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ORIGINAL ARTICLE

Ischemic postconditioning inhibits apoptosis after renal ischemia/reperfusion injury in rat

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Summary

Ischemic postconditioning is a phenomenon that intermittent interruptions of blood flow in the early phase of reperfusion can protect organ from ischemia/reperfusion (I/R) injury. In the present study, we investigated whether the protective effect of ischemic postconditioning was associated with modulation of apoptosis after renal I/R injury. Rats were subjected to 45 min of renal ischemia, both with and without treatment with ischemic postconditioning. Serum urea nitrogen and creatinine levels, phosphorylation of Akt and ERK1/2 and apoptosis were compared after renal injury. Our data showed that ischemic postconditioning attenuated the renal dysfunction and cell apoptosis induced by I/R and increased phosphorylation of Akt and ERK1/2. The results indicated that ischemic postconditioning decreased apoptosis and improved renal function. This protective effect may be related with the levels of Akt and ERK1/2 activation. These findings may have major implications in the treatment of renal transplantation.

Introduction

Renal ischemia, whether caused by shock or during surgery or transplantation, is a major cause of acute renal failure (ARF). Although reperfusion is essential for the survival of ischemic tissue, there is good evidence that reperfusion itself causes additional injury [1]. Recent studies demonstrated that tubular cell apoptosis emerged as a primary and major contributor to the pathophysiology of renal ischemia/reperfusion (I/R) and determined the outcome of renal damage [2,3]. Moreover, Daemen *et al.* [4–6] indicated that a significant component of inflammation was induced by apoptosis after renal I/R injury. They also demonstrated that blockade of apoptosis prevented renal inflammation after I/R. Thus, the ideal preventative or therapeutic approach would indeed target apoptosis after I/R.

Recent studies indicated that ischemic postconditioning significantly reduced cardiac infarct size and inhibited inflammation and apoptosis [7–17]. In addition, a recent exciting clinical report demonstrated that ischemic postconditioning, after coronary angioplasty and stenting, protected the human heart during acute myocardial infarction [13]. We previously demonstrated that ischemic postconditioning attenuated renal damage after I/R injury *in vivo* [18]. However, it remained to be determined whether the protective effects of ischemic postconditioning were associated with modulation of apoptosis *in vivo*. The major purpose of this study was to determine whether ischemic postconditioning inhibited apoptosis after renal I/R injury.

Materials and methods

Animal preparation

Adult male Wistar rats (250–280 g) were from the Center of Experimental Animals in Medical College, Wuhan University. This project was approved by the committee of experimental animals of Wuhan University, and the procedures were carried out according to the routine

animal-care guidelines. All experimental procedures complied with the Guidelines for the Care and Use of Laboratory Animals. Animal preparation was performed as previously described [18]. Briefly, rats were anesthetized with pentobarbital (45 mg/kg) and placed on a homeothermic table to maintain core body temperature at 37 °C. The midline laparotomy was made and the left kidney was subjected to 45 min of ischemia followed by reperfusion after right nephrectomy.

Experimental protocols

Animals were divided into two different treatment schedules: Schedule A was designed to evaluate the effect of ischemic postconditioning on apoptosis after renal I/R injury; Schedule B was designed to evaluate the role of Akt and ERK1/2 in postconditioning-induced renal protection.

In schedule A, animals were randomly separated into three groups: (i) sham-operated control group; (ii) I/R group: kidneys were subjected to 45 min of ischemia followed by reperfusion; and (iii) ischemic postconditioning group: kidneys were subjected to six cycles of 10 s of reperfusion followed by 10-s ischemia immediately after 45 min of ischemia [18] (Fig. 1). Rats were killed at 24, 48, and 72 h after I/R injury. Sham operations were performed in a similar manner, except that the renal vessels were clamped. Each group comprised 24 rats, and eight rats were used at each time. Serum urea nitrogen (BUN) and creatinine (Cr) levels were evaluated at 24, 48, and 72 h after I/R injury, and other parameters were evaluated at 24 h after I/R injury.

In schedule B, animals were randomly divided into three groups: (i) sham-operated control group; (ii) I/R group: kidneys were subjected to 45 min of ischemia followed by reperfusion; and (iii) ischemic postconditioning group: kidneys were treated with ischemic postconditioning immediately after 45 min of ischemia (as in group 3, schedule A). Ischemic kidneys were harvested at 5, 15 and 60 min (all $n = 6$) after starting reperfusion in both groups and sham kidneys were harvested at 60 min ($n = 6$) after sham surgery.

Preservation of kidneys

The left kidney was removed under fully maintained anesthesia. After removal, the kidney was fixed in 10% phosphate-buffered formalin or immediately frozen, and stored at −80 °C for different determinations.

Serum assays

To assess 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).

Histologic examination

The kidney was fixed in 10% neutral-buffered formalin, paraffin embedded and sectioned at 4-μm thick according to the standard procedure. The sections were deparaffinized and hydrated gradually, and examined by hematoxylin–eosin staining. Morphologic assessment was performed by an experienced renal pathologist who was unaware of the treatment. A grading scale of 0–4, as outlined by Jablonski *et al.* [19], was used for the histopathologic assessment of I/R-induced damage of the proximal tubules.

DNA fragmentation analysis

Genomic DNA was extracted from renal cortices ($n = 4$ for each group) using Easy DNA extraction kit (Fermentas Life Sciences, Hanover, MA, USA). Ten micrograms of DNA were electrophoresed on a 2% agarose gel. Frag-

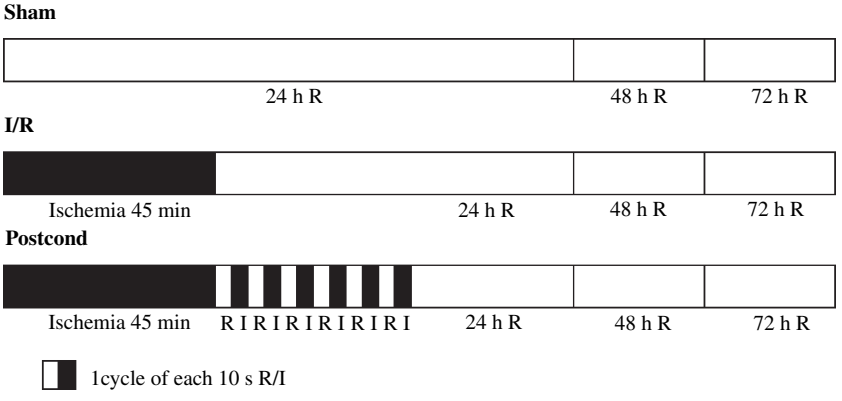

Figure 1 Experimental protocol used to determine the effect of ischemic postconditioning after ischemia (I) and reperfusion (R). sham, sham-operated rats; I/R, rats were subjected to left renal ischemia followed by reperfusion; Postcond, rats treated with ischemic postconditioning after ischemia (beginning of reperfusion).

mented DNA was visualized by ethidium bromide under an UV light source.

Caspase-3 activity assay

Activities of caspase-3 were measured using commercialized caspase-3 activity kit (Beyotime Institute of Biotechnology, Shanghai, China). In brief, renal cortices ($n = 4$ for each group) were homogenized in lysis buffer. The lysate was centrifuged at 20 000 *g* for 10 min at 4 °C, and supernatants were incubated for 1 h at 37 °C with 10 μl caspase-3 substrate (Ac-DEVDpNA) (2 mM). Substrate cleavage was measured with a spectrofluorometer at 405 nm.

Western blot analysis

The cytosolic/mitochondrial protein samples for Western blot analysis were prepared as described with some modifications [20]. Whole-cell lysates were obtained by homogenizing the renal sample ($n = 6$ for each group) 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 4 °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. In addition, the phospho-protein samples for Western blot analysis were extracted from renal cortices based on previous methods [21]. 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, USA). 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: Bcl-2, cytochrome c, and Bax (1:200; Santa Cruz Inc., Santa Cruz, CA, USA), caspase-3 and poly (ADP-ribose)-polymerase (PARP) (1:1000; Santa Cruz Inc.), β-actin and COX IV (1:5000; Abcam Inc.), total AKT (T-Akt), phospho-AKT (P-Akt), total ERK1/2 (T-ERK1/2) and phospho-ERK1/2 (P-ERK1/2) (1:1000; Cell Signaling Inc., Boston, MA, USA). After extensive rinsing with TBST buffer, the membranes were incubated with secondary antibodies (1:2000; Santa Cruz Inc.) for 1 h at room temperature and then developed with the use of an enhanced chemiluminescence system (ECL kit; Pierce Biotechnology Inc., Rockford, IL, USA).

Statistical analyses

All data are expressed as mean values ± SEM. The Kolmogorov–Smirnov test was applied to test for a normal distribution. The mean values of the different groups were compared using one-way ANOVA Student–Newman–Keuls test. All statistical analyses were performed with the SPSS statistical package (SPSS 13.0 for Windows; SPSS, Inc., Chicago, IL, USA). Significant differences were accepted when *P*-values were <0.05.

Results

Ischemic postconditioning improves renal dysfunction

The renal functional parameters of rats were significantly different at 24 and 48 h after I/R injury. Rats subjected to I/R injury showed significant increases in BUN and Cr compared with sham-operated rats. The renal function changes induced by I/R were significantly improved by ischemic postconditioning treatment. (Fig. 2a and b).

Ischemic postconditioning 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, ischemic postconditioning relieved these severe renal damages (Fig. 3). According to Jablonski scores, 45-min renal ischemia followed by 24 h reperfusion resulted in severe acute tubular necrosis. Quantitative analysis showed a dramatically decreased score in ischemic postconditioning group compared with I/R group (Fig. 2c).

Ischemic postconditioning attenuates cell apoptosis after renal I/R

Apoptosis was evaluated by DNA fragmentation analysis and caspase-3 proteolytic activity. The typical DNA laddering pattern and increased caspase-3 activity were observed in I/R group. Ischemic postconditioning treatment decreased I/R-induced DNA fragmentation and caspase-3 activity (Fig. 2d and e).

The mitochondrial and cytosolic expressions of cytochrome c were significantly decreased and increased, respectively, in I/R group compared with the sham group. The cytochrome c release was significantly attenuated by ischemic postconditioning treatment (Fig. 4). Renal I/R injury, also, decreased cytosolic Bax level and enhanced mitochondrial Bax content compared with the sham group. Whereas ischemic postconditioning treatment

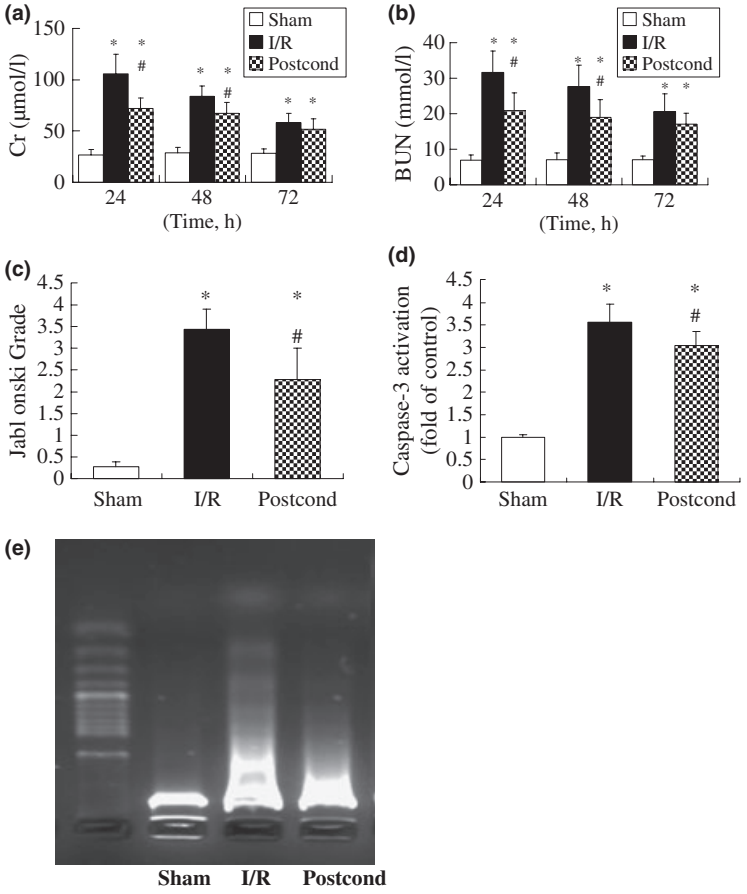

Figure 2 Ischemic postconditioning attenuated renal I/R injury. (a) Effect of ischemic postconditioning on the serum Cr concentrations after 45-min ischemia. (b) Effect of ischemic postconditioning on the serum BUN concentrations after 45-min ischemia. The renal functional parameters of rats were significantly different at 24 h and 48 h after I/R injury. Compared with sham group, I/R group showed significant increases in BUN and Cr. However, ischemic postconditioning treatment significantly decreased BUN and Cr levels at 24 and 48 h after I/R. Bars represent mean values ± SE (n = 8); *P < 0.05 versus sham, #P < 0.05 versus I/R. (c) Jablonski scores for histologic appearance of acute tubular necrosis from sham, I/R, ischemic postconditioning groups. (d) Caspase-3 activity. Ischemic postconditioning treatment reduced caspase-3 activity. Bars represent mean values ± SE (n = 4); *P < 0.05 versus sham, #P < 0.05 versus I/R. (e) DNA fragmentation analysis revealed typical laddering of fragmentated DNA in I/R group. Ischemic postconditioning treatment decreased the laddering pattern.

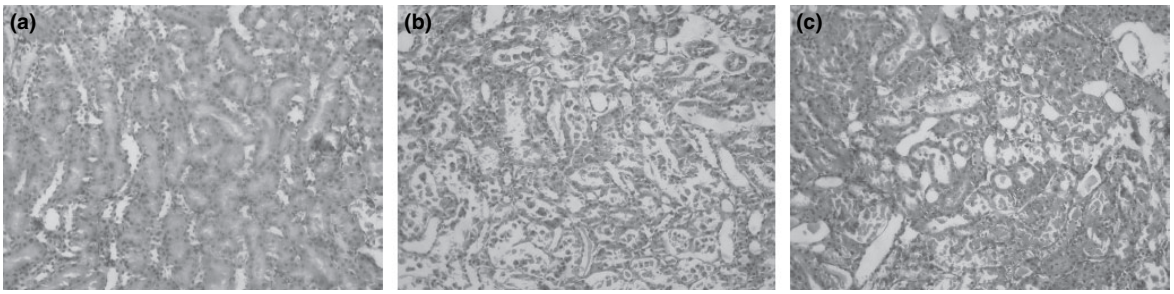

Figure 3 Histologic evaluations of renal tissues. (a–c) Representative kidney sections obtained 24 h after sham surgery or I/R. (a) Section from sham-operated rat. (b) Section from rat subjected to I/R. (c) Section from rat subjected to I/R and pretreated with ischemic postconditioning. All hematoxylin and eosin ×200.

reduced mitochondrial Bax levels and restored cytosolic Bax levels (Fig. 4). In addition, the procaspases-3 and Bcl-2 levels were significantly reduced in I/R group compared with sham group. However, ischemic postconditioning treatment restored the levels of Bcl-2 and inhibited the decrease of procaspases-3 (Fig. 5).

Poly (ADP-ribase)-polymerase has been known as a substrate of activated caspase. In our experiment, a significant amount of active 85 kDa-sized PARP was detected in I/R group, whereas the sham group showed 116 kDa-sized intact PARP. Ischemic postconditioning treatment significantly attenuated the amount of active 85 kDa-sized PARP (Fig. 5).

Effect of ischemic postconditioning on Akt and ERK1/2 activation

To determine Akt and ERK1/2 activity, we measured the phosphorylation of Akt and ERK1/2 by Western blot analysis. The 45-min ischemia followed by 5-min reperfusion

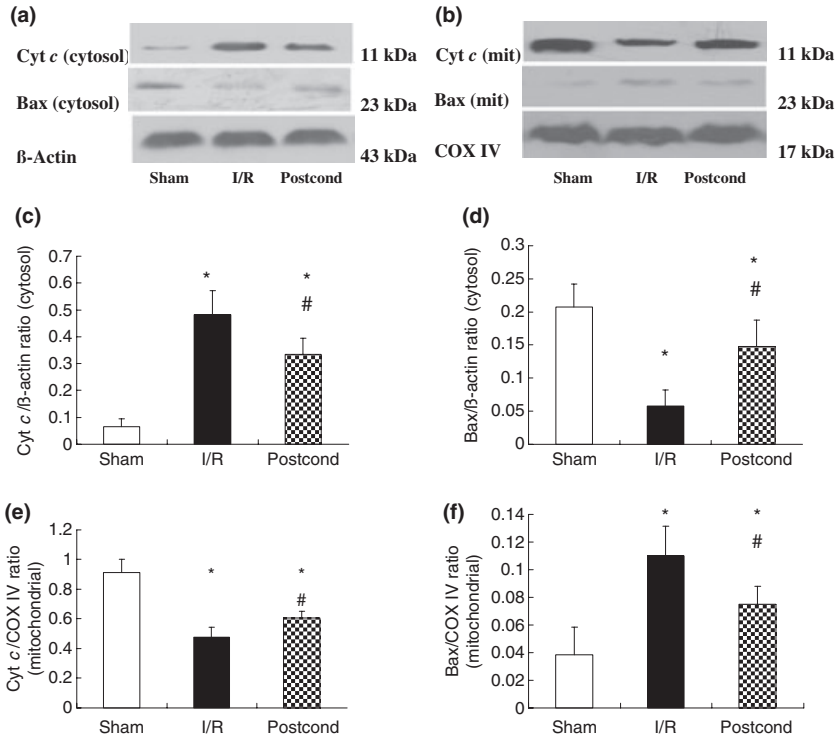

Figure 4 Cytochrome c and Bax expressions at 24 h after reperfusion. Ischemic postconditioning treatment significantly reduced the accumulation of cytochrome c in the cytoplasm and the translocation of Bax to the mitochondria after injury. (a) Representative blots showing the effect of ischemic postconditioning treatment on cytochrome c and Bax expressions in the cytoplasm. (b) Representative blots showing the effect of ischemic postconditioning treatment on cytochrome c and Bax expressions in the mitochondria. (c) The relative band densities of cytosolic cytochrome c to the mean value of the control. (d) The relative band densities of cytosolic Bax to the mean value of the control. (e) The relative band densities of mitochondrial cytochrome c to the mean value of the control. (f) The relative band densities of mitochondrial Bax to the mean value of the control. Bars represent mean values ± SE (n = 6); *P < 0.05 versus sham, #P < 0.05 versus I/R.

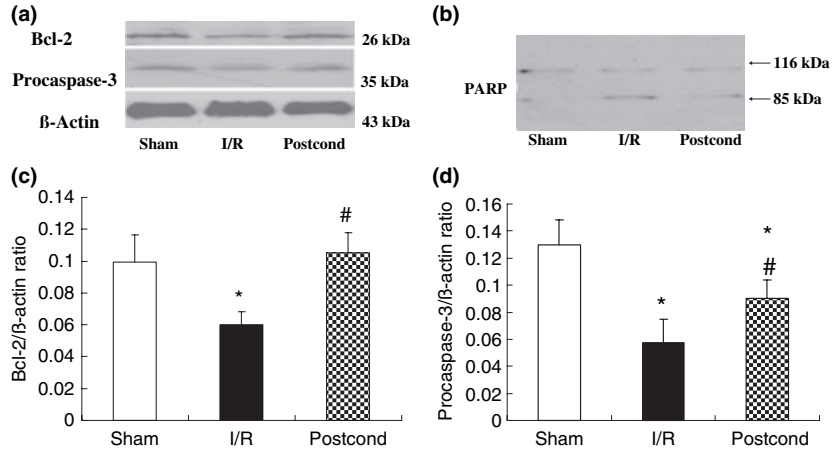

Figure 5 Procaspases-3, Bcl-2 and PARP expressions at 24 h after reperfusion. (a) Representative blots showing the expression of procaspases-3 and Bcl-2. I/R injury reduced procaspases-3 and Bcl-2 levels compared with the sham group. However, ischemic postconditioning treatment restored the levels of Bcl-2 and inhibited the decrease of procaspases-3. (b) Representative blots showing the levels of intact (116 kDa) and cleaved (85 kDa) PARP in kidney of sham, I/R and ischemic postconditioning groups. (c) The relative band densities of Bcl-2 to the mean value of the control. (d) The relative band densities of procaspases-3 to the mean value of the control. Bars represent mean values ± SE (n = 6); *P < 0.05 versus sham, #P < 0.05 versus I/R.

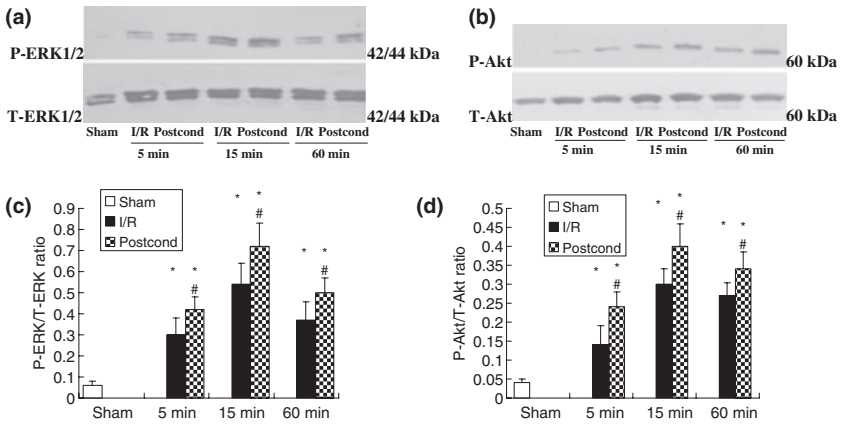

Figure 6 The phosphorylation of Akt and ERK1/2 at 5, 15 and 60 min after reperfusion. Ischemic postconditioning treatment increased postischemic phosphorylation of Akt and ERK1/2 at 5, 15 and 60 min. (a) Representative blots showing the phosphorylation of ERK1/2. (b) Representative blots showing the phosphorylation of Akt. (c) The relative band densities of P-ERK1/2 to the T-ERK1/2. (d) The relative band densities of P-Akt to the T-Akt. Bars represent mean values ± SE (n = 6); *P < 0.05 versus sham, #P < 0.05 versus I/R.

markedly activated Akt and ERK1/2. Akt and ERK1/2 activity peaked at 15 min and remained at a relatively high level until 60 min. Ischemic postconditioning increased postischemic phosphorylation of Akt and ERK1/2 at 5, 15, and 60 min (Fig. 6).

Discussion

Ischemic preconditioning is the phenomenon in which a prior ischemic stress renders the organ resistant to a subsequent ischemic insult [22]. Although extensive researches have demonstrated that ischemic preconditioning reduced renal I/R damage, ischemic preconditioning is clinically feasible only when the occurrence of ischemia is predictable [23]. Compared to ischemia, the onset of reperfusion is more predictable. Ischemic postconditioning is a simple and harmless method which provides a new tool to protect organ from I/R injury, for example, heart [7–17], brain [24], and liver [25]. Results from these studies suggested that the early moments of reperfusion were important in the pathogenesis of postischemic injury, and that manipulation of this early reperfusion phase reduced I/R injury. This study supported and extended our previous findings, in that ischemic postconditioning attenuated renal damage after I/R [18]. Moreover, we demonstrated for the first time that ischemic postconditioning inhibited apoptosis and upregulated activity of Akt and ERK1/2 after renal I/R injury.

Renal apoptosis is an important factor in the development of ARF after I/R injury [2,3]. In response to oxidative load in the mitochondria, the outer membrane of mitochondria becomes permeabilized, resulting in the translocation of Bax from cytosol to the mitochondria and the release of cytochrome c normally confined to the mitochondrial intermembrane space. Those pro-apoptotic proteins translocation is controlled by the Bcl-2 family proteins [26–29]. Release of cytochrome c into the cytosol leads to the formation of the apoptosome, a complex comprised of apoptotic protease-activating factor-1 (Apaf-1), procaspase-9 and ATP. The apoptosome permits the autoactivation of procaspase-9, which is followed by the activation of procaspase-3 [26,30,31]. Active caspase-3 activates the caspase activated DNase, leading to DNA fragmentation. Recent studies demonstrated that ischemic postconditioning exerted an anti-apoptotic effect on heart both *in vivo* and *in vitro* [12,14]. Consistent with these reports, our results showed that ischemic postconditioning significantly inhibited apoptosis caused by renal I/R injury, which was proved by DNA fragmentation and activated caspase-3. In order to further clarify the mechanism of ischemic postconditioning protection, we investigated the expressions of key apoptotic-related molecules. Ischemic postconditioning increased the levels of anti-apoptotic Bcl-2 protein and inhibited Bax translocation to the mitochondria and cytochrome c release from the mitochondria, thus attenuating the downstream caspase and PARP activation. Our data suggested that the mitochondrial pathway was an important target for ischemic postconditioning.

Through our study, ischemic postconditioning had an anti-apoptotic effect *in vivo*, but it was still unclear how ischemic postconditioning inhibited apoptosis after renal I/R injury. The opening of the mitochondrial permeability transition pore (mPTP) is associated with apoptosis by releasing cytochrome c and other pro-apoptotic factors in response to excessive production of oxidants [32–34]. Several studies have demonstrated that the activation of pro-survival phosphatidylinositol 3-kinase

(PI3K)–Akt and mitogen-activated protein kinase p42/p44 extra-cellular signal-regulated kinases 1 and 2 (ERK1/2) protected kidney against I/R injury [35–39]. These kinases may inhibit mPTP opening by (i) preventing the translocation of the mPTP-inducing pro-apoptotic protein Bax to the mitochondria [40] or (ii) activating endothelial nitric oxide synthase, which may inhibit mPTP opening via nitric oxide [41]. Recent studies indicated that the activation of Akt and/or ERK1/2 played an important role in the cardiac protection of ischemic postconditioning [14–17].Our study produced similar results and indicated that ischemic postconditioning upregulated activity of Akt and ERK1/2 after renal I/R injury. Therefore, we speculated that, at reperfusion, the upregulation of Akt and ERK1/2 induced by ischemic postconditioning might protect the kidney by inhibiting the opening of mPTP.

In our research, we only tested six cycles of 10 s of reperfusion followed by 10 s ischemia. The interval (10 s) referred to previous literature [8,9,14,18]. Whether the ischemic postconditioning plays its role in an 'on–off' style or a 'dose-dependent' one were not fully elucidated in this study. Ten seconds may not afford maximal protective effect against renal I/R injury if ischemic postconditioning was a 'dose-dependent' one. Thus the exact number of optimal interval and cycles may need to be investigated in further study. On the other hand, we observed an inherent interconnection between the effects of ischemic postconditioning treatment on tissue salvage and the protein signals *in vivo* study. To confirm these results *in vitro* studies are needed.

In conclusion, our study demonstrated for the first time that ischemic postconditioning possessed anti-apoptotic properties after renal I/R injury. Ischemic postconditioning seemed to trigger the upregulation of Akt and ERK1/2 principally known to attenuate the pathogenesis of apoptosis. Therefore, the intervention of ischemic postconditioning is very simple and useful, which targets the first few minutes of reperfusion. Someday it will be clinically applicable in the treatment of renal transplantation.

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Authorship

HC: designed and performed research; wrote the paper. XL: designed research. BX: performed research; analyzed data. BZ, JZ, HZ and ZC: collected data.

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