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ScienceDirect
Biochemical and Biophysical Research Communications 359 (2007) 628–634
BBRC
www.elsevier.com/locate/ybbrc

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

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Received 16 May 2007
Available online 29 May 2007

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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doi:10.1016/j.bbrc.2007.05.129

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).