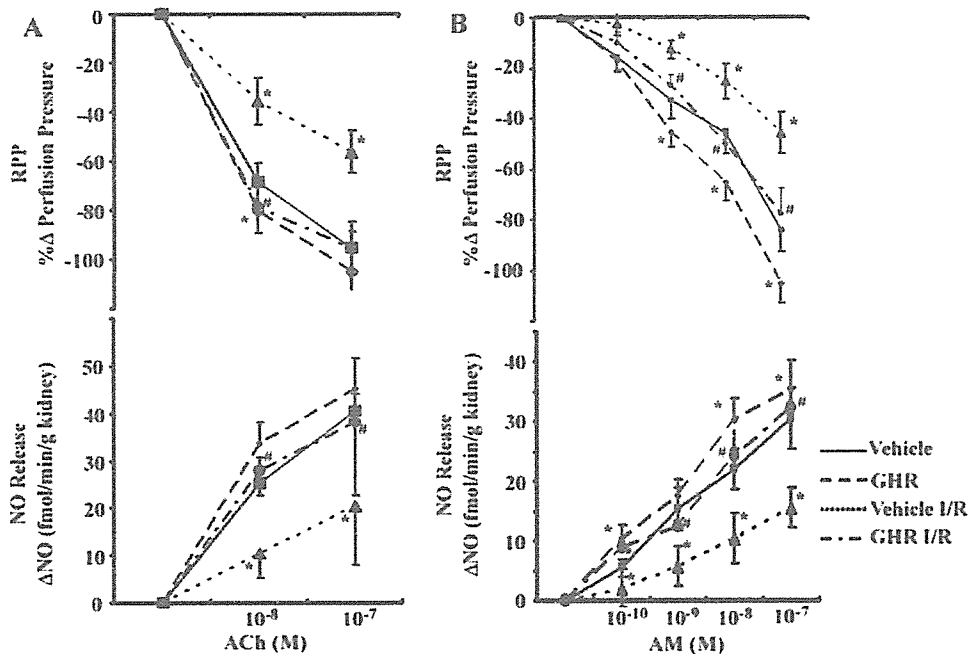

Figure 1. Effects of acetylcholine (ACh; A) and adrenomedullin (AM; B) on renal perfusion pressure (RPP) and nitric oxide (NO)-releasing activity in the vehicle, ghrelin, vehicle–ischemia/reperfusion (I/R) and ghrelin-I/R groups. NO concentration in the venous effluent was measured by luminol chemiluminescence assay. *$P < 0.05$ *versus* vehicle; #$P < 0.05$ *versus* vehicle-I/R. Bars indicate means ± SEM; $n = 8$.

To examine the involvement of the NO-cGMP pathway, we measured cGMP in the kidneys of mice in the two groups. The renal content of cGMP was significantly greater in the ghrelin group (Figure 2).

Effects of Ghrelin on Ischemia/Reperfusion Injury of the Kidney

None of the mice died of iARF when the renal arteries were clamped for 45 min. Figure 3 shows the renal histology stained with periodic acid-Schiff reagent. In the vehicle group, remarkable damage, particularly in the tubuli, was observed. Renal damage included detachment of epithelial cells of the tubuli, interstitial edema, and many tubular cell casts. Bowman's space was also remarkably expanded. The kidneys of the mice that were administered ghrelin were also damaged, but the extent of the injuries was less than that of injuries observed in the control mice. The renal injury scores of the four groups are shown in Figure 4. The ischemia/reperfusion (I/R) procedures resulted in significantly greater increases in the injury scores, and administration of ghrelin reduced renal damage (vehicle 0.6 ± 0.1, vehicle I/R 5.3 ± 1.5, ghrelin 0.5 ± 0.1, ghrelin I/R 2.5 ± 0.8).

The result of these histologic studies was supported by the measurement of renal excretory function. Twenty-four hours

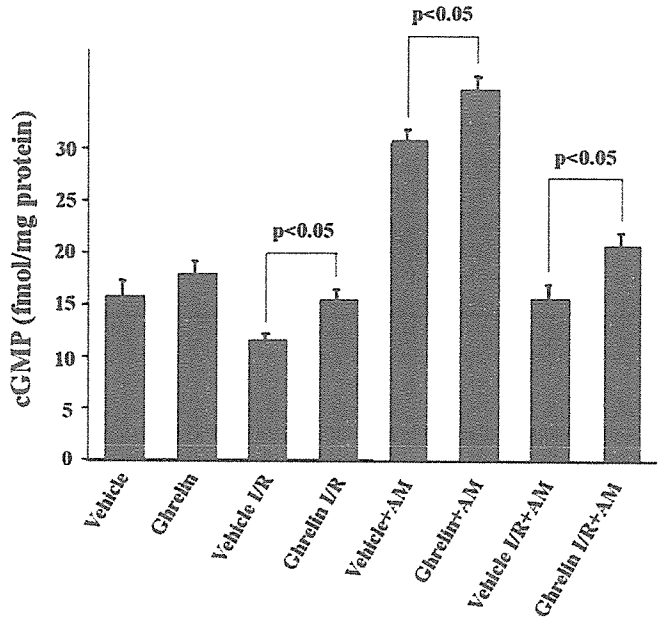

Figure 2. cGMP production in isolated kidneys from mice. The kidneys were stimulated with 10^{-8} M AM, and cGMP extracted from the kidneys was measured by ELISA. Bars indicate means ± SEM; $n = 8$.

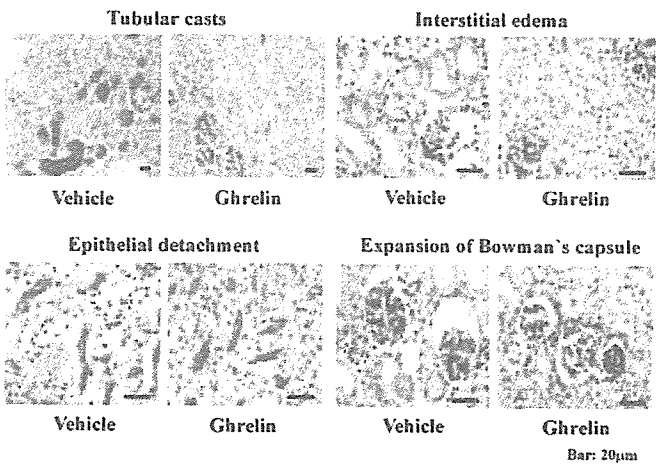

Figure 3. Photographs of renal tissue stained with periodic acid-Schiff reagent. Tubular cell casts, interstitial edema, epithelial detachment, and expansion of Bowman's capsule were observed in the kidneys that were treated with vehicle or ghrelin.

after reperfusion, the concentration of serum BUN and creatinine was markedly elevated in the two I/R groups. The degree of impairment of renal function was significantly smaller in the ghrelin group than in the vehicle group (Figure 5). When we injected ghrelin just before ischemia and 8 h after reperfusion, the serum levels of BUN and creatinine and the renal injury score increased in the two groups, and there were no significant differences between the two groups (BUN 181 ± 21 *versus*

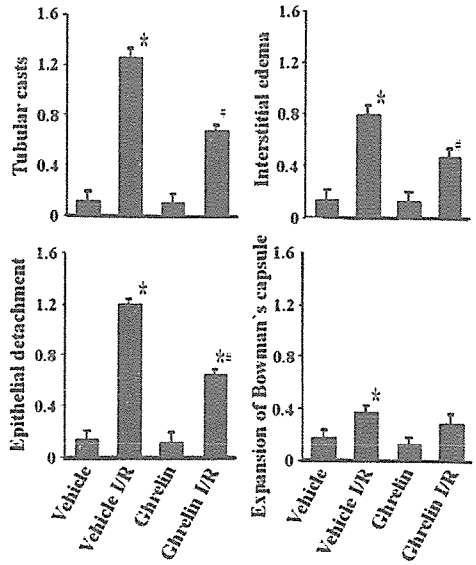

Figure 4. Four types of renal injury scores of vehicle-, ghrelin-, vehicle-I/R–, and ghrelin-I/R–treated mice. *$P < 0.05$ *versus* vehicle; #$P < 0.05$ *versus* vehicle-I/R. Bars indicate means ± SEM; $n = 8$.

176 ± 7, NS; Cr 1.9 ± 0.3 *versus* 2.1 ± 0.1, NS; renal injury score 6.7 ± 0.2 *versus* 7.1 ± 1.3, NS)

Antiapoptotic Effect of Ghrelin

Figure 6 shows apoptosis of renal tubular cells detected by the TUNEL staining method. In both groups with I/R-induced renal injury, apoptosis of proximal tubular cells was particularly prominent. However, administration of ghrelin resulted in a significantly decreased number of apoptotic cells in the kidneys, as compared with vehicle administration.

Survival Rate of Mice with iARF

When the renal arterial clamping period was 45 min, none of the mice died. However, after 60 min of ischemia, most mice that were administered the vehicle solution died by 36 h after reperfusion. Treatment with ghrelin substantially increased the survival of the mice (Figure 7).

Effect of Ghrelin on the IGF-1/IRS Pathway

To explore the mechanism for the renal protective effect of ghrelin, we examined the direct vascular effect of ghrelin. However, ghrelin did not substantially influence the vascular tone in the isolated aorta or isolated perfused kidney. We also examined the effect of ghrelin on apoptosis of cultured human umbilical vein endothelial cells caused by serum deprivation. We did not detect an antiapoptotic action of ghrelin in cultured cells (data not shown).

Next, we examined the indirect effects of ghrelin. Because ghrelin may upregulate IGF-1 *via* stimulation of GH, we measured serum IGF-1 concentration in these mice. Furthermore, to examine the role of the IGF-1/IRS pathway in ghrelin-induced renal protection, we repeated the same experiment using IRS-2

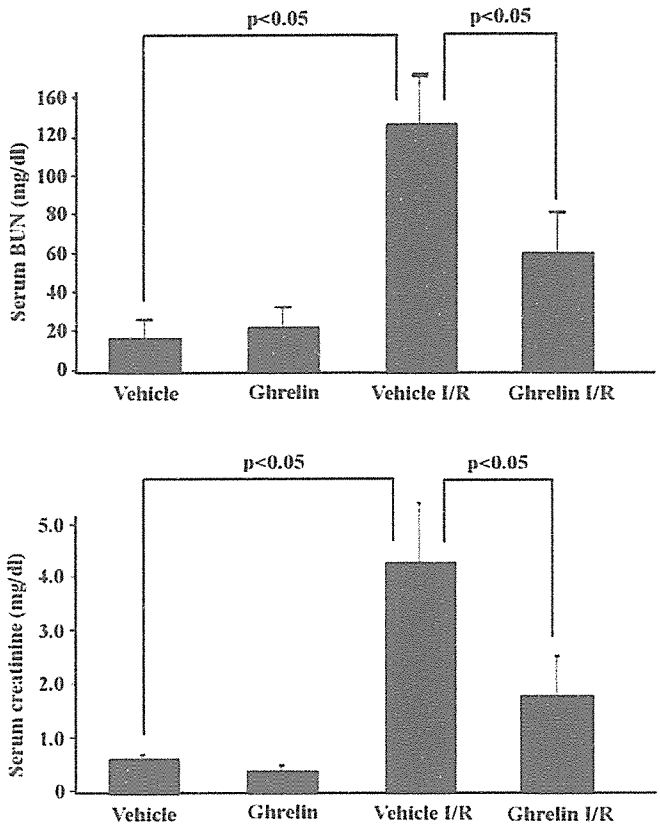

Figure 5. Serum levels of urea nitrogen and creatinine in sham-operated mice and mice that were subjected to renal I/R. Bars indicate means ± SEM; $n = 8$.

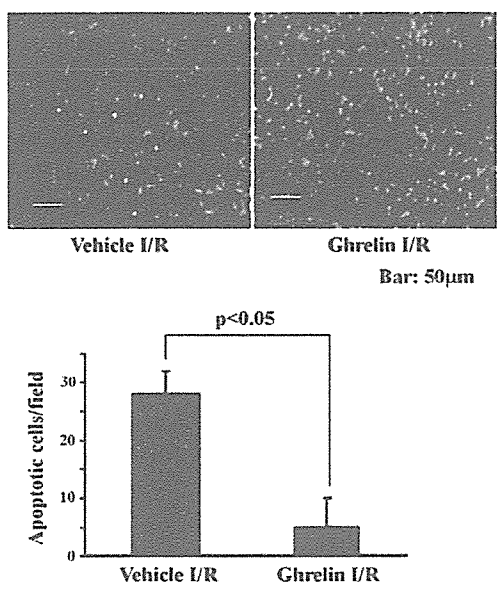

Figure 6. Photographs of apoptotic tubular cells and the numbers determined by terminal deoxynucleotidyl transferase mediated dUTP nick end-labeling (TUNEL) technique. TUNEL-positive cells are shown in yellow. Bars indicate means ± SEM; $n = 8$.

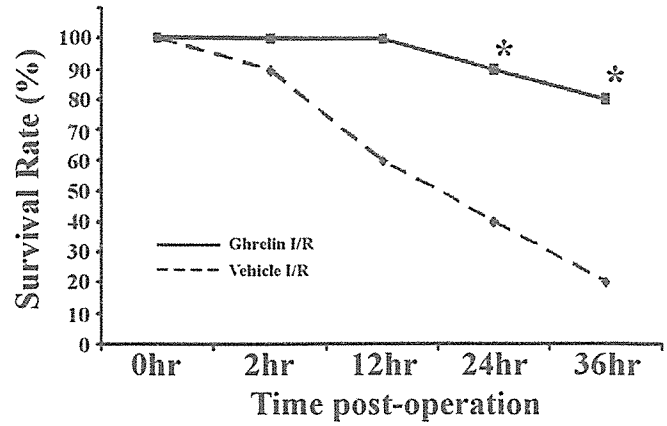

Figure 7. Survival rates of mice after ischemic acute renal failure (iARF) induced by 60 min of clamping of bilateral renal arteries in the vehicle and ghrelin groups. $^*P < 0.05$ *versus* vehicle-I/R; $n = 10$.

KO mice. As a result, serum IGF-1 concentration was significantly higher in the ghrelin group than in the vehicle group (Figure 8).

Ischemia for 45 min and reperfusion for 24 h caused iARF also in IRS-2 KO mice. Serum BUN and creatinine levels were markedly high in both treated mice. Their levels were slightly lower in the ghrelin group than in the vehicle group, but the differences were not statistically significant (Figure 9). With regard to histologic analysis, both groups of mice showed marked renal damage. The renal injury scores were almost similar between the two groups. Furthermore, the baseline perfusion pressure of the kidney obtained from IRS-2 KO mice was almost the same between the two groups (vehicle 110.6 ± 5.4 *versus* ghrelin 111.3 ± 7.5 mmHg; NS). There was no significant difference in ACh-induced endothelium-dependent vasorelaxation of isolated perfused kidneys between the vehicle-treated group and the ghrelin-treated group (Figure 9), indicating lack of renal protective effects of ghrelin in IRS-2 KO mice.

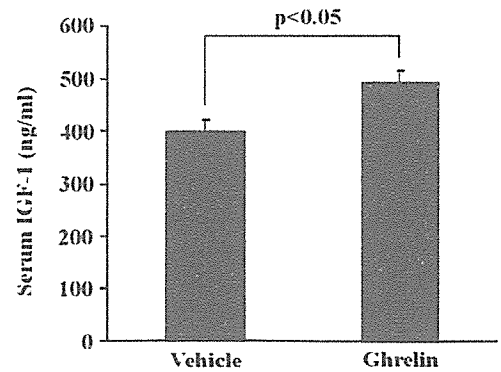

Figure 8. Serum IGF-1 concentrations in vehicle- and ghrelin-treated mice. Bars indicate means ± SEM; $n = 6$.

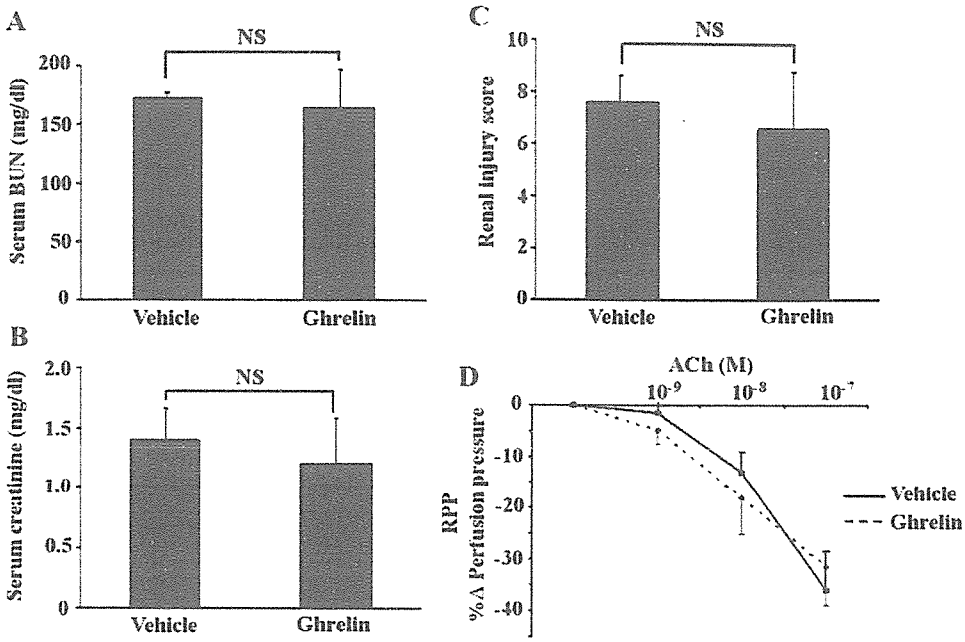

Figure 9. Serum levels of urea nitrogen (A) and creatinine (B), renal injury scores (C), and the effect of ACh on RPP (D) in iARF of IRS-2 knockout mice that were treated with vehicle and ghrelin. Renal injury scores are the sums of four injury scores (expansion of Bowman's space, interstitial edema, epithelial detachment, and tubular cell casts). Bars indicate means ± SEM; $n = 4$.

Discussion

In this study, we showed that ghrelin improved renal tissue damage and renal excretory function in the mice with iARF. These beneficial effects of ghrelin were associated with renal endothelium-dependent vasodilation and increases in NO/cGMP formation, suggesting an improvement of vascular endothelial function in the kidneys. However, no favorable effects of ghrelin were observed in IRS-2 KO mice, although the circulating IGF-1 level was significantly increased by ghrelin administration.

The detailed mechanisms by which ghrelin mitigates iARF are not clear. Previous reports showed that ghrelin treatment increased serum GH and IGF-1 concentrations (2,5) and that GH and IGF-1 exerted a tissue-protective action through endothelial NO formation (11,12). IGF-1 releases NO *via* activation of phosphatidyl inositol-3 kinase (PI3K) and its downstream effector Akt (22–24). Before activation of PI3K, it is necessary that IGF-1 bind to IGF-1 receptor and phosphorylates IRS (25). The IRS proteins are phosphorylated by insulin and IGF-1 stimulation, and four members of this family have been identified (IRS-1, IRS-2, IRS-3, and IRS-4). Through the analysis of IRS KO mice, IRS-1 and IRS-2 have been found to play major roles in the determination of insulin resistance. It has been shown that insulin resistance in IRS-1 and IRS-2 KO mice was related to the skeletal muscle and the liver, respectively (15,26). These IRS proteins are thought to exert a compensatory effect (27). The functions of these IRS proteins in the kidney have not been investigated fully as yet. However, it was reported that the expression level of IRS-2 mRNA in the kidney was more abundant than that of IRS-1 (28). Moreover, it has been suggested that IRS-2 but not IRS-1 may have a vascular protective effect on neointimal formation when the artery is mechanically injured (29). Therefore, we used IRS-2 KO mice to investigate whether the renal protective effect of ghrelin, especially at the vascular level, depended on the IGF-1/IRS-2 signaling pathway. The results showed that ghrelin had no effect on iARF in IRS-2 KO mice. The isolated kidneys of IRS-2 KO mice with iARF showed markedly attenuated responses to ACh. It is possible that insulin resistance in IRS-2 KO mice interferes with the responses to ghrelin independent of its GH stimulation. However, serum levels of BUN and creatinine and the renal injury score were the same in the vehicle and ghrelin treatment groups, suggesting that not only endothelium-dependent but also endothelium-independent actions of ghrelin may be altered in the IRS-2 KO mice. Furthermore, ghrelin improved endothelial function and renal function in iARF mice, which showed marked endothelial dysfunction. Although it is not clear whether IRS-1 has compensatory effects in the kidney, our results suggest that the signaling pathway between IGF-1 and IRS-2 plays a critical role in the renal protective effect of ghrelin. However, a GH/IGF-1–independent cardiovascular effect of ghrelin has also been suggested. Wiley *et al.* (30) reported that ghrelin had a vasodilatory effect on the isolated human internal mammary artery precontracted with endothelin-1 and that its effect was endothelium-independent. Moreover, subcutaneous injection of ghrelin for 3 wk improved ACh-induced vasodilation in GH-deficient rats, indicating a GH-independent action of ghrelin on the vascular endothelium. Physiologic activity of ghrelin is mediated by an interaction between ghrelin and GHSR (1). Recently, several groups reported that GHSR existed

in the pituitary, myocardium, aorta, and kidney and that various tissues, including the kidney, expressed the ghrelin gene (13). Furthermore, Mori *et al.* (31) reported that ghrelin was produced locally in the kidney, suggesting a direct effect of ghrelin on the kidney. However, in this study, we failed to show an improvement of renal function in IRS-2 KO mice by treatment with ghrelin. Thus, it is highly likely that the effect of ghrelin on the kidney is largely mediated by an IGF-1 signaling pathway.

The most rational dosage of ghrelin is still unclear. In this study, to examine whether this therapeutic regimen is rational, we injected ghrelin six times before and three times after ischemia. This injection schedule was based on the report by Nagaya *et al.* (5), in which they examined the effects of ghrelin in rats with heart failure and showed the cardiac-protective effect of ghrelin. Thus, we think that only one injection is not sufficient to protect renal function from iARF and the treatment protocol that was used by our group and others is appropriate to protect ischemic organ damage. It is possible that the continuous effect of ghrelin during the reperfusion period may be essential.

In this study to investigate the beneficial effect of ghrelin on renal endothelium-dependent vasodilation, we stimulated isolated perfused kidneys with ACh and AM. ACh and AM are known to have an endothelium-dependent vasodilating action, and we have already shown that AM induced vasorelaxation in an endothelium-dependent manner *via* the NO-cGMP pathway (16,32). In this study, we showed that treatment with ghrelin improved endothelium-dependent vascular responses to ACh and AM, but we did not observe a direct vasodilatory action of ghrelin in the renal artery of the isolated kidney. It seems well established that improvement of endothelial function is associated with an improvement of I/R injury at least in rodents (33,34). These results indicate that the renal protective effects of ghrelin may be mediated by an improvement of endothelial function through an IGF-1 signaling pathway.

Induction of apoptosis is one of the major causes of tissue damage after I/R injury (35,36). Several reports pointed out the existence of apoptotic cells and upregulation of Fas after I/R injury, particularly apoptosis of renal tubular epithelial cells (37). Inhibition of cellular apoptosis by ghrelin itself has not been investigated. However, the antiapoptotic activity of IGF-1 has been reported in various models, such as the unilateral ureteral obstruction model, ultraviolet radiation model, and I/R injury model (22,36,38). It is known that the tissue-protective effects of GH and IGF-1 are mediated by the PI3K/Akt pathway (22). Activated PI3K/Akt increases the release of NO and shows various effects, including antiapoptotic activity (23,24). Ghrelin binds to GHSR and upregulates the GH concentration in an intracellular calcium–dependent manner, resulting in increases of the serum IGF-1 level. In this study, ghrelin increased the serum level of IGF-1 and decreased the number of apoptotic renal tubular cells after I/R injury. It is possible for ghrelin to act as a tissue survival factor through the IGF-1/IRS-2 signaling pathway such as vascular endothelial growth factor, which also activates PI3K/Akt.

Our assay system is based on the chemiluminescent reaction of organ-derived NO with the luminol-H_2O_2 system, and this chemiluminescence is due to the formation of peroxynitrite from NO and H_2O_2. In previous studies (18,19), to confirm whether the changes of chemiluminescence and RPP were related to endothelium-derived NO, we examined the effect of inhibition of endothelial function using CHAPS, deoxycholic acid, or L-NMMA. After infusion of either agent, ACh-induced NO signal and vasorelaxation were diminished. However, infusion of exogenous NO increased NO chemiluminescence and decreased RPP. To exclude the possibility of superoxide as a precursor of peroxynitrite, we infused superoxide dismutase, but this caused no significant changes in chemiluminescence, denying the possibility of the involvement of organ-derived superoxide. Furthermore, there was a lag time of 5 to 15 s to mix the venous effluent and chemiluminescence agents. This lag time was too long for superoxide or a hydroxyradical but not for NO to be detected. Therefore, this assay system sensitively detected endothelium-derived NO production but not superoxide.

To demonstrate the effect of ghrelin on iARF, we used an I/R model. *In vivo* tissue injury induced by I/R is believed to be mediated by local inflammation and various inflammatory cytokines such as TNF-α and IL-1β. In addition, the production of reactive oxygen species in the kidney during reperfusion is suggested. Very high concentrations of NO, usually derived from inducible NO synthase (iNOS), are also considered to be toxic. The involvement of iNOS expression in iARF is still a matter of controversy. In a previous study, we did not detect iNOS expression in the kidneys with iARF from rats (33). One group investigated the antioxidant effect of ghrelin using an I/R model of the isolated rat heart. In that study, ghrelin suppressed the production of malondialdehyde, one of the markers of oxidative stress, in the myocardium in a dose-dependent manner (39). It has been reported that NO has a renal protective effect against superoxide anion (40,41). AM-induced cGMP production in the kidney with iARF was increased by ghrelin, suggesting an increase in NO availability and a decrease in oxidative stress. Further studies are required to clarify whether ghrelin itself or IGF-1–mediated NO release has an antioxidant activity in the kidney.

In conclusion, 45 min of ischemia and 24 h of reperfusion induced severe iARF in mice. However, administration of ghrelin before and during ischemia improved vascular endothelial function and renal excretory function and decreased the renal tissue damage and apoptosis of the tubular cells. The increment of IGF-1 and the subsequent activation of the IGF-1 signaling pathway play more important roles regarding the renal protective effect of ghrelin than the direct effect of ghrelin. Moreover, ghrelin has an appetite-increasing activity (42) and exerts some other favorable actions on energy metabolism, particularly in the anorexic condition, implicating a clinical application of this peptide in patients with iARF.

Acknowledgments

This study was supported by Grant-in-Aid 13557061 (awarded to Y.H.) and by a grant from Advanced and Innovational Research Program in Life Sciences (awarded to Y.H.) from the Ministry of Education,

Culture, Sports, Science and Technology of Japan. This work was also supported by a Research Grant for Cardiovascular Diseases (12A-2) from the Ministry of Health, Labor and Welfare (awarded to Y.H.).

We thank Etsuko Taira, Marie Morita, and Reiko Sato for technical assistance.

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