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Effect of NADPH oxidase inhibitor-apocynin on the expression of Src homology-2 domain-containing phosphatase-1 (SHP-1) exposed renal ischemia/reperfusion injury in rats

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ABSTRACT

This study was designed to evaluate whether NADPH oxidase inhibitor (apocynin) preconditioning induces expression of Src homology-2 domain-containing phosphatase-1 (SHP-1) to protect against renal ischemia/reperfusion (I/R) injury (RI/RI) in rats. Rats were pretreated with 50 mg/kg apocynin, then subjected to 45 min ischemia and 24 h reperfusion. The results indicated that apocynin preconditioning improved the recovery of renal function and nitroso-redox balance, reduced oxidative stress injury and inflammation damage, and upregulated expression of SHP-1 as compared to RI/RI group. Therefore our study demonstrated that apocynin preconditioning provided a protection to the kidney against I/R injury in rats partially through inducing expression of SHP-1.

1. Introduction

Ischemia/reperfusion (I/R)-induced injury was a primary reason of acute organ dysfunction and that usually occured in association with pathophysiological conditions, including primary surgery, trauma, large area burns, and acute hypovolemia due to heavy fluid and blood loss [1]. Although reperfusion after ischemia generally improved the recovery of organ dysfunction caused by ischemia, in some instances, reperfusion may aggravate the damage associated with ischemia [2].

Renal ischemia/reperfusion injury (RI/RI) was mediated by multiple pathophysiological mechanisms of which free radical damage and NO were two key events [3,4]. During RI/RI, free radicals were increased and NO was reduced, resulting in extensive damage and apoptosis of tubular epithelial cells [5,6]. Among these factors, Src homology-2 domain-containing phos-phatase-1 (SHP-1) was involved in the pathophysiology of damage and apoptosis through multiple signaling pathways [7–9].

Src homology-2 domain-containing phosphatase-1 (SHP-1) was first identified [10] as a protein tyrosine phosphatase containing two tandem SH2 domains later shown to have targeting and regulatory functions [11]. Literatures demonstrated that the intracellular protein tyrosine phosphatase (PTP) known as SHP-1 may regulate macrophage activation [8]. Previous study also demonstrated that SHP-1, a PTP that was critical in abating cell response to growth factors, was elevated in renal cortex by diabetes [12]. SHP-1 had been shown to inhibit vascular endothelial growth factor (VEGF) actions in renal glomeruli of diabetic rats and mice [13]. However, Ischemia/reperfusion (I/R) injury produced a lot of oxygen free radicals (OFR) and these elevated level of OFR can inhibit the phosphatase activity of SHP-1, and the function of SHP-1 was restrained to promote the release of OFR [7,8]. During the early IR period of lung transplantation, the activity of PTP was reduced [14]. Present study indicated that RI/RI can produce lots of OFR [6] and inflammatory factors [15] which can inhibit the activity of SHP-1, and loss of activity of SHP-1 can further exacerbate the damage of RI/RI [9,16–18]. These researches suggested that this renal protective effect was at least in part by activation of SHP-1. The aforementioned results manifested that SHP-1 played different roles in different tissues or organs during I/R injury.

On the other hand, SHP-1 negatively regulated Toll-like receptor-mediated production of proinflammatory cytokines by inhibiting activation of the transcription factor NF-kappaB [9]. It was known that Toll-like receptors (TLRs) were a family of transmembrane proteins that in addition to binding to a range of

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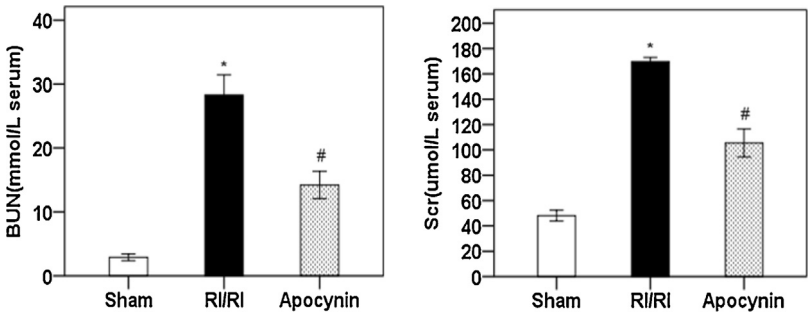

Fig. 1. Serum levels of BUN and Scr. The blood of sham, RI/RI, RI/RI + apocynin groups rats was collected after reperfusion 24 h and level of BUN and Scr measured. Results expressed as mean ± SD. (A) a significant increase from sham group was denoted by"*" ($p < 0.01$), a significant decrease from RI/RI group, by"#" ($p < 0.01$); (B) a significant increase from sham group was denoted by"*" ($p < 0.01$), a significant decrease from RI/RI group, by"#" ($p < 0.01$)

microbial products can also recognize endogenous ligands termed danger-associated molecular patterns (DAMPs) [19]. After TLR activation an intracellular cascade of events occured resulting in the release of NF-κB from IκB, allowing NF-κB translocation from cytoplasm to nucleus where it mediated the increase in inflammatory cytokine gene expression leading to pro-inflammatory responses [20–23]. Documents confirmed that TLR4/NF-κB p65 signal pathway was activated to promote renal damage on kidney ischemia reperfusion injury [19], and SHP-1 can negatively regulated TLR4 signal pathway to inhibit the product of downstream proinflammatory cytokines (TNF-α) [9].

Apocynin was a naturally occurring methoxy-substituted catechol that effectively inhibits NADPH oxidase through preventing the assembly of its multi-subunits [24]. Protective effects of apocynin had been observed when administered before ischemia (preconditioning) [25]. Increased attention had been paid to the protective effects of apocynin on I/R injury to the testicle, heart, neuro, brain, and kidney. In testicular I/R injury, preconditioning with apocynin reduced the production of oxygen free radicals and improved these histological alterations [26]. Preconditioning with apocynin also reduced myocyte inflammatory response and cell apoptosis after myocardial I/R-induced injury [27,28]. Preconditioning with apocynin in animal models had also been reported to provide protection through reducing the production of oxygen free radicals from I/R-induced renal injury [29]. Present literatures also demonstrated that apocynin can ameliorate nitroso-redox unbalance and regulate TLR4 expression on myocardial ischemia reperfusion injury [27].

Thus, apocynin may exert protective effects against ischemia/reperfusion injury in many organs, including the kidney. Also, in different ischemia/reperfusion models, the role of SHP-1 varied. However, to date, no studies had inspected the effects between apocynin and SHP-1 on renal ischemia/reperfusion injury. Therefore, in this preliminary study, we inquired into SHP-1 how to participate in the possible protective effects of apocynin preconditioning on renal ischemia/reperfusion injury in rats.

2. Materials and methods

2.1. Animals and reagents

Sprague-Dawley (SD) rats (180–220 g) were supplied by the animal research center of Lanzhou university medical college, Lanzhou, China. All the procedures and care administered to the animals had been approved by the institutional ethic committee. All reagents and drugs were purchased from Sigma.

2.2. Animal experimental design

In the pilot experiment, 5 rats were in sham group and 15 rats underwent RI/RI. 12 h ($n = 5$), 24 h ($n = 5$) and 48 h ($n = 5$) after reperfusion, the rats were euthanized, and kidneys were collected. In the formal experiments, 30 SD rats were randomly assigned to three groups (each group had 10 rats): sham, RI/RI and RI/RI + apocynin. The rats in RI/RI + apocynin groups injected apocynin (50 mg/kg) through intraperitoneal injection 30 min before reperfusion. The doses were selected based on previous reports [30].

2.3. Surgical procedures of RI/RI

Rats were anesthetized with 1% pentobarbital sodium (60 mg/kg) through injecting in enterocoelia. The enterocoelia was opened and the bilateral renal artery and vein were separated. The artery and vein were occluded for 45 min by two artery clamps, and the success was confirmed by observing the color changing of the kidneys. The artery clamp was removed 45 min later and renal artery and vein were allowed reperfusion for 24 h. The blood samples were collected via abdominal aorta, and centrifuged at 3600 × *g* for 10 min to harvest the sera. The left kidneys of animals were removed immediately. Renal homogenate from each group were used to stored at −20 °C for the analyses below, respectively.

2.4. Measurement of serum BUN and Scr

The methods had been described previously [4]. Briefly, the blood of abdominal aorta was collected after reperfusion 24 h and centrifuged at 3600 × *g* for 10 min to harvest the sera. The BUN and Scr of serum were measured by *O*-phthalaldehyde and Picric acid method.

2.5. Measurement of SOD activity and MDA content

The levels of SOD and MDA in tissues were measured to assess lipid peroxidation as described previously [4]. The kidney specimen was collected after reperfusion 24 h and the blood of tissues was washed cleanly, homogenized (100 mg) in ice normal saline and made into 10% homogenate, then centrifuged at 3600 × *g* for 10 min to harvestthe supernatant. The SOD activity and MDA content were measured by xanthine oxidase and thiobarbituric acid method.The absorbance was measured at 550 and 532 nm. The level of lipid peroxides was expressed as U of SOD/mg prot and nmol of MDA/mg tissue.

2.6. Measurement of TNF-α content

The levels of TNF-α in tissues were measured to assess inflammatory injury as described previously [31]. The samping process

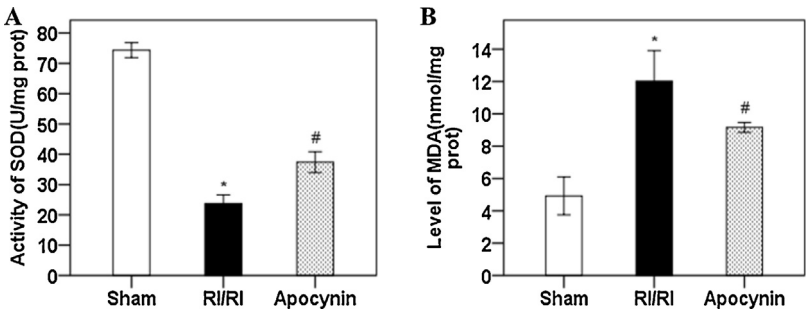

Fig. 2. The activity of SOD and the level of MDA in renal tissues. The renal tissues of sham, RI/RI, RI/RI + apocynin groups rats were collected after reperfusion 24 h and activity of SOD and level of MDA measured. Results expreed as mean ± SD. (A) a significant decrease from sham group was denoted by"*" ($p < 0.01$), a significant increase from RI/RI group, by"#" ($p < 0.01$); (B) a significant increase from sham group was denoted by"*" ($p < 0.01$), a significant decrease from RI/RI group, by"#" ($p < 0.01$)

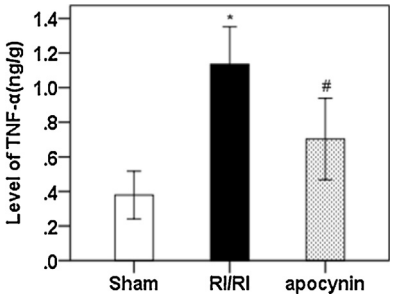

Fig. 3. The level of TNF-α in renal tissues. The renal tissues of sham, RI/RI, RI/RI + apocyningroups rats were collected after reperfusion 24 h and level of TNF-α measured. Results expreed as mean ± SD. A significant increase from sham group was denoted by"*" ($p < 0.01$), a significant decrease from RI/RI group, by"#" ($p < 0.01$)

was the same as above and the TNF-α content was measured by double antibody sandwich ELISA method. The absorbance was measured at 495 nm. The level of inflammatory injury was expressed as ng of TNF-α/g tissue.

2.7. Measurement of NO content and iNOS activity

The methods had been described previously [3]. The samping process was the same as above, the NO content and iNOS activity were measured by nitrale reduetase and absorption photometry. The absorbance was measured at 550 nm and 530 nm. The levels of NO and iNOS were expressed as nmol of NO/g and U of iNOS/mg tissue.

2.8. Western blot analysis

The methodology had been described previously [20,32,33]. Firstly, we demonstrated that the expression of SHP-1 in the kidney of rats with RI/RI was downregulated. 5 rats in sham group and 15 rats underwent RI/RI. 12 h, 24 h and 48 h after reperfusion, the kidneys removed after the rats were culled. Renal tissues were homogenized in protein lysate buffer. The homogenates were resolved on polyacryl-amide SDS gels, and electrophoretically transferred to polyvinylidene difluoride membranes. The membranes were blocked with 3% BSA, incubated with primary Abs against active SHP-1, and subsequently with alkaline phosphatase-conjugated secondary Abs. They were developed by 5-b-romo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium. Blots were stained with anti-GAPDH Ab, and the levels of proteins were normalized with respect to GAPDHb and density. In the formal experiments, the kidney specimen was collected after reperfusion 24 h and was homogenized in protein lysate buffer. The homogenates were resolved on polyacryl-amide SDS gels, and electrophoretically transferred to polyvinylidene difluoride membranes. The membranes were blocked with 3% BSA, incubated with primary Abs against active SHP-1, TLR4, NF-κB, and subsequently with alkaline phosphatase-conjugated secondary Abs. They were developed by 5-b-romo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium. Blots were stained with anti-GAPDH Ab, and the levels of proteins were normalized with respect to GAPDHb and density.

2.9. Statistical analysis

All the data was expressed as mean values ± standard deviation (SD). Statistical analysis between groups was carried out using ANOVA with post-hoc testing. A value of less than 0.01 ($p < 0.01$) was used for statistical significance.

3. Result

3.1. Serum level of BUN and Scr

The serum level of BUN in RI/RI rats was significantly higherthan that in sham group ($p < 0.01$). The levels of BUN ranged from 2.54

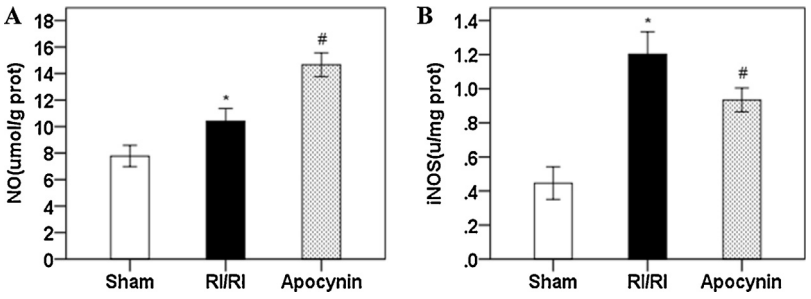

Fig. 4. The level of NO and the activity of iNOS in renal tissues. The renal tissues of sham,RI/RI, RI/RI + apocynin groups rats were collected after reperfusion 24 h and level of NO and activity of iNOS measured. Results expreed as mean ± SD. (A) a moderately increase from sham group was denoted by"*" ($p < 0.05$), a significant increase from RI/RI group, by"#" ($p < 0.01$); (B) a significant increase from sham group was denoted by"*" ($p < 0.01$), a significant decrease from RI/RI group, by"#" ($p < 0.01$)

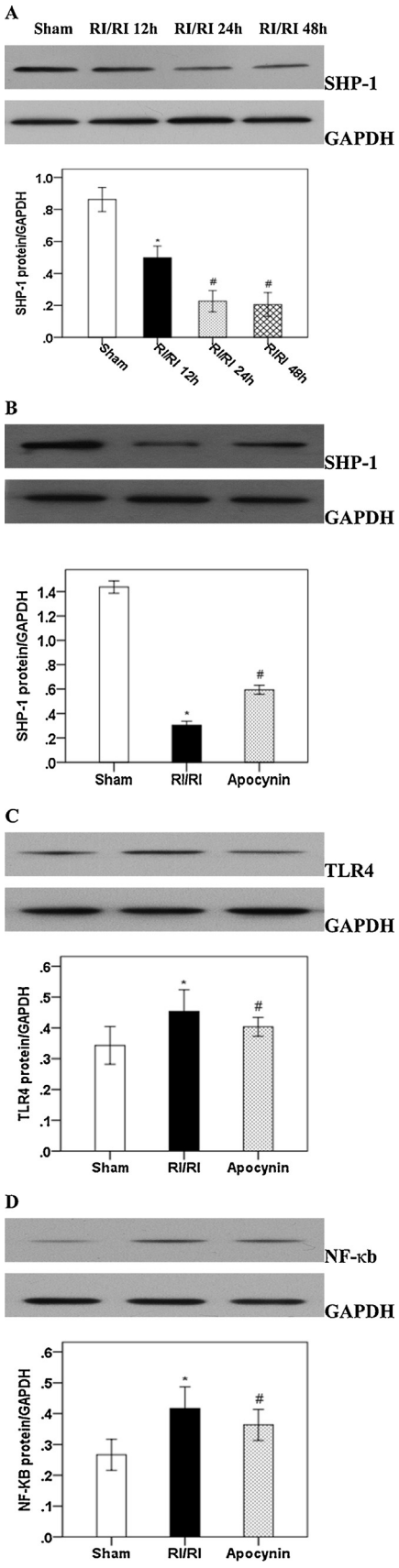

Fig. 5. The expression of SHP-1, TLR4 and NF-κB in renal tissues. In the pilot experiment, the renal tissues of sham, RI/RI (12 h), RI/RI (24 h), RI/RI (48 h) groups rats were collected and the expression of SHP-1 measured. Results expreed as mean ± SD. (A) a significant decrease from sham group was denoted by "*" ($p < 0.01$), a further significant decrease from RI/RI group, by "#" ($p < 0.01$); (B) in the formal experiments, the renal tissues of sham, RI/RI, RI/RI + apocynin groups rats were collected after reperfusion 24 h and measured the expression of SHP-1. Results expreed as mean ± SD. A significant decrease from sham group was denoted by "*" ($p < 0.01$), a significant increase from RI/RI group, by "#" ($p < 0.01$); (C) in the formal experiments, the renal tissues of sham, RI/RI, RI/RI+apocynin groups rats were collected after reperfusion 24 h and measured the expression of TLR4. Results expreed as mean ± SD. A significant increase from sham group was denoted by "*" ($p < 0.01$), a significant decrease from RI/RI group, by "#" ($p < 0.01$); (D) in the formal experiments, the renal tissues of sham, RI/RI, RI/RI + apocynin groups rats were collected after reperfusion 24 h and the expression of NF-κB measured. Results expreed as mean ± SD. A significant increase from sham group was denoted by "*" ($p < 0.01$), a significant decrease from RI/RI group, by "#" ($p < 0.01$)

to 3.18 μmol (2.91 ± 0.25 μmol) in sham group, whereas the level of BUN reached 28.29 ± 1.58 μmol in RI/RI group. Administration of RI/RI + apocynin group significantly reduced the levels of BUN (14.21 ± 1.07 μmol) compared with RI/RI group ($p < 0.01$) (Fig. 1A). The serum level of Scr in RI/RI rats was significantly higher than that in sham group ($p < 0.01$). The levels of Scr ranged from 45.34 to 50.04 μmol (48.12 ± 2.10 μmol) in sham group, whereas the level of Scr reached 169.72 ± 1.59 μmol in RI/RI group. Administration of RI/RI + apocynin group significantly reduced the levels of Scr (105.44 ± 5.56 μmol) compared with RI/RI group ($p < 0.01$) (Fig. 1B).

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

The activity of SOD in renal tissues which was induced by RI/RI was significantly less than that in sham group ($p < 0.01$). The activity of SOD in renal tissues reached 74.31 ± 1.23 U in sham group, whereas the activity of SOD in renal tissues reached 23.68 ± 1.46 U in RI/RI group. Administration of RI/RI + apocynin group significantly increased the activity of SOD (37.39 ± 1.73 U) in renal tissues, compared with the activity of SOD in renal tissues by RI/RI group induced ($p < 0.01$) (Fig. 2A). The level of MDA in renal tissues which was induced by RI/RI was significantly higher than that in sham group ($p < 0.01$). The level of MDA in renal tissues reached 4.93 ± 0.59 nmol in sham group, whereas the level of MDA in renal tissues reached 12.02 ± 0.95 μmol in RI/RI group. Administration of RI/RI + apocynin group significantly reduced the level of MDA (9.16 ± 0.15 μmol) in renal tissues, compared with the level of MDA in renal tissues by RI/RI group induced ($p < 0.01$) (Fig. 2B).

3.3. The level of TNF-α in renal tissues

As shown in Fig. 3, the level of TNF-α in renal tissues which was induced by RI/RI was significantly higher than that in sham group ($p < 0.01$). The level of TNF-α in renal tissues reached 0.3785 ± 0.0023 ng in sham group, whereas the level of TNF-α in renal tissues reached 1.135 ± 0.0036 ng by RI/RI group induced. Administration of RI/RI + apocynin significantly reduced the level of TNF-α (0.7024 ± 0.0039 ng) in renal tissues, compared with the level of TNF-α in renal tissues by RI/RI group induced ($p < 0.01$).

3.4. The level of NO and the activity of iNOS in renal tissues

The level of NO in renal tissues which was induced by RI/RI was moderately higher than that in sham group ($p < 0.05$). The level of NO in renal tissues reached 7.78 ± 0.40 μmol in sham group, whereas the level of NO in renal tissues reached 10.40 ± 0.48 umol by RI/RI group induced. Administration of RI/RI + apocynin group significantly increased the level of NO (14.66 ± 0.45 μmol) in renal tissues, compared with the level of NO in renal tissues by RI/RI group induced ($p < 0.01$) (Fig. 4A). The activity of iNOS in renal tissues which was induced by RI/RI was significantly higher than that in sham group ($p < 0.01$). The activity of iNOS in renal tissues reached 0.45 ± 0.05 U in sham group, whereas the activity of iNOS in renal tissues reached 1.20 ± 0.07 U by RI/RI group induced. Admin-

istration of RI/RI + apocynin group significantly reduced the activity of iNOS (0.93 ± 0.04 U) in renal tissues, compared with the activity of iNOS in renal tissues by RI/RI group induced ($p < 0.01$) (Fig. 4B).

3.5. The expression of SHP-1, TLR4 and NF-κB in renal tissues

In the pilot experiment, expression of SHP-1 in renal tissues which was induced by renal ischemia/reperfusion for 12 h, 24 h and 48 h was significantly less than that in sham group ($p < 0.01$–0.05), and the expression of SHP-1 was not significant difference between 24 h and 48 h in ischemia/reperfusion ($p > 0.05$) (Fig. 5A); In the formal experiments, expression of SHP-1 in renal tissues which was induced by RI/RI was significantly less than that in sham group ($p < 0.01$). Administration of RI/RI + apocynin significantly upregulated expression of SHP-1 in renal tissues, compared with expression of SHP-1 in renal tissues by RI/RI group induced ($p < 0.01$) (Fig. 5B). In the formal experiments, expression of TLR4 in renal tissues which was induced by RI/RI was significantly higher than that in sham group ($p < 0.01$). Administration of RI/RI + apocynin significantly downregulated expression of TLR4 in renal tissues, compared with expression of TLR4 in renal tissues by RI/RI group induced ($p < 0.01$) (Fig. 5C); In the formal experiments, expression of NF-KB in renal tissues which was induced by RI/RI was significantly higher than that in sham group ($p < 0.01$). Administration of RI/RI + apocynin significantly downregulated expression of NF-κB in renal tissues, compared with expression of NF-κB in renal tissues by RI/RI group induced ($p < 0.01$) (Fig. 5D);

4. Discussion

In this preliminary inspection we demonstrated that a main preconditioning regimen involving injected apocynin provided some protection against RI/RI in rats. These protective roles were showed by less pronounced increases in serum BUN and Scr contents after I/R injury. Further, these protective roles appeared to be due, at least in part, to fortified renal expression of SHP-1, the expression of which was reduced after I/R injury in rats that did not adopt preconditioning with apocynin. In addition, in the pilot experiment, we demonstrated that the expression of SHP-1 in renal tissues was really reduced in I/R for different time. Although the detailed mechanisms underlying these roles remain to be illuminated, it was known that SHP-1 was involved in regulating signaling cascades related to cellular apoptosis and oxidative stress injury.

RI/RI was a common pathophysiological phenomenon and a primary reason of acute renal failure, which was associated with a high mortality [1]. Patients who underwent RI/RI may be subjected to chronic renal dysfunction of variable severity. RI/RI may occur during kidney surgery, or as a consequence of blood loss, hypotensive shock, or extracorporeal shock-wave lithotomy [1]. Thus, weakening or preventing RI/RI had been of significant clinical interest.

Previous studies with apocynin had shown protective roles in the testicle, heart, and kidney [23–26]. These protective roles mainly involved generalized melioration of nitroso-redox unbalance and oxidative stress injury, although the particular cells and molecular mechanisms involved either varied or were unknown. In our study, apocynin can ameliorate renal function and reduced oxidative stress injury, the result was consistent with the literature [29]. In addition, apocynin can reduce the activity of iNOS on myocardial ischemia reperfusion injury [34]. Our study demonstrated that apocynin can reduce the activity of iNOS and increase the level of NO (eNOS induced, it was consistent with the literature [35] to protect renal injury against ischemia reperfusion induced. Therefore, apocynin can ameliorate nitroso-redox unbalance to protect kidney on RI/RI.

TLRs were a family of pattern-recognition receptors that enable the recognition of conserved structural motifs in a broad array of pathogens [20]. Several pieces of evidence confirmed that TLR4-deficient mice were protected against ischemic kidney damage and injury [36]. TLR4 signals through MyD88, leading to subsequent downstream activation of NF-κB and MAPK signaling pathways [37]. These signaling cascades are involved in the induction of proinflamma-tory cytokines and chemokines [38]. NF-κB was normally sequestered in an inactiveform in the cytoplasm bound to IκB proteins, an interaction that regulated its activity [20–22]. Multiplestimuli can activate NF-κB signaling by degradation of IκB and release of the NF-κB p65–p50 dimer, which translocated to the nucleus, binds to κB binding sites on DNA, and regulated transcriptional activation of the target genes [39–43]. NF-κB was an important nuclear transcription factor in eukaryotic cells, which existed in almost all the cells [20]. Inhibiting NF-κB activation or knocking out p50 subunit of NF-κB was protection and developed smaller infarct volume [20]. A critical role of NF-κB was to regulate the genes of inflammatory mediators such as TNF-α, all of which play an important role in ischemic renal damage [20,21]. Literature showed that apocynin can reduce the expression of TLR4 on myocardial ischemia reperfusion injury [34]. Our study demonstrated that apocynin can downregulate the expression of TLR4 on renal ischemia reperfusion injury. It hinted that apocynin can modulate the TLR4 of TLR4/MAPK/NF-κB signaling pathway to decrease the inflammatory damage on RI/RI.

SHP-1 was an intracellular protein tyrosine phosphatase (PTP) which was widely expressed in the body, SHP-1 can negatively regulated TLR4 to modulate TLR4/MAPK/NF-κB signaling pathway [9]. It hinted that inflammatory damage was reduced partially via upregulating the expression of SHP-1 on RI/RI. Our study demonstrated that apocynin can upregulate the expression of SHP-1 on RI/RI. These results illustrated that apocynin can decrease the inflammatory damage partially through upregulating the expression of SHP-1 to regulate TLR4/MAPK/NF-κB signaling pathway on RI/RI.

This research had several restrictions. For this preliminary inspection, only a small number of rats were used each group, which may have influenced the statistical capacity of our analyses. Further, we did not accomplish any histological examination to analyze the renal damage. Finally, we did not accomplish any evaluations of cell apoptosis, which would have been the mostly likely mediators of the surveyed roles in the kidneys of rats with RI/RI.

In conclusion, we found that apocynin preconditioning improved renal function in rats following RI/RI, and upregulated the expression of renal SHP-1. Further studies were needed to explore whether the renal protective roles of apocynin preconditioning were due to partially upregulated expression of SHP-1 in the kidney.

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