Original Article

Ghrelin attenuates UUO-induced renal fibrosis via attenuation of Nlrp3 inflammasome and endoplasmic reticulum stress

Lilu Ling¹, Min Yang¹, Wei Ding²*, Yong Gu¹*

¹Division of Nephrology, The Fifth People’s Hospital of Shanghai, Fudan University, 128 Ruili Road, Shanghai 200240, China; ²Division of Nephrology, Shanghai Ninth People’s Hospital, School of Medicine, Shanghai Jiaotong University, 639 Zhizaoyu Road, Shanghai 200011, China. *Equal contributors.

Received July 16, 2018; Accepted December 23, 2018; Epub January 15, 2019; Published January 30, 2019

Abstract: Background/aims: All chronic kidney disease (CKD) can eventually develop into renal fibrosis. We explored the renoprotective effects of a gastric peptide, ghrelin, and investigated whether endoplasmic reticulum stress (ERS) and the NLR family pyrin domain-containing 3 (NLRP3) inflammasome mediate the protective effect of ghrelin in unilateral ureteral obstruction (UUO). Methods: Male C57BL/6J mice were divided into vehicle- or ghrelin-treated sham-operated groups and vehicle- or ghrelin-treated UUO groups. The kidneys were harvested on postoperative day 14. Renal fibrosis was evaluated by periodic acid-Schiff, Masson trichrome, and immunohistochemical (IHC) staining. To assess renal fibrosis, α-smooth muscle actin and type I collagen were detected. NLRP3 inflammasome and ERS activation were also detected via western blotting. The effect of ghrelin on cultured renal cells was further confirmed in HK-2 cells. Results: Compared with the sham mice, UUO mice developed obvious renal fibrosis; pathological and IHC staining showed increased matrix accumulation and elevated ERS, NLRP3 inflammasome was activated both in vivo and in vitro. Ghrelin significantly attenuated collagen fibril accumulation and apoptosis by reducing NLRP3 inflammasome activation and ERS in obstructed kidneys. Conclusions: Ghrelin may attenuate UUO-induced renal fibrosis by inhibiting the NLRP3 inflammasome and ERS in vivo. Therefore, ghrelin might be an effective strategy for preventing CKD.

Keywords: Ghrelin, renal fibrosis, NLRP3 inflammasome, endoplasmic reticulum stress

Introduction

Renal fibrosis is a common pathway of chronic kidney disease (CKD) that ultimately leads to end-stage renal failure. Various primary glomerular diseases such as ureteral obstruction and diabetes can induce inflammatory cell infiltration in the renal interstitium, tubular epithelial cell apoptosis, and myofibroblast accumulation, which can promote pro-fibrotic factors, increasing the production of extracellular matrix (ECM) and reducing its degradation. Subsequently, renal interstitial fibrosis develops and damages renal function [1]. At present, slowing the prognosis by controlling the risk factors that aggravate the deterioration of renal function and renal fibrosis is not satisfactory in the clinic [2]. Accordingly, preventing and curing renal fibrosis effectively presents a major challenge.

Endoplasmic reticulum stress (ERS) is caused by the accumulation of unfolded proteins or false folded protein in the ER, which results in an imbalance of calcium in the cell and ER instability [3]. Accumulating evidence indicates that ERS plays an active role in the development of acute and chronic kidney disease; in particular, it contributes to glomerular and tubular damage in CKD [4]. Furthermore, inhibiting ERS can alleviate renal fibrosis progression [5]. ERS promotes renal fibrosis through multiple signaling pathways, such as epithelial-mesenchymal transition, transforming growth factor-β, and oxidative stress. In rat models, ERS causes podocyte injury and apoptosis, leading to increased proteinuria and the development of renal fibrosis [6]. Therefore, targeted inhibition of ERS may be a promising therapeutic strategy against renal fibrosis.
The inflammasome is a polyprotein complex mainly composed of intracellular pattern recognition receptors, the adaptor ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain), and pro-caspase-1 [7]. The inflammasome is believed to be the cellular machinery triggering the maturation of pro-inflammatory cytokines such as interleukin-1β (IL-1β) to engage the innate immune defenses. Several types of inflammasomes have been identified; among them, the NLR family pyrin domain-containing protein 3 (NLRP3) inflammasome is currently the most fully characterized [8]. Activation of the NLRP3 inflammasome directly causes renal tubular epithelial injury [9]. NLRP3 inflammasome expression is increased in models of unilateral ureteral obstruction (UUO) [10], ischemia-reperfusion (I/R) [11], and lupus nephritis [12]. NLRP3 gene knockout (NLRP3-/-), which blocks NLRP3 inflammasome activation, can significantly attenuate kidney damage.

Ghrelin is a brain peptide found in 1999 by Kojima et al. [13]. Acting on the growth hormone (GH) secretagogue receptor (GHSR), ghrelin can accelerate GH secretion, participate in energy metabolism, improve gastrointestinal function, and protect the cardiovascular system [14]. A recent study demonstrated that the exogenous injection of ghrelin protected against acute kidney injuries [15]. In 2004, Rodríguez et al. [16] found that the serum ghrelin levels of patients with ESRD were significantly increased as compared to the controls. Borges and co-workers have suggested that the increased total ghrelin levels in patients with CKD is mainly due to the impaired renal function of ghrelin removal and degradation, which may be one of the main reasons for the apparent increase in patients with CKD [17]. However, to our knowledge, no direct evidence shows that ghrelin can alleviate renal fibrosis, and the underlying mechanisms of ghrelin alleviation of renal fibrosis remain unexplored. Considering the role of ghrelin in inhibiting the release of inflammatory factors, we assumed that ghrelin and the NLRP3 inflammasome might be linked. To test this hypothesis, we evaluated the effects of ghrelin on UUO-induced renal dysfunction, fibrosis, and inflammation and verified the mechanisms of ghrelin-mediated ERS and NLRP3 inflammasome signaling in renal fibrosis.

Materials and methods

Reagents and antibodies

Ghrelin was purchased from Sigma Chemical (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO). Antibodies against α-SMA, Collagen-I, fibronectin and GAPDH, were obtained from Abcam (Cambridge, UK). Mouse monoclonal anti-Nlrp3 antibody was purchased from adipoGen (San Diego, CA). Rabbit polyclonal anti-IL-18, IL-1β and anti-caspase-1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against GRP78, Caspase12, IRE1α, CHOP, ATF4, PERK, phospho-PERK, eIF-2α, phospho-eIF-2α, BAX, BCL-2, phospho-Erk, Erk and β-actin were purchased from Cell Signalling Technology.

Animal studies

The study protocols were reviewed and approved by the Institutional Animal Care and Use Committee at Fudan University, China. C57BL/6J mice (male, 20-25 g, 8 weeks old) were housed in a light-, temperature-, and humidity-controlled environment under standard conditions with a 12-h light-dark cycle and free access to water and feed. The mice were divided into four groups (n = 6 per group): Sham (control); UUO; UUO+ghrelin; ghrelin. For the experiments, the mice were anesthetized, an incision was made in the abdominal midline, and the left proximal ureter was exposed and ligated with 6-zero silk sutures. In sham-operated mice, the ureters were manipulated but not ligated. Ghrelin (100 μg/kg/day) was administered by daily subcutaneous injection from the day of surgery until the end of the experiment [18]. The mice were anesthetized, and kidney sections were fixed in 10% formalin and paraffin-embedded. In addition, kidney samples were immediately frozen in liquid nitrogen and stored at -80°C for further analyses.

Cell culture

Human renal proximal tubular cells (HK-2 cells) were cultured in DMEM/F12 medium, supplemented with 10% bovine serum at 37°C and 5% CO₂ in a humidified incubator. In vitro experiments, cells were incubated with ghrelin (10 nM) for 1 h, then treated with AngII (10⁻⁸ mol/L) for further 24 h [19].
Histopathologic examination

The kidneys were sectioned (4 μm thickness), processed, and stained with periodic acid-Schiff (PAS) and Masson trichrome [20]. After PAS staining, the severity of glomerular injury was evaluated using light microscopy in accordance with the following semi-quantitative grades: grade 0, normal; grade 1, < 25% segmental lesion; grade 2, 25%-50% segmental lesion; grade 3, 50%-75% segmental lesion; grade 4, 75%-100% segmental lesion. At least 20 glomeruli were analyzed per group. Following Masson trichrome staining, the area of tubulointerstitial fibrosis was measured in 10 random fields under ×400 magnification.

Immunohistochemical staining

Kidney tissues were fixed with PBS containing 4% paraformaldehyde and embedded in paraffin. Sections were cut 3-μm. Immunohistochemical staining was performed using primary antibody against type I collagen (1:200) and fibronectin (1:200) overnight at 4°C. The sections were subsequently incubated with the corresponding secondary antibodies at room temperature for 1 h. Diaminobenzidine was
Ghrelin attenuates renal fibrosis

Western blotting

Harvested renal and cell proteins were boiled at 98°C in Laemmli buffer with 5% β-mercaptoethanol for 5 min, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride membrane, blocked with 5% milk in Tris-buffered saline-Tween 20 (10 mM Tris-HCl, pH 8.0; 150 mM NaCl; 0.5% Tween 20) for 1 h [22], and probed with primary antibodies against NLRP3, caspase-1, IL-1β, IL-18, α-SMA, GRP94, CHOP, BAX, Bcl-2, ATF4, PERK, phospho-PERK, EIF2α, GRP78, Caspase12, IRE1α, phospho-Erk, Erk and phospho-EIF2α (all, 1:1000) at 4°C overnight. The membranes were incubated with horseradish peroxidase-labelled secondary antibodies for 1 h at 37°C, then the blots were visualized with the ECL-plus detection system (Merck Millipore, USA), and band density was quantified by ImageJ (http://imagej.nih.gov/ij/).

Statistical analysis

Data are expressed as the mean ± standard error of the mean (SEM). Comparisons between groups were performed with one-way analysis.
Ghrelin attenuates renal fibrosis

Results

Ghrelin attenuated renal injury in UUO mice

We evaluated renal fibrosis by Masson trichrome staining. The UUO group had increased glomerular and interstitial fibrosis; ghrelin treatment attenuated these changes (Figure 1A). Consistent with this finding, quantitative determination of the tubulointerstitial fibrosis index revealed that UUO induced marked renal fibrosis compared with the Sham group; however, ghrelin treatment inhibited the UUO-induced renal fibrosis (Figure 1A, 1B). Similarly, mesangial matrix expansion was evident in the glomeruli of the UUO group. Compared with the Sham group, mice in the UUO group had significantly larger PAS-positive mesangial matrix areas (Figure 1A). However, treatment with ghrelin significantly attenuated the renal injury and decreased the glomerular injury score (Figure 1C).

Ghrelin alleviated the expression of pro-fibrosis factors and renal fibrosis induced by UUO

To investigate whether ghrelin regulates UUO-induced renal fibrosis, we quantified the amount of type I collagen, fibronectin, and α-SMA by immunohistochemical staining and western blotting. At 14 days after UUO surgery, there was an obvious increase in collagen deposition in the renal interstitial area in UUO group, which ghrelin treatment significantly reduced renal fibrosis. Consistent with these pathological changes, the kidney cortices of UUO mice had significantly increased expression levels of fibronectin. However, this increase was attenuated in ghrelin-treated UUO mice (Figure 2). UUO induced marked type I collagen and α-SMA protein expression, while ghrelin administration decreased it (Figure 3). Ghrelin inhibited renal fibrosis in uuo mice, may most

Figure 3. Ghrelin alleviated α-SMA and type I collagen expression in UUO mice. (A) Representative immunoblots of type I collagen and α-SMA. (B, C) Mean quantitative analysis of relative expression of type I collagen (B) and α-SMA (C) in the experimental groups. **P < 0.01 versus Sham group; *P < 0.05 versus UUO group; #P < 0.01 versus UUO group. n = 6.

Figure 4. Ghrelin alleviated P-Erk activation in UUO mice. A. Representative immunoblots of P-Erk. B. Mean quantitative analysis of relative expression of P-Erk. **P < 0.01 versus Sham group; #P < 0.01 versus UUO group. n = 6.
Ghrelin attenuates renal fibrosis likely by inhibiting the activation of Erk pathway (Figure 4).

Ghrelin decreased renal cell apoptosis during UUO

As apoptosis is an important detrimental outcome of progressive kidney disease, we detected the effect of ghrelin on renal cell apoptosis and dysfunction in response to UUO by evaluating apoptotic renal cells in a UUO mouse model (Figure 5). As expected, UUO increased renal cell apoptosis, and ghrelin prevented the UUO-mediated Bcl-2 downregulation and Bax upregulation, which subsequently increased the Bcl-2/Bax expression ratio (Figure 5). These results all indicate that ghrelin protected renal function by attenuating apoptosis in UUO mice.

Ghrelin inhibited NLRP3 inflammasome activation both in vivo and in vitro

To investigate the role of ghrelin on NLRP3 inflammasome activation in UUO-induced renal injury in vivo, we detected NLRP3, caspase-1, IL-1β, and IL-18 levels using western blotting. The UUO group had significantly upregulated renal NLRP3 and caspase-1 protein levels, while treatment with ghrelin markedly inhibited NLRP3 inflammasome activation (Figure 6). Similarly, renal IL-1β and IL-18 protein levels were increased in the UUO group. However, the administration of ghrelin decreased the levels of these inflammatory cytokines (Figure 6). To confirm the protective role of ghrelin in UUO, we detected whether it could inhibit Ang II-induced NLRP3 inflammasome activation in HK-2 cells. As shown in Figure 6C, 6D, treatment with ghrelin significantly prevented NLRP3 inflammasome activation in Ang II-induced HK-2 injury.

Growing evidence suggests that ERS is involved in renal injury [18, 19]. Therefore, based on the findings discussed above, we hypothesized that ghrelin may be involved in the protective effects against renal injury by inhibiting ERS in UUO mice. Compared with the Sham group, mice in the UUO group had increased expression of the ERS-related protein (Figures 7, 8), and ghrelin treatment decreased ERS marker levels significantly (Figures 7, 8). The ERS-mediated apoptosis pathway was also involved; in particular, the UUO group had markedly increased CHOP and caspase 12 protein levels, and treatment with ghrelin attenuated these changes (Figures 8, 9).

Discussion

This study provides evidence of the overall effects and underlying mechanisms of the ghrelin in the pathogenesis of obstructive nephropathy. First, we showed that UUO induced significant renal damage in mice, as characterized by the increased glomerular injury score, tubulointerstitial fibrosis index, collagen, and ECM deposition in the mouse kidneys. Moreover, mice in the UUO group had increas-
Ghrelin attenuates renal fibrosis

Inflammasomes are cytoplasmic multiprotein complexes that were first proposed by the Tschopp research group in 2002 [23]. Inflammasomes are responsible for caspase-1 activation, the subsequent production of the cytokines IL-1β and IL-18, and the initiation of the inflammatory cell death program termed pyroptosis [24]. Four main types of inflammasomes have been found, including NLRP1, NLRP3, NLRC4, and they are absent in melanoma [25]. So far, the NLRP3 inflammasome has been the most extensively studied. The NLRP3 inflammasome is typically composed of a NLRP3 inflammasome sensor, ASC, and the precursor pro-caspase-1 [26]. Considering the inflammatory response is involved in cellular dysfunction during CKD, and an increasing amount of evidence has demonstrated that NLRP3 inflammasome activation is an important pathogenic factor in multiple kidney diseases [27], inflammation appears to be an important destructive process that mediates injury in the kidneys. In addition, NLRP3 inflammasome activation is involved in UUO-induced renal inflammation and leads to renal fibrosis. A recent study also demonstrated that an NLRP3-specific inflammasome inhibitor improved crystal-induced renal inflammation and fibrosis significantly [28]. Consistent with previous studies, the present study shows that the NLRP3 inflammasome activation was increased both in vivo and in vitro. Moreover, its downstream cytokines including mature IL-1β and IL-18 were increased.

ed renal cell apoptosis, NLRP3 inflammasome activation, and increased ERS activation. We found that treatment with ghrelin had protective effects against renal fibrosis, renal cell apoptosis, NLRP3 inflammasome activation, and ERS. Therefore, ghrelin may be a potential therapeutic target for reducing UUO-induced kidney damage and renal fibrosis.

Figure 6. Ghrelin attenuated activation of the NLRP3 inflammasome in vivo and in vitro. (A, B) Expression of renal NLRP3 protein via western blot (A) and (B) relative protein levels normalized against β-actin in mice. **P < 0.01 versus Sham group; *P < 0.05 versus UUO group; **P < 0.01 versus UUO group. (C, D) Expression of NLRP3 protein via western blot (C) and (D) relative protein levels normalized against β-actin in HK-2 cells. All values are means ± SEM (n = 6). *P < 0.01 versus Con (control) group; **P < 0.01 versus control group; *P < 0.05 versus Ang-II group; **P < 0.01 versus Ang-II group.
Ghrelin attenuates renal fibrosis

also increased in UUO mice. Taken together, these results suggest that the NLRP3 inflammasome may contribute to UUO-induced renal injury. Treatment with ghrelin markedly attenuated kidney disease and decreased NLRP3 inflammasome activation both in vivo and vitro.

The ER is an important cell organelle, and is a site responsible for protein synthesis, folding, and maturation; it participates in Ca²⁺ storage, signal transduction, lipid, and glucose metabolism; and regulates protein synthesis, folding, and congregation after synthesis. When the body is exposed to oxidative stress, ischemia, hypoxia, malnutrition, viral infection, and calcium imbalance, unfolded proteins or false folded proteins accumulate in the ER cavity. This accumulation causes cellular calcium imbalance and ER instability, which results in ERS and the unfolded protein response [29]. Previous studies have indicated that ERS plays a critical role in CKD progression [30]. CHOP, an ERS activation marker, is commonly expressed at low levels and is robustly activated in a wide variety of organs as part of the natural stress response. Here, we show that UUO substantially increases CHOP expression, and demonstrate that ghrelin alleviates the ERS response by markedly reducing the ERS signaling pathway in UUO mice.

Ghrelin is a hormone produced by specific cells in the stomach. It can cause increased pituitary secretion.
Ghrelin attenuates renal fibrosis

of GH. Research on the role of ghrelin has mainly focused on GH secretion and regulation of the balance between growth and energy, and it can promote gastric acid secretion and increase appetite and body weight. At the same time, the universality of its receptor distribution indicates that ghrelin may have a wider biological effect. Recombinant ghrelin was successfully used to alleviate cachexia, anorexia, and gastrointestinal symptoms in patients with cancer and chronic disease [31-33]. In addition, recent studies have shown that ghrelin could attenuate myocardial I/R injury by inhibiting ERS [34]. Recent research has also suggested that ghrelin can alleviate the renal fibrosis caused by UUO, but the mechanism is unknown [35]. In present study, ghrelin alleviated inflammation-induced injury by downregulating the NLRP3 inflammasome, and inhibiting UUO-driven production of IL-18 and IL-1β, suggesting that redundant inflammatory pathways are involved in UUO-induced CKD. Furthermore, ghrelin can block the ERS pathway. The above results suggest that ERS and the NLRP3 inflammasome both contribute to UUO-induced renal injury. Ghrelin may attenuate UUO-induced renal fibrosis by inhibiting the NLRP3 inflammasome and ERS activation.

Conclusions

Our results indicate that ghrelin plays an important role in attenuating UUO-induced renal injury in vivo, possibly by alleviating collagen deposition and preventing NLRP3 inflammasome and ERS activation. Therefore, ghrelin might be an effective strategy for ameliorating obstruction-induced renal injury and the progression of CKD.

Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China 81270822 (to Yong Gu), 81570661 (to Yong Gu), 81400714 (to Min Yang), Shanghai Pujiang Program 17PJ0021 (to Wei Ding), and the Major State Basic Research Development Program of China 2012CB517700 (to Yong Gu).

Disclosure of conflict of interest

None.

Address correspondence to: Yong Gu, Division of Nephrology, The Fifth People’s Hospital of Shanghai, Fudan University, 128 Ruili Road, Shanghai 200240, China. E-mail: yonggu@vip.163.com; Wei Ding, Di-
Ghrelin attenuates renal fibrosis

References


Ghrelin attenuates renal fibrosis


