Beneficial effects of ischemic preconditioning on renal **hemodynamics in ischemia-reperfused rat kidneys: Role of two nitric oxide synthase isozymes in ischemic preconditioned kidneys**

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Abstract:

Background: Ischemic preconditioning (IP) reduces oxidative damage during ischemia/reperfusion (I/R). IP elicits vasodilation following ischemia-perfusion in the microvasculature; however, there are few precise data concerning the rate of blood flow following IP-mediated I/R in tissues. Nitric oxide (NO) plays an important role in vasodilation; however, the type of nitric oxide synthase (NOS) expression that influences IP has not yet been determined. Therefore, the present study aimed to determine the effect of IP on renal hemodynamics during I/R in rat renal I/R models and analyze NOS expression in IP and IP-mediated reperfused kidneys. Methods: Renal hemodynamics, function and tissue NOS-enzyme expression were estimated in the following animal groups: Group1; bilateral renal ischemia for 30-min and reperfusion group, Group 2: 40-min ischemia group, Group 3; 5-min ischemia (IP) prior to 30-min ischemia, Group 4; 40-min ischemia with IP, and sham group.

Results: IP had beneficial effects on renal hemodynamics and function. We found that renal hemodynamics and glomerular filtration rate were significantly increased in IP-mediated kidneys. Western blotting analysis revealed increased expression levels of NOS-3 and NOS-2 in IP-mediated kidneys. Conclusion: These results suggest that IP-induced constitutive NOS may elicit a vasodilating effect in ischemia-reperfused rat kidneys.

Keywords: ischemia/reperfusion injury, ischemic preconditioning, kidney, rat, nitric oxide, renal blood flow, renal function

Introduction

Renal ischemia/reperfusion (I/R) injury causes severe tissue damage, affecting hemodynamic, functional, and pathophysiologic characteristics, and influences distant organ damage [1]. Clinically, acute renal reperfusion injury is often seen in renal surgery, renal transplantation, and vascular surgery in the thoracic or abdominal aorta and renal artery, in which renal blood flow is temporarily interrupted. Renal I/R exacerbates the glomerular filtration rate (GFR), tubular sodium reabsorption, and renal blood flow (RBF) and increases the renal vascular resistance[2, 3]. These functional impairments indicate the involvement of diminished intrarenal reflow due to vasoconstriction and an increase in renal vascular resistance [4]. Although reactive oxygen free radicals produced in reperfused kidneys may act as crucial pathogenic agents for microvascular endothelial cells, which results in diminished reflow in I/R kidneys [5], administration of several free radical scavenging agents in I/R kidneys lead to an incomplete functional recovery [6].

Ischemic preconditioning (IP) is a phenomenon in which brief periods of ischemia prior to I/R diminish the tissue damage caused by I/R injury, which was first observed in dog myocardium by Murry and co-workers [7]. The protective effects of IP against I/R injury have subsequently been documented in several organs, including the liver [8], brain [9], and small intestine [10]. In this study, we attempted to determine the effects of IP on I/R kidneys and demonstrated that a single 5-min period of IP prior to ischemia can ameliorate functional damage and impaired hemodynamics after I/R in rat kidneys [11]. Although the underlying mechanisms of IP are not yet fully understood, several studies suggest that these

mechanisms mainly involve vasodilating effects, including the production of nitric oxide (NO), which has the ability to prevent the detrimental action of leucocytes and platelets rather than having an anti-oxidant effect [12], which may result in the amelioration of diminished reflow in I/R kidneys. In the past two decades, several studies have reported the benefits of IP on I/R kidneys, which include amelioration of the GFR, tubular fractional excretion of sodium, and serum creatinine level; however, the precise hemodynamics in I/R kidneys during IP treatment have not yet been fully determined.

The present study aimed to determine the effects of IP on renal hemodynamics, including the effective renal plasma flow (ERPF) using conventional para-aminohippurate (PAH) clearance, renal cortical blood flow via direct measurement using a Doppler flow meter, and GFR by inulin clearance in I/R rat kidneys. Based on our previous study, we performed IP in a single 5-min period prior to ischemia [11]. In the present study, we set up two ischemic period groups of 30-min and 40-min ischemia to determine whether the renal ischemic period influences renal hemodynamics and functional damage, and whether IP improves renal damage in both groups.

NO, a strong vasodilator, is an effective agent in I/R organs. Our previous study showed that the nitric oxide synthase (NOS) inhibitor, NG - nitro - L - arginine methyl ester, diminished the effect of IP in I/R rat kidneys [12], indicating that NO plays an important role in the beneficial mechanisms of the IP. However, whether a short period of ischemia can induce NOS expression has not been determined yet. The second purpose of the present study was to identify the production of two types of NOS isozymes after a short period of ischemia during IP in rat

kidneys and determine the specific type of NOS enzyme that influences the IP effect in I/R kidneys.

Material and Methods

Experiment 1

Animals

Male Wistar rats (250–280 g), obtained from Charles River Japan (Tokyo, Japan), were housed individually at constant temperature (24–26 °C) and a 12 h dark-light cycle was maintained in the animal room. All rats were allowed free access to a basic diet and distilled water.

Surgical procedures

All rats were anesthetized by an intraperitoneal injection of 50 mg/kg sodium pentobarbital (Abbot Laboratories, IL, USA). Throughout the procedure, the body temperature was maintained at 36–38 °C using a thermostat pad. A polyethylene catheter, PE-50 (Bio Research Center, Tokyo, Japan), which was cut obliquely to insert into small blood vessels, was placed in the right femoral artery.

The catheter was connected to a pressure transducer (PaS-111; Star Medical, Tokyo, Japan) to measure the blood pressure and collect blood samples during the experiment. Another PE-50 catheter was inserted into the right femoral vein to infuse 0.9% NaCl (20 mL/kg/h), 3% inulin (5 mL/kg/h; Sigma, St. Louis, MO), and 1% PAH (5 mL/kg/h; Sigma, St. Louis, MO). A silicon tube (1.02 mm ID, 2.03 mm OD; Bio Research Center, Tokyo, Japan) was placed in the urinary bladder to collect urine samples. Through a midline abdominal incision, the bilateral kidneys and renal arteries were exposed. Thereafter, a laser Doppler flowmeter

probe (ALF-2100; Advance Co., Tokyo, Japan) was placed on the surface of the left kidney. The probe was fixed by a stainless-steel arm with a microclip throughout the experiment to continuously measure the RBF. After a period of 60-min 0.9% NaCl infusion, 20-min urine and 2 mL blood samples were collected to measure the urine and serum inulin concentrations, respectively. Following the first clearance test, 5-min IP (5-min bilateral renal arterial occlusion with microvascular clips; C-2-V, S&T Marketing, Neuhausen, Switzerland) was performed. Thirty minutes after the IP procedure, the bilateral renal arteries were occluded for a period of 30-min or 40-min. After 60-min of reperfusion, a second clearance test was performed (Figure 1).

Analytical methods

Inulin concentrations in serum and urine were measured via the method described by Davidson and Sackner [13] using anthrone as a substrate (Sigma, Deisenhofen, Germany). PAH concentrations were measured using a standard colorimetric assay [14]. GFR and ERPF were calculated from inulin clearance and PAH clearance, respectively [15]. RBF was calculated as follows: RBF = ERPF/1-hematocrit/100. Renal vascular resistance (RVR) was calculated as follows: RVR= (mean arterial pressure-5)/RBF [16].

Aminal Groups:

Animals were then divided into five groups as follows:

Sham group: sham operation was performed 10 min after saline injection (n=5).

Group 1: 30-min ischemia group (n=6): bilateral renal arteries were occluded for

30 min without IP.

Group 2: 40-min ischemia group (n=6): bilateral renal arteries were occluded for 40 min without IP.

Group 3: After IP, the bilateral renal arteries were occluded for 30 min (n=6).

Group 4: After IP, the bilateral renal arteries were occluded for 40 min (n=6).

Ethics Statement

All procedures involving animals were performed in accordance with the ethical standards of the National Research Council Guide for the Care and Use of Laboratory Animals. (Ulm University No. 655 and The University of Tokyo, Faculty of Medicine)

Experiment 2

Western blotting analysis of NOS-2 and NOS-3

Tissue samples were collected from rat kidneys. Lane 1: sham-operated rat kidneys. Lane 2: 5-min I/R in rat kidneys. Lane 3: 30-min I/R in rat kidneys with IP. Lane 4: 30-min I/R in rat kidneys without IP. Each sample was prepared for western blotting analysis. Western blotting analysis of the expression levels of NOS-2 and NOS-3 in reperfused kidneys was conducted as per the procedure described in previous studies [17]. Approximately 200 mg of the tissue samples were collected and protein concentrations were determined using Bradford assay. Equal amounts of protein (200 μg) were electrophoresed on 8% sodium dodecyl sulfate polyacrylamide gels and blotted onto nitrocellulose filters (Thermo Fisher Scientific, Tokyo, Japan). The filters were blocked with skim milk and incubated

with NOS-2 and NOS-3 rabbit polyclonal antibodies (1/5000; Bioss Inc., Boston, MA, USA) for 60-min at 24 °C. Equal protein loading was verified after the membranes were stripped with monoclonal anti-beta-actin mouse IgG antibodies (1/2000; Sigma.,St.Louis, MI, USA). Membranes were washed three times with Tris-buffered saline containing 0.1% Tween 20 and then incubated with a horseradish peroxidase-labeled goat anti-rabbit polyclonal antibody (1/1000; GE Healthcare, Amersham, UK). Finally, membranes were developed via autoradiography using an enhanced chemiluminescence western blotting system (GE Healthcare, Amersham, UK).

Statistical Analysis

All data are expressed as the mean±SE. The values from each group were compared using one-way analysis of variance with Scheffe's correction for multiple comparisons. All statistical analyses were performed using J-STAT® software (http://toukeijstat.web.fc2.com/), and probability (p) values of < 0.05 were considered significant.

Results

Changes in RBF

Changes in RBF throughout the experiment are shown in Figure 2. In Groups 2 and 3, RBF in reperfused kidneys gradually decreased with time and did not recover to the pre-ischemic state. A significant elevation of RBF was observed in the IP-treated reperfused kidneys (reperfusion: 30–60 min) in the 30-min and 40-min ischemia groups ($p<0.01$ vs 30-min I/R, and 40-min I/R). In the groups that underwent IP, a significant decrease in RBF following reperfusion was noted in

the rat kidneys in the 40-min ischemia group compared with that in the 30-min ischemia group ($p<0.01$).

Effects of IP on ERPF

Effects of IP on ERPF are shown in Figure 3. There was a significant decrease in ERPF in the ischemia-reperfused kidneys. A significant decrease in ERPF was observed in the 40-min ischemia group (2.3±0.3 mL/min) as compared with the 30-min ischemia group (3.3±0.4 mL/min)(* $p<0.01$). Groups that underwent IP showed a significant increase in ERPF (# $p<0.01$ vs 30-min I and ## $p<0.05$ vs 40-min I) after reperfusion when compared with groups that did not undergo this procedure (Group 3: 5.3±0.8 mL/min; Group 4: 4.6±0.6 mL/min).

Effects of IP on GFR

GFR in the five groups is shown in Figure 4. A significant decrease in GFR was observed in Group 2 (0.5±0.1 mL/min) and Group 3 (0.3±0.1 mL/min) compared with that in the sham-operated control group (2.2±0.2 mL/min; $p<0.01$). An extension of the ischemic period significantly decreased the GFR ($p<0.05$) in the 40-min ischemia group compared with that in the 30-min ischemia group. The renal IP procedure significantly reduced functional damage ($p<0.01$) in the 30-min (1.4±0.2 mL/min) and 40-min (1.0±0.2 mL/min) ischemia groups.

Effects of IP on RVR

Renal vascular resistance (RVR) was calculated by the method mentioned above. RVR in the five groups is shown in Figure 5. A significant increase in RVR was showed in Group 2 (30-min I/R) and Group 3 (40-min I/R) compared with that in the sham-operated control group ($p<0.01$ vs sham). The renal IP

significantly ameliorated RVR in both ischemia groups (Group 4 and 5: *p<0.05 vs 30-min I/R, ***p<0.05 vs 40-min I/R).

NOS-2 and NOS-3 expression

In experiment 2

We analyzed the expression levels of NOS-2 and NOS-3 in animals subjected to a brief 5-min ischemic influx followed by 30-min I/R in rat kidneys, as shown in Figure 6. A 5-min IP period did not lead to NOS-2 expression but showed a stronger NOS-3 expression (Lane 2). A similar result was observed in the normal kidney tissue (Lane 1); however, NOS-3 protein expression in the ischemic preconditioned kidney was stronger than that in the normal kidney. In contrast, kidneys subjected to an IP period followed by I/R injury (Lane 3) or I/R treatment (Lane 4) showed increased expression levels of NOS-2 and NOS-3.

Discussion

The pathogenesis of acute renal failure by I/R injury is known to produce excessive reactive oxygen species, including oxide, hydrogen peroxide, hydroxide, and peroxynitrite, which are produced by the interaction of superoxide with NO [18]. Superoxide-induced membrane changes may be caused by neutrophil migration, resulting in increased vascular permeability and intravascular coagulation, which diminishes microcirculation [19]. To diminish I/R injury, different methods of administration of several types of antioxidant, anti-inflammatory, and vasodilating agents have been tried, and the beneficial effects have been reported. We previously reported that production of metallothionein, a

cysteine-rich antioxidant and endogenous metal detoxification protein, induced by zinc administration prior to renal I/R, diminishes renal functional damage in a rat I/R model [3]. The inhibitory effect of the adenosine triphosphate (ATP)-sensitive potassium channel blocking agents on vascular permeability is well known.

Pompermayer and co-workers [20] reported that glibenclamide, an ATP-sensitive potassium channel blocker, diminished renal I/R injury. Diminished renal hemodynamics in I/R kidneys are ameliorated by erythropoietin administration, which increases the phosphorylation of endothelial NOS (eNOS)[21, 22]. Wang [23] reported that ozone oxidative preconditioning protected kidney cells against hypoxia/reoxygenation injury using a renal tubular epithelial cell line.

IP has received much attention because of its significant effect on renal hemodynamics in I/R injuries. In the present renal I/R model, RBF continued to diminish in the 40-min ischemia group during the 60-min reperfusion period. In contrast, RBF in the 30-min ischemia group tended to recover to the initial level of reperfusion. It is evident that the recovery of hemodynamics in reperfused kidneys is dependent on the ischemic period. A decrease in blood flow to the ischemia-reperfused tissues in the myocardium was first reported by Kloner and co-workers [24], who coined the “no-reflow” phenomenon. As diminished reflow is also observed following reperfusion in the rat kidney, it appears that diminished reflow is not a tissue-specific phenomenon. In this study, we demonstrated that a brief period of ischemia prior to I/R ameliorated the diminished RBF and renal plasma flow during the reperfusion period. Although prolonged ischemia for 40-min resulted in more severe renal damage, it is worth noting that IP improved

renal hemodynamics at the same level as the 30-min renal ischemia group. Thus, the beneficial effects of IP may be more pronounced in the acute phase of renal hemodynamics following reperfusion.

NO plays an important role in the beneficial effects on hemodynamics in I/R tissues[12, 25]. The anti-apoptotic, anti-inflammatory, and vasodilative activities of NO have been demonstrated in numerous studies. Under severe oxidative stress induced by I/R injury, tissue blood flow diminishes due to vasoconstriction induced by endothelial cell damage, and over-regulation of NO induced by IP plays a crucial role in the restoration of blood flow via its vasodilating effect[12, 26]. In the present study, western blotting analysis showed increased expression levels of NOS-2 (inducible NOS: iNOS) and NOS-3 (eNOS) following I/R. Moreover, a short period of ischemia following IP could induce eNOS production. Therefore, it may be stated that IP-induced eNOS production may have beneficial effects on the hemodynamics in I/R kidneys. Our results are supported by the results of Yamasowa et al., who reported that eNOS-mediated NO production was important in IP-mediated protection of I/R kidneys in uninephrectomized mice [27]. Interestingly, a short period of ischemia did not induce NOS-2 in the present study. In contrast, Park et al. showed that iNOS plays an important role in the protective effect of IP in mouse I/R kidneys [25]. Kapitsinou and Haase [28] provided an explanation for the discrepant results by stating that the difference in NOS isoforms produced by IP may be due to differences in experimental protocols. For instance the ischemic period as IP protocol reported by Park et al[25], was 15-minute, on the contrary Yamashita et al[29], reported that 3 cycles of 2-minute ischemia followed by 5-minute

reperfusion prior to the I/R elicited the beneficial effect on reperfused renel function by inducing eNOS but iNOS. The study concerning the interaction between eNOS and iNOS in I/R tissue was reported by Muscari et al[30]. who investigated iNOS and eNOS expression in the Langendorff-perfused rat hearts and found that the amount of eNOS protein decreased ,but total NOS acitivity was not reduced and both NOS activity were significantly higher in IP than I/R tissues. These findings suggested that a parallel compensatory stimulation of this enzyme acitivity occurred.

Although in the present study, we did not investigate the contribution of other NOS isoform, neuronal NO synthase (nNOS) to renal hemodynamics, it is essential to note the effect of nNOS on renal microcirculation and function. Tojo and co-workers reported that nNOS in the macula densa acts as a modulator of tubuloglomerular feedback and regulates the afferent arterior diameter in drug induced hypertensive rats [31]. However, the specific NOS isoform produced by IP that contributes to tissue protection is still ambiguous and requires further investigation.

In conclusion, the present study showed that IP ameliorates diminished renal reflow and hemodynamics, including ERPF and GFR, during reperfusion in a rat kidney I/R model. The analysis of NOS enzymes revealed that IP induces the production of NOS-3, but not NOS-2, which may influence the beneficial effects of IP. Further studies are required to determine which factors influence renal hemodynamics, including NO, and whether different IP procedures, including

remote IP, may be involved in inducing the production of NOS enzymes.

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Conflicts of Interest

There are no conflicts of interest in the present study.

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Figure legends

Figure 1 Experimental protocol: CT: clearance test, IP: ischemic preconditioning

Figure 2 Changes in renal blood flow in the left kidney during ischemic preconditioning following ischemia-reperfusion in experiment 1.

Compared with sham operated rats (○), renal blood flow (RBF) gradually diminished during reperfusion in the ischemia groups (△: 30-min bilateral renal ischemia and reperfusion; □: 40-min bilateral renal ischemia and reperfusion), whereas it was significantly increased during reperfusion in the ischemic preconditioning (IP) groups (▲ IP+30-min ischemia group; single 5-min period of bilateral renal ischemia following 30-min ischemia and reperfusion; ■ IP+40-min ischemia group; single 5-min period of bilateral renal ischemia following 40-min ischemia and reperfusion, *p<0.01, vs. ▲ :IP+30-min bilateral renal ischemia and reperfusion groups, **p<0.01 vs ■ IP+40-min ischemia group). A significant decrease in RBF following reperfusion was noted in the rat kidneys in the 40-min ischemia group compared with that in the 30-min ischemia group (# p<0.01).

Figure 3 Effect of ischemic preconditioning (IP) on the effective renal plasma flow (ERPF) in ischemia-reperfused rat kidneys.

The sham control value indicates the ERPF in pre-injured untreated kidneys (*p<0.01, sham-operated animals and IP groups).

Figure 4 Effect of ischemic preconditioning (IP) on glomerular filtration rate (GFR) in ischemia-reperfused rat kidneys.

40-min ischemia significantly decreased the GFR in reperfused kidneys as compared with the GFR after 30-min ischemia reperfusion in rats (*p<0.01 vs. sham, † p <0.01 vs. 30-min I/R).

IP significantly ameliorated renal function in both 30-min and 40-min ischemia-reperfusion (I/R) groups ((## p<0.01 vs 30-min I and # p<0.05 vs 40-min I).

Figure 5 Nitric oxide synthase (NOS) expression following ischemic preconditioning (IP) and ischemia/reperfusion in rat kidneys.

Lane 1: sham-operated control kidneys; Lane 2: ischemic preconditioned kidneys (single 5-min ischemic kidneys); Lane 3: ischemic preconditioned 30-min ischemia-reperfused kidneys; Lane 4: 30-min ischemia-reperfused kidneys. No NOS-2 expression and relatively strong NOS-3 expression levels were observed in Lane 2. NOS-2 expression was observed in both I/R (Lane 3) and IP+I/R (Lane 4) kidneys.

PC: positive control