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Attenuation of reperfusion injury by renal ischemic postconditioning: The role of NO

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Abstract

Ischemic postconditioning (Postcond) is defined as rapid intermittent interruptions of blood flow in the early phase of reperfusion and mechanically alters the hydrodynamics of reperfusion. Although Postcond has been demonstrated to attenuate ischemia/reperfusion (I/R) injury in the heart and brain, its roles to renal I/R injury remain to be defined. In the present study, we examined the role of Postcond in I/R injury in a right-nephrectomized rat model. Postcond prevents the renal dysfunction and cell apoptosis induced by I/R and increases nitric oxide (NO) release and renal NO synthase (endothelial, eNOS and inducible, iNOS) expression. In contrast, enhancement of endothelin-1 (ET-1) in the kidney after the reperfusion was markedly suppressed by Postcond. These findings indicate that Postcond can inhibit renal I/R injury. The protective effect of Postcond is closely related to the NO production following the increase in eNOS and iNOS expression and the suppressive effect of ET-1 overproduction.

Keywords: Ischemic postconditioning; Kidney; Ischemia; NO; eNOS; iNOS; ET-1

Ischemic will result in the injury of tissue when the blood is interrupted, but more severe tissue injury comes when blood flow is restored. Ischemia/reperfusion injury often occurs in clinical practice and is associated with high morbidity and mortality. It is important to improve the ability of organs to tolerate ischemic injury.

There are different ways to deal with I/R injury, for example, ischemic preconditioning. Ischemic preconditioning (IP) is the phenomenon that a prior ischemic stress renders the organ resistant to a subsequent ischemic insult [1,2]. It is now well demonstrated that IP shows the tolerance of kidney to a subsequent I/R injury [3–8]. Although IP has successful attenuated I/R injury, its utilization as clinical strategy is largely limited because we cannot predict the onset of ischemia. However, the onset of reperfusion is more predictable. Recent development in cardiac physiology has indicated that Postcond is an interesting mechanisms to against reperfusion injury.

Postconditioning is defined as rapid intermittent interruptions of blood flow in the early phase of reperfusion and mechanically alters the hydrodynamics of reperfusion [9]. It seems to be preconditioning treatment, the mechanical interventions with multiple and interacting components marshaled against reperfusion injury by endogenous protective mechanisms. Zhao et al. [10] documented and defined the protective effect of 'ischemic postconditioning' in a canine model and the infarct reduction was comparable to the group treated with ischemic preconditioning, which is considered as 'gold standard' of cardio protection [11]. Postconditioning has been studied in heart [12–16], brain [17], and liver [18]. Several studies have indicated that the activation of the pro-survival PI3K-Akt pathway plays an important role in the protection [12,19].

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In I/R injury, NO may have a dual role. On the one hand, it reacts with superoxide anions and turns into the cytotoxic oxidant peroxynitrite. On the other hand, it attenuates neutrophil events, reduces infarct size and alleviates coronary vascular endothelial injury [20,21]. In addition, Yang et al. [22] have indicated that NO is involved in the cardio protection of postconditioning.

Currently, it is unclear whether the ischemic postconditioning can protect kidney against ischemic/reperfusion injury in vivo. The major purposes of this study were: (1) to determine whether ischemic–reperfusion injury can be overcome by Postcond, (2) to determine whether Postcond can activate the expression of iNOS and eNOS, and (3) to elucidate the role of endogenous NO in ischemic postconditioning-induced renal protection.

Materials and methods

Animal preparation and experimental design. Adult male Wistar rats (250–280 g) were used in studies. Rats were maintained in an air-filtered and temperature-conditioned (20–22 °C) and light-controlled (12 h light/dark cycle) room with a relative humidity of 50–52%. Rats were fed with standard commercial pellets and water ad libitum. Briefly, adult male Wistar rats were anesthetized with pentobarbital intraperitoneally (45 mg/kg) and allowed to breathe room air spontaneously. After 500 U of heparin (intraperitoneally), a 10 min stabilization period and maintaining the body temperature at 37 °C, a midline laparotomy was performed. A right nephrectomy was performed, and the left renal artery and vein were isolated; 30 min with no further surgical intervention was allowed for circulatory re-adjustment after right nephrectomy.

These rats were separated into 3 groups: (1) sham-operated control group ($n = 8$), (2) ischemic–reperfusion (I/R) group ($n = 10$): 45 min ischemia followed by 24 h reperfusion, and (3) ischemic Postcondtioning (Postcond) group ($n = 10$): 6 cycles of 10 s of reperfusion followed by 10 s global ischemia immediately after I/R; the left renal artery and vein were occluded with a non-traumatic clamp. At the end of the ischemic period, the clamp was released and blood reperfused. In sham-operated control rats, the kidney was treated identically, except for clamping.

To evaluate the effect of pharmacological blockade of NOS activities on Postcond-mediated renal protection, *N*-nitro-L-arginine methyl ester (L-NAME 10 mg/kg, i.v.), a non-selective NOS inhibitor, was pretreated 5 min before the onset of reperfusion. Blood samples (1 mL) were taken from the inferior vena cava for the measurement of urea nitrogen (BUN), creatinine (Cr) and NO.

The left kidney was removed under fully maintained anaesthesia and the animals were allowed to exsanguinate. 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 analyses, adopting standard techniques using an Olympus AU 2700 Analyzer (Olympus Optical Co. Ltd., Tokyo, Japan).

Measurement of NO content in serum. Serum concentration of nitrite was assayed using a NO detection kit (Nanjing Jiancheng Bioengineering Institute). To determinate the contents of NO, the blood samples were centrifuged at 3000 rpm for 10 min at 4 °C. The supernatant was stored at −20 °C. We analysis results according to the manufacturer's guide.

Histological examination. The tissues were 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 HE staining, immunohistochemistry, and TUNEL technique, respectively. Morphological assessment was performed by an experienced renal pathologist who was unaware of the treatment. A grading scale of 0–4, as outlined by Jablonski [23], was used for the histopathological assessment of ischemia and reperfusion-induced damage of the proximal tubules.

Apoptosis detection by TUNEL method. TUNEL assay was performed to detect apoptosis in situ cell death according to the manufacturer's instructions (TUNEL kit Beijing Zhongshan Biotechnology Co., Ltd.). The results of staining were analyzed and evaluated with American Image-Pro Plus software. The percentage of positive cells with TUNEL staining in five 400× sights served as apoptosis index (AI). Negative control was investigated by omitting terminal deoxynucleoferase in the label solution.

Immunohistochemical. Sections were performed according to the reagents of immunohistochemical assay (Gene Tech). Finally, eNOS and iNOS protein expression in the groups were analyzed and evaluated with the intensity of staining (negative, mild or strong) in five 400× sights on microscopic examination.

RNA isolation and RT-PCR. Total RNA (2 μg) was isolated by TRIzol reagent (Invitrogen) and reverse transcription was performed with the Revert Aid TM H Minus M-uLV Reverse Transcriptase kit (Fermentas Life Sciences) according to the manufacturer's instructions. PCR was performed with primers for ET-1 (F: TACTTCCCACAAAGACCACA; R: CGGACAGATGTTCTTGCTAA; 425 bp; GenBank Accession No. NM012548) and β-actin (F: TCATGAAGTGTGACGTTGACATCCGT; R: CCTAGAAGCATTTGCGGTGCACGATG; 285 bp; GenBank Accession No. NM031144). β-Actin was used as an internal control for stable expression (housekeeping gene) in all experiments. PCR was performed by use of a Gene Cycler (Bio-Rad). Initial denaturation was done at 94 °C for 5 min followed by 35 cycles of amplification. Amplification protocol was repeated cycles of denaturation (30 s, 94 °C), annealing (30 s; 56 °C), extension (1 min, 72 °C) and final extension (7 min, 72 °C). PCR products were electrophoresed through 2% agarose gels containing ethidium bromide (0.5 μg/ml). Gels were visualized under UV light, photographed and optical densities of the bands were analyzed using the Quantity One software (Bio-Rad).

Western blot analysis. Proteins were extracted from kidney as previously described [24]. Briefly, protein samples were separated on 12.5% SDS–PAGE gels (40 μg/lane) and then transferred to a nitrocellulose membrane (Bio-Rad). The membrane was blocked with 5% non-fat dry milk in TBST buffer (10 mmol/L Tris–HCl, 0.15 mol/L NaCl, and 0.05% Tween 20, pH 7.2) and then incubated with the rabbit polyclonal anti-eNOS or anti-iNOS antibody (Santa Cruz, 1:500) or mouse monoclonal anti-β-actin antibody (Santa Cruz; 1:500) for overnight at 4 °C. After extensive rinsing with TBST buffer, the blots were incubated with HRP-conjugated anti-rabbit secondary antibodies (Santa Cruz) and developed with the use of an enhanced chemiluminescence system (ECL kit, Pierce Biotechnology Inc.) and captured on light-sensitive imaging film (Kodak).

Materials. All drugs and chemicals were obtained from Sigma Chemical Co. (St. Louis, Missouri). The L-NAME was dissolved in 0.9% saline.

Statistical analyses. All data are expressed as means ± SEM. The means of the different groups were compared using one-way ANOVA Student–Newman–Keuls test. Immunohistochemistry analysis of eNOS and iNOS expression among the groups were compared using Fisher's exact test. The level of significance for all comparisons was set at $P < 0.05$.

Results

Postcond protects the kidney from ischemia/reperfusion injury

The renal functional parameters of rats subjected to I/R were significantly different among the groups. Compared with sham-operated control rats, I/R rats showed significant increases in BUN and Cr, but renal function changes induced by I/R were significantly attenuated in Postcond rats (Fig. 1A and B).

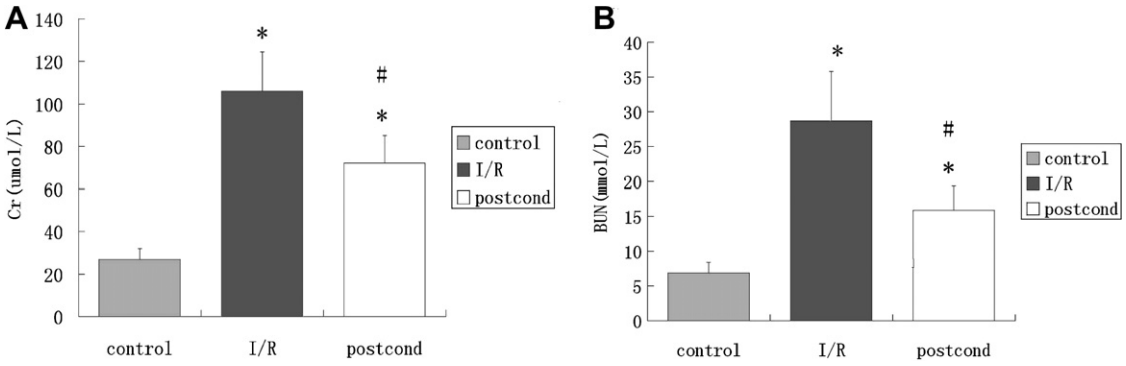

Fig. 1. Effect of ischemic postconditioning modification on the renal functional. As compared with sham-operated control rats, I/R rats showed significant increases in BUN and Cr, but renal function changes induced by I/R were significantly attenuated in Postcond rats. (A) The effects of ischemic postconditioning on the serum Cr concentrations after 45 min of ischemia followed by 24 h of reperfusion. (B) The effects of ischemic postconditioning on the serum BUN concentrations after 45 min of ischemia followed by 24 h of reperfusion. Bars represent means ± SE ($n \geqslant 8$); $^{*}p < 0.05$ versus control, $^{\#}p < 0.05$ versus I/R.

Postcond increases NO concentrations in serum

Compared with sham-operated control rats, the contents of NO were significantly elevated in I/R and Postcond rats. Furthermore, this increase was prominent in Postcond group (Fig. 2A).

Postcond improves renal morphology

Histopathological examination revealed that I/R induced severe lesions in the kidney of rats and Postcond improved renal morphology. The 45 min ischemia followed by 24 h reperfusion resulted in significant renal injury as evidenced by tubular necrosis, medullary hemorrhage, congestion, and development of proteinaceous casts. These severe renal damage could be relieved by Postcond (data not shown). According to Jablonski scale histology grading scores, an experienced renal pathologist who was unaware of the treatment performed morphological assessment (data not shown).

Influence of Postcond on apoptosis

TUNEL staining assay showed that positive apoptotic cells appeared with brown stain (data not shown). TUNEL assay showed an increase in the number of apoptotic cells after I/R and a decrease from ischemic postconditioning group (Fig. 2B).

Postcond increases iNOS and eNOS expression in the kidney

To determine whether Postcond induces iNOS and eNOS expression, we measured the level of iNOS and eNOS by Western blot analysis. The 45 min ischemia followed by 24 h reperfusion increases iNOS and eNOS protein expression, moreover this increase was much more prominent in Postcond group (Fig. 3).

We localized iNOS and eNOS expression by immunohistochemical techniques. iNOS is intensively expressed in the glomerulus and proximal tubules and eNOS is

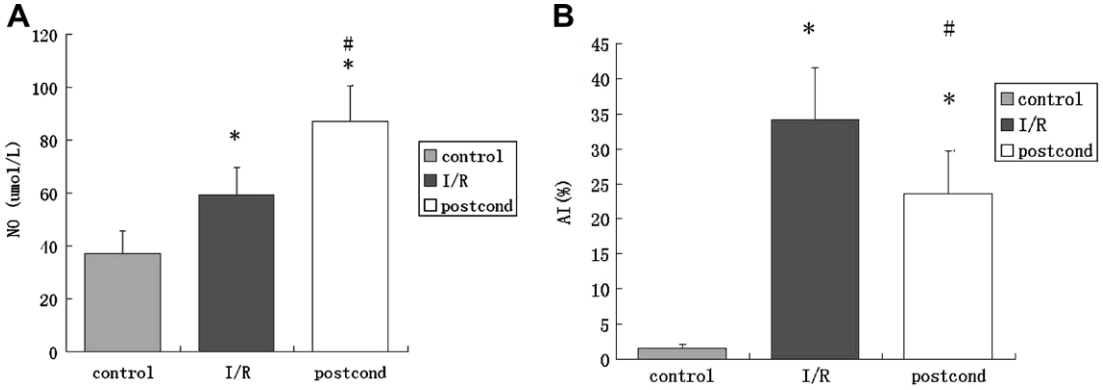

Fig. 2. (A) The effects of ischemic postconditioning on the serum NO concentrations after 45 min of ischemia followed by 24 h of reperfusion. The contents of NO were elevated in I/R and Postcond rats, moreover this increase was prominent in Postcond group. (B) The effects of ischemic postconditioning on the apoptosis index (AI) after 45 min of ischemia followed by 24 h of reperfusion. TUNEL assay showed a decrease in the number of apoptotic cells from ischemic postconditioning group. Bars represent means ± SE ($n \geqslant 8$); $^{*}p < 0.05$ versus control, $^{\#}p < 0.05$ versus I/R.

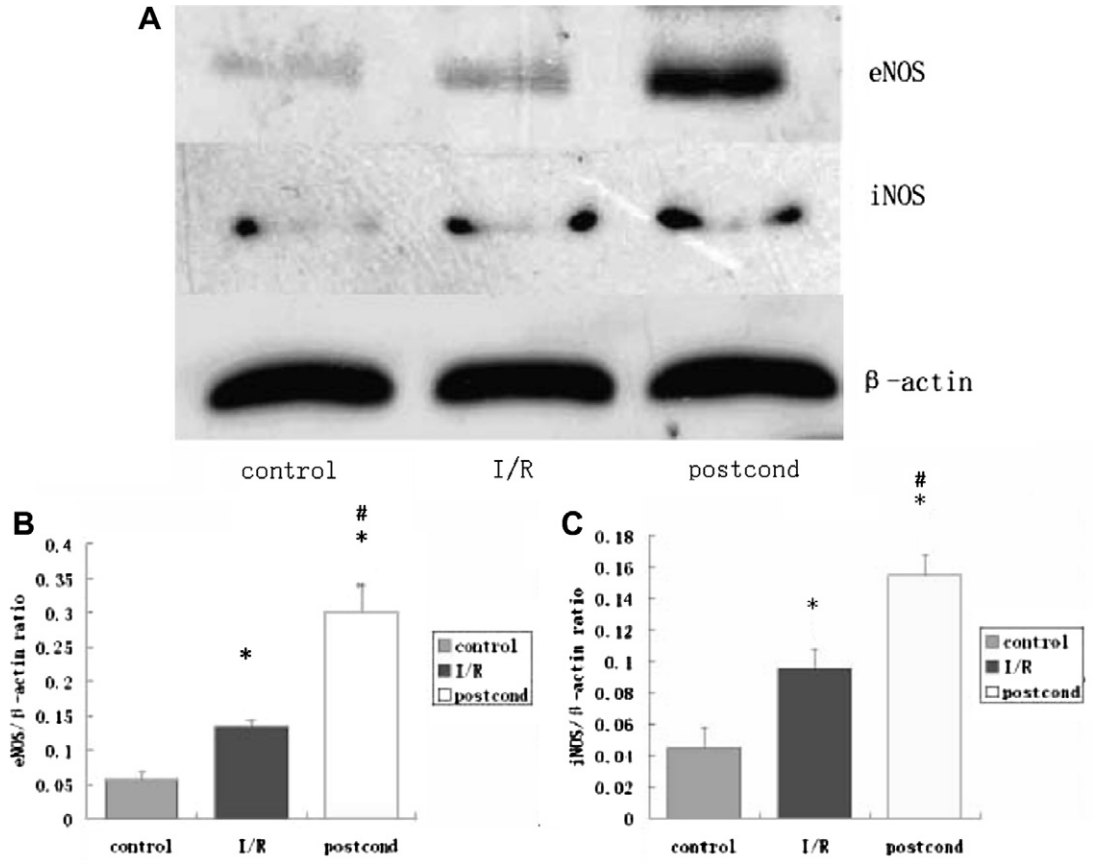

Fig. 3. The effects of ischemic postconditioning on eNOS and iNOS expression in the kidney after 45 min of ischemia followed by 24 h of reperfusion. I/R increased iNOS and eNOS protein expression, but this increase was much more prominent in Postcond group. (A) Representative Western blots showing the effect of Postcond treatment on eNOS and iNOS expression. (B) The relative band densities of eNOS to the mean value of the control. (C) The relative band densities of iNOS to the mean value of the control. Bars represent means ± SE ($n = 6$); $^{*}p < 0.05$ versus control, $^{\#}p < 0.05$ versus I/R.

intensively expressed in the renal vascular endothelium. Consistent with the Western blot analyses, I/R and Postcond group increased the renal expression of eNOS compared to control kidney sections and eNOS was expressed more intensely in Postcond group. The iNOS immunohistochemical staining showed an increased expression in I/R group and Postcond group than in sham-operated control sections. However, the iNOS intensity was lower in I/R group than in Postcond group (data not shown).

mRNA expression of ET-1

To investigate the difference of mRNA expression of ET-1, we measured the level of ET-1 by RT-PCR. The PCR products were separated on agarose-gel and the relative expression of ET-1 to β-actin are shown. The mRNA level of ET-1 expression in I/R group is significantly greater than sham-operated control rats. However, the expression of ET-1 was significantly lower in Postcond group. Moreover, the suppressive effect of Postcond treatment on ET-1 mRNA level could be abolished by NOS inhibitor (Fig. 4).

L-NAME abolished the renal protection by Postcond

The pretreatment with L-NAME, a non-selective NOS inhibitor, almost completely abolished the renal protective effect of Postcond against I/R-induced renal dysfunction (data not shown).

Discussion

Although ischemic preconditioning is powerful against I/R injury intervention and has been introduced almost 20 years [1], it is impossible to ask patients to accept pretreatment. Ischemic is out of the clinician's control. However, ischemic postconditioning could be applied in setting of reperfusion.

Postcond has been originally described in vivo dog model [10] and shown to protect the ischemic heart in various experimental models. It is a simple and harmless method which provides a new tool to protect organ from I/R injury, for example, heart and liver. This Postcond-induced protection has been the topic of intense research interest in cardiac research for the last 4 years [12–16]. However, it is unclear whether the ischemic postcondition-

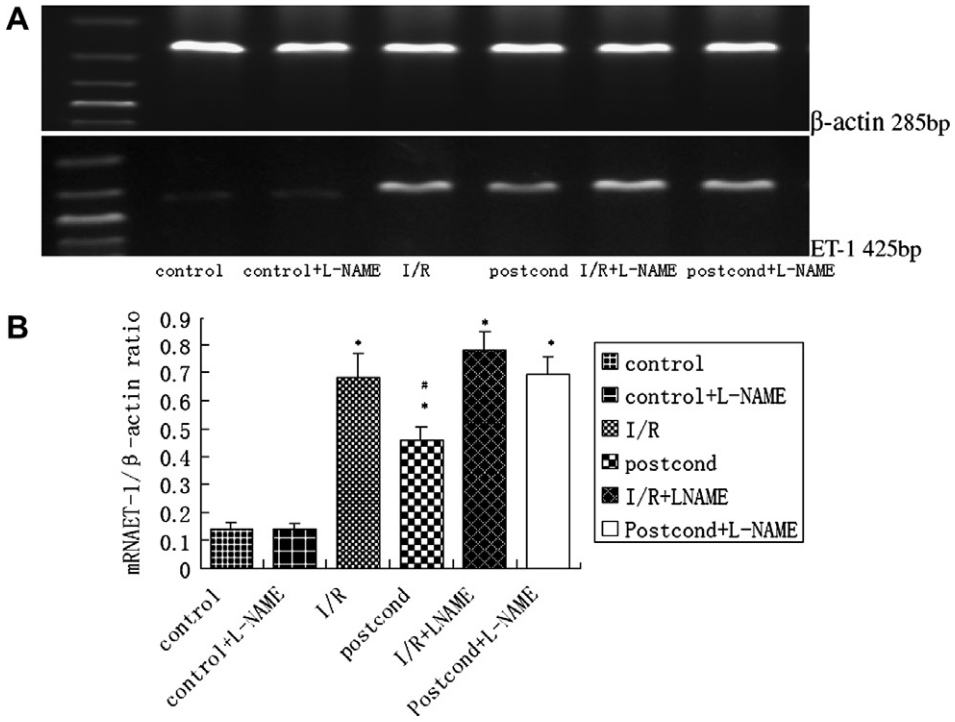

Fig. 4. The effects of ischemic postconditioning and L-NAME on ET-1 mRNA expression in the kidney after 45 min of ischemia followed by 24 h of reperfusion. The mRNA level of ET-1 expression in I/R group is significantly greater than control group. However, the expression of ET-1 was significantly lower in Postcond group. Moreover the suppressive effect of Postcond treatment on ET-1 mRNA level could be abolished by NOS inhibitor (A) Representative RT-PCR results showing mRNA expression of ET-1 and actin. (B) The relative band densities of ET-1to the mean value of the control. Bars represent means ± SE ($n = 6$) ;$^{*}p < 0.05$ versus control, $^{\#}p < 0.05$ versus I/R.

ing can protect kidney against I/R injury. In our study, we demonstrate for the first time that ischemic postconditioning also works in kidney via expressing iNOS and eNOS which increase NO.

Recent studies indicated that NO is involved in the cardio protection of postconditioning [22]. It has been reported that the protection of NO via the cGMP pathway and NO may be functioning in many levels (signaling, anti-inflammatory) [19]. However, it should be mentioned that the role of NO and NOS in I/R is ambiguous. On the one hand, I/R activate NOS [25] and increase the expression of NOS proteins in kidney [6,26,27]. NO can induce injury via lipid peroxidation, DNA damage, and pro-apoptotic effects, which are included in I/R injury [28]. On the other hand, many studies have demonstrated that the increased activity of NOS is associated with reduced I/R-induced injury and an increase of blood flow in the ischemic region [29]. Non-selective NOS inhibitors are known to worsen the post-ischemic renal function, whereas L-arginine (NO precursor) can reverse the NOS inhibitor-induced deterioration of renal function [30]. NO may have a protective effect in vasodilatation, inhibition of platelet plug formation, anti-apoptotic action and reduction of the inflammatory response. Thus, cellular effects of NO may depend on its concentration, site of release and duration of action and low levels of NO may be protective but higher levels may be detrimental [28]. However, anti-inflammatory mechanism of NO may not been the sole mechanism. Whether the involvement of NO in K-ATP activation and closure of the mitochondrial permeability transition pore (mPTP) will need further investigations.

There is a limited amount of information in the literature regarding renal ischemic postconditioning. Our study provides evidence to demonstrate the beneficial effects of renal ischemic postconditioning in vivo. Interestingly, previous studies by Zhao et al. [10] first reported that brief episodes of ischemia performed at the onset of reperfusion after a prolonged ischemia provided a powerful protection. The results from that study suggested that the early moments of reperfusion were important in the pathogenesis of post-ischemic injury, and that manipulation of this early reperfusion phase could reduce these downstream physiological consequences of ischemia–reperfusion injury. In the present study, we confirmed 45 min of renal ischemia followed by 24 h of reperfusion resulted in irreversible injury in cell apoptosis, morphology, and renal functional. We found that Postcond significantly reduced BUN, Cr and apoptosis and improved renal morphology. This renal protection offered by ischemic postconditioning is in agreement with the data reported in cardiac [12–16], brain [17] and liver [18].

Our results clearly indicated that the NO levels in serum and protein expression of eNOS and iNOS were markedly elevated in kidney exposed to I/R with Postcond at 24 h after the reperfusion. In this study, when L-NAME, a non-selective NOS inhibitor, was administered prior to

the Postcond treatment, renal-protecting effects by Postcond were abolished. Thus, the present data strongly suggest that eNOS and iNOS-mediated endogenous NO production is an important mechanism of this protection in Postcond treatment. Similarly, it has been reported that the protection of renal IP is mediated by the NO [6,31,32].

It has been confirmed that ET-1 is closely related to the I/R [32–34]. Moreover, there is growing evidence that NO releaser and endogenous NO can inhibit the production of ET-1 [35–37]. In addition, it has been reported that NO mediated by renal IP can inhibit overproduction of ET-1 induced by the I/R [32]. Taken together, it seems likely that the up-regulation of endogenous NO may attenuate the I/R injury by the inhibition of ET-1. In our study, there were significant increases in renal ET-1 mRNA expression in I/R rats which can be attenuated by the Postcond treatment. Moreover the suppressive effect of Postcond on ET-1 could be abolished by NOS inhibitor. Thus it may be suggested that Postcond can suppress the I/R-induced ET-1 overproduction, which is one of the mechanisms that endogenous NO is against the renal I/R damage. According to our data, the increase of eNOS and iNOS expression and NO production parallels with a decline of ET-1 mRNA expression in Postcond treatment. Thereby, an enhanced eNOS and iNOS expression seems to play an important role in the attenuation of ET-1 overproduction.

In conclusion, the current study demonstrates that Postcond can attenuate renal ischemic–reperfusion injury and NOS inhibition by pharmacological inhibitors can abolish the protective effects of Postcond in a rat model. In addition, our findings suggest that the protective effect of Postcond is closely related to the enhanced level of expression of iNOS and eNOS which result the increasing concentrations of endogenous NO after the reperfusion; the suppressive effect of ET-1 overproduction may be partly involved in the renal protective mechanisms of Postcond. Therefore, the intervention of Postcond is very simple and useful, which targets the first few minutes of reperfusion. Someday it will be clinically applicable at the time of renal transplantation.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.05.129.

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