Original Paper

The Nrf2/HO-1 Pathway Mediates the Antagonist Effect of L-Arginine On Renal Ischemia/Reperfusion Injury in Rats

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Key Words

Renal ischemia/reperfusion injury • Oxidative stress • Nrf2/HO-1 • L-arginine/NO

Abstract

Background/Aims: Ischemia/reperfusion (I/R) is the most common cause of acute renal injury. I/R-induced oxidative stress is involved in the development of acute renal injury, which can be reversed by supplementation with L-arginine, a precursor of nitric oxide (NO). This study was conducted to evaluate alterations in the expression of transcription factors [nuclear factor kappa B (NF-κB), nuclear factor-E2-related factor-2 (Nrf2), and heme oxygenase 1 (HO-1)] and heat shock protein 70 (HSP70) in the kidney of I/R-induced injury rats. **Methods:** Sprague-Dawley (SD) rats were subjected to bilateral renal ischemia for 45 min followed by reperfusion for 24 h. Group 1, Sham; group 2, I/R; group 3, L-arginine; and group 4, L-arginine+zinc protoporphyrin (ZnPP). The levels of serum creatinine (Scr), blood urea nitrogen (BUN), serum nitric oxide (NO), histic malondialdehyde (MDA) and reactive oxygen species (ROS) and superoxide dismutase (SOD) activity were determined, and the expression levels of Nrf2, HO-1, NF-κB, and HSP70 were evaluated. **Results:** The treatment of rats with L-arginine produced a significant reduction in the levels of BUN, Scr, MDA and a significant enhancement in the level of NO and in the activity of SOD compared to renal I/R groups. The expression levels of Nrf2, HO-1, and HSP70 were strongly increased, and the expression of NF-κB and production of ROS were significantly decreased in the L-arginine group compared to that of the I/R group. ZnPP increased renal damage and displayed effects opposite to those of L-arginine. **Conclusion:** These findings suggested that L-arginine/NO reduces renal dysfunction associated with I/R of the kidney and may act as a trigger to regulate the NF-κB, HSP70 and Nrf2/HO-1 signaling cascades.

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Introduction

Renal ischemia/reperfusion (I/R) results in considerable alterations in cell function and metabolism and in the structural regularity of the proximal tubule [1-7]. Renal I/R is an important effect of renal dysfunction in renal transplantation, surgical revascularization of the renal artery, partial nephrectomy, and treatment of suprarenal aortic aneurysms [8]. The mechanisms underlying I/R-induced renal injury are complex, and many steps are still unclear [9-11].

Reactive oxygen species (ROS) generated during renal ischemia and reperfusion are one of the major causes of renal damage [12]. Exogenous antioxidants aimed at reducing ROS injury have been studied in the treatment of I/R-induced renal damage; however, they have not been used widely because of side effects, including DNA damage [13]. Evidence indicates that stimulation of endogenous antioxidant systems might provide a strategy to achieve renoprotection from I/R injury [14]. NF-E2-related factor (Nrf2), an inducible transcription factor, regulates multiple cellular antioxidant systems that limit oxidative stress during I/R-induced renal damage [15]. Under normal conditions, Nrf2 is degraded by the Kelch-like ECH-associated protein-1 (Keap1)-dependent pathway [16]. Once activated, Keap1-Nrf2 binding is disrupted, and Nrf2 transactivates antioxidant response element (ARE)-driven genes in the nucleus, including superoxide dismutase (SOD), glutathione peroxidase (GSH-PX) and heme oxygenase-1 (HO-1) [17]. Nrf2 has been proposed to be the hub of defense against oxidative stress in the pathophysiology of renal ischemia/reperfusion injury (RI/RI) [15].

Nuclear factor-kappa B (NF- κ B), a transcription factor, has complex roles in renal functions as well as in cell cycle changes when cellular stress was provoked, ROS was produced and DNA damage occurred [18]. NF- κ B is involved in the genesis of several inflammatory diseases including RI/RI [18]. The activation of NF- κ B is extremely important for both oxidative stress and inflammatory signaling pathways in RI/RI.

Endogenous heat shock proteins (HSPs), categorized into various subfamilies based on their molecular weight, have their expression induced upon tissue stress as a cellular protection mechanism [19]. HSP70 is a chaperone protein that has a key role in stress tolerance [20]. Expression of HSP70 is an endogenous protective mechanism that protects against the progression of I/R [19]. Previous studies have noted that HSP levels, particularly those of HSP70, were highly sensitive to I/R injury in rats kidneys [21].

Nitric oxide (NO), a soluble free radical gas, is an extremely important endogenous vasodilator that has a variety of physiological and pathological effects in biological structures [22, 23]. NO is synthesized from L-arginine and molecular oxygen by the enzyme nitric oxide synthase (NOS) [22, 24] and is produced by the endothelial cells to relax the preglomerular arteries to improve renal blood flow [25, 26]. Some studies have reported that L-arginine/NO alleviates RI/RI [27, 28]. The purpose of this study was to determine the role of L-arginine (a precursor of NO) on RI/RI via the Nrf2/HO-1 signaling cascades, to determine whether the upregulation of Nrf2 is involved in the protective mechanism by L-arginine, and to determine whether Nrf2 expression or activity can be partly modulated by pharmacological or genetic intervention.

Materials and Methods

Animals and reagents

Sprague-Dawley (SD) rats (180-220 g) were supplied by the Animal Research Center of Jiaxing University Medical College, Jiaxing, China. All procedures and the care administered to the animals was approved by the institutional ethic committee. All reagents and drugs were purchased from Sigma.

Animal experimental design

A total of 40 SD rats were randomly assigned to 4 equal-sized groups: (1) Sham group; (2) I/R group: 45 min of ischemia followed by 24 h reperfusion; (3) I/R+L-arginine group (L-arginine group): the same surgical procedure as in group 2 was performed except that L-arginine (300 µg/kg/min i.v.) was infused for 20 minutes by an infusion pump starting at 30 min prior to ischemia; (4) I/R+L-arginine+ZnPP group (ZnPP group): zinc protoporphyrin (ZnPP, 20 mg/kg, a HO-1 inhibitor) was intraperitoneally administered 24 h prior to ischemia, L-arginine treatment as administered to the L-arginine group. The doses were selected based on previous reports [29, 30].

Surgical procedures of I/R

The surgical methods have been described previously [31]. Rats were anesthetized with 1% pentobarbital sodium (50 mg/kg) by intraperitoneal injection. The abdomen was opened, and the bilateral renal artery and vein were separated. The artery and vein were occluded for 45 min by two arterial clamps, and success was confirmed by observing the change in color of the kidney. The arterial clamp was removed after 45 min and the renal artery and vein were allowed to reperfuse the kidney for 24 h. Blood samples were collected from the abdominal aorta, and centrifuged at 3600×g for 15 min to harvest the sera. The left kidneys of the animals were removed and sectioned in half lengthways. One-half of these kidneys were homogenized in ice-cold normal saline to create a 10% homogenate solution, then centrifuged at 3600 ×g for 15 min to harvest the supernatant. The other half of the kidneys were used for conventional hematoxylin-eosin (HE) staining.

Histopathologic evaluation

The renal tissues were fixed in formalin, paraffin-embedded, sliced into 4 µm sections, stained with hematoxylin-eosin (HE) staining, and observed under an optical microscope. Briefly, twenty-four areas corresponding to the kidney proximal tubules were graded for the degree of renal injury based on the following parameters: hemorrhage, cytoplasmic vacuole formation, tubular dilatation, and tubular cell necrosis. Specifically, one whole deep coronal section was measured under the microscope and graded according to the extent of injury, based on the percentage of kidney area affected. Higher scores represented more severe damage, with the maximum score being 4 [0, histopathological changes <10%; 1, (10%–25%); 2, (25%–50%); 3, (50%–75%); and 4, (75%–100%)]. The mean score for each parameter was decided and subjected to statistical analysis [32–34].

Measurement of serum BUN, Scr, and NO on RI/RI

The blood BUN and Scr levels were examined using the o-phthalaldehyde and picric acid methods [35]. The serum NO level was measured by the nitrate reductase method [36]. Briefly, the blood samples of different groups were obtained after reperfusion. The sera were separated and measured with a Hitachi automatic biochemical analyzer 7060c (Hitachi, Japan) employing a kit provided by Roche Diagnostics (Mannheim, Germany).

Measurement of SOD activity and MDA content

The levels of SOD and MDA in renal tissues were used to assess lipid peroxidation. The SOD activity and MDA content were measured at 550 and 532 nm using the xanthine oxidase and thiobarbituric acid methods [35]. The levels are expressed as U of SOD/mg protein and nmol of MDA/mg protein.

ROS level assessment

The methods used to measure ROS levels have been described previously [37]. After 24 h of reperfusion, rats were anesthetized with 1% pentobarbital sodium (50 mg/kg), and CM-H₂DCFDA (100 µg) was injected into the renal circulation [38]. Forty-five minutes after CM-H₂DCFDA injection, the kidney was harvested and placed in 4% paraformaldehyde for approximately 24 h, transferred to 20% sucrose for 12 h and then frozen in liquid nitrogen. Cryostat sections were cut at –20 °C, and the sections were placed on Star-Frost adhesive slides and air-dried for 3 min [37]. Sections were washed in PBS and stained with DAPI for fluorescence microscopy qualitative evaluation of ROS. ROS quantitation in isolated mitochondria was performed using the amplex red H₂O₂/peroxidase assay kit through spectrofluorometer and emission wavelengths of 544 and 590 nm, respectively [38].

Immunohistochemical examination

Sections with a thickness of 5 μ m were obtained from the left kidney segments and mounted on poly-l-lysine coated slides [39]. The sections were immersed in 0.3% H_2O_2 for 15 minutes and washed with PBS. The sections were then incubated with rabbit anti-HSP70 polyclonal antiserum (Abcam, UK) for 1 hour (1:100 dilution). After the primary incubation and three rinses in PBS, sections were incubated in biotinylated goat anti-rabbit IgG (Invitrogen, CA, USA) for 10 minutes (1:2000 dilution). Following the incubation with the chromagen substrate solution for 10 minutes, all sections were washed in PBS and distilled water, mounted in glycerol and examined later under the microscope. Renal sections staining positive for HSP70 were assessed and compared among groups.

Western blot analysis

The western blot methodology has been described previously [40]. Briefly, renal tissues were homogenized in protein lysate buffer. The homogenates were resolved on polyacrylamide SDS gels and electrophoretically transferred to polyvinylidene difluoride membranes. The membranes were blocked with 3% BSA, then incubated with primary Abs against active HO-1 or NF- κ B, and subsequently with alkaline phosphatase-conjugated secondary Abs. The membranes were developed with 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium. Blots were probed with an anti- β -actin Ab, and the levels of proteins were normalized to β -actin expression.

Statistical analysis

All data are expressed as the mean $\pm$ standard deviation (SD). Analysis of variance (ANOVA) test and Student's t test were used to evaluate statistical significance. A value of less than 0.01 ($p < 0.01$) was considered statistically significant.

Results

Histopathologic evaluation

Tubular cell necrosis, cytoplasmic vacuoles, hemorrhage, and tubular dilatation were observed in histological specimens in the I/R group but were absent in the sham group. Histological alterations were significantly reduced in specimens from the L-arginine group compared to the I/R group. Histological alterations were moderately reduced in ZnPP group compared with the I/R group (Fig. 1).

Serum level of BUN and Scr

The serum levels of BUN and Scr in the I/R group were significantly higher than in Sham group ($p < 0.01$). The levels of BUN ranged from 7.3 to 8.8 mmol (8.1 ± 0.8 mmol) in the Sham group rats, whereas the BUN level reached 57.0 ± 4.4 mmol in the I/R group rats. Administration of L-arginine significantly reduced the BUN levels (23.8 ± 3.6 mmol), whereas administration of ZnPP moderately reduced the BUN levels (32.3 ± 4.1

Fig. 1. Histopathologic evaluation of kidney injury and quantitative injury scores. Note: a, sham group; b, I/R group; c, L-arginine group; d, ZnPP group. * $p < 0.01$ vs. Sham group; ** $p < 0.01$ vs. I/R group; *** $p < 0.01$ vs. L-arginine group. Light microscopy images ($\times 400$).

Fig. 2. Serum level of BUN and Scr. Note: * $p < 0.01$ vs. Sham group; ** $p < 0.01$ vs. I/R group; # $p < 0.01$ vs. L-arginine group.

mmol) compared with the I/R group rats ($p < 0.01$).

The Scr levels ranged from 159.2 to 170.5 μmol ($164.4 \pm 5.7 \mu\text{mol}$) in the Sham group rats, whereas the Scr level reached $521.9 \pm 13.5 \mu\text{mol}$ in the I/R group rats. Administration of L-arginine significantly reduced the Scr levels ($215.7 \pm 10.2 \mu\text{mol}$), whereas administration of ZnPP moderately reduced the Scr levels ($298.3 \pm 11.3 \mu\text{mol}$) compared with the I/R group rats ($p < 0.01$) (Fig. 2).

The activity of SOD and the level of MDA in renal tissues

SOD activity in renal tissues was significantly less in the I/R group than in the Sham group ($p < 0.01$). The activity of SOD in renal tissues reached $77.3 \pm 5.1 \text{ U}$ in the Sham group, whereas the activity of SOD in renal tissues of the I/R group was $17.7 \pm 2.2 \text{ U}$. Administration of L-arginine significantly increased SOD activity ($34.3 \pm 3.6 \text{ U}$) in renal tissues compared with the activity of SOD in renal tissues in the I/R group ($p < 0.01$), whereas administration of ZnPP moderately increased the activity of SOD ($25.9 \pm 3.3 \text{ U}$) compared to the I/R group ($p < 0.01$).

The level of MDA in renal tissues was induced by I/R and was significantly higher than that in the Sham group ($p < 0.01$). The level of MDA in renal tissues reached $6.4 \pm 0.5 \text{ nmol}$ in the Sham group, whereas the level of MDA in the renal tissues of the I/R group reached $13.7 \pm 0.5 \text{ nmol}$. Administration of L-arginine significantly reduced the level of MDA ($8.7 \pm 0.4 \text{ nmol}$) in renal tissues compared with the level of MDA in renal tissues of the I/R group ($p < 0.01$), whereas administration of ZnPP moderately reduced the level of MDA ($10.6 \pm 0.8 \text{ nmol}$) compared to the I/R group ($p < 0.01$) (Fig. 3).

Serum level of NO

The serum level of NO in I/R rats was higher than that in the Sham group ($p < 0.01$). The serum level of NO reached $5.7 \pm 0.4 \mu\text{mol/L}$ in the Sham group, whereas the serum level of NO reached $8.5 \pm 0.4 \mu\text{mol/L}$ in the I/R group. Administration of L-arginine significantly

Fig. 3. SOD activity and MDA levels in renal tissues induced by I/R. Note: * $p < 0.01$ vs. Sham group; ** $p < 0.01$ vs. I/R group; # $p < 0.01$ vs. L-arginine group.

increased the serum level of NO ($15.8 \pm 0.7 \mu\text{mol/L}$) compared with the serum level of NO in the I/R group ($p < 0.01$), whereas administration of ZnPP moderately increased the serum level of NO ($11.3 \pm 0.9 \mu\text{mol/L}$) compared to the I/R group ($p < 0.01$) (Fig. 4).

ROS expression

Renal ROS expression was measured using fluorescence microscopy. Mitochondrial ROS was greater in the I/R group than in the Sham group ($p < 0.01$). The L-arginine group exhibited significantly reduced

Fig. 4. Serum NO levels. Note: * $p < 0.01$ vs. Sham group; ** $p < 0.01$ vs. I/R group; # $p < 0.01$ vs. L-arginine group.

Fig. 5. Expression and quantitation of ROS. Note: a, Sham group; b, I/R group; c, L-arginine group; d, ZnPP group. * $p < 0.01$ vs. Sham group; ** $p < 0.01$ vs. I/R group; # $p < 0.01$ vs. L-arginine group.

Fig. 6. Expression and quantitation of HSP70. Note: a, Sham group; b, I/R group; c, L-arginine group; d, ZnPP group. * $p < 0.01$ vs. Sham group; ** $p < 0.01$ vs. I/R group; # $p < 0.01$ vs. L-arginine group.

ROS compared with the I/R group ($p < 0.01$), whereas administration of ZnPP moderately downregulated expression of ROS compared to the I/R group ($p < 0.01$) (Fig. 5).

Immunohistochemical analysis

The expression of HSP70 in renal tissues that was induced by I/R was slightly higher than that in the Sham group ($p < 0.01$). Administration of L-arginine significantly upregulated expression of HSP70 in renal tissues compared with expression of HSP70 in the I/R group ($p < 0.01$), whereas administration of ZnPP moderately upregulated expression of HSP70 compared to the I/R group ($p < 0.01$) (Fig. 6).

The expression of HO-1 and NF- κ B in renal tissues

Expression of HO-1 in renal tissues was induced by I/R compared to the Sham group ($p < 0.01$). Administration of L-arginine significantly upregulated expression of HO-1 in renal tissues compared with expression of HO-1 in the I/R group ($p < 0.01$), whereas administration of ZnPP moderately upregulated expression of HO-1 compared to the I/R group ($p < 0.01$).

Expression of NF- κ B in renal tissues was significantly induced by I/R compared to that in the Sham group ($p < 0.01$). Administration of L-arginine significantly downregulated

Fig. 7. Expression and quantitation of HO-1 (a) and NF- κ B (b). Note: * $p < 0.01$ vs. Sham group; ** $p < 0.01$ vs. I/R group; # $p < 0.01$ vs. L-arginine group.

expression of NF- κ B in renal tissues compared with expression in the I/R group ($p < 0.01$), whereas administration of ZnPP moderately downregulated expression of NF- κ B compared with the I/R group ($p < 0.01$) (Fig. 7).

Discussion

Renal histopathologic evaluation, renal dysfunction, and oxidative stress were used to ascertain the establishment of RI/RI. RI/RI led to renal dysfunction as reflected by elevated serum BUN and Scr levels and by tissue SOD and MDA concentrations [41]. Previous literature has shown that pretreatment of rats subjected to I/R with L-arginine significantly increased kidney antioxidant status and NO levels at 24 after reperfusion [27, 28]. Our studies also showed that intravenous treatment with L-arginine significantly protected rat kidneys against renal I/R-induced injury in terms of renal dysfunction, renal pathological changes, oxidative stress and nitric oxide imbalance. These results indicate that L-arginine offers a potential therapy in preventing and treating renal ischemic damage in clinical use.

ROS were generated during renal ischemia and reperfusion and were one of the major causes of renal damage [41]. Studies have demonstrated that NO protects against renal ischemic damage through its antioxidative activity in vivo [29]. Previous research has shown that NO attenuated the level of MDA [29], which is a key end product of endogenous membrane lipid peroxidation. Thus far, Nrf2 has been shown to be the most important transcription factor regulating endogenous antioxidant system, and its activation can exert renoprotective effects against renal ischemic damage [42]. Moreover, Nrf2 is a key regulator of the antioxidative defense pathway [43], and it is activated on RI/RI [44]. Normally, Nrf2 is present in the cytosol; however, stress conditions lead to it being translocated to the nucleus. In addition, Nrf2 can be degraded via the Keap1-dependent pathway [16], and Nrf2 activation may raise NO levels [45]. In this study, we found that Nrf2 translocated to the nucleus in response to NO derived from L-arginine, a precursor of NO. Furthermore, upregulation of Nrf2 translocation increased HO-1 expression in response to L-arginine. These findings provide evidence for a relationship between Nrf2 and adaptive responses. In addition, they suggested that the decreased MDA levels, the increased SOD activity, and the increased HO-1 protein expression observed in L-arginine-treated RI/RI may reflect the coordinated expression of ARE-driven genes mediated by Nrf2. The ARE has been shown to be activated by a wide variety of agents belonging to nine chemical classes. Nitric oxide-related species may represent yet another chemical class responsible for ARE activation [46].

The induction of HO-1 was an adaptive cellular defense response against oxidative stress, protecting cells in pathophysiological states including RI/RI, the HO-1 inhibitor (ZnPP) displayed opposite effects [44, 47]. Similar to our data, Shokeir et al [44], also showed decreased expression of Nrf2 and HO-1 as serum MDA increased in experimentally induced RI/RI. Supplementation with L-arginine, a precursor of NO, partially recovered the expression of HO-1, a cytoprotective antioxidant enzyme, through activation of Nrf2 expression. Some studies have shown that the Nrf2 activator promotes translocation of Nrf2 and HO-1 expression [48]. Furthermore, it had been reported that HO-1 upregulation reduced apoptosis and HO-1 inhibition aggravated apoptosis [48]. Our study demonstrated that L-arginine could partially promote Nrf2 activation to decrease injury by upregulating Nrf2 and HO-1 expression.

ROS production in response to RI/RI has been shown to stimulate the signal transduction pathway that activates expression of the transcription factor NF- κ B, leading to the expression of numerous genes involving in inflammation (i.e., cytokines, chemokines, growth factors, immune receptors, cellular ligands, and adhesion molecules) [49]. Nuclear factor-kappa B plays a critical role in the pathophysiology of RI/RI, the accompanying inflammation and oxidative stress [49]. These conditions have been previously reported to activate NF- κ B expression as a whole body response to IR/IR [43], as in the present experiment. Studies with NO supplementation have also shown the inhibition of NF- κ B protein abundance in kidney tissues [50].

Hsp70 is a molecular chaperone that plays a critical role in the recovery of cells from stress [51]. Induction of heat shock protein 70 (HSP70) is important in the tolerance of ischemia-reperfusion (I/R) injury [52]. Studies have confirmed that drug-induced increases in HSP70 can relieve kidney damage and reduce renal cortical cell necrosis, while at the same time strengthening renal tubular epithelial cell proliferation and repair by increasing nucleus antigen expression [52]; these results are consistent with our findings in the present experiment. Heat shock proteins (HSPs) are crucial for the maintenance of cellular homeostasis during normal cell growth and for survival during and after various cellular stresses [53]. The HSP70 family functions as molecular chaperones and reduces stress-induced denaturation and aggregation of intracellular proteins [53]. In addition to its chaperoning activities, HSP70 has been suggested to exert its cytoprotective action by protecting mitochondria and by interfering with the stress-induced apoptotic program. Studies with NO supplementation have also shown the upregulation of HSP70 protein abundance in kidney tissues [36].

Conclusion

RI/RI was accompanied by inflammatory factors and oxidative stress as well as enhanced NF- κ B expression and suppression of Nrf2, HO-1 and HSP70 expression. L-arginine (a precursor of NO) partially suppressed the increase in NF- κ B expression and enhanced Nrf2, HO-1 and HSP70 expression. NO appeared to reduce the severity of oxidative stress in response to RI/RI by activating an antioxidative defense signaling pathway. In addition, L-arginine partially promoted Nrf2 activation to decrease RI/RI associated injury by indirectly upregulating Nrf2/HO-1 expression and increasing NO levels.

Disclosure Statement

All the authors declared no competing interests.

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