Endothelial Nitric Oxide Contributes to the Renal Protective Effects of Ischemic Preconditioning

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ABSTRACT
We determined whether endothelial nitric oxide synthase (eNOS) plays an important role in the renal protective effect of ischemic preconditioning (IP) against the ischemia/reperfusion-induced acute renal failure (ARF) by using eNOS-deficient (eNOS−/−) and wild-type (eNOS+/+) mice. Ischemic ARF was induced by occlusion of the left renal artery and vein for 45 min followed by reperfusion, 2 weeks after contralateral nephrectomy. IP, which consists of three cycles of 2-min ischemia followed by 5-min reperfusion, was performed prior to 45-min ischemia. In eNOS+/+ mice, IP treatment markedly attenuated the ischemia/reperfusion-induced renal dysfunction and significantly improved histological renal damage such as tubular necrosis, proteinaceous casts in tubuli, and medullary congestion. Constitutive nitric oxide synthase activity in the kidney without IP was markedly decreased 6 h after reperfusion, but this decreased response was not observed in eNOS−/− mice with IP treatment. The improvement of renal dysfunction in eNOS−/− mice with IP treatment was abolished by pretreatment with Nω-nitro-L-arginine, a nonselective NOS inhibitor, whereas aminoguanidine, an inducible NOS inhibitor, had no effect. Finally, no protective effects of IP on ischemia/reperfusion-induced renal dysfunction and histological damage were observed in eNOS−/− mice. These findings strongly support the view that eNOS-mediated NO production plays a pivotal role in the protective effect of IP on ischemia/reperfusion-induced ARF.

Prior exposure to brief periods of tissue ischemia leads to a state of increased tolerance to the effects of subsequent ischemia/reperfusion (I/R)-induced injury. This phenomenon was referred to as ischemic preconditioning (IP) by Murry et al. (1986), who first demonstrated this benefit in the dog heart. Thereafter, IP has been extended to several organs including the brain (Heurteaux et al., 1995), liver (Peralta et al., 1999), skeletal muscle (Schroeder et al., 1996), and kidney (Lee and Emala, 2000). Several studies on cardiac IP have demonstrated that myocardial protection by IP does not exceed 3 h (called early preconditioning or first window) but reappears 12 to 24 h after IP treatment and lasts about 72 h (known as delayed preconditioning or second window) (Kuzuya et al., 1993; Yellon and Baxter, 1995; Bolli, 2000). Although the precise mechanisms by which IP reduces the I/R-injury remain obscure, several factors have been reported to contribute to IP-mediated tissue protection. In cardiac IP, the translocation and activation of myocardial protein kinase C (PKC), which is caused by increased production of adenosine and A1 receptor activation, play an important role in myocardial protection (Downey et al., 1993). Activated PKC is known to regulate the cytosolic concentration of calcium and by the ATP channel, ATP-sensitive potassium channel, ATP-sensitive potassium channel. NO, nitric oxide; NOS, nitric oxide synthase; nNOS, neuronal NOS; eNOS, endothelial NOS; iNOS, inducible NOS; cNOS, constitutive NOS; ARF, acute renal failure; NO-ARG, Nω-nitro-L-arginine; BUN, blood urea nitrogen; Pcr, plasma creatinine concentration; Uosm, urinary osmolality; UF, urine flow.

ABBREVIATIONS: I/R, ischemia/reperfusion; IP, ischemic preconditioning; PKC, protein kinase C; K+ATP channel, ATP-sensitive potassium channel; NO, nitric oxide; NOS, nitric oxide synthase; nNOS, neuronal NOS; eNOS, endothelial NOS; iNOS, inducible NOS; cNOS, constitutive NOS; ARF, acute renal failure; NO-ARG, Nω-nitro-L-arginine; BUN, blood urea nitrogen; Pcr, plasma creatinine concentration; Uosm, urinary osmolality; UF, urine flow.
presence of cofactors such as FAD/FMD, NADPH, and tetrahydrobiopterin; therefore, eNOS and nNOS are known as constitutive NOS (cNOS) (Knowles and Moncada, 1994). On the other hand, iNOS is a calcium-independent synthase whose activity appears to rely on its protein expression induced by transcription factors such as nuclear factor-κB (Knowles and Moncada, 1994). In the heart, it has been reported that iNOS and/or eNOS are involved in the cardioprotective effect of IP, both in early and delayed preconditioning (Bolli et al., 1997; Takano et al., 1998; Bell and Yellon, 2001; Bell et al., 2002; Shinmura et al., 2002). Although there is some information on the possible involvement of NO in renal IP (Torras et al., 2002), it remains obscure which type of NOS isoform is involved in the renal protective effect of IP. One available piece of evidence has been reported by Ogawa et al. (2001), who obtained findings that the renal protective effect of IP against the I/R-induced injury was abolished by N\textsuperscript{G}-nitro-L-arginine, a nonselective NO inhibitor and was enhanced by L-arginine treatment. Furthermore, they found that IP alone does not lead to iNOS protein expression, whereas the I/R with or without IP treatment enhanced the protein expression, suggesting that NO production mediated by iNOS activity may contribute to the renal protective effect of IP. However, the above study was evaluated on the renal hemodynamic function in the acute phase of reperfusion injury (30–60 min after the reperfusion). Thus, the role of NO/NOS system in delayed preconditioning in the kidney remains to be elucidated.

Most recently, we have found that the protective effect of IP on I/R-induced acute renal failure (ARF), which is observed 24 h after reperfusion, is associated with renal NO production following the increase in eNOS protein expression after reperfusion (Yamashita et al., 2003). However, there is no direct evidence that eNOS is responsible for the renal protective effect of IP. To confirm this, we evaluated the effects of pharmacological blockade and the genetic deficiency of eNOS in IP-mediated renal protection from I/R-induced injury.

**Materials and Methods**

**Animals and Experimental Design.** C57bl/6J wild-type and eNOS−/− mice (20–25 g, 11–13 weeks old, The Jackson Laboratory, Bar Harbor, ME) were used. Animals were housed in a light-controlled room with a 12-h light/dark cycle and were allowed ad libitum access to food and water. Experimental protocols and animal care methods in the experiments were approved by the Experimental Animal Committee at Osaka University of Pharmaceutical Sciences. Two weeks before the study, the right kidney was removed through a small flank incision under pentobarbital anesthesia (50 mg/kg i.p.). After a 2-week recovery period, these mice were separated into three groups: sham-operated control, I/R group (untreated ARF); 45-min ischemia followed by 24-h reperfusion, and IP treatment group (IP + ARF): three cycles of 2-min ischemia followed by 5-min reperfusion prior to I/R. The mice were anesthetized with pentobarbital (50 mg/kg i.p.), and the left kidney was exposed through a small flank incision. The left renal artery and vein were occluded with a non-traumatic clamp. At the end of the ischemic period, the clamp was released to allow reperfusion. In sham-operated control mice, the kidney was treated identically, except for clamping. Animals were housed in metabolic cages at 24 h after reperfusion; 24-h urine samples were taken, and blood samples were drawn from the thoracic aorta at the end of the urine collection period. The plasma was separated by centrifugation. These samples were used for measurements of renal function parameters. The kidneys were excised and examined using a light microscope.

In separate experiments, left kidneys were obtained just after IP treatment and at 0, 6, and 24 h after reperfusion, and NOS activities were measured. To evaluate the effects of the pharmacological blockade of NOS activities on IP-mediated renal protection, N\textsuperscript{G}-nitro-L-arginine (NO-ARG, 10 mg/kg i.v.), a nonselective NOS inhibitor, or aminoguanidine (10 mg/kg i.v.), an iNOS inhibitor, was pretreated 5 min before starting IP as a slow bolus injection (1 ml/kg) into the external jugular vein, and renal functional parameters were determined as described. The doses of these drugs were determined based on previous studies (Toda et al., 1993; Ortiz et al., 1996).

**Histological Studies.** The excised kidneys were preserved in phosphate-buffered 10% formalin, embedded in paraffin wax, and cut into thin sections (4 μm) according to conventional techniques. The sections were stained with hematoxylin and eosin. Histopathological changes were analyzed for tubular necrosis, proteinaceous casts, and medullary congestion, as suggested by Slez et al. (1974). Tubular necrosis and proteinaceous casts were graded as follows: no damage (0), mild (+1, unicellular, patchy isolated damage), moderate (+2, damage less than 25%), severe (+3, damage between 25 and 50%), and very severe (+4, more than 50% damage). The degree of medullary congestion was defined by: no congestion (0), mild (+1, vascular congestion with identification of erythrocytes by 400× magnification), moderate (+2, vascular congestion with identification of erythrocytes by 200× magnification), severe (+3, vascular congestion with identification of erythrocytes by 100× magnification), and very severe (+4, vascular congestion with identification of erythrocytes by 40× magnification). Evaluations were made in a blind manner.

**Renal Functional Parameters.** Blood urea nitrogen (BUN) and creatinine levels in plasma (Pcr) were determined using a commercial assay kit, BUN-test-Wako and Creatinine-test-Wako (Wako Pure Chemicals, Osaka, Japan), respectively. Urinary osmolality (Uosm) was measured by freezing-point depression (Fiske Associates, Uxbridge, MA).

**Measurement of NOS Activity.** Ca\textsuperscript{2+}-dependent and -independent NOS activities (eNOS and iNOS activities, respectively) were determined by measuring the conversion of L-[3H]arginine to L-[3H]citrulline using a NOS detection kit (NOS detection kit BOX-2; Sigma-Aldrich, St. Louis, MO). Briefly, whole frozen kidneys were homogenized in ice-cold homogenization buffer containing protease inhibitors. Homogenates were centrifuged (30 min at 5000g) to remove tissue debris, and the supernatant was utilized for the measurement of NOS activities. The samples were incubated in assay buffer containing 0.323 μM L-arginine, 600 μM CaCl\textsubscript{2}, and 1 mM NADPH, in the presence of an excess amount of calmodulin, at 37°C for 30 min. After stopping the reaction, the samples were centrifuged (3 min at 3000 rpm), and radioactive activities of L-[3H]citrulline were measured using a liquid scintillation counter (TRI-CARB; Packard, Tokyo, Japan). To determine Ca\textsuperscript{2+}-independent NO (iNOS) activity, the assay was conducted in the presence of 10 mM EDTA without CaCl\textsubscript{2}. Ca\textsuperscript{2+}-dependent NOS (eNOS) activity was calculated by subtracting iNOS activity from total NOS activity.

**Drugs.** NO-ARG (Peptide Institute Inc., Osaka, Japan) and aminoguanidine (Tocris Cookbook Inc., Bristol, UK) were dissolved in 0.9% saline.

**Statistical Analysis.** Values are expressed as the mean ± S.D. Relevant data were processed by InStat (GraphPad Software for Science; GraphPad Software Inc., San Diego, CA). For statistical analysis, we used the unpaired Student’s t test for two-group comparison and one-way analysis of variance followed by Dunnett’s tests for multiple comparisons. Histological data were analyzed using the Mann-Whitney test. For all comparisons, differences were considered significant at P < 0.05.
Results

Effects of IP Treatment on I/R-Induced Renal Dysfunction. As shown in Fig. 1, the renal functional parameters of mice subjected to 45-min ischemia showed marked deterioration, as measured 24 to 48 h after reperfusion. Compared with sham-operated control mice, I/R (untreated ARF) mice exhibited significant increases in BUN, Pcr, and urine flow (UF) and significant decreases in Uosm. However, I/R-induced changes in renal functional parameters were markedly attenuated by IP treatment (IP/ARF).

Effects of IP Treatment on I/R-Induced Histological Renal Damage. Histological examination revealed severe lesions in the kidney of untreated ARF mice (48 h after the 45-min ischemia and reperfusion). These changes were characterized by tubular necrosis (outer zone outer stripe of medulla), proteinaceous casts in tubuli (inner zone of medulla), and medullary congestion and hemorrhage (outer zone inner stripe of medulla). IP treatment markedly improved the development of all these lesions (Table 1). Typical photographs are shown in Fig. 2.

Measurement of NOS Activity in the Kidney. Changes of NOS activity were evaluated in kidneys exposed to I/R with or without IP treatment. As shown in Fig. 3A, IP treatment alone and 45-min ischemia with or without IP treatment exhibited no changes in eNOS activity compared with sham-operated mice. eNOS activity was significantly reduced at 6 h after reperfusion in I/R mice without IP, but the reduced level recovered at 24 h after reperfusion. On the other hand, the I/R-induced reduction of cNOS activity at 6 h after reperfusion was not observed in the kidney with IP

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**Table 1**

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Proteinaceous casts in tubuli</th>
<th>Medullary congestion</th>
<th>Tubular necrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated ARF (n = 6)</td>
<td>3.00 ± 0.91</td>
<td>3.33 ± 0.51</td>
<td>3.33 ± 0.81</td>
</tr>
<tr>
<td>IP + ARF (n = 6)</td>
<td>2.17 ± 0.76*</td>
<td>2.33 ± 0.51</td>
<td>2.50 ± 0.83*</td>
</tr>
</tbody>
</table>

* P < 0.05, compared with untreated ARF mice.
treatment. As shown in Fig. 3B, iNOS activities were not detected in kidneys of the sham-operated control, IP treatment alone and 45-min ischemia with or without IP treatment. However, at 6 and 24 h after reperfusion, there were notable increases in iNOS activities, which were markedly suppressed by the IP treatment.

Effects of NOS Inhibitors on Renal Protection by IP Treatment. We next examined the effects of the pharmacological blockade of NOS activities on IP-mediated renal protection. As shown in Fig. 4, the pretreatment with NO-ARG, a nonselective NOS inhibitor, almost completely abolished the renal protective effects of IP against I/R-induced renal dysfunction. On the other hand, aminoguanidine, a preferential inhibitor of iNOS, had no effect on the IP-induced improvement of renal dysfunction.

I/R-Induced Renal Dysfunction and Effects of IP Treatment in eNOS<sup>−/−</sup> Mice. As shown in Fig. 5, there was marked impairment of renal function in eNOS<sup>−/−</sup> mice subjected to 45-min ischemia, as measured 24 to 48 h after reperfusion, showing a tendency to further deterioration compared with wild-type mice. In contrast to the case in wild-type animals (Fig. 1), IP treatment failed to improve I/R-induced renal dysfunction. In addition, some mice exposed to 45-min ischemia and reperfusion died between 24 to 48 h (two of eight mice in both groups, respectively).

I/R-Induced Histological Renal Damage and Effects of IP Treatment in eNOS<sup>−/−</sup> Mice. Histological examination revealed severe lesions in the kidney of untreated eNOS<sup>−/−</sup> mice (48 h after 45-min ischemia and reperfusion), as seen in wild-type animals. However, in contrast to the findings observed in wild-type animals, IP treatment of eNOS<sup>−/−</sup> mice did not attenuate I/R-induced histological damage (Fig. 6; Table 2).

Discussion

Our recent study using rats demonstrated that the protective effect of IP on I/R-induced renal dysfunction and tissue injury correlated with eNOS protein expression and NO production in the kidney (Yamashita et al., 2003). This study

![Fig. 3. Renal cNOS (A) and iNOS (B) activities in a sham-operated control, IP alone, and I/R mice with or without (untreated ARF) IP treatment. Each value represents the mean ± S.D. †, P < 0.01, compared with sham-operated mice. †, P < 0.01, compared with I/R mice without IP pretreatment (untreated ARF). N.D., not detected.](image)

![Fig. 4. Effects of NO-ARG or aminoguanidine on BUN (A), Pcr (B), UF (C), and Uosm (D) of I/R mice with IP treatment (IP + ARF). At 24 h after reperfusion, 24-h urine was collected. Each value represents the mean ± S.D. †, P < 0.05; ‡, P < 0.01, compared with IP + ARF.](image)
was performed to determine whether the eNOS/NO system is responsible for IP-mediated renal protection. We obtained evidence that an IP-mediated improvement on postischemic renal injury at 24 to 48 h after reperfusion was observed in wild-type mice, but not in eNOS \( -/- \) mice, in which there was somewhat augmented renal dysfunction in the postischemic kidney. Pretreatment with NO-ARG, a nonselective NOS inhibitor, abolished the protective effect of IP, whereas amnoguanidine, an iNOS inhibitor, failed to affect the IP-mediated renal protection. Thus, it seems likely that the eNOS/NO system plays a crucial role in the IP effect on I/R-induced renal injury.

Lieberthal et al. (1991) found that impairment of renal hemodynamics in rats with hypovolemic shock induced by hemorrhage was to some extent overcome by the inhibition of NO production. The NO synthase inhibitor was reported to prevent hypoxia/reoxygenation injury in rat proximal tubules, thereby suggesting that NO is synthesized in proximal tubules and is involved in tubular hypoxia/reoxygenation injury (Yu et al., 1994). In contrast, Chintala et al. (1993) noted that the inhibition of NO production with an NO synthase inhibitor significantly deteriorated the renal function of the postischemic kidney in anesthetized rats, whereas pretreatment with the NO precursor l-arginine abolished the NO synthase inhibitor-induced deterioration of renal function. Similar improvement by l-arginine against the decreased renal function in ischemic ARF was noted by Schramm et al. (1994), although they observed no detrimental effect of the NO synthase inhibitor. We found that I/R-induced renal dysfunction and tissue injury were markedly attenuated by preischemic treatment with FK-409, a spontaneous NO releaser (Matsumura et al., 1998). Thus, the pathophysiological roles of NO in ischemic ARF are controversial. Recent studies clearly demonstrated that renal I/R injury was efficiently attenuated by genetic deficiency or the pharmacological blockade of iNOS (Ling et al., 1999; Walker et al., 2000; Chatterjee et al., 2002), hypothesizing that iNOS-generated NO mediates I/R-induced renal damage. One possibility is that a toxic peroxynitrite, a reactive substance of NO with superoxide anion, may cause renal damage via direct oxidant injury and protein tyrosine nitration (Walker et al., 2000; Chatterjee et al., 2002), hypothesizing that iNOS-generated NO mediates I/R-induced renal damage. One possibility is that a toxic peroxynitrite, a reactive substance of NO with superoxide anion, may cause renal damage via direct oxidant injury and protein tyrosine nitration (Walker et al., 2000; Chatterjee et al., 2002), hypothesizing that iNOS-generated NO mediates I/R-induced renal damage. One possibility is that a toxic peroxynitrite, a reactive substance of NO with superoxide anion, may cause renal damage via direct oxidant injury and protein tyrosine nitration (Walker et al., 2000; Chatterjee et al., 2002), hypothesizing that iNOS-generated NO mediates I/R-induced renal damage. One possibility is that a toxic peroxynitrite, a reactive substance of NO with superoxide anion, may cause renal damage via direct oxidant injury and protein tyrosine nitration (Walker et al., 2000; Chatterjee et al., 2002), hypothesizing that iNOS-generated NO mediates I/R-induced renal damage. One possibility is that a toxic peroxynitrite, a reactive substance of NO with superoxide anion, may cause renal damage via direct oxidant injury and protein tyrosine nitration (Walker et al., 2000; Chatterjee et al., 2002), hypothesizing that iNOS-generated NO mediates I/R-induced renal damage. One possibility is that a toxic peroxynitrite, a reactive substance of NO with superoxide anion, may cause renal damage via direct oxidant injury and protein tyrosine nitration (Walker et al., 2000; Chatterjee et al., 2002), hypothesizing that iNOS-generated NO mediates I/R-induced renal damage.
associated with the imbalance of eNOS and iNOS activities, and the improvement of this imbalance may be involved in the IP-mediated renal protective effects.

The precise mechanisms by which IP treatment prevents the loss of eNOS activity after reperfusion are unclear. Changes in eNOS protein expression seem to be at least partly related to those of cNOS activities. Most recently, Muscari et al. (2004) demonstrated that cardiac IP inhibits the loss of eNOS protein expression and enhances its activity in rat hearts exposed to I/R. We also observed that eNOS protein expression was increased in the postischemic rat kidney with IP treatment (Yamashita et al., 2003). Further studies are required to clarify the mechanisms underlying the IP-induced increase in eNOS protein expression.

The signaling mechanisms underlying the NO-mediated IP effect have been discussed. In isolated rat hearts, Lochner et al. (2000) indicated the importance of the NO-guanylyl cyclase-cyclic GMP pathway in the cardioprotective effects of IP by using NO donors and inhibitors of NOS and guanylyl cyclase. It has been demonstrated that the activation and translocation of PKC during cardiac IP is NO dependent (Ping et al., 1999). The mitochondrial K<sub>ATP</sub> channel, which is contributive to IP-mediated myocardial protection (Auchampach and Gross, 1999), could be activated by an NO donor in ventricular myocytes (Sasaki et al., 2000). In addition, it has been reported that hepatic IP is mediated by the inhibitory action of NO in endothelin-1 overproduction induced by I/R (Peralta et al., 1996). There is growing evidence that endothelin-1 is closely related to the development of I/R-induced ARF (Yamashita et al., 1996). There is growing evidence that endothelin-1 is closely related to the development of I/R-induced ARF (Yamashita et al., 1996).

The protective role of adenosine in inducing nitric oxide synthesis in rat liver has been reported (Lee HT and Emala CW, 2000) Protective effects of renal ischemic preconditioning and adenine pretreatment: role of A<sub>1</sub> and A<sub>2B</sub> receptors. Am J Physiol 278:F383–F387.

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References


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