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Remote ischemic per-conditioning protects against renal ischemia-reperfusion injury via suppressing gene expression of TLR4 and TNF- α in rat model

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Abstract

Background: The pathogenesis of renal ischemic reperfusion injury (IRI) involves both inflammatory processes and oxidative stress in the kidney. This study determined whether remote ischemic per-conditioning (RIPerC) is mediated by toll-like receptor 4 (TLR4) signaling pathway in rats. **Materials and Methods:** Renal I/R injury was induced by occluding renal arteries for 45 min followed by 24 h reperfusion. RIPerC included four cycles of 2 minutes ischemia of left femoral artery followed by 3 minutes reperfusion performed at the start of renal ischemia. Rats were grouped into sham, I/R, and RIPerC. At the ending of reperfusion period, urine, blood and tissue samples were gathered. **Results:** I/R created kidney dysfunction, as ascertained by significant decrease in creatinine clearance, and significant increase in sodium fractional excretion. This was occurred with a decrease in the activities of glutathione peroxidase, catalase and superoxidase dismutase with an increment in malondialdehyde levels, mRNA expression levels of Toll-like receptor 4 (TLR4) and tumor necrosis factor- α (TNF- α) and histological damages in renal tissues. RIPerC treatment diminished all these changes. **Conclusion:** This study demonstrated that RIPerC has protective effects on the kidney after renal I/R, which might be related with inhibition of TLR4 signaling pathway and augmentation of anti-oxidant systems.

Key words: Renal ischemia-reperfusion, Remote ischemic per-conditioning, Toll-like receptor 4, TNF- α , Sodium fractional excretion, Creatinine clearance, Oxidative stress

Introduction

It is obvious that renal ischemia-reperfusion injury (IRI) leads to histological damages and functional disorders due to regaining of blood flow after an ischemic period in the kidney. Ischemic kidney injury is generally a severe postoperative complication of organ transplantation (de Haan et al. 2017; Lagny et al. 2015; Panah et al. 2018) and cardiac surgery (Lagny et al. 2015). During ischemia phase, ATP depletion leads to cytosolic calcium and sodium overload by inhibiting of Na/Ca²⁺ antiporter and Na/K/ATPase function. Reperfusion exacerbates these events, because excess calcium induces cell apoptosis through activating intracellular calcium-dependent-enzymes such as phospholipase A2 (Orrenius et al. 2003).

The pathogenesis of renal IRI involves various mechanisms including inflammatory processes facilitated by the inherent immune response. One of the most important molecules that mediates inflammatory pathways, is TLR4, which is a pattern recognition receptor with a chief role in the inflammation. Many evidences have represented that TLR4 has a role in mediating IRI (Shimamoto et al. 2006; Wu et al. 2007; Zhai et al. 2004). TLR4 and its ligands are involved in triggering multiple downstream inflammatory reactions, including the activation of pro-inflammatory cytokines and chemokines such as TNF- α (Liu and Ding 2016). Excessive activation of TLR4 is close related to renal IRI (Chen et al. 2011; Li et al. 2013; Wu et al. 2007). Studies have shown that inhibition of TLR4-mediated inflammation is efficient in treatment of renal IRI (Liu et al. 2010; Qi et al. 2015).

One of the most effective conditioning strategies for prevention of IRI, is ischemic pre- conditioning (RIPerC). In RIPerC, several cycles of ischemia and reperfusion applied during the ischemic insult, render distant organ and tissues more resistant to the IRI. Prior studies express that several mechanisms, including augmenting antioxidant activity and decreasing inflammation in the damaged tissues, may be accountable for renal protective effects of RIPerC against IRI (Jiang et al. 2015).

However, the precise mechanisms remain unclear. TLR4 is highly regarded as an upstream sensor of multiple intracellular signaling pathways including TLR4/TNF- α signaling. It is suggested that TNF- α is one of the principal proinflammatory cytokines which is upregulated following IRI (Wu et al. 2010). This upregulation is attenuated in experiments with TLR4-deficient animal IRI models (Wu et al. 2010). However, to the best of our knowledge, there are currently no published data regarding whether RPerC involves changes in TLR4 gene expression. Therefore, this experiment was performed to examine the underlying mechanism of RPerC with the focus on TLR4/TNF- α signaling in an in vivo rat model.

Materials and methods

Experimental animals

All of the experimental methods complied to the guidelines for the care and handling of animals provided by the Ethics Committee of Shiraz University of Medical Sciences (Code:12000). The animals were cared for in accordance with Guide to the Care and Use of Experimental Animals (Vol. 1, 2nd ed., 1993, available from the Canadian Council on Animal Care). Adult male Sprague-Dawley rats (weighing 260–280 g) were conserved at room temperature ($22 \pm 1^\circ\text{C}$) with 12-hr dark and light cycle. They were provided with food and water ad libitum.

Surgical procedures

Each animal was anaesthetized by sodium pentobarbital (60 mg/kg, i.p.), and then was placed on a heating surgical table to maintain the animal body temperature at $37 \pm 1^\circ\text{C}$. After a median abdominal laparotomy, left and right renal artery and vein were attentively cleared and disconnected from each other under the surgical microscope. The rats were randomly divided into three groups ($n = 10$): (1) sham group, renal arteries were not occluded; (2) I/R group, both renal arteries were

occluded for 45 min using atraumatic clamp; (3) RIPerC group, rats were subjected to 45 min ischemia by occluding both left and right renal artery and four cycles of 2 minutes ischemia of left femoral artery followed by 3 minutes reperfusion applied at the starting of renal ischemia.

At the end of the surgery, rats were housed in metabolic cages and their urine was collected over a period of 24 h. At the end of the reperfusion period of 24 h, rats were anaesthetized and blood samples were obtained from the inferior vena cava. Rats were slayed (under deep anesthesia) and their kidneys were removed and weighed immediately. Two coronal sections were cut from the middle part of the kidney. One piece was immediately frozen under liquid nitrogen for molecular studies and oxidative stress assays; the second was fixed in %10 formalin for histological assessments.

Assessment of renal function

The urine sample collected throughout the 24 hr reperfusion period was evaluated gravimetrically and urine flow rate was calculated. Plasma and urinary samples were measured for creatinine by means of an autoanalyser (Prestige, Biolis 24I, Japan) and were used in conjunction with urine flow rate to calculate creatinine clearance (C_{Cr}). Plasma and urine samples were evaluated for Na^+ with a flame photometer (JENWAY, U.K.) and used to assess the fractional excretion of Na^+ (FE_{Na}). C_{Cr} and FE_{Na} were used as indicators of renal glomerular function and tubular dysfunction respectively (Chatterjee et al. 2004).

Assessment of renal redox markers

Malondialdehyde levels

Reaction of malondialdehyde (MDA), as an end product of lipid peroxidation, with thiobarbituric acid (TBA) produces the pink colour. MDA level in the kidney tissue homogenates was determined spectrophotometrically with a maximum absorption at 532 nm wavelength (Heath and Packer 1986).

Antioxidant enzyme activity in kidney tissue homogenates

Glutathione peroxidase activity (GPX) activity was assayed spectrophotometrically. The assay mixture consisted of 3 mM NADPH, 20 mM cumene hydroperoxide, 40 mM reduced glutathione (GSH), tris buffer (pH 7.6), and 10 U/ml glutathione reductase (GR). Cumene hydroperoxide was used as the substrate. The enzymatic activity was calculated using the molar extinction coefficient for NADPH ($6.22 \mu\text{mol}^{-1} \times \text{cm}^{-1}$ at 340 nm). Catalase (CAT) activity was evaluated spectrophotometrically (Aebi 1984). The assay mixture (final volume 3 mL) consisted of 1 mL of 30 mmol/L H_2O_2 diluted in 50 mol/L sodium phosphate buffer (pH 7.0), 100 μL of the sample with 1.9 mL phosphate buffer, which directly prepared in cuvette. The decrease in absorbance at 240 nm was monitored for 1 min. CAT enzymatic activity was calculated using the H_2O_2 extinction (coefficient $0.036 \mu\text{mol}^{-1} \times \text{cm}^{-1}$). Superoxide dismutase (SOD) activity in kidney tissue homogenates was determined based on the autoxidation of epinephrine at alkaline pH, which results in building $\text{O}_2^{\bullet-}$ in the solution (Ighodaro and Akinloye 2017; Misra and Fridovich 1972). Reaction of $\text{O}_2^{\bullet-}$ with epinephrine leads to adrenochrome formation. On the other hand, SOD reaction with the $\text{O}_2^{\bullet-}$ slows down the rate of adrenochrome formation. Briefly, reaction mixtures containing 925 μL 0.05 M bicarbonate buffer ($\text{Na}_2\text{CO}_3/\text{NaHCO}_3$, pH 10.25) with 10^{-4} M EDTA and 50 μL kidney tissue homogenates were added to the cuvette. One minute after the addition of 25 μL of 0.01 M epinephrine in 0.02 M HCl, light absorbance by adrenochrome was read at 485 nm every 30 seconds for 10 minutes, using spectrophotometer. Serial dilution from standard SOD (Sigma) were prepared in potassium phosphate buffer (0.1 M, pH 7.4). The inhibition percent of epinephrine auto-oxidation

was plotted against various concentrations of standard SOD. The line equation was used to calculate the concentration of each samples. The value of enzyme, which creates 50% inhibition of epinephrine auto-oxidation, considered as one unit of the enzyme activity.

Molecular analysis

RNA isolation and real-time polymerase chain reaction (Real-Time PCR)

Total RNA of kidney tissue was isolated using RNA extraction kit (Cinnagen Inc., Iran). To determine the purity, integrity, and concentration of RNA, the optical density 260/280 and agarose gel (1%) electrophoresis were measured. Then, total RNA was converted to complementary DNA (cDNA) using RevertAid™ first strand cDNA synthesis kit (Fermentas Inc.). Real-Time PCR was performed with the protocol of RealQ Plus 2x Master Mix Green (Ampliqon Inc.) on a real-time PCR system (StepOne™ Instrument, Applied Biosystems, ABI, USA). Real-Time PCR for expression analysis of the primer pairs for TLR4 and TNF- α were designed, as shown in Table 1. The B2m housekeeping gene was also used as the internal control of real-time PCR reactions. The real-time PCR conditions were set for 10 minutes at 94°C followed by 40 cycles of 15 seconds at 94°C, 60 seconds at 60°C and extension steps. After each real-time PCR run, gel electrophoresis and melting curve analysis were accomplished to confirm specific amplification of targets. The amplification signals of 155 different samples were normalized to PGK Ct (cycle threshold). Relative gene expression was calculated using delta-delta CT ($2^{-\Delta\Delta CT}$) method, which demonstrated as fold change in data analysis.

Histopathological evaluation

For histological study the left kidney samples were fixed in formalin (10% phosphate-buffered), dehydrated in different concentrations of ethanol. Thereafter, renal samples were embedded in

paraffin and cut into serial slices of 5 μm thickness. After mounting on slides, tissue slices stained with hematoxylin and eosin. In a blinded fashion, each renal section was histopathologically scored in at least 10 randomly selected non-overlapping fields under a light microscope. Renal histopathological changes were quantified for the degree of reduced number of RBC (red blood cell) in the glomerular capillaries, enlargement of Bowman's space, loss of brush borders, necrosis and exfoliation of the cells into the proximal tubular lumen, formation of tubular casts, infiltration of lymphocytes, dilatation of the lumen of medullary blood vessels with congestion and increased number of RBC in their lumen. Sections were scored according to the changes involved as follows: 0 (no lesions); 1 with (less than 20%), 2 (20%–40%), 3 (40%–60%), 4 (60%–80%), 5 (greater than 80%). The sum of all numerical scores in each group was taken as the total histopathological score.

Statistical analysis

Present data were expressed as mean $\pm$ SD and analyzed by one-way ANOVA followed by Tukey's post hoc using SPSS statistics software package (SPSS for Windows v. 22.0, Chicago, IL). Comparisons of histopathological data between groups were calculated by Kruskal-Wallis multiple comparison test. Mann–Whitney U test was used for binary comparisons. Statistical significance was considered as P value below 0.05.

Results

RIPerC protects I/R- induced changes in the kidney function

As shown in figure 1, I/R group showed a significant enhancement in the plasma concentration of creatinine ($[\text{Cr}]_p$) and urea nitrogen ($[\text{UN}]_p$) (Fig. 1A and 1B), a decrement in clearance of creatinine (C_{Cr}) (Fig. 1C) and an increment in sodium fractional excretion (FE_{Na}) (Fig. 1D) compared to the

sham group. In addition, RPerC caused improvement in C_{Cr} (Fig. 1C) and reduction in $[Cr]_p$, $[UN]_p$, and FE_{Na} in the I/R group (Fig. 1A, 1B and 1D).

RPerC attenuates I/R-induced changes of redox markers in the kidney tissue

According to figure 2A, renal I/R created a significant increase in the MDA levels of kidney in comparison with the sham group. But, RPerC pretreatment significantly decreased MDA content in renal tissue of I/R group.

Moreover, renal I/R produced a significant reduction in the GPX activity of the kidney tissue (Fig. 2B) in comparison to the sham group. However, RPerC induced a significant enhancement in the kidney tissue GPX activity compared to that of I/R group (Fig. 2B).

As shown in figures 2C and 2D, renal I/R led to significant decreases in renal tissue CAT and SOD activity. But, enzymatic activity of CAT and SOD in the kidney tissue was increased by the RPerC strategy in comparison to the I/R group (Fig. 2C and D).

RPerC attenuates I/R-induced changes in the gene expression of TLR4 and TNF- α in the kidney tissue

Figure 3A shows that renal I/R created a significant increase in the expression level of TLR4 in the kidney tissue in comparison to the sham group. RPerC decreased the expression of TLR4 in the kidney tissue (Fig.3A).

In addition, the expression of TNF- α in the kidney tissue (Fig. 3B) increased significantly in the IR group as compared with the sham group. TNF- α expression in the kidney tissue of RPerC group was significantly reduced in comparison to that of I/R group (Fig. 3B).

RIPerC decreases I/R-induced renal histopathological changes

There was no damage in the kidney tissue of the sham group (Fig. 4A1 and A2). But, in the I/R group, there were major changes in the renal tissue. The ischemic kidneys showed reduced number of RBC in the glomerular capillaries, enlargement of Bowman's space, loss of brush borders, necrosis and exfoliation of the cells into the proximal tubular lumen (Fig. 5B1), formation of tubular casts, infiltration of lymphocytes, dilatation of the lumen of medullary blood vessels with congestion and increased number of RBC in their lumen (Fig. 5B2). However, RIPerC treatment improved renal damages in the RIPerC group (Fig 4C1 and C2) compared to I/R group (Fig 5).

DISCUSSION

It has been established that subsequent to renal IRI, TLR4 expression is elevated in the kidney which triggers an inflammatory response that deteriorates tissue injury (Bergler et al. 2012). One of the main proinflammatory cytokines upregulated following IRI is TNF- α (Wu et al. 2007). In this study, renal IRI was induced in rats to assess the contribution of the TLR4/TNF- α signaling pathway in the renal protective effects of RIPerC against renal IRI using an in vivo model. The results indicated that RIPerC can attenuate renal dysfunction and structural injury via intensification of antioxidant system and suppression of the inflammatory response subsequent to IRI in the ischemic kidneys. RIPerC decreased the mRNA level of inflammatory factors, TLR4 and TNF- α , in kidney samples of the rat model. Our findings support the speculation that RIPerC attenuates renal damage by inhibiting activity of TRL4 and TNF- α after renal I/R injury in rats.

RIPerC, as one of the effective and newest protective techniques, alleviates renal (Brito et al. 2017; Ogawa et al. 2001; Sedaghat et al. 2016) and liver IRI (Costa et al. 2014). The mechanisms of

renoprotection of RPerC are complex and are not fully understood. Two main pathophysiological mechanisms of IRI comprise oxidative stress reaction and inflammatory responses (Bonventre and Yang 2011).

Under stress conditions such as renal I/R, after prolonged ischemia, blood flow reperfusion leads to production of reactive oxygen species (ROS) from damaged mitochondria (Granger and Kvietys 2015). An unbalance between ROS production and defensive capacity of the cells leads to oxidative stress (Birben et al. 2012; Tarantino et al. 2011). Adhering to these hypotheses and that remote ischemic conditioning protects against endothelial I/R injury through ROS and NO-dependent mechanisms in the acute myocardial IRI model (Hauerslev et al. 2018), our results showed that RPerC provided protection against oxidative stress. Elevated MDA level, which has been frequently observed in animal models of renal I/R, is definitive feature of I/R-induced tissue damage (Sehirli et al. 2003). The present study evaluated some of oxidative stress parameters in the renal tissue. RPerC significantly abolished the increase of MDA level and reduction in the activity of antioxidant enzymes (CAT, GPX and SOD) compared with I/R group, which suggested that RPerC intensified antioxidant system.

This study showed that RPerC alleviated renal IRI by decreasing plasma creatinine and BUN concentration and attenuating renal cell necrosis. Light microscopy for I/R group confirmed that there was denudation of basement membrane which would lead to the back-leak of filtered fluid (Kribben et al. 1999). In addition, the formation of intra-tubular casts would obstruct the tubular lumens and raise tubular pressure up to Bowman's capsules, which developed the enlargement in the I/R group. Thus, the composition of decreased renal blood flow (as indicated by reduced number of RBC in the glomerulus), back-leak and increased Bowman's space pressure were presumably to be accountable for the reduced C_{Cr} as an indicator of GFR subsequent to the ischemia in I/R group

(Brady et al. 2004; Kribben et al. 1999). TLR4 signaling plays a critical role in renal I/R injury and has a major role in activating the inflammatory reaction (Akira and Takeda 2004). The great expression of TLR4 which was seen in kidney samples of I/R group is probably a main key to inflammation. Indeed, Pulskens et al. employing TLR4^(-/-) mice demonstrated a protective effect on the function of kidney, production of chemokines and infiltration of granulocytes (Pulskens et al. 2008). Besides, overexpression of TLR4 and up-regulation of TNF- α by renal I/R in diabetic rats has been demonstrated (Jiang et al. 2016), indicating that the reduction in kidney function mediated TLR4 activation and augmented the inflammation. RPerC inhibited the expression of TLR4 in the animals undergoing renal I/R injury. These results propose that RPerC confers protection from renal I/R injury via TLR4. In the heart, TLR4 activation can modify responses leading to cell stress and inflammation during diabetes and ischemic events (Dong et al. 2012). Also, amelirative effect of Artesunate, which exhibits protective effect on chronic kidney diseases, on diabetic nephropathy is mediated by suppressing TLR4/NF- κ B/NLRP3 inflammasome pathway (Sun et al. 2018). Furthermore, TNF- α levels were decreased when RPerC was applied for I/R injury in rats. TNF- α is other inflammatory cytokine interfering in the pathological process of IRI (Song et al. 2017). As a proinflammatory factor, TNF- α is closely related to extensive tubular damage (Patel et al. 2005; Ramesh and Reeves 2002). In the current work, light microscopy for I/R group showed loss of brush border and exfoliation of the epithelial cells into the lumen in the cortical proximal tubules, which would lead to diminished proximal tubular reabsorption. Besides, the severe tubular necrosis observed in the pars recta and mTAL of the I/R group could also contribute to major increment of FE_{Na} in I/R group. RPerC decreased TNF- α expression to enable survival after I/R renal injury. In line with our results, Sedaghat et al. reported the improvement of kidney function by ischemic preconditioning which was associated by decreased TNF- α expression (Sedaghat et al. 2016). In the current study, histopathological evaluation showed that RPerC reduced the morphological changes

in comparison with the sections from I/R group which represented necrosis, loss of brush borders and exfoliation of the epithelial cells into the lumen of the proximal tubules, leukocyte infiltration, vascular congestion and tubular cast formation in the medulla. It is in line with another study showing that TLR4-deficient mice are protected against renal I/R injury as proved by diminished tubular dysfunction or damage, decreased proinflammatory cytokines, and less leukocyte cumulation in TLR4- mice compared to their wild-type controls suffering renal I/R (Liu et al. 2014). Therefore, these evidences suggest a possibility that RPerC attenuates 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 RPerC on inflammation may be partly through the suppression of TLR4 signaling pathway. It is notable that other methods of remote ischemic conditioning arouse a systemic protective reaction involving modification of immune cells through reduction in the levels of inflammatory cytokines (Dezfulian et al. 2017; Joseph et al. 2017; Kim et al. 2014; Peralta et al. 2001). Thus, according to the result of current work and findings of others (Dezfulian et al. 2017; Joseph et al. 2017; Kim et al. 2014; Peralta et al. 2001), it is concluded that RPerC probably induces functional changes in leukocytes, which perfuse into the ischemic kidney with modulated pattern of gene expression and function.

In summary, RPerC strongly diminishes the renal tissue damage, possibly subsequent to intensification of antioxidant system and suppression of the inflammatory response following IRI in the ischemic kidneys. It also decelerated the decrease in GFR and Na reabsorption, and consequently attenuated the increments in the plasma concentration of creatinine and urea nitrogen. These findings strongly suggest that decreased gene expression of TLR4 and TNF- α may mediate renal protective effects of RPerC.

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Disclosure

The authors declare no competing financial interests.

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Fig. 1 Effects of RPerC on (A) plasma creatinine concentration, (B) plasma urea nitrogen concentration, (C) creatinine clearance (C_{Cr}) and (D) fractional excretion of sodium (FE_{Na}). ** $P < 0.01$, *** $P < 0.001$ vs sham group; §§ $P < 0.01$, §§§ $P < 0.001$ vs I/R group.

Fig. 2 Effects of RPerC on the (A) malondialdehyde (MDA) content, (B) glutathione peroxidase (GPX) activity, (C) catalase (CAT) activity, (D) superoxide dismutase (SOD) activity in the kidney tissue. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs sham group; § $P < 0.05$, §§ $P < 0.01$, §§§ $P < 0.001$ vs I/R group vs RPerC group.

Fig. 3 Effects of RPerC on the level of gene expression of (A) TLR4 and (B) $TNF-\alpha$ in the kidney tissue. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs sham group; § $P < 0.05$, §§§ $P < 0.001$ vs I/R group.

Fig. 4 Representative kidney light microphotographs in the sham (A1, A2), I/R (B1, B2), RPerC (C1, C2) groups. Renal sections stained with H&E ($\times 400$) were evaluated for histological changes at the end of 24 hour's reperfusion including: B1: enlargement of Bowman's space (white arrow), necrosis and exfoliation of the proximal tubular cells into the lumen (black arrow); B2: including necrosis and exfoliation of the proximal tubular cells (pars recta) into the lumen (white arrow), and outer medullary vascular congestion (black arrow).

Fig. 5 Effects of RPerC on the total histological score in the renal tissue. *** $P < 0.001$ vs sham group; §§§ $P < 0.05$ vs I/R group.

Graphical Abstract caption:

Diagram showing the experimental protocol.

Table 1: Primers used in the current experiment.

Genes	Primer Sequences	Sizes (bp)
TNF- α	Forward: 5'- ATGGGCTCCCTCTCATCAGT-3'	106
	Reverse: 5'- GCTTGGTGGTTTGCTACGACG-3'	
TLR4	Forward: 5'- TATCCAGAGCCGTTGGTGTATCT-3'	85
	Reverse: 5'- AATGAAGATGATGCCAGAGCG-3'	
B2m	Forward: 5'- CGTGCTTGCCATTCAGAAA -3'	244
	Reverse: 5'- ATATACATCGGTCTCGGTGG -3'	

Abbreviation: bp, base pair

Figure 1.

Figure 1

182x190mm (300 x 300 DPI)

Figure 2.

Figure 2

175x190mm (300 x 300 DPI)

Figure 3

Figure 3

294x160mm (300 x 300 DPI)

Figure 4

Figure 4

164x190mm (300 x 300 DPI)

Figure 5

157x169mm (300 x 300 DPI)