Ghrelin protects mice against endotoxemia-induced acute kidney injury

Wei Wang,1 Shweta Bansal,1 Sandor Falk,1 Danica Ljubanovic,2 and Robert Schrier1

1Department of Medicine, University of Colorado Denver, Aurora, Colorado; and 2Department of Pathology, Dubrava University Hospital, Zagreb, Croatia

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Wang W, Bansal S, Falk S, Ljubanovic D, Schrier R. Ghrelin protects mice against endotoxemia-induced acute kidney injury. Am J Physiol Renal Physiol 297: F1032–F1037, 2009. First published July 22, 2009; doi:10.1152/ajprenal.00044.2009.—Acute kidney injury (AKI) in septic patients drastically increases the mortality to 50–80%. Sepsis is characterized by hemodynamic perturbations as well as overwhelming induction of proinflammatory cytokines. Since ghrelin has been shown to have anti-inflammatory properties, we hypothesized that ghrelin may ameliorate renal protection during endotoxemia-induced AKI. Studies were conducted in a normotensive endotoxemia-induced AKI model in mice by intraperitoneal injection of 3.5 mg/kg LPS. Serum ghrelin levels were increased during endotoxemia accompanied by increased ghrelin receptor (GHSR-1a) protein expression in the kidney. Ghrelin administration (1.0 mg/kg sc 6 h and 30 min before and 14 h after LPS) significantly decreased serum cytokine levels (TNF-α, IL-1β, and IL-6) and serum endothelin-1 levels which had been induced by LPS. The elevated serum nitric oxide (NO) levels and renal inducible NO synthase expression were also decreased by ghrelin. Renal TNF-α levels were also increased significantly in response to LPS and ghrelin significantly attenuated this increase. When administrated before LPS, ghrelin protected against the fall in glomerular filtration rate at 16 h (172.9 ± 14.7 vs. 90.6 ± 15.2 μl/min, P < 0.001) and 24 h (147.2 ± 20.3 vs. 59.4 ± 20.7 μl/min, P < 0.05) as well as renal blood flow at 16 h (1.65 ± 0.07 vs. 1.47 ± 0.04 ml/min, P < 0.01) and 24 h (1.56 ± 0.08 vs. 1.22 ± 0.03 ml/min, P < 0.05) after LPS administration without affecting mean arterial pressure. Ghrelin remained renal protective even when it was given after LPS. In summary, ghrelin offered significant protection against endotoxemia-induced AKI. The renal protective effect of ghrelin was associated with an inhibition of the proinflammatory cytokines. Of particular importance was the suppression of TNF-α both in the circulation and kidney tissues. Thus, ghrelin may be a promising peptide in managing endotoxemia-induced AKI.

Materials and methods

Animals. The experimental protocol was approved by the Animal Ethics Review Committee at the University of Colorado Health Sciences Center. C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME). Male mice aged 8–10 wk were used throughout the study. Mice were maintained on a standard rodent chow and had free access to water.

Materials. Chemicals were purchased from Sigma (St. Louis, MO) unless otherwise specified. LPS (Escherichia coli 011:B4) was purchased from LIST Biological Laboratories (Campbell, CA). Recombinant rat ghrelin was purchased from Anaspec (San Jose, CA). Normal saline was used to dissolve both LPS and ghrelin.

Animal protocol. Mice were injected intraperitoneally (ip) with 3.5 mg/kg dose of LPS. In the pre-LPS treatment 16-h group, rat ghrelin was given 1.0 mg/kg sc subcutaneously (sc) 6 h and 30 min before and 14 h after LPS administration. In the pre-LPS treatment 24-h group, rat ghrelin was given 1.0 mg/kg sc 1 h before and 3 and 6 h after LPS administration. In post-LPS treatment group, rat ghrelin was given at 1 and 14 h after LPS. Functional studies were done at 16 h after LPS injection.

Measurement of serum ghrelin level. Blood was taken through cardiac puncture 16 h after LPS (3.5 mg/kg) ip injection. Serum ghrelin levels in mice were measured using rat/mouse ghrelin ELISA kit from Millipore (Billerica, MA).

Ghrelin receptor, GHSR-1a, inducible nitric oxide synthase, and high-mobility group box 1, and β-actin protein expression in the kidney. Kidneys were removed at 16 h after LPS (3.5 mg/kg) ip injection. Western blots were used to measure the protein expression described earlier (33, 34). The primary antibodies for GHSR-1a, inducible nitric oxide synthase (iNOS), high-mobility group box 1 (HMGB1), and β-actin were rabbit polyclonal antibodies from Alpha-diagnostics International (San Antonio, TX), Cell Signaling Technology (Danvers, MA), and Abcam (Cambridge, MA) and diluted at 1:100, 1:1,000, 1:1,000, and 1:1,000, respectively. The secondary antibodies were conjugated to horseradish peroxidase. Antigenic detection was made by enhanced chemiluminescence (Amersham, Arlington Heights, IL) with exposure to x-ray film. Densitometry was measured and normalized to β-actin.

Measurement of serum NO levels. Blood was taken through cardiac puncture 16 h after LPS (3.5 mg/kg) ip injection. Serum NO levels were measured using nitrate/nitrite colorimetric assay kit from Cayman (Ann Arbor, MI).
Measurement of renal cortical cGMP. Kidneys were taken at 16 h after LPS (3.5 mg/kg) ip injection. Renal cortical tissues were homogenized in 0.5% Triton X-100 in 0.1 M HCl and cGMP was measured using enzyme immunoassay kit from Assay Designs (Ann Arbor, MI).

Measurement of serum ET-1 levels. Blood was taken through cardiac puncture 16 h after LPS (3.5 mg/kg) ip injection. Serum ET-1 levels were measured using EIA kit from Assay Designs.

Measurement of serum and renal cytokine levels. Blood was taken through cardiac puncture 16 h after LPS (3.5 mg/kg) ip injection. Kidneys were frozen in the liquid nitrogen at the same time and were counted five times on pulse for 5 s each. They were centrifuged at 14,000 g at 4°C and the supernatant was reserved for protein concentration measurement and cytokine assay. Serum and renal cytokine levels were detected using Bio-Plex cytokine assay kit from Bio-Rad. Renal cytokine levels were normalized to protein concentration in the lysate.

Measurement of glomerular filtration rate, renal blood flow, and mean arterial pressure. The animals were anesthetized with pentobarbital sodium (60 mg/kg) and placed on a thermostatically controlled surgical table. A tracheotomy was performed in all mice. Catheters (custom pulled from PE-250) were placed in the jugular vein for maintenance infusion and in the carotid artery for blood pressure measurement. The kidney was exposed by a left subcostal incision and was dissected free from perirenal tissue, and the renal arteries were isolated for the determination of renal blood flow (RBF) using a blood flow meter and probe (0.5 v; Transonic Systems, Ithaca, NY) as described by Traynor and Schnermann (29). Mean arterial pressure (MAP) was measured via a carotid artery catheter connected to a TranspacIV transducer and monitored continuously using Windaq Waveform recording software (Dataq Instruments). An intravenous maintenance infusion of 2.25% bovine serum albumin in normal saline at a rate of 0.25 µl·g body wt−1·min−1 was started 1 h before experimentation; 0.75% FITC-inulin was added to the infusion solution for the determination of glomerular filtration rate (GFR) as described by Lorenz and Gruenstein (23). A bladder catheter (PE-10) was used to collect urine. Two 30-min collections of urine were obtained under oil and weighed for volume determination. Blood for plasma inulin determination was drawn between urine collections. FITC in plasma and urine samples was measured using CytoFluor plate reader (PerSeptive Biosystems, Foster City, CA).

Histological examination. Paraformaldehyde (4%)-fixed and paraffin-embedded kidneys were sectioned at 4 µm and stained with periodic acid-Schiff (PAS) by standard methods. All histological examinations were performed by the renal pathologist without knowledge of the intervention. Histological changes due to acute tubular necrosis (ATN score) were evaluated in the outer stripe of the outer medulla and the cortex on PAS-stained tissue and were quantified by counting the percentage of tubules that displayed cell necrosis, loss of brush border, cast formation, and tubule dilatation as follows: 0 = none, 1 = 1–10%, 2 = 11–25%, 3 = 26–45%, 4 = 46–75%, and 5 = >76%. At least 10 fields (×200) were reviewed for each slide.

Statistical analysis. Values are expressed as mean ± SE. Multiple comparisons were assessed by ANOVA using the post hoc Newman-Keuls test. P < 0.05 was considered statistically significant.

RESULTS

Serum ghrelin levels and renal ghrelin receptor protein expression during endotoxemia. Serum ghrelin levels increased significantly 16 h after the ip injection of LPS at 3.5 mg/kg (138.3 ± 17.6 vs. 25.0 ± 9.2 pg/ml, P < 0.01; Fig. 1). The ghrelin treatment group had a dramatic increase in serum ghrelin (3,078.1 ± 697.3 pg/ml, P < 0.001 vs. both control and LPS groups; Fig. 1). The protein expression of GHS-R in the kidneys was also increased during endotoxemia (Fig. 2). Ghrelin treatment decreased the renal GHS-R expression (Fig. 2).

Effect of ghrelin on renal iNOS protein expression and serum NO levels during endotoxemia. Renal iNOS protein expression (Fig. 3A) was significantly induced at 16 h after LPS administration which was accompanied by a significant induction in serum NO levels (236.6 ± 16.3 µM, n = 5 vs. 4.2 ± 0.4 µM, n = 5, P < 0.001; Fig. 3B). Ghrelin significantly attenuated the induced renal iNOS expression (n = 5; Fig. 3A) as well as serum NO levels (175.2 ± 9.6 µM, n = 5, vs. 236.6 ± 16.3, n = 5, P < 0.05; Fig. 3B).

Effect of ghrelin on renal cortical cGMP levels during endotoxemia. Renal cortex was collected 16 h after LPS administration. LPS increased renal cortical cGMP levels compared with the control group (0.41 ± 0.03 vs. 0.11 ± 0.02 pmol/mg, P < 0.001; Fig. 4). Ghrelin decreased renal cGMP levels induced by LPS (0.18 ± 0.03 vs. 0.41 ± 0.03 pmol/mg, P < 0.001; Fig. 4).

Effect of ghrelin treatment on ET-1 levels during endotoxemia. Serum ET-1 increased significantly 16 h after the ip administration of 3.5 mg/kg LPS compared with the control group (4.96 ± 0.87 vs. 1.02 ± 0.38 pg/ml, P < 0.01; Fig. 5). Treatment of ghrelin significantly decreased the serum ET-1 level compared with the LPS group (1.93 ± 0.93 vs. 4.96 ± 0.87 pg/ml, P < 0.01; Fig. 5) which brought the serum ET-1 level 26% lower.

GHSR-1α
β-actin

Fig. 2. Renal ghrelin receptor (GHSR-1α) protein expression during endotoxemia. Kidneys were harvested at 16 h after LPS. GHSR-1α protein expression was examined using Western blot. Relative densitometry is expressed as mean ± SE.
levels to those in control group (1.93 ± 0.93 vs. 1.02 ± 0.38 pg/ml, P = NS; Fig. 5).

Effect of ghrelin on serum cytokine concentrations during endotoxemia. Blood was collected at 16 h after LPS injection in mice. LPS induced a significant increase in serum TNF-α (46.1 ± 6.7 pg/ml, n = 6 vs. 5.2 ± 0.7 pg/ml, n = 4, P < 0.001; Fig. 6A), IL-1β (56.7 ± 5.0 pg/ml, n = 6 vs. 14.3 ± 2.3 pg/ml, n = 4, P < 0.01; Fig. 6B), and IL-6 (250.1 ± 31.0 vs. 12.6 ± 2.6 pg/ml, n = 4, P < 0.001; Fig. 6C) levels compared with the controls. The administration of ghrelin decreased serum TNF-α (25.3 ± 4.7 pg/ml, n = 6 vs. 46.1 ± 6.7 pg/ml, n = 6, P < 0.01; Fig. 6A), IL-1β (35.6 ± 3.6 pg/ml, n = 6 vs. 56.7 ± 5.0 pg/ml, n = 6, P < 0.05; Fig. 6B), and IL-6 (103.6 ± 37.0 pg/ml, n = 6 vs. 250.1 ± 31.0 pg/ml, n = 4, P < 0.01; Fig. 6C) levels.

Effect of ghrelin on renal cytokine concentrations during endotoxemia. Kidneys were collected at 16 h after LPS. LPS induced a significant increase in renal TNF-α levels (1,783.6 ± 384 pg/mg, n = 7 vs. 72.9 ± 19.5 pg/mg, n = 6, P < 0.05; Fig. 6D). Ghrelin significantly decreased the level induced by LPS (491.0 ± 281.0 pg/mg, n = 7 vs. 1,783.6 ± 384.0 pg/mg, n = 7, P < 0.05; Fig. 6D). However, renal IL-1β and IL-6 levels were not significantly increased during endotoxemia and ghrelin did not alter these levels (Fig. 6, E and F).

Effect of ghrelin on renal HMGB1 expression. The kidneys were harvested at 16 h after LPS. There were no changes among the control, LPS, and LPS with ghrelin groups (data not shown).

Effect of ghrelin on renal function in wild-type mice during endotoxemia. Sixteen hours after ip injection of 3.5 mg/kg LPS a significant decrease in GFR occurred. Administration of 1 mg/kg ghrelin at 6 h and 30 min before and 14 h after LPS injection significantly improved GFR (172.9 ± 14.7 μl/min, n = 13 vs. 90.6 ± 15.2 μl/min, n = 11, P < 0.001; Fig. 7A) and RBF (1.65 ± 0.07 ml/min, n = 12 vs. 1.47 ± 0.04 ml/min, n = 10, P < 0.01; Fig. 7B). In the meantime, MAP remained unchanged (85.6 ± 2.1 mmHg, n = 13 vs. 83.3 ± 7.8 mmHg, n = 11, P = NS; Fig. 7C). GFR deteriorated further at 24 h after 3.5-mg/kg LPS administration and ghrelin was able to attenuate it (147.2 ± 20.3 μl/min, n = 6 vs. 59.4 ± 20.7 μl/min, n = 4, P < 0.05; Fig. 8A) when administrated 1 h before and 3 and 6 h after LPS. RBF was also improved by ghrelin (1.56 ± 0.08 ml/min, n = 6 vs. 1.22 ± 0.03 ml/min, n = 4, P < 0.05; Fig. 8B) while MAP remained unchanged (86.0 ± 4.1 mmHg, n = 6 vs. 83.8 ± 5.8 mmHg, n = 4, P = NS; Fig. 8C). Ghrelin also improved GFR (137.6 ± 24 vs. 35.3 ± 16.1 μl/min, P < 0.05; Fig. 9A) and RBF (1.57 ± 0.1 vs. 1.15 ± 0.15 μl/min, P < 0.05; Fig. 9B) even when it was given after LPS (1 and 14 h post-LPS). There was no effect on MAP either (88.3 ± 3.2 vs. 83.0 ± 2.4 mmHg, P = NS; Fig. 9C). Ghrelin alone had no effect on GFR, MAP, or RBF compared with the baseline controls (data not shown).

Effect of ghrelin on renal histology during endotoxemia. Renal histology was examined and ATN score was conducted. There was no significant tubular necrosis in our normotensive endotoxemia model compared with the normal controls. Therefore, the pathogenesis primarily involves renal vasoconstriction.
This is very similar to studies in humans with sepsis and AKI in which neither renal apoptosis nor necrosis was found (15).

**Effect of ghrelin on body weight during endotoxemia.** We weighed the animals before and after the treatments. Both control and ghrelin treatment groups lost weight and there were no significant differences on the weight loss between the groups.

**DISCUSSION**

Sepsis has been identified as the most common cause of AKI in intensive care units. Moreover, the combination of sepsis and AKI is associated with a very high mortality (27, 28). There is, therefore, an important need to identify potential therapeutic interventions with the potential to attenuate sepsis-related AKI. Endotoxin is known to cause most of the hemodynamic and inflammatory responses that occur in the gram-negative sepsis. The present study was undertaken to examine the effect of ghrelin on a normotensive model of endotoxemia-related AKI in mice and its underlying mechanisms. In this model, ghrelin afforded significant protection against AKI in mice either given before or after LPS.

Ghrelin is an endogenous ligand for the GHSR. It has a strong stimulatory effect on growth hormone secretion in the rat and in the human (2, 10). GHSR was thought to be exclusively expressed in the brain (12). However, it was later found to be expressed in many tissues including kidneys (26). Serum ghrelin levels have been reported to be decreased or increased after LPS injection depending on the timing of disease course (5, 13, 31). In the present study, the serum ghrelin levels increased at 16 h after exposure to LPS. This was accompanied by significant increase in renal GHSR protein expression. The elevated receptor levels were compatible with a potential therapeutic benefit of exogenous ghrelin. This was proved by the fact that recombinant ghrelin dramatically increased serum ghrelin levels and offered significant protection against endotoxemia-induced AKI. Upregulation of cardiovascular GHSR has also been
reported during sepsis in rats (37), thus a beneficial effect of ghrelin on the heart is also possible.

Most important are the potential mechanisms whereby ghrelin exerts this protective effect on endotoxin-related AKI. Proinflammatory cytokines induced by endotoxin, such as TNF-α, participate in the magnification of the endotoxemic response mediated by the release of other cytokines and active substances (4, 25). Results from our laboratory demonstrated the importance of inhibiting TNF with either soluble TNF receptor (18) or pentoxifylline (34) in affording protection against endotoxin-related AKI. In these studies inhibiting TNF was associated with a decrease in other cytokines, renal iNOS, and serum NO, thus indicating a pivotal role of TNF in inflammatory and hemodynamic responses to endotoxin. In the present study, the effect of ghrelin was shown to not only decrease serum TNF during endotoxemia, but also other serum cytokines, namely IL-1β and IL-6, endothelin, and renal iNOS and serum NO were suppressed by ghrelin treatment. Thus, the multifactorial pathogenetic response to endotoxemia preferably necessitates a multifactorial intervention to afford protection against AKI.

The resultant increases in renal iNOS and plasma NO with endotoxin are known to contribute to the systemic arterial vasodilation which results in activation of the sympathetic nervous and renin-angiotensin system (32). These responses have been shown in our endotoxemia model of AKI to contribute to the renal vasoconstriction (32). In this regard, a protective effect of renal denervation was shown to attenuate the AKI during endotoxemia (32). In the present study, ghrelin was demonstrated to attenuate the renal effect of NO to stimulate renal cGMP despite the fact that ghrelin increased RBF during endotoxemia. This finding is relevant since in vitro studies demonstrated the effect of NO to mediate injury in proximal tubules (39).

TNF is also known to stimulate endothelin (3, 21, 24) and ghrelin has been shown to improve tissue perfusion during septic shock by downregulating endothelin (36). In the present study, the suppression of the increased endothelin with ghrelin was associated with an increase in GFR and RBF.

Perhaps most important is the effect of TNF to stimulate an inflammatory response. Mortality in sepsis has been shown to correlate with the degree of upregulation of IL-6 (15). IL-1β has also been shown to be a potent proinflammatory cytokine (7, 8). In the present study, ghrelin was shown to suppress the upregulation of both IL-6 and IL-1β during endotoxemia. Thus, in addition to any hemodynamic benefit of ghrelin in protecting against endotoxin-induced AKI, the agent’s anti-inflamatory properties are no doubt critical. In the present study, when cytokines in the kidneys were examined, only TNF-α levels were significantly induced by LPS administration and ghrelin significantly decreased this induction. Renal IL-1β and IL-6 were not affected by either LPS or ghrelin. As in results from our and other laboratories (6, 34), the present results incriminate a pivotal role of TNF-α in the hemodynamic and inflammatory responses to endotoxemia which lead to AKI. Thus, while the mechanisms involved in the protective effect of ghrelin are no doubt multifactorial, the main effect may relate to the attenuation of circulating and renal local levels of TNF-α.

![Fig. 8. Effect of pre-LPS treatment of ghrelin on GFR, RBF, and MAP during endotoxemia at 24 h. Mice were injected ip with 3.5-mg/kg dose of LPS. Rat ghrelin was given 1.0 mg/kg sc 1 h before and 3 and 6 h after LPS administration. Functional studies were done at 16 h after LPS injection. GFR (A), RBF (B), and MAP (C) were measured at 16 h after LPS injection. GFR was measured using FITC-inulin clearance. RBF was measured using a blood flow meter and probe. MAP was measured through carotid artery. Values are expressed as mean ± SE.](http://ajprenal.physiology.org/)

![Fig. 9. Effect of post-LPS treatment of ghrelin on GFR, RBF, and MAP during endotoxemia. Mice were injected ip with 3.5-mg/kg dose of LPS. Rat ghrelin was given at 1 and 14 h after LPS. GFR, RBF, and MAP were measured at 16 h after LPS injection. GFR was measured using FITC-inulin clearance. RBF was measured using a blood flow meter and probe. MAP was measured through carotid artery. Values are expressed as mean ± SE.](http://ajprenal.physiology.org/)
Of interest, ghrelin’s anti-inflammatory effects have also been shown to inhibit sympathetic activation which is known to be increased in sepsis (38) and endotoxemia (32). Although an effect of ghrelin has been reported to inhibit the late inflammatory factor, HMGB (30), there was no effect observed in the present study.

In summary, sepsis and endotoxemia are associated with AKI and involve a multifactorial pathogenesis. Thus, any beneficial intervention must also be multifactorial. In this regard, the present and previous experimental studies indicate a key role of TNF in the hemodynamic, hormonal, and inflammatory responses to endotoxemia. Ghrelin appears to possess properties to attenuate these responses and thereby afford protection against endotoxin-related AKI. The protective effect of ghrelin was observed when administered before or after endotoxin, thus contributing to the potential clinical benefit in sepsis-related AKI.

REFERENCES


