LABORATORY STUDY

Ischemic post-conditioning attenuates renal ischemic reperfusion injury via down-regulation of toll-like receptor 4 in diabetic rats

Bo Tao Jiang*, Qing Zhi Chen*, Zong Hua Guo, Wei Zou, Xiong Chen and Wen Liang Zha

Department of Urology, Central Hospital of Xianning City, The First Affiliated Hospital of Hubei University of Science and Technology, Xianning, Hubei Province, PR China; Department of Respiratory Medicine, Central Hospital of Xianning City, The First Affiliated Hospital of Hubei University of Science and Technology, Xianning, Hubei Province, PR China; Department of Pediatrics, Central Hospital of Xianning City, The First Affiliated Hospital of Hubei University of Science and Technology, Xianning, Hubei Province, PR China

ABSTRACT

Background: Ischemia/reperfusion (I/R) injury, which is commonly seen in the field of renal surgery or transplantation, is a major cause of acute renal failure (ARF). The ischemic ARF in diabetic rats is much more severe than that in the normal rats exposed to the same ischemic time. Ischemic post-conditioning (IPO) is a phenomenon by which intermittent interruptions of blood flow in the early phase of reperfusion can protect organs from I/R injury. To determine whether the renal protection effect of IPO mediates by toll-like receptor 4 (TLR4) signaling pathway in diabetic rats.

Methods: Streptozotocin-induced diabetic rats were randomly divided into three groups: sham operation group, I/R group, and IPO group. Except sham operation group, rats were subjected to 30 min of renal ischemia, both with and without treatment with IPO, then reperfusion 24 h. Light microscope and transmission electronic microscope were used to observe structural changes of renal tubule. RT-PCR was used to measure TLR4 and tumor necrosis factor-alpha (TNF-α) mRNA expression level, renal TLR4 and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) protein expression was detected by Western blot.

Results: The results demonstrated that IPO markedly decreased renal ischemic injury caused by I/R and inhibited the proinflammatory expression levels of TLR4, TNF-α, and NF-κB, all of which up-regulated by I/R in diabetic rats.

Conclusion: Taken together, our results suggest that proper IPO may have protective effect on the ischemic injury mediated by renal I/R, which might be associated with inhibition of TLR4 signaling pathway in diabetic rats.

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Introduction

Acute renal failure (ARF) is a major clinical problem that affects up to 5% of all hospitalized patients and has a mortality rate of 40% in this vulnerable patient population. Ischemia/reperfusion (I/R) injury is the leading cause of ARF in native and allograft kidneys.1–3 There is good clinical evidence that diabetes is an important risk factor for ARF and diabetic patients also are at higher risk of failing to recover from acute kidney injury (AKI) than non-diabetic patients, indicating that either the degree of injury is worse or the repair mechanisms are impaired (or both). Inflammation after renal I/R is one of the major pathways that lead to the process of cell death. Deprived of oxygen-carrying blood, cellular respiration is impaired with irreversible damage occurring virtually in every organelle and subcellular system of the affected cells.4–7 Early reperfusion remains the most effective strategy to limit organ damage. However, reperfusion of the kidney has the potential to cause lethal cell death similar to that observed in the heart later.8 Despite extensive research on reperfusion injury treatment in the past several decades, few protectants have been successfully adopted from basic research into clinical application. Novel protective strategies such as ischemic post-conditioning (IPO) applied at the time of reperfusion are required to target this injury. IPO is defined as a series of rapid intermittent interruptions of blood flow in the early phase of reperfusion that mechanically alters the hydrodynamics of reperfusion.9,10 Recent studies including clinical reports indicated that IPO significantly reduced cardiac infarct size and inhibited inflammation and apoptosis.11,12 We previously demonstrated that IPO attenuated renal damage after I/R injury by inhibiting apoptosis and inflammation.9,13,14 The mechanisms by which redox stress can

CONTACT Bo Tao Jiang doctortjbt@163.com Department of Urology, Central Hospital of Xianning City, The First Affiliated Hospital of Hubei University of Science and Technology, Xianning 437000, Hubei Province, PR China

*These authors contributed equally to this work.

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activate an inflammatory response have not been fully elucidated. There is accumulating evidence that pattern recognition receptors of the innate immune system such as toll-like receptors (TLRs) may be involved in mediating I/R-induced inflammation in non-diabetic rats.\textsuperscript{15} But in the clinical transplantation donors obviously had some kind of comorbidity such as diabetes or hypertension. It remained to be determined whether the protective effects of IPO were associated diabetes in vivo. In this study, we estimated the effects of IPO on inflammation after I/R in diabetic rats by TLR4 and downstream signaling events.

**Subjects and methods**

**Experimental animals and experimental design**

Adult male Sprague-Dawley rats (250–280 g) were from Hubei Laboratory Animal Research Center (Hubei, PR China). All animal experiments were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication No. 8023), and all efforts were made to minimize animal suffering to reduce the number of animals used. The rats were rendered diabetic by a single intraperitoneal injection of freshly prepared streptozotocin (STZ; 55 mg/kg body wt; Sigma) dissolved in sodium citrate buffer (pH 4.5). Experiments were performed two weeks following STZ administration. Blood glucose levels were determined using tail blood samples. Rats were maintained in an air-filtered, temperature-conditioned (20–22 °C), and light-controlled (12 h light/dark cycle) room with a relative humidity of 50–52%. Rats were fed with standard commercial pellets and water ad libitum. Briefly, adult diabetic rats were anesthetized with pentobarbital intraperitoneally (45 mg/kg) and allowed to breathe room air spontaneously. After 500 U heparin (intraperitoneally), a 10 min stabilization period, and maintenance of the body temperature at 37 °C, a midline laparotomy was performed. The left kidney was subjected to 30 min of ischemia followed by reperfusion after right nephrectomy.

Rats were divided randomly into three groups: (i) sham-operated control group (n = 10), (ii) I/R group (n = 10): 30 min renal ischemic followed by 24 h reperfusion, and (iii) IPO group (n = 10): IPO was performed after 30 min renal ischemic (beginning of reperfusion). For the IPO study, reperfusion was established for 10 s after which the renal vessels were occluded again for 10 s, followed by another five cycles of the same period of reperfusion and occlusion. Rats were killed at 24 h after I/R injury. Furthermore, if there was evidence of renal thrombus on extraction of renal tissue, the animal was excluded from the study. Serum urea nitrogen (BUN) and creatinine (Cr) levels were evaluated at 24 h after I/R injury, and other parameters were evaluated at 24 h after I/R injury.

**Serum assays**

To assess serum Cr and BUN, blood samples were collected, centrifuged and kept at −20 °C until required for analyses, adopting standard techniques using an Olympus AU 2700 Analyzer (Olympus Optical Co. Ltd, Tokyo, Japan).

**Histological examination**

Rats were deeply anesthetized with chloral hydrate and the left kidney was removed, followed by fixed in 10% phosphate-buffed formalin. Tissues were sectioned at a thickness of 4 μm thick according to the standard procedure. The sections were deparaffinized and hydrated gradually, and examined by hematoxylin and eosin.

**Transmission electron microscopic examination**

Small pieces of renal cortical tissue (about 1 mm\(^3\)) were rapidly fixed for 1.5 h in 3% cacodylate buffered glutaraldehyde (pH 7.4). The tissue was post-fixed for 2 h in 2% aqueous osmium tetroxide at 4 °C, dehydrated in ascending grades of ethanol, cleared in toluene for 10 min, and embedded in Epon resin. Semi-thin sections were stained with 0.5% toluidine blue in borax and examined by light microscopy. Ultrathin sections were obtained from the selected blocks, mounted on copper grids, stained with uranyl acetate for 20 min and lead citrate for another 20 min, and examined in a JEM-1011 electron microscope (JEOL, Tokyo, Japan), which were taken by an observer who was unaware of the treatment assignment.

**Real-time polymerase chain reaction analysis**

Samples from three groups were used for experiments. Total RNA (2 μg) was isolated by Trizol reagent (Invitrogen, Carlsbad, CA) and reverse transcription was performed with the PrimeScript RT Master Mix (Takara Bio Inc., Shiga, Japan) according to the manufacturer’s instructions. Real-time polymerase chain reaction (PCR) was performed with primers for TRL4 and tumor necrosis factor-α (TNF-α). β-actin was used as an internal control for stable expression (housekeeping gene) in all experiments (Table 1). PCR was conducted at 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s, 60 °C for 34 s, and 95 °C for 15 s. The amount of messenger RNA for
each gene was normalized by β-actin, and the relative expression levels were calculated using the 2^{ΔΔCt} as reported previously.16

**Western blot analysis**

The cytosolic/mitochondrial protein samples for Western blot analysis were prepared as described with some modifications. Whole-cell lysates were obtained by homogenizing the renal sample with a homogenizer in five volumes of buffer (20 mm Hepes, 1.5 mm MgCl₂, 10 mm KCl, 1 mm EDTA, 1 mm EGTA, 250 mm sucrose, 0.1 mm PMSF, 1 mm dithiothreitol (DTT), and proteinase inhibitor cocktail tablets; pH 7.9). Samples were further centrifuged at 750 g at 4°C for 15 min to separate the sample into supernatant A and pellet A. Supernatant A, containing the cytosolic/mitochondrial protein, was further centrifuged at 16,000 g for 30 min at 44°C to separate supernatant B from pellet B. Supernatant B was used as the cytosolic fraction and pellet B was used as the mitochondrial fraction after resuspension in buffer. The protein samples were separated on 10 or 12% SDS-PAGE gels (20–50 μg/Lane) and then transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA). The membrane was blocked with 5% nonfat dry milk in TBST buffer and then incubated with primary antibodies overnight at 4°C. The primary antibodies and concentrations were as follows: TLR4 NF-κB (1:500; Santa Cruz Inc., Santa Cruz, CA), β-actin (1:5000; Abcam Inc., Bristol, England), after extensive rinsing with TBST buffer, washed three times in TBST (50 mmol/L Tris–HCl, pH 7.5, 150 mmol/L of NaCl, 0.01% Tween 20). The membranes were incubated with secondary antibodies (1:2000; Santa Cruz Inc., Santa Cruz, CA) for 1 h at room temperature and then developed with the use of an enhanced chemiluminescence system (ECL kit; Pierce Biotechnology Inc., Rockford, IL). The relative density was quantified by using Scion Image software (Bio-Rad Laboratories, Inc., Hercules, CA).

**Statistical analyses**

All data are expressed as mean ± SEM. The Kolmogorov–Smirnov test was applied to test for a normal distribution. The mean of the different groups were compared using one-way ANOVA Student–Newman–Keuls test. Significant differences were accepted when p values were <0.05.

**Results**

Ischemic post-conditioning improves renal dysfunction

Renal functional parameters of rats were significantly different among groups. Rats subjected to I/R injury showed significant increases in Cr and BUN compared with sham-operated rats. Renal function changes induced by I/R were significantly improved by treatment IPO (Figure 1(A,B)).

Ischemic post-conditioning improves the morphologic features after renal I/R

Renal I/R resulted in significant renal injury as evidenced by tubular necrosis, medullary hemorrhage, congestion, and development of proteinaceous casts.
In contrast, IPO relieved these severe renal damages (Figure 2(A,B)). According to Jablonski scores, severe 30 min renal ischemia followed by 24 h reperfusion resulted in severe acute tubular necrosis. Quantitative analysis showed a dramatically decreased score in IPO group compared with I/R group (Figure 2(C)). In the I/R group, electron microscopy revealed that there was separation of tight junctions, widening of intercellular spaces and obvious swelling of the mitochondria. However, there were some cells with lower levels of intracellular vacuolization, pyknosis, and mitochondrial swelling in the proximal tubule in the IPO group (Figure 3(A–C)).

Figure 2. Histologic evaluations of renal tissues (A–D). Representative kidney sections obtained 24 h after sham surgery or I/R. (A) Section from sham-operated diabetic rat. (B) Section from diabetic rat subjected to I/R. (C) Section from diabetic rat subjected to I/R and treated with IPO. All hematoxylin and eosin ×200. (D) Jablonski scores for histologic appearance of acute tubular necrosis from sham, I/R, and IPO groups. Bars represent mean values ± SE (n = 10); *p < 0.05 versus sham, #p < 0.05 versus I/R.

Figure 3. Transmission electron microscopy (A–C). (A) Normal proximal tubular epithelial cells show characteristic microvilli and mitochondrial apparatus. (B) Loss of microvilli and numerous vacuolization in tubular epithelial cells of I/R suggests mitochondrial breakdown and irreversible degeneration. (C) IPO show considerably less vacuolization, preservation of microvilli, and maintenance of internal cellular architecture, ×10,000 for A, B, and C.
Ischemic post-conditioning down-regulates mRNA expression of TRL4 and TNF-α

To investigate the mRNA expression of TRL4 and TNF-α, we measured the levels of TRL4 and TNF-α by RT-PCR. The PCR products were separated on agarose gel and the relative expressions of TRL4 and TNF-α to β-actin were shown. At 24 h of reperfusion after renal ischemic, the mRNA levels of TRL4 and TNF-α were significantly higher in I/R group than those in sham-operated group. Treatment with IPO decreased the mRNA levels of TRL4 and TNF-α in the ischemic renal tissue (Figure 4(A,B)).

Ischemic post-conditioning inhibits the expression of TRL4 and NF-κB

Western blot analysis showed a significant increase in TRL4 and NF-κB in I/R group compared with sham-operated group. In IPO group, the level of TRL4 and NF-κB protein decreased compared with that of I/R group (Figure 5(A–C)).

Discussion

This study showed that IPO markedly decreased renal ischemic injury caused by I/R, and inhibited the expression levels of TLR4, TNF-α, and NF-κB after renal I/R injury in diabetic rats. Our findings support the speculation that IPO attenuates renal damage by inhibiting activity of TRL4 and NF-κB after renal I/R injury in diabetic rats.

Ischemic pre-conditioning is the phenomenon that a prior ischemic stress renders the organ resistant to a subsequent ischemic insult. Although extensive researches have demonstrated that ischemic pre-conditioning reduced renal I/R damage, renal ischemic pre-conditioning is clinically feasible only when the occurrence of ischemia is predictable.18–20 IPO is a simple and harmless method which provides a new tool to protect organ from I/R injury in brain, liver, heart, and kidney.13,21–23 The early moments of reperfusion are important in the pathogenesis of post-ischemic injury. A previous study has demonstrated that IPO reduces renal I/R injury in non-diabetic rats.15 However, whether deleterious mechanisms were attenuated or whether beneficial mechanisms were triggered by IPO is still unclear. Diabetes has been recognized as a risk factor for the development of AKI in a variety of clinical settings, such as radio-contrast nephropathy or following cardiopulmonary bypass operations.24,25 The increased susceptibility of the kidney to AKI has been previously

Figure 4. Representative real-time PCR of TLR4 and TNF-α in renal tissues after 30 min of ischemia followed by 24 h of reperfusion. (A) Effects of IPO on the mRNA level of TLR4. (B) Effects of IPO on the messenger ribonucleic acid (mRNA) level of TNF-α. Bars represent mean values ± SE (n = 10); *p < 0.05 versus sham, #p < 0.05 versus I/R.

Figure 5. TLR4 and NF-κB expressions after 30 min of ischemia followed by 24 h of reperfusion (A–C). (A) Representative blots showing the effect of IPO treatment on the TLR4 and NF-κB expressions. (B) The relative band densities of TLR4 to the mean value of the control. (C) The relative band densities of NF-κB to the mean value of the control. Bars represent mean values ± SE (n = 10); *p < 0.05 versus sham, #p < 0.05 versus I/R.
reported in patients with diabetes mellitus and experimental models of this disease. However, the mechanisms underlying the enhanced vulnerability of the kidney to ischemia in diabetes have not been clearly established.

The ischemic renal damage is largely attributed to oxidative stress, inflammation, and endothelial dysfunction. Inflammation has been demonstrated to play a key role in diabetes and ischemic AKI. TLRs are part of the innate immune system and their stimulation can cause activation of NF-κB, leading to cellular dysfunctions, decreased contractility, and activation of apoptosis. TNF-α is another inflammatory cytokine participating in the pathological process of ischemic/reperfusion. Under normal circumstance, TNF-α is corrected with immune responses and could repair the cells of urinary system. In the heart, TLR4 activation can modulate responses driving to cell stress and inflammation during diabetes, hypoxia or ischemic events. In this study, rats with IPO show less renal damage than rats with I/R. The level of TLR4 increased in renal tissue after 30 min of renal ischemic, which was accompanied by the high level of multiple inflammatory cytokines such as TNF-α and NF-κB. Previous work has shown that TLR4-deficient mice, like MyD88-deficient mice, are also protected from renal I/R injury as demonstrated by reduced tubular dysfunction or damage, reduced proinflammatory cytokines, and less leukocyte accumulation in TLR4- or MyD88-deficient mice compared to their wild-type controls undergoing renal I/R. Therefore, these evidences suggest a possibility that IPO attenuate renal TNF-α production through TLR4-dependent mechanisms, which contributes to the protection against I/R-induced ARF. Our study also revealed that the suppressed effects of IPO on inflammation may be partly through the suppression of TLR4 signaling pathway. Furthermore IPO did not affect the blood sugar levels not shown in this article.

In conclusion, optimal IPO could decrease renal TLR4 expression to protect kidney from ischemic injury in diabetic rats. Part of the proinflammatory effects in the ischemic kidney may be mediated by TLR4 expression, and a better understanding of the factors involved in IPO attenuating renal I/R is essential for the development of innovative therapeutic strategies to interfere with acute renal injury in diabetic patients. Further studies are required to obtain more conclusive results and the TLR4 pathway as target for new therapeutic treatment aimed to prevent or delay ARF.

Disclosure statement
The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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