Original Article
Fibroblast growth factor-2 ameliorates acute renal ischemia and reperfusion injury through ERK1/2-PARP-1 signaling pathway

Wenbin Li, Xiuhua Lian, Lijun Tang, Meiyu Cui, Dongmei Xu
Department of Nephrology, Shandong Provincial Qianfoshan Hospital, Shandong University, Jinan, P. R. China
Received February 4, 2016; Accepted April 27, 2016; Epub June 1, 2016; Published June 15, 2016

Abstract: The aim of this study is to analyze the effect of basic fibroblast growth factor-2 (FGF-2) signaling pathway on acute renal failure (ARF) induced by ischemia and reperfusion (IR), and its relationship with poly (adenosine diphosphate-ribose) polymerase-1 (PARP-1). FGF-2 recombinant protein (r-FGF) was injected into C57BL/6 mouse kidneys after bilateral ischemia. Biochemical indicators of renal dysfunction were measured and kidney histopathology was examined to assess renal injury. Enzyme-linked immunosorbent assay was used to measure the production of cytokines. Subgroups of mice were treated with pharmacological inhibitors of PARP-1 and ERK1/2. Phosphorylation of ERK1/2 and activation of PARP-1 were assessed by Western blotting. IR induction caused ARF by changing the histopathology of kidney and increasing the levels of creatinine and blood urea nitrogen in the blood. Injection of r-FGF increased phosphorylation of ERK1/2 and activation of PARP-1. Pharmacological inhibition of ERK1/2 decreased the activation of PARP-1, indicating that activation of FGF-ERK1/2 pathway was necessary for the activity of PARP-1. By contrast, inhibitor of PARP-1 had little effect on the phosphorylation of ERK1/2. The present study demonstrates that FGF-2 ameliorates acute renal ischemia and reperfusion injury through ERK1/2-PARP-1 signaling pathway.

Keywords: Acute renal ischemia and reperfusion injury, fibroblast growth factor-2, poly (adenosine diphosphate-ribose) polymerase-1 (PARP-1)

Introduction
Acute renal failure (ARF) is a complex disorder that includes a heterogeneous pathogenic phase characterized by organ dysfunction [1], an extension phase featured by inflammatory responses [2], and a resolution phase in which cellular repair typically occurs [1]. One of the major causes of ARF is acute renal ischemia-reperfusion (IR) injury due to hemorrhage, severe injury, shock, or kidney transplantation, and IR injury leads to acute tubular necrosis. Renal ischemia initiates a complex and interrelated sequence of events, resulting in the injury and death of renal cells. Although essential for the survival of ischemic renal tissue, reperfusion causes additional damage [3]. IR of the kidney contributes to renal dysfunction and injury associated with ischemic ARF.

Fibroblast growth factor-2 (FGF-2) is an angiogenic growth factor involved in renal growth and regeneration. FGF-2 is expressed in epithelial and endothelial cells, and mononuclear leukocytes in kidneys under normal and pathological conditions [4, 5]. In addition to its angiogenic activity, FGF-2 is also considered a powerful renal tubular regenerative growth factor [6, 7]. Previous studies show that single intrarenal injection of FGF-2, given simultaneously during the induction of acute renal ischemic injury, accelerates the regeneration and recovery of renal tubules in rats [8, 9]. However, other studies in human and rodents show that renal accumulation of FGF-2 can cause tubular proliferative and fibrogenic lesions [10, 11], and that FGF-2 is involved in fibrogenesis induced by hypertension and IR injury [12]. Some clinical studies show that elevated plasma levels of FGF-2 [13, 14] are possible risk factors for ARF. To date, it is unclear how FGF-2 affects the renal outcome of ARF patients.

FGFs are known to exert their multiple functions through MAPK/ERK pathway [15, 16]. Poly...
FGF-2 ameliorates renal injury via ERK1/2-PARP-1

(adenosine diphosphate-ribose) polymerase-1 (PARP-1), which mediates ERK1/2-initiated transcripational regulation [17], may be one of the downstream effectors of ERK. PARP-1 is a highly conserved DNA binding nuclear protein that catalyzes the covalent attachment of poly (adenosine diphosphate-ribose) units (PAR) to itself and other nuclear target proteins involved in replication, DNA repair, and cell death [3, 18]. It has been shown to be regulated by ERK2 [17, 19]. It is shown that inhibition of PARP activity after ischemia ameliorates the course of ARF [20]. In FGF-induced specification of human embryonic stem cells that involves ERK activation, PARP-1 activity is increased [21]. We therefore hypothesize that PARP-1 may mediate the effect of FGF-2 on ARF. In the present study, we develop a mouse model to explore the relationship between FGF-2 and PARP-1 in the pathogenesis of acute kidney IR injury.

Materials and methods

Animals

Male C57BL/6 mice (25-30 g) obtained from the Shandong Provincial Qianfoshan Hospital Laboratory Animal Center were maintained under standardized conditions and fed a standard rodent diet, with free access to tap water. Animal welfare and experimental procedures were carried out in accordance with the Guidelines for the Care and Use of Laboratory Animals (Ministry of Science and Technology of China, 2006), after approval by the Animal Ethics Committee of Shandong Provincial Qianfoshan Hospital.

To construct mouse model of acute kidney IR injury, the mice in IR group were anesthetized by intraperitoneal injection of pentobarbital (50 mg/kg). Additional pentobarbital was given as needed. The surgery was conducted on a homoeothermic pad, maintaining rectal temperature at 36.8-37.2°C. Midline laparotomy was made to expose kidneys and both of the renal pedicles were clamped with micro-aneurysm clips to block blood flow for 18 min. While clamps were in position, the left kidney was injected with FGF-2 recombinant protein (r-FGF) (30 mg/kg) in parenchymal medullary area [IR (r-FGF) group]. The right kidney was injected with the same volume of normal saline (0.9% NaCl), and used as control [IR (NS) group]. After injection, the micro-aneurysm clips were released for reperfusion. Mice in sham operation group received similar procedure except for renal ischemia. Mice were allowed to recover in a room at warm temperature levels.

PARP inhibitors PJ34 (N-(6-oxo-5,6-dihydropyranthridin-2-yl)-N, dimethylacetamide; Sigma-Aldrich, St. Louis, MO, USA) is effective in ameliorating various inflammatory processes in murine and rodent models [22]. The precise half-life for PJ34 in mice is not known, but is approximately 2 h in rats [23]. IR (NS) group received intraperitoneal administration of 1.0 mL of 0.9% saline solution at 30 min before reperfusion and at 2 h, 6 h, 12 h, and 24 h of reperfusion. IR (PJ34) group received intraperitoneal injection of 10 mg/kg PJ34 dissolved in 1.0 mL of 0.9% saline at 30 min before reperfusion, and at 2 h, 6 h, 12 h, and 24 h of reperfusion at the same time periods.

PD0325901, a potent, highly specific non-ATP-competitive inhibitor of ERK1/2, was dissolved in 20% sulfobutylether-β-cyclodextrin (SBED) solution at a concentration of 10 mg/ml, and filter-sterilized through a 0.2-μm filter. Animals in oral treatment groups received vehicle alone or PD0325901 by gavage at a dose of 5 ml/kg, and were necropsied approximately 24 h after dosing. Mice were sacrificed by exsanguination under heavy anesthesia at 72 h of reperfusion (sham operation group, n = 8; IR group, n = 8; IR (PJ34) group, n = 4; IR (PD0325901) group, n = 4).

Blood chemical analysis

Blood was collected at 24 h and 72 h after injury for the measurement of renal function. Mice were anesthetized with inhaled isoflurane. Blood samples were drawn from postcava, collected in heparin-coated Eppendorf tubes and centrifuged at 1,800 rpm for 10 min before the mice were sacrificed. Blood creatinine and blood urea nitrogen (BUN) levels were measured by an autoanalyzer (LX20; Beckman Coulter, Brea, CA, USA).

Enzyme-linked immunosorbent assay (ELISA)

A complex network of renal mechanisms were involved in the pathophysiology of kidney IR injury that led to structural changes and production of cytokines such as tumor necrosis factor α (TNF-α) and interleukins (ILs) [24].
TNF-α, IL-6 and monocyte chemotactic protein 1 (MCP-1) could be explored as predicting factors of organ dysfunction [25]. Kidneys were homogenized (90 sec) in 4 volumes of ice-cold 0.15 M KC1 with 0.2% Triton X-100. The homogenates were centrifuged at 10,000 rpm for 30 min at 4°C, and the supernatants were used for cytokine assay. Respective ELISA kits were used to detect IL-6 (Jiancheng Biotech Inc., Nanjing, China), MCP-1 (Leinco Technologies, Inc., St. Louis, MO, USA) and TNF-α (Jiancheng Biotech Inc., Nanjing, China).

ELISA plates were read with Spectromax-250 plate reader (Molecular Devices, CA, USA). The values were extrapolated from standard curves and normalized to total protein concentration, which was determined with bicinchoninic acid protein assay reagent kit (Pierce Biotechnology, Thermo Fisher Scientific, Waltham, MA, USA) [26].

Hematoxylin and eosin (HE) staining
Tissues were fixed in 10% phosphate-buffered formalin and embedded in paraffin. Sections (5 μm) were processed for HE staining. Morphological analyses were undertaken by an experienced pathologist blinded to tissue sources. The following parameters were chosen to indicate morphological damages to the kidney after IR: injury (loss of brush border, formation of tubular casts, tubular dilatation) and tubular necrosis. Injury and necrosis were quantified in 10 randomly chosen 40 × microscopic image fields in cortex, corticomedullary junction, and medulla area, respectively. Results were expressed as injury tubular number/total tubular number or necrosis tubular number/total tubular number per field.

Western blotting
Western blotting was performed according to previously published methods [27]. Tissues extracted from mouse kidneys were washed with phosphate-buffered saline and resuspended in lysis buffer (50 mM Tris-HCl pH 8, 0.1 mM EDTA, 0.5% Triton X-100, and 12.5 mM mercaptoethanol) for 30 min on ice. Pellet was eliminated and sample buffer (50 mM Tris-HCl pH 6.8, 6 M urea, 6% mercaptoethanol, 3% sodium dodecyl sulfate, and 0.003% bromo-
FGF-2 ameliorates renal injury via ERK1/2-PARP-1

Phenol blue (pH 7.5) was added to the supernatant. Proteins were resolved on 12% sodium dodecyl sulfate-polyacrylamide gels and transferred onto Immun-Blot polyvinylidene fluoride membrane (Bio-Rad, Hercules, CA, USA). The blots were blocked with 3% nonfat milk in Tris-buffered saline for 2 h, washed with phosphate-buffered saline/Tween 20, and incubated overnight with ERK1/2 (Santa Cruz, Dallas, TX, USA), phosphor-ERK1/2 (Thr202/Tyr204) (Santa Cruz, Dallas, TX, USA), PARP-1 (Thermo Fisher Scientific, Waltham, MA, USA), PAR (Thermo Fisher Scientific, Waltham, MA, USA), or α-Tubulin (Sigma-Aldrich, St. Louis, MO, USA) antibodies. Primary antibodies were used in 1:1000 dilution. The membranes were washed six times of 5 min in Tris-buffered saline (pH 7.5) containing 0.2% Tween 20 before addition of goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:3000 dilution; Bio-Rad, Hercules, CA, USA). Bands were visualized by electrochemiluminescence (ECL PLUS; Amersham Biosciences, Piscataways, NJ, USA), and the pixel volumes of the bands were determined using NIH Image J software (http://rsb.info.nih.gov/ij/).

Statistical analysis

The results were analyzed with Student's t-test when comparing means between two groups. All data were expressed as means ± SEM. Differences with P < 0.05 were considered statistically significant.

Results

IR induction causes ARF by changing the histopathology of kidney and increasing the levels of BUN and creatinine in the blood

To examine renal function and histopathology after IR injury, blood chemical analysis and HE staining were performed. BUN and creatinine levels were significantly elevated in IR group of mice at 24 h and 72 h compared with time-matched sham operation group of mice (P < 0.05) (Figure 1A and 1B). HE staining revealed that IR mice had the loss of brush border, vacuolation, and desquamation of epithelial cells of the proximal tubules and glomerular shrinkage, in contrast to sham operation group (Figure 1C). These results suggest that IR induction causes ARF by changing the histopathology of
FGF-2 ameliorates renal injury via ERK1/2-PARP-1

Figure 3. Effect of r-FGF on renal histopathology. (A) Representative histological micrographs of renal tissues at 72 h after surgery. Kidneys were fixed in 10% formalin, embedded into paraffin and 5-μm thin sections were cut with microtome. Sections were stained with hematoxylin and eosin (HE). Original magnification, × 400. Figures
FGF-2 ameliorates renal injury via ERK1/2-PARP-1

were randomly chosen. (B, C) Quantification of (B) tubular injury and (C) tubular necrosis in cortex, corticomedullary junction and medulla area. A minimum of 10 fields for each kidney slide were examined to evaluate the severity of changes. Results were expressed as injury tubular number/total tubular number or necrosis tubular number/total tubular number per field. The four groups were sham operation (NS) group (kidneys with injection of saline in mice with sham surgery, n = 8), sham operation (r-FGF) group (kidneys with injection of r-FGF in mice with sham surgery, n = 8), IR (NS) group (kidneys with injection of saline in mice with IR surgery, n = 8), and IR (r-FGF) group (kidneys with injection of r-FGF in mice with IR surgery, n = 8). Data are expressed as means ± S.E.M. *, P < 0.05 compared with sham operation (NS) group at the same time point; #, P < 0.05 compared with sham operation (NS) group at the same time point.

Figure 4. Effect of r-FGF on PARP-1 activity and phosphorylation of ERK1/2 in kidneys. A. Representative Western blots of PAR, PARP-1, cleaved PARP-1, phosphorylated ERK1/2 and total ERK1/2 in kidneys at 72 h after surgery. B. Quantification of Western blots of PAR, PARP-1, and cleaved PARP-1 proteins against α-Tubulin. C. Quantification of Western blots expressed as phosphorylated ERK1/2/total ERK1/2 ratio. The four groups were sham operation (NS) group (kidneys with injection of saline in mice with sham surgery, n = 8), sham operation (r-FGF) group (kidneys with injection of r-FGF in mice with sham surgery, n = 8), IR (NS) group (kidneys with injection of saline in mice with IR surgery, n = 8), and IR (r-FGF) group (kidneys with injection of r-FGF in mice with IR surgery, n = 8). Data are expressed as means ± S.E.M. *, P < 0.05 compared with sham operation (NS) group; #, P < 0.05 compared with IR (NS) group.

Injection of r-FGF enhances the levels of inflammatory cytokines in kidney, and aggravates injury and necrosis in sham operation group and IR group, respectively

To test the effect of r-FGF on kidney injury, ELISA and HE staining were employed. Compared sham operation (NS) group, the protein levels of TNF-α, IL-6, and MCP-1 in kidney tissues from IR (NS) group were significantly increased (P < 0.05). Injection of r-FGF resulted in significant elevation of local inflammatory cytokine levels in both sham operation group and IR group (P < 0.05 for both) (Figure 2). HE staining showed that IR injury caused severe damage to epithelial cells of tubule especially in the corticomedullary junction area. Loss of brush border and detachment of epithelial cells from the basement membrane caused tubular injury and necrosis in cortex, corticomedullary junction and medulla area (Figure 3A). In kidney and increasing the levels of BUN and creatinine in the blood.

Injection of r-FGF enhances the levels of inflammatory cytokines in kidney, and aggravates injury and necrosis in sham operation group and IR group, respectively.
FGF-2 ameliorates renal injury via ERK1/2-PARP-1

addition, r-FGF caused significantly more severe histological injury in normal kidneys from sham operation group (P < 0.05) (Figure 3B). In addition, tubular necrosis at corticomedullary junction and medulla area caused by IR was significantly more severe in kidneys with injection of r-FGF than those in IR (NS) group (P < 0.05) (Figure 3C). These results indicate that injection of r-FGF enhances the levels of inflammatory cytokines in kidney, and aggravates injury and necrosis in sham operation group and IR group, respectively.

Injection of r-FGF elevates the activity of PARP-1 and the phosphorylation of ERK1/2

To investigate the effect of r-FGF on the activity of PARP-1 and phosphorylation of ERK1/2, Western blotting was carried out. Immunoblotting analysis indicated that PARP-1 activity, revealed by an antibody against PAR, was increased in IR (NS) group compared with sham operation (NS) group (P < 0.05), whereas the level of PARP-1 protein expression was not altered. PARP-1 activity was also significantly increased in kidneys of sham operation (r-FGF) group and IR (r-FGF) group compared to sham operation (NS) group and IR (NS) group, respectively (P < 0.05) (Figure 4A and 4B). In addition, phosphorylation of ERK1/2 was increased in kidneys during acute renal IR injury in IR (NS) group compared with that in sham operation (NS) group (P < 0.05). Injection of r-FGF increased the phosphorylation of ERK1/2 in sham operation (r-FGF) group and IR (r-FGF) group compared with sham operation (NS) and IR (NS) group, respectively (P < 0.05) (Figure 4A and 4C). These results suggest that injection of r-FGF elevates the activity of PARP-1 and the phosphorylation of ERK1/2.

PARP-1 activity is regulated by FGF-ERK1/2 signaling pathway during acute IR injury

To determine whether PARP-1 activity is regulated by FGF-ERK1/2 pathway in acute renal...
Figure 6. Effect of PJ34 on renal histopathology. A. Representative histological micrographs of renal tissues at 72 h after surgery. Kidneys were fixed in 10% formalin, embedded into paraffin and 5-μm thin sections were cut with microtome. Sections were stained with hematoxylin and eosin. Original magnification, × 400. Figures were randomly chosen. B. Quantification of tubular injury in cortex, corticomedullary junction and medulla area. C. Quantification of tubular necrosis in cortex, corticomedullary junction and medulla area. A minimum of 10 fields for each kidney slide.
were examined to evaluate the severity of changes. Results were expressed as injury tubular number/total tubular number or necrosis tubular number/total tubular number per field. The four groups were IR (NS) group (kidneys with injection of saline in mice with IR surgery, n = 4), IR (r-FGF) group (kidneys with injection of r-FGF in mice with IR surgery, n = 4), PJ34 (NS) group (kidneys with injection of saline in mice with IR surgery and treatment with PJ34, n = 4), and PJ34 (r-FGF) group (kidneys with injection of r-FGF in mice with IR surgery and treatment with PJ34, n = 4). Data are expressed as means ± S.E.M. * P < 0.05 compared with IR (r-FGF) group.

Discussion

Renal glomerular and tubular structures are capable of trapping large quantities of FGF-2 from blood circulation [28]. FGFs exert their biological effects by binding to and activating their receptors. These interactions result in a series of molecular events such as mitosis, differentiation and migration [22]. FGF receptors are localized throughout the whole cell and, mainly in tubular segments of the inner stripe of the outer medulla as observed in some cases [28]. These findings can explain the proliferative and apoptotic changes induced by FGF-2 during ARF. It is suggested that the accumulation of FGF-2 in the kidney can cause these changes. Therefore, we manipulated FGF-2 levels in the kidney, and evaluated the effect and downstream mechanism of FGF-2 in kidney IR injury.

It is reported that additional FGF-2 does not cause significant changes in normal tissues [29]. However, our experiments showed that injection of r-FGF facilitates diverse inflammatory responses by promoting the production of inflammation-relevant proteins, including IL-6, TNF-α, and MCP-1. These proteins play significant roles in ARF [30-32]. In the present study, treatment with r-FGF also causes tubular injury in kidneys from sham operation group. Different ways to treat animals with r-FGF may lead to different results. A study shows that FGF-2 can induce significant mitogenic changes in the presence of preexisting vascular injury [29]. In our experiments, TNF-α, IL-6 and MCP-1 protein levels that are up-regulated by IR injury, are further elevated in kidneys injected with r-FGF compared with kidneys treated with normal saline. In addition, the extent of renal damages caused by IR injury is aggravated by r-FGF injection. These results demonstrate the destructive effect of r-FGF in the pathogenesis of ARF.

It has been shown that FGF-2 induces the proliferation of cultured human renal tubular epithelial cells by activating an ERK-dependent pathway [33]. Therefore, we speculate that renal tubular injury induced by FGF-2 is at least partially mediated by an ERK-dependent pathway. The present study shows a sharp increase in the phosphorylation of ERK1/2 in kidneys injected with r-FGF compared to those injected with normal saline after IR surgery. These results indicate that ERK1/2 is a predominant downstream molecule activated by FGF signaling. PARP-1, a conserved nuclear enzyme that catalyzes the polymerization of adenosine diphosphate-ribose units to target proteins, plays a role as DNA damage sensor in different DNA repair pathways [34]. As it is regulated by ERK1/2 during cell proliferation and differentiation [18, 34], PARP-1 emerges as a potential mediator. IR injury leads to excessive PARP-1 activation [3]. PARP overactivation consumes large quantities of NAD+, slowing down the rate of ATP formation and eventually leading to functional impairment or cell death [35]. Excessive PARP activation in IR injury is also found in a variety of tissues, organs, and models [18]. Our
data have shown that kidney IR injury results in the activation of nuclear enzyme PARP. PARP-1 expression remains constant, but PAR activity is increased as the accumulation of local r-FGF, during which the phosphorylation ERK1/2 is also enhanced. The temporal course of PAR activity suggests that PARP-1 may be downstream of FGF-ERK pathway.

Various PARP inhibitors and PARP-1 specific non-pharmacological inhibition have resulted in basically identical effects in different systems [17, 36]. Our results demonstrate that treatment with PJ34 removes most of the auto-PARylating activity from PARP-1, but does not affect p-ERK1/2 levels in kidneys. In addition, PJ34 ameliorates histological lesions in acute kidney IR injury. To determine whether PARP-1 activity is regulated by FGF-ERK1/2 pathway in kidney injury, we measured the level of PAR, the enzymatic product of PARP-1, in the presence of PD0325901, an inhibitor of ERK1/2. Interestingly, PD0325901 treatment significantly reduces the level of PAR formation. However, the amount of PARP-1 protein is not altered by treatment with PD0325901. Taken together, these results suggest that PARP-1 activity is regulated by FGF-ERK1/2 signaling pathway during acute kidney IR injury, and is responsible for histopathological lesions in kidneys. To summarize, renal lesions caused by IR injury may be aggravated by local r-FGF injection, but can be alleviated by pharmacological inhibition of PARP-1. The present study demonstrates that PARP-1 activity is regulated by FGF-ERK1/2 pathway in acute renal IR injury.

Acknowledgements

This work was supported by Shandong National Health and Family Planning Commission Foundation of China (No. 2015WS0224).

Disclosure of conflict of interest

None.

Address correspondence to: Dongmei Xu, Department of Nephrology, Shandong Provincial Qianfoshan Hospital, Shandong University, No. 16766 Jingshi Road, Jinan 250014, Shandong Province, P. R. China. Tel: 86-13395312810; E-mail: xudongmei63@163.com

References


FGF-2 ameliorates renal injury via ERK1/2-PARP-1

