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Original Article

Remote ischemic preconditioning protects the kidney against injury after ischemia and reperfusion through activation of hypoxia-inducible factor 1

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Abstract: Clinical studies suggest that remote ischemic preconditioning (RIPC) in the arm or leg may protect the heart, brain and kidney against injury after ischemia and reperfusion. However, the detailed mechanism remains unclear. A recent study indicates that Hypoxia-Inducible Factor 1 (HIF-1) activates IL-10 gene transcription and is required for remote ischemic preconditioning of the heart. In this study, we investigate the role of HIF-1 in the protection effect of RIPC in the kidney after ischemia and reperfusion injury (IRI). RIPC was induced by blocking unilateral femoral artery for 5 min followed by 5 min of reperfusion for a total of five cycles. IRI was induced by blocking bilateral renal artery for 45 min followed by 120 min of reperfusion. The HIF specific inhibitor YC-1 was injected through tail vein with the dose of 2 mg/kg before surgery operation. Results showed that renal functions including serum creatinine, cystatin C and tubular injury score after IRI in rats with RIPC pretreatment were significantly better than in those without RIPC treatment. Renal expression of SOD and p-AKT were also elevated after RIPC. Our study also showed that RIPC treatment itself obviously induced the accumulation of HIF-1 in rat renal tubular epithelial nucleus and further induced upregulation of mRNA levels of target genes of HIF, such as EPO, VEGF and HO-1. Taken together, these data indicate that the protective effect of RIPC against IRI might be achieved through the activation of HIF.

Keywords: Remote ischemic preconditioning (RIPC), ischemia and reperfusion injury (IRI), hypoxia-inducible factor 1 (HIF-1)

Introduction

Compelling evidence indicates that the incidence of acute kidney injury (AKI) is rapidly increasing. With the greater recognition of AKI and the improvement of medical technology, AKI-associated mortality rate is decreasing slightly, but remains unacceptably high [1]. Therefore, prevention and treatment of AKI is still an urgent problem. Cardiovascular surgery is one of the important causes of AKI. As reported by Bastin et al. [2], the incidence of AKI after cardiac surgery is up to about 25%. Although recently the discovery of various biomarkers makes early diagnosis of cardiovascular surgery-related acute kidney injury (CSA-AKI) become possible, there is no effective protection and management of AKI [3, 4]. For elective cardiovascular surgery, prevention of postoperative AKI is of great importance.

Ischemic preconditioning (IPC) was first proposed by Murry et al. [5]. It is a phenomenon in which previous application of a short, temporary ischemic pretreatment can protect an organ against subsequent prolonged ischemia injury. It was found afterwards that remote ischemic preconditioning (RIPC) with brief episodes of ischemia-reperfusion applied in distant tissues or organs results in the protective effect in the heart and render the myocardium resistant to a subsequent sustained episode of ischemia [6], which makes it possible in clinical application. Clinical observations indicate that RIPC has the same protective effect as IPC in ischemia injuries of the heart, brain, kidney and other vital organs [7-10]. In clinical applications, it is a simple, economic and efficient method to alleviate ischemic damage to vital organs by pressing the cuff to the patient limbs. Several clinical observations demonstrated the protec-

tive effect of RIPC in renal ischemia injury. Research by Zimmerman et al. [11] showed that RIPC prevented acute kidney injury in patients undergoing cardiopulmonary bypass-assisted cardiac surgery. RIPC was accomplished by an automated thigh tourniquet consisting of three 5-min intervals of lower extremity ischemia separated by 5-min intervals of reperfusion. After surgery AKI occurred significantly lower in remote ischemic preconditioned patients than in control group. Another study stated that in patients with a non-ST-segment elevation myocardial infarction undergoing Percutaneous Coronary Intervention (PCI), those who received RIPC by serial balloon inflations and deflations were conferred protection against AKI with lower AKI incidence and the 30-day rate of death or re-hospitalization than the control group [12]. However, it was also reported that there was no benefit of RIPC which protected renal function and reduced the incidence of AKI [13]. Thus, it is necessary to further valuate the clinical feasibility and effectiveness of RIPC. The mechanism how RIPC exerts effect on kidney in ischemia situation is very complex and still remains unclear.

Hypoxia-inducible factor (HIF) plays a pivotal role in the transcriptional response to changes in inadequate oxygen availability. HIF is a heterodimer transcription factor which consists of an unstable α subunit and a stable β subunit. Under hypoxic conditions, members of the prolyl hydroxylase domain (PHD) family, the key factor involved in the process of α subunit degradation, are deactivated, resulting in HIFα accumulation, dimerization with a HIFβ subunit, translocation to the nucleus, and transcriptional activation of targeted genes, including genes involved in erythropoiesis, angiogenesis, autophagy, and energy metabolism [14, 15]. In AKI, HIF activation by various factors has strong renoprotective effect, including relieving decrease of renal functions and reducing histopathological damage. Pharmacological activation of HIF by small molecules that inhibit HIF hydroxylases protected mouse kidneys against ischemia-reperfusion injury [16]. HIF induction by cobalt chloride administration led to renoprotective gene expression and thereby ameliorated ischemic injury of the kidney in rats [17]. It has been demonstrated that HIF-1 plays an essential role in cardioprotection that is induced by RIPC [18]. However, the role of HIF in protection of renal IRI by RIPC is almost blank. In this study, we investigated whether RIPC had protective effect on rat kidney injury induced by ischemia and reperfusion and try to explore the role of HIF in this process.

Materials and methods

Animals and study design

Male SD rats weighting 160-180 g were purchased from Shanghai Jiesijie Laboratory Animal Co., Ltd (Shanghai, China). All rats were maintained in a temperature- and light-controlled environment in accordance with the Principles of Laboratory Animal Care and Use. All animal experiments were approved by the Institutional Animal Care and Use Committee of Nanjing Medical University. Rats were randomly divided into four groups: 1. SHAM group (n=10): After opening the abdominal cavity, separate perirenal adipose tissue, expose both kidneys but without any treatment and suture abdominal incision after 45 min; 2. IRI group (n=10): Rat kidneys received ischemia-reperfusion injury. The detailed process was described afterward; 3. RIPC group (n=10): Rats received RIPC which was immediately followed by ischemia-reperfusion injury. The detailed RIPC treatment was described afterward; 4. YC-1 group (n=10): The specific HIF-1 inhibitor YC-1 (Sigma, 2 mg/kg) was intravenously administered before rats received RIPC and the following IRI. Rats were anesthetized by intraperitoneal injection of 10% chloral hydrate (5 mL/kg). Body temperature was maintained at 37°C by performing surgery on a heating pad (Yuyan Instruments Co., Ltd., Shanghai, China). 24 h after induction of IRI, blood samples were obtained from the heart and levels of serum creatinine and cystatin C were determined by an automatic biochemical analyzer (Olympus AU5400 analyzer, Olympus Diagnostics). Kidney tissue was obtained after saline infusion for histologic analysis.

Induction of IRI

After confirmation of anesthesia, rats were placed on a heating operation pad. Hair of the abdominal region was removed and povidone-iodine was applied for sterilization. After midline abdominal incision, the free intestinal tis-

sue was gently pulled out and put on saline-soaked gauze to expose the kidney. Perirenal fat tissue was separated by blunt dissection. The renal pedicles were exposed and a microvascular clamp (Yuyan Instruments Co., Ltd., Shanghai, China) was applied to clip bilateral renal artery. The kidneys were confirmed to be completely clipped by observing the exterior color change. After 45 min, the clamps were removed and reperfusion was applied. After reperfusion, Roxithromycin (5 mg/kg) and Diclofenac sodium (1 mg/kg) was given in the peritoneal cavity and the abdomen incision was closed in layers.

Induction of RIPC

RIPC was induced in advance of IRI. Hair of the thigh region was removed and povidone-iodine was applied for sterilization. After incision, the subcutaneous tissue and fascia were separated by blunt dissection to expose femoral artery and vein. Microvascular clamps were applied to clip bilateral femoral artery for 5 minutes ischemia followed by a 5-minute reperfusion (clamps open). The RIPC stimulus consisted of 5 clip-release cycles (total duration 50 minutes). In animals randomized to the RIPC control group, five 5-minute of clip-release cycles were also applied, but without the following IRI treatment.

Real-time PCR

Total RNA was isolated from rat kidney tissue with Trizol (Invitrogen) according to the manual. cDNA was synthesized from 1 μg of total RNA using a reverse transcription kit (Thermo Scientific). 18S rRNA was served as an internal control. Primer sequences were as follows: HIF-1α forward: 5'-AGCAATTCTCCAAGCCCTCC-3', HIF-1α reverse: 5'-TTCATCAGTGGTGGCAGTTG-3', EPO forward: 5'-CGGAACTGTAATCCACGC-CA-3', EPO reverse: 5'-CATTCTCCAGGCCCTGT-GTT-3', VEGF forward: 5'-TGGACCCTGGCTTTA-CT-GCTG-3', VEGF reverse: 5'-GGCAATAGCTGC-GCTGGTAGA-3', HO-1 forward: 5'-AGGTGCACAT-CCGTGCAGAG-3', HO-1 reverse: 5'-TCCAGGG-CCGTATAGATATGGTACA-3'; 18 S forward: 5'-CA-TGATTAAGAGGGACGGC-3', 18 S reverse: 5'-TT-CAGCTTTGCAACCATACTC-3'. Quantitative real-time PCR was performed in duplicate with 0.2 μM primers, 1 μL cDNA and the SYBR Green Real-time PCR Master Mix (Roche Applied Science) in a total volume of 10 μL. Reactions were run at 95°C for 60 s, followed by 40 cycles of 15 s at 95°C, 15 s at 60°C and 45 s at 72°C. The StepOne Plus Real-time PCR system from ABI was used for analysis. Results were expressed as cycle threshold (Ct) and calculated as ΔCt, which were normalized to endogenous control 18S rRNA.

Western blot

Rat kidney tissue was homogenized and lysed in RIPA buffer (KeyGEN BioTECH, China). A liquots of tissue lysates containing 30 μg of total proteins were subjected to SDS-PAGE, transferred to a PVDF membrane and immunoblotted with anti-total AKT, p-AKT, SOD, and GAPDH antibodies. Antibodies were all purchased from Abcam Shanghai Trade Co., LTD. The ECL plus western blotting detection system (Bio-Rad, USA) was used for signal detection. Densitometric analysis was performed with Image J software.

Histological examination

Rat kidney tissue was fixed with formalin and embedded in paraffin. Sections in 3 μm thick were stained with hematoxylin-eosin (HE). Histological injury was mainly evaluated by quantitative measurement of tubular injury by assessment of specific changes in 10 individual and random selected fields with light microscopy. The number of necrotic cells, loss of brush border, cast formation, and tubule dilation were estimated. A percentage of the area affected was used for damage scoring [19]. All evaluations were made without knowledge of the sample identity. Three pathologists respectively made the histological assessments of injury and the average score was obtained as the final result.

Immunohistochemistry

Sections in 3 μm thick were grilled, dewaxed, rehydrated and heated in microwave oven with high power for 12 min in antigen retrieval citrate solution (pH 6.0). Sections were then incubated with polyclonal rabbit anti-HIF1α antibody (abcam) at a 1:1000 dilution overnight at 4°C followed by detection with Novolink™ Polymer Detection System (Novocastra). Sections were

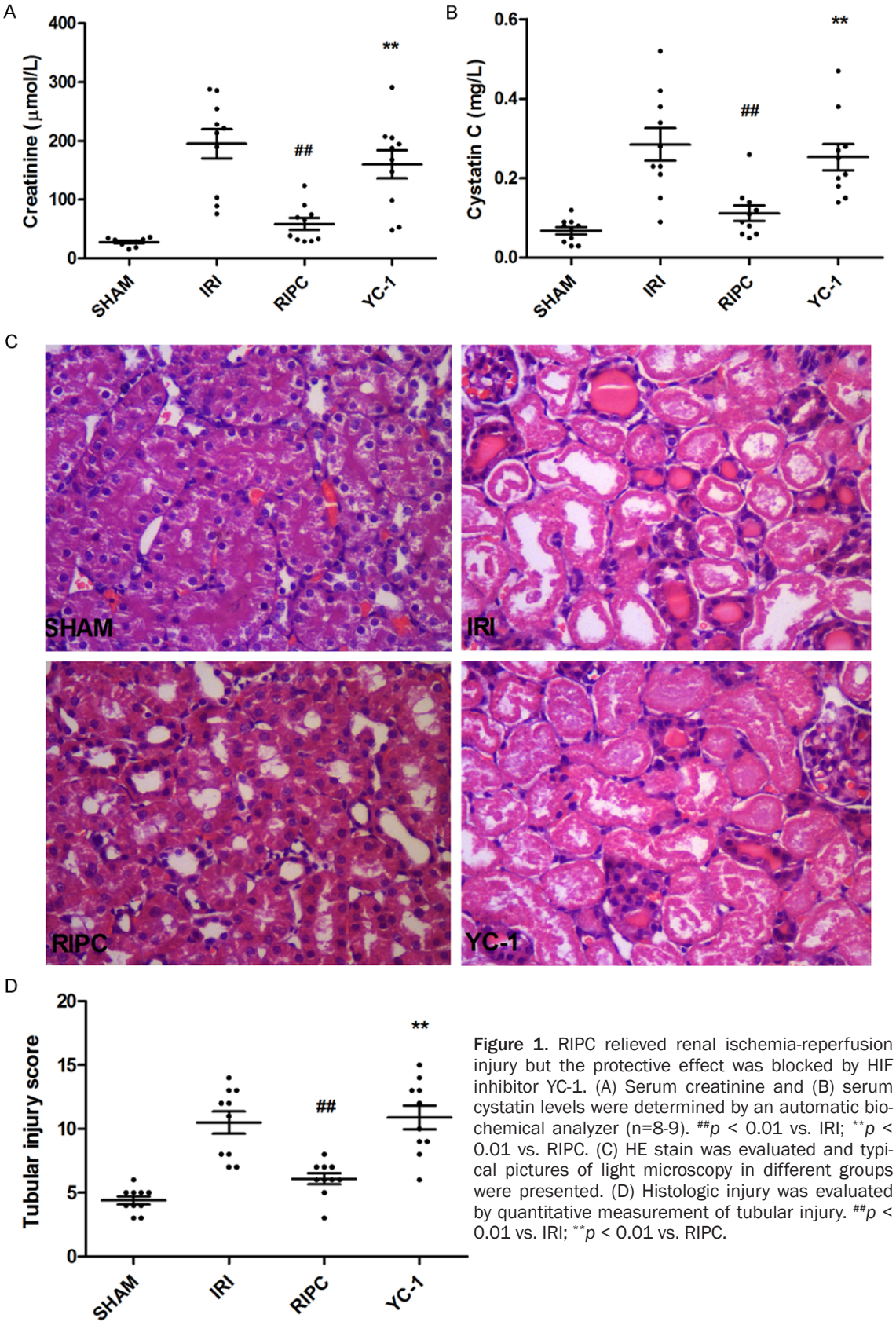

Figure 1. RIPC relieved renal ischemia-reperfusion injury but the protective effect was blocked by HIF inhibitor YC-1. (A) Serum creatinine and (B) serum cystatin levels were determined by an automatic biochemical analyzer (n=8-9). $^{\#\#}p < 0.01$ vs. IRI; $^{**}p < 0.01$ vs. RIPC. (C) HE stain was evaluated and typical pictures of light microscopy in different groups were presented. (D) Histologic injury was evaluated by quantitative measurement of tubular injury. $^{\#\#}p < 0.01$ vs. IRI; $^{**}p < 0.01$ vs. RIPC.

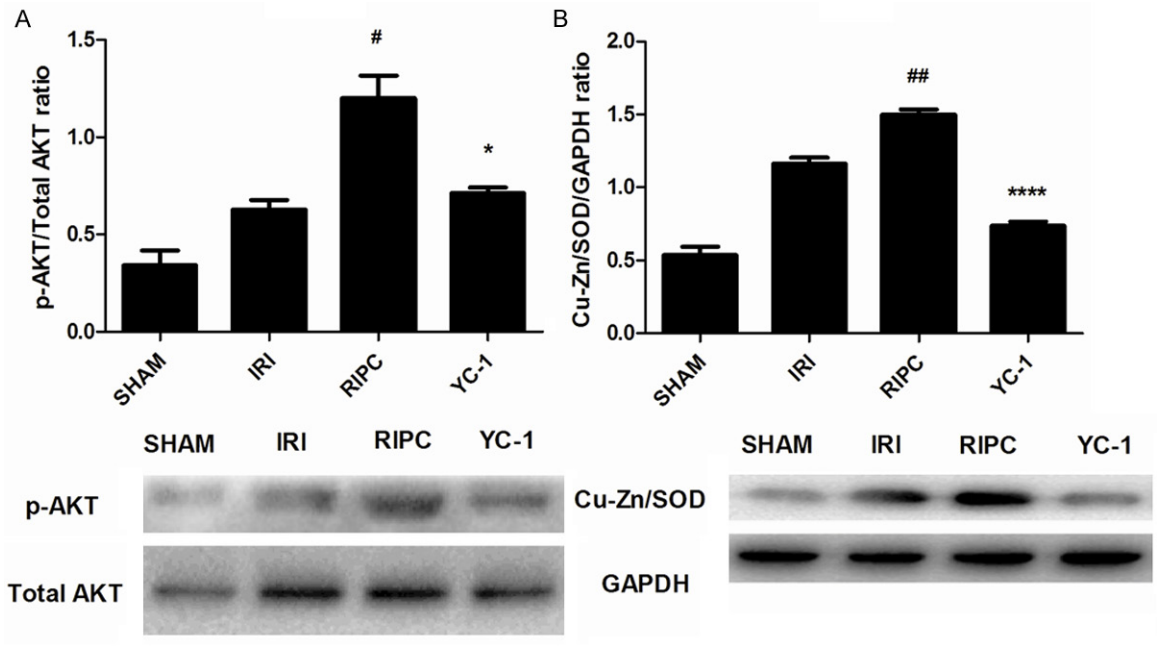

Figure 2. RIPC activated phosphorylation of AKT and induced SOD production. (A) Total protein of rat renal tissue in different groups were extracted and subjected to immunoblot using p-AKT and total AKT antibodies, respectively. Representative blots from three independent experiments were shown and the relative expression of p-AKT and total AKT were quantified by density analysis. #$p < 0.05$ vs. IRI; *$p < 0.05$ vs. RIPC. (B) The same samples as described in (A) were subjected to immunoblot assays of SOD and GAPDH. ##$p < 0.01$ vs. IRI; ****$p < 0.0001$ vs. RIPC.

stained with HE, dehydrated, and mounted in neutral baisam with coverslips.

Statistics

Data are presented as mean ± SEM from at least three experiments. Statistical significance was evaluated using student's t-test for two groups, or ANOVA for multiple groups. A value of $p < 0.05$ was considered statistically significant. Data analysis was carried out using GraphPad Prism 5.0.

Results

RIPC relieved renal ischemia-reperfusion injury and the protective effect was blocked by HIF inhibitor YC-1

In RIPC group, five RIPC ($[5 \times I_5R_5]^{fem}$) was subjected to rats before renal ischemia-reperfusion injury. After 24 h, blood and renal tissue were obtained for further analysis. As shown in **Figure 1A** and **1B**, serum creatinine level and cystatin C level were both significantly decreased in RIPC pretreated rats than in those without RIPC pretreatment. Pathology analysis showed that in RIPC group, renal tissue damage was mitigated (**Figure 1C**) and tubular pathological injury score was reduced (**Figure 1D**). These results demonstrated that RIPC relieved tissue injury caused by renal IRI and protected renal functions. However, when the specific HIF inhibitor YC-1 was administratered in advance of RIPC, the protective effect of RIPC disappeared. Serum creatinine level and cystatin C level in YC-1 group were significantly increased compared with RIPC grpup (**Figure 1A** and **1B**). The pathologic damage in YC-1 group was also more severe than in RIPC group (**Figure 1C** and **1D**). The above results indicated that RIPC treatment obviously relieved tissue injury induced by renal IRI and protected renal functions, while the protective effect was significantly weakened when the in vivo HIF system was suppressed, suggesting that RIPC may exert its protective effect by activating the HIF system.

AKT activation in renal tissue after RIPC treatment

Activation of AKT signaling pathway is an important part through which RIPC exerts organ protective effect in ischemia-reperfusion injury [20]. In this study, phosphorylation of AKT in serine 473 was significantly enhanced in renal tissue after RIPC treatment, while was obvious-

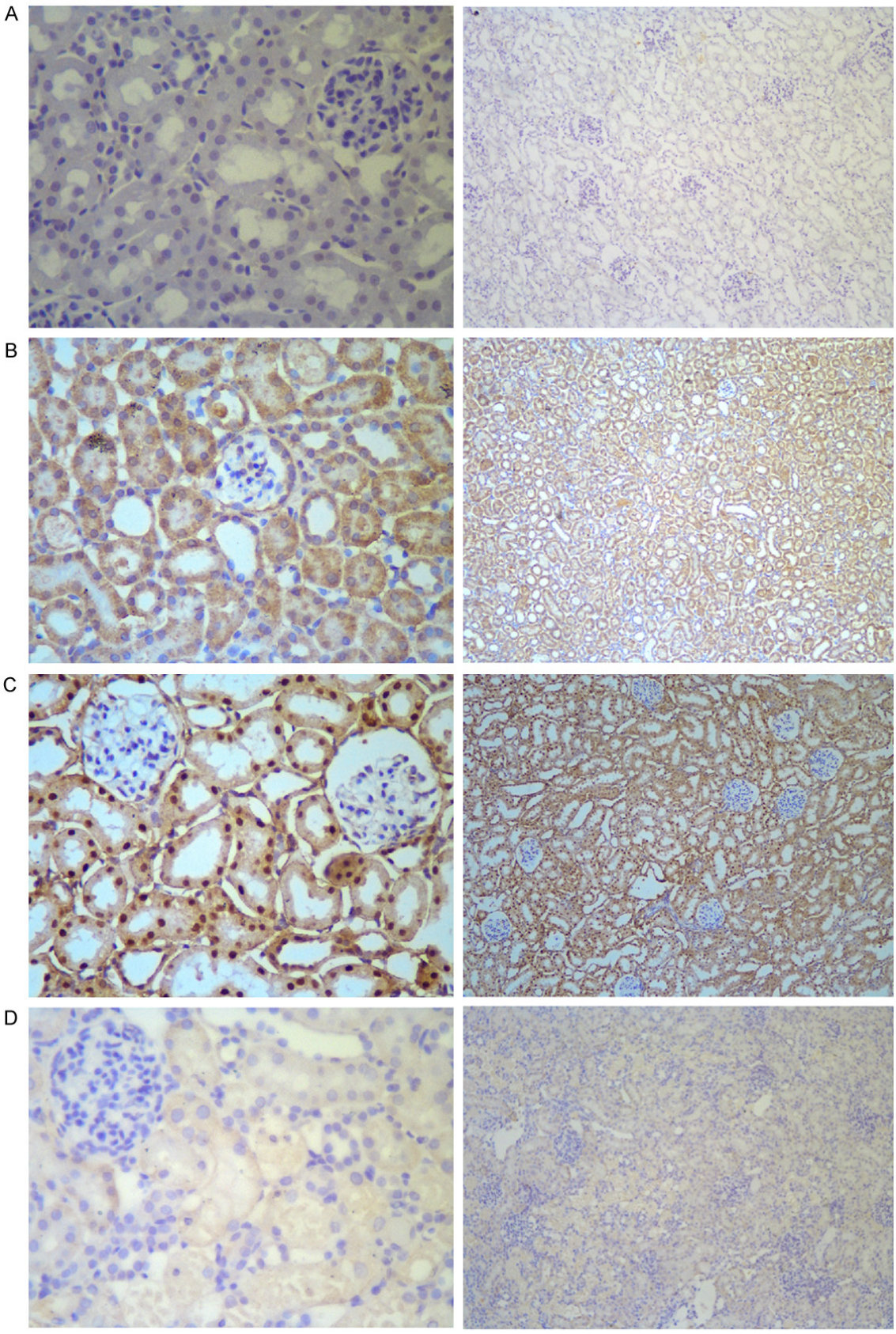
A
B
C
D

Figure 3. RIPC activated HIF-1α Expression in renal tubular epithelial cells. Kidney sections were subjected to immunohistochemistry using anti-HIF1α antibody. Typical pictures in different groups were presented. A. There was no expression of HIF-1α in SHAM group. B. 24 h after RIPC and IRI treatment, HIF1α could be observed in renal tubular epithelial cell plasma. C. Immediately after a single RIPC treatment($[5 \times I^5R^5]^{fem}$) without IRI, kidney tissue was obtained and treated for immunohistochemistry. HIF1α was obviously accumulated in nucleus of renal tubular epithelial cells. D. HIF inhibitor YC-1 blocked production of HIF1α in renal tubular epithelial cell. Left: 400×; Right: 100×.

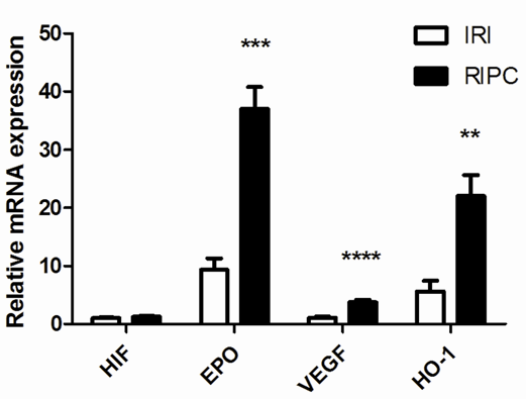

Figure 4. RIPC activated mRNA expression of target genes of HIF in kidney tissue. Total RNA was extracted after IRI induction with or without RIPC pretreatment (n=10). mRNA expression of targeted genes of HIF in kidney was assayed by real-time PCR. mRNA levels of EPO, VEGF, and HO-1 was significantly upregulated after RIPC treatment. *$p < 0.05$ vs. IRI; **$p < 0.01$ vs. IRI; ***$p < 0.001$ vs. IRI.

ly repressed by the specific HIF inhibitor YC-1 (**Figure 2A**).

Expression of SOD in renal tissue after RIPC treatment RIPC

In renal IRI, SOD is an important protective factor which can effectively eliminate oxygen free radicals and reduce the direct tissue damage caused by reactive oxygen species (ROS). Under IRI condition, transient production of a large number of SOD was induced due to acute hypoxia. As shown in **Figure 2B**, RIPC treatment could yield a significant increase in SOD production. However, this effect was also repressed by the specific HIF inhibitor YC-1.

Accumulation of HIF in kidney after RIPC treatment

Previous studies have shown that after RIPC treatment the kidney has a significant ability to resist IRI, and the effect is dependent on activation of HIF system [21]. As a transcript factor, HIF enters into the nucleus to regulate the expression of many target genes. Cu/Zn-SOD is one of the adjustable target genes regulated by HIF. In this study, it was observed that expression of SOD was significantly increased in renal tissue after RIPC, which was down-regulated by the HIF inhibitor YC-1. Based on these results, we speculate that RIPC may directly induce activation of HIF system through femoral artery ischemia-reperfusion cycles in rat kidney so as to play a protective role in IRI. Results of immunohistochemical analysis showed that HIF-1α accumulated in renal tubular epithelial cell cytoplasm (**Figure 3B**) in renal tissue of RIPC group. As IRI created a hypoxic environment in the kidney which induced the production of HIF and 24 hours later HIF-1α in renal tissue could be degraded by ubiquitination of the activity-regained prolyl hydroxylase (Phd), rats were treated with RIPC ($[5 \times I_5R_5]^{fem}$) only, after which the renal tissue were immediately obtained and expression of HIF-1α was assayed by immunohistochemical analysis. It was observed in **Figure 3C** that HIF-1α aggregated in nucleus of tubule epithelial cells, suggesting that a separate treatment of RIPC in the femoral artery could induce the activation of HIF in renal tissue and play a role in renal protection.

mRNA expression of target genes of HIF in kidney after RIPC treatment

The activated HIF dimer which is released into cytoplasm can adjust multiple downstream target genes and play a role in resistance to ischemic injury, protecting renal functions. As shown in **Figure 4**, mRNA expression of target genes of HIF in kidney after RIPC treatment was assayed by real-time PCR. Although there was no prominent change in mRNA level of HIF itself after RIPC treatment, mRNA levels of the main target genes adjusted by HIF such as EPO, VEGF, and HO-1 was significantly upregulated ($p < 0.001$), further demonstrating that RIPC treatment might activate HIF system and its transcriptional adjustable activity.

Discussion

It has been thirty years since the concept of RIPC came into being. In animal experiments, RIPC exhibits strong protective effect on isch-

emia induced damage in organs. However, RIPC remains controversial in clinical validity and so far has not been widely used. In this study, we confirmed that RIPC could significantly protect the kidney against renal IRI and conserve renal functions. Administration of RIPC treatment in advance of renal IRI could effectively reduce the increase of serum creatinine and cystatin C levels, increase production of SOD, alleviate pathological injury in renal tissue. Although the mechanism that IPC and RIPC protect ischemia organs still remains unclear, the activation of AKT signal pathway is widely accepted to be the central link [22]. In this study, phosphorylation of AKT was significantly enhanced in renal tissue after RIPC treatment, while was obviously repressed by the specific HIF inhibitor YC-1, giving a hint that HIF plays a key role in protection of AKI by RIPC treatment. Further study demonstrated that the activated HIF dimmers could be detected in nucleus early after a separate RIPC treatment ($[5 \times I_5R_5]^{fem}$) towards unilateral femoral artery in rats. Results of RT-PCR showed that the HIF system activated by RIPC treatment possessed the transcriptional regulatory activity, with a significant up-regulation of mRNA levels of target genes of HIF, including EPO, VEGF and HO-1, confirming the important role of HIF in RIPC- mediated protection against renal IRI. In RIPC induced hypoxic conditions, although mRNA level of HIF itself was not altered, the accumulation of active HIF in the nucleus due to less degradation further activated the upexpression of downstream genes.

HIF is a heterodimeric transcription factor which plays an important role in the process of hypoxia adjustment. It is well known that preactivation of HIF system in kidney can protect the kidney against AKI induced by various factors. Previous studies proposed that HIF played an important role in the protective mechanism of RIPC in myocardial ischemia [18, 23]. Our study also demonstrated that HIF participated in the process of protection against renal IRI by RIPC. Although activation of HIF provides a powerful protective effect in AKI, selection of HIF activation manner is still a problem in clinical. And whether excessive HIF activation may cause tumor growth and fibrosis is inconclusive currently. The commonly used HIF agonist cobalt dichloride has strong toxicity and is not applicable to humans. New specific prolyl hydroxylase inhibitors are still under development and not yet available in the market. Aanesthetic xenon is one of the more promising options, with a strong role in activating HIF and is very suitable for prevention of cardiovascular postoperative AKI [24]. However, the high price limits its clinical application. In contrast, RIPC treatment by simply pressing the cuff on patient limbs has significant advantages before cardiovascular surgery. It is non-invasive and does not increase the financial burden of patients. Further, it does not induce the potential side effects caused by overexpression of HIF. Therefore, RIPC might has great applicable value in clinical.

RICP is an exciting future strategy, but more work is needed before wide application. In animal experiments the role of RIPC is unanimously approved, but its effect is controversial in clinical studies. Some researchers reported that RIPC could not reduce the incidence of cardiovascular postoperative AKI [25, 26]. The reason for this phenomenon is varied. In addition to the different experimental conditions and methods of each research team, the anatomical differences between humans and rats are inevitable. In the process of femoral artery ligation and reperfusion in mice, a transient decrease in renal vascular flow can be monitored, which causes the renal tissue in transient ischemia [27], suggesting that short-term hypoxic state may activate the HIF system in the kidney and thus result in a series of protective effect. In animal experiments the commonly used RIPC treatment is to expose femoral artery and clamp it using vascular clips. In terms of human bodies, body weight, kidney volume and distance between peripheral artery and renal vessels are significantly higher than the size of rats. Therefore, changes in renal plasma flow and so-causing HIF activation in renal tissue after human limbs cuff pressure may be used as a reference to value whether RIPC treatment could play a protective role. Moreover, whether increasing cuff pressure and extending blocking time and cycles can enhance the protective effect of RIPC remains to be explored in more clinical studies.

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Disclosure of conflict of interest

None.

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