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-treated mice (%Δ renal perfusion pressure by $10^{-7}$ M ACh $-63.5 \pm 3.7$ versus $-41.2 \pm 5.5\%$; $P < 0.05$). This change was associated with significant increases of nitric oxide release in the kidneys of ghrelin-treated mice ($10^{-7}$ M ACh $35.5 \pm 5.8$ versus $16.9 \pm 3.5$ fmol/g kidney per min; $P < 0.05$). Serum concentration of urea nitrogen (53 versus 28; $P < 0.05$) and renal injury score were significantly lower in the ghrelin group (2.5 versus 5.3; $P < 0.01$). Tubular apoptotic index was significantly lower in the ghrelin group (5 versus 28; $P < 0.05$). 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.

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(Yokohama, Japan). The IRS-2<sup>−/−</sup> mice were maintained on the original C57BL6/CBA hybrid background and were prepared by IRS-2<sup>−/−</sup> mouse intercrosses (15).

iARF

iARF was induced in 8- to 12-wk-old BALB/C and IRS-2<sup>−/−</sup> 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 dissolved 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).

Isolated Perfused Kidney

Male BALB/C and IRS-2<sup>−/−</sup> 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<sub>2</sub>/5% CO<sub>2</sub> and contained 10<sup>−6</sup> mol/L angiotensin II and 10<sup>−5</sup> mol/L indo- methacin to maintain the renal perfusion pressure (RPP) at approximately 100 mmHg. After a 60-min equilibrium period, graded doses of acetylcholine (ACh; 10<sup>−8</sup> to 10<sup>−7</sup> M) and adrenomedullin (AM; 10<sup>−10</sup> to 10<sup>−7</sup> M) were added to the buffer at 10-min intervals, and RPP was monitored through a pressure transducer (DateX-Ommeda 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<sub>2</sub>O<sub>2</sub>, 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 by an 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<sup>−8</sup> 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 re-dissolved in a buffer solution. The cGMP content was assayed using an ELSA 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 is 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 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).
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

### Table 1. Baseline characteristics of mice that had iARF and were treated with vehicle or ghrelina

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>BW (g)</th>
<th>KW (g)</th>
<th>KW/BW (%)</th>
<th>Baseline RPP (mmHg)</th>
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<tr>
<td>BALB/C</td>
<td></td>
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<td></td>
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<tr>
<td>sham + vehicle</td>
<td>8</td>
<td>27.6 ± 2.6</td>
<td>0.137 ± 0.042</td>
<td>0.496 ± 0.125</td>
<td>79.6 ± 5.5</td>
</tr>
<tr>
<td>sham + ghrelin</td>
<td>8</td>
<td>28.6 ± 2.1</td>
<td>0.132 ± 0.058</td>
<td>0.462 ± 0.117</td>
<td>87.6 ± 4.2</td>
</tr>
<tr>
<td>iARF + vehicle</td>
<td>8</td>
<td>25.9 ± 3.5</td>
<td>0.173 ± 0.079b</td>
<td>0.668 ± 0.096b</td>
<td>110.3 ± 10.9c</td>
</tr>
<tr>
<td>iARF + ghrelin</td>
<td>8</td>
<td>27.8 ± 2.2</td>
<td>0.159 ± 0.059d</td>
<td>0.572 ± 0.143e</td>
<td>95.3 ± 7.5ef</td>
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<tr>
<td>IRS-2 KO</td>
<td></td>
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<tr>
<td>iARF + vehicle</td>
<td>4</td>
<td>29.5 ± 2.1</td>
<td>0.158 ± 0.009</td>
<td>0.54 ± 0.04</td>
<td>110.6 ± 5.4</td>
</tr>
<tr>
<td>iARF + ghrelin</td>
<td>4</td>
<td>28.8 ± 0.8</td>
<td>0.165 ± 0.006</td>
<td>0.57 ± 0.03</td>
<td>111.3 ± 7.5</td>
</tr>
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aValues are means ± SEM. BW, body weight; KW, kidney weight; RPP, renal perfusion pressure; KO, knockout.
bP < 0.05 versus sham + vehicle.
cP < 0.01 versus sham + vehicle.
dP < 0.05 versus sham + ghrelin.
eP < 0.05 versus iARF + vehicle.
fP < 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.

Figure 2.
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 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
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 110.6/110.0 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.

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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.

Figure 8. Serum IGF-1 concentrations in vehicle- and ghrelin-treated mice. Bars indicate means ± SEM; n = 6.
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 phosphatidylinositol-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). 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-H2O2 system, and this chemiluminescence is due to the formation of peroxynitrite from NO and H2O2. 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 hydroxyl radical 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.

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