Int J Clin Exp Pathol 2015;8(2):1128-1140
www.ijcep.com /ISSN:1936-2625/IJCEP0004501

Original Article

Effects of ischemic preconditioning on the systemic and renal hemodynamic changes in renal ischemia reperfusion injury

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Received December 6, 2014; Accepted February 3, 2015; Epub February 1, 2015; Published February 15, 2015

Abstract: Background: Ischemic preconditioning (IPC) could protect against subsequent renal ischemia reperfusion injury (IRI). However, the mechanisms underlying IPC remain far from complete. Hence, we explored the effects of IPC on the renal and systemic hemodynamic changes, renal function and morphology, as well the involvement of endothelial and inducible nitric oxide synthase (eNOS/iNOS), and nitric oxide (NO). Methods: Male Sprague-Dawley rats were randomly divided into five groups after right-side nephrectomy: Sham group (surgery without vascular clamping); IRI group (the left renal artery was clamped for 45 min); IPC group (pretreated with 15 min of ischemia and 10 min of reperfusion); IPC + vehicle group (administrated with 0.9% saline 5 min before IPC); and IPC + N^G-nitro-L-arginine methylester (L-NAME) group (pretreated with L-NAME 5 min prior to IPC). The renal and systemic hemodynamic parameters, renal function and morphology, as well as eNOS, iNOS, and NO expression levels in the kidneys were measured at the indicated time points after reperfusion. Results: IPC rats exhibited significant improvements in renal function, morphology, and renal artery blood flow (RABF), without obvious influence on the systemic hemodynamics and renal vein blood flow. Increased eNOS, iNOS, and NO expression levels were detected in the kidneys of IPC rats 24 h after reperfusion. Furthermore, the beneficial effects were fully abolished by the administration of L-NAME. Conclusions: The results suggest that IPC contributes to early restoration of RABF, probably through eNOS/iNOS-mediated NO production, thereby alleviating the renal dysfunction and histological damage caused by IRI.

Keywords: Ischemic preconditioning, ischemia reperfusion injury, renal/systemic hemodynamics, eNOS/iNOS, nitric oxide

Introduction

Renal ischemia reperfusion injury (IRI) is encountered in many clinical settings including kidney transplantation, nephron-sparing surgery, and complex cardiovascular surgeries, which could significantly increase the morbidity, mortality, and medical resource utilization [1, 2]. Renal IRI is characterized by an initial restriction of renal blood supply and the subsequent perfusion restoration. During the process, a robust inflammation and oxidative response occurs, and leads to the injury of microvascular endothelium and renal tubular epithelium [3, 4]. Efforts have been denoted to identify potential therapeutic interventions with the consistent advances in understanding the pathophysiological mechanisms of renal IRI [5, 6], and ischemic preconditioning (IPC) has been proposed as an effective therapeutic approach to enhance ischemia tolerance and preserve renal function [4, 7].

IPC was initially reported by Murry et al. as brief sublethal episodes of ischemia and reperfusion that protect the myocardium against lethal isch-

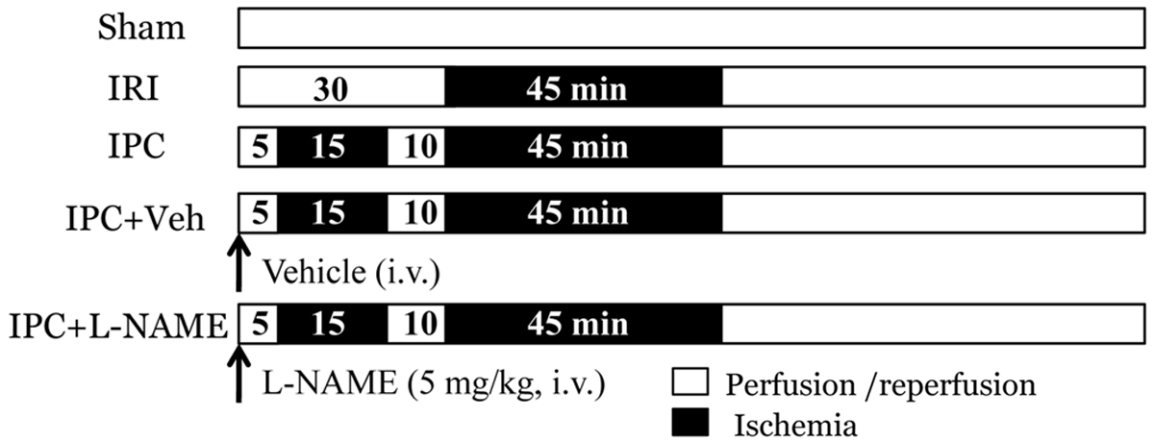

Figure 1. Schematic illustration of the main experimental protocols. IRI, ischemia reperfusion injury; IPC, ischemic preconditioning; Veh, vehicle (0.9% saline); and L-NAME, NG-nitro-L-arginine methylester (a nonselective NO synthase inhibitor).

emia insult [8]. The beneficial effects of IPC have been subsequently confirmed in various organs, including the heart [9], brain [10], liver [11], and kidneys [12]. The protective effects of IPC against kidney IRI have been extensively studied [12], and proven to be partially mediated by reducing inflammation and enhancing the mobilization and recruitment of endothelial progenitor cells (EPCs) [13-15]. Although the renoproctective effects of IPC have been successfully validated, the underlying mechanisms remain elusive, which limited the translation of IPC in the clinical settings to some extent [7].

Sustained reductions in renal blood flow (RBF) following renal IRI have been demonstrated in numerous studies [16, 17], which were caused by microcirculation disturbance and impaired renal vascular reactivity and hampered the early recovery of injured kidneys [4, 18, 19]. IPC has been proven to maintain the microcirculation balance effectively [14, 20]; however, the effects of IPC on renal hemodynamics and its underlying mechanisms remain to be elucidated. Hence, we conducted the present study to comprehensively explore: 1) the effects of IPC on renal function and morphology; and 2) its impact on systemic and renal hemodynamics and the potential underlying mechanisms.

Materials and methods

Animals

Male Sprague-Dawley rats weighing 280 g to 320 g were bred and housed in the Experiment Animal Center of Nanjing First Hospital, Nanjing Medical University. The rats were housed in individual cages under controlled conditions (a 12 h dark/12 h light cycle and 20°C to 25°C room temperature) and with free access to standard laboratory chow and water. All procedures were approved by the Ethics Committee for the Use of Experimental Animals at Nanjing Medical University and were conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health (NIH).

Experiment design and surgical procedures

Anesthesia was performed using sodium pentobarbital (50 mg/kg, i.p.), a transverse 1 cm to 2 cm lumbotomy incision was made, and the right kidney was removed. During the surgery, body temperature was monitored using a rectal probe and maintained at approximately 37°C using a heat lamp. After housed in separated cages and closely watched up for another two weeks, the rats were randomly assigned to five groups using a random number table generated by SPSS software (version 20.0; SPSS Institute, Chicago, IL, USA). Group 1 was the sham-operated group, in which the left renal artery was carefully separated without clamping. Group 2 was the IRI group, in which the left renal arteries were occluded using a nontraumatic vascular clamp for 45 min. Group 3 was the IPC group, in which the left renal arteries of rats were clamped for 15 min and reperfused for another 10 min prior to the subsequent 45 min occlusion. Group 4 was the IPC + vehicle (Veh) group, in which 0.9% saline was injected

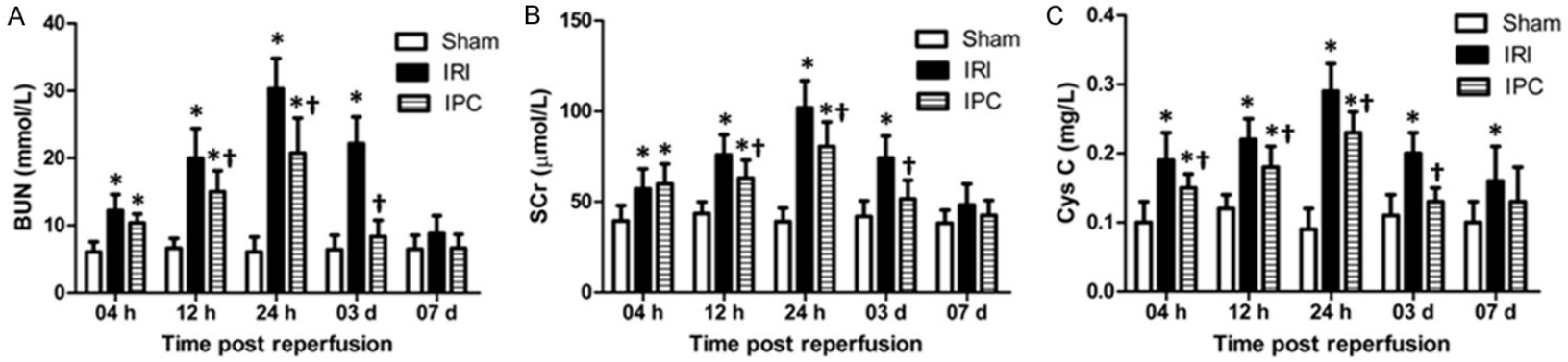

Figure 2. Time-dependent changes in the renal function of different groups. A. Blood urine nitrogen (BUN, mmol/L); B. Serum creatinine (SCr, μmol/L); C. Cystatin C (Cys C, mg/L). Columns represent mean ± SD. *significant difference vs. Sham group ($P < 0.05$); †significant difference vs. IRI group ($P < 0.05$).

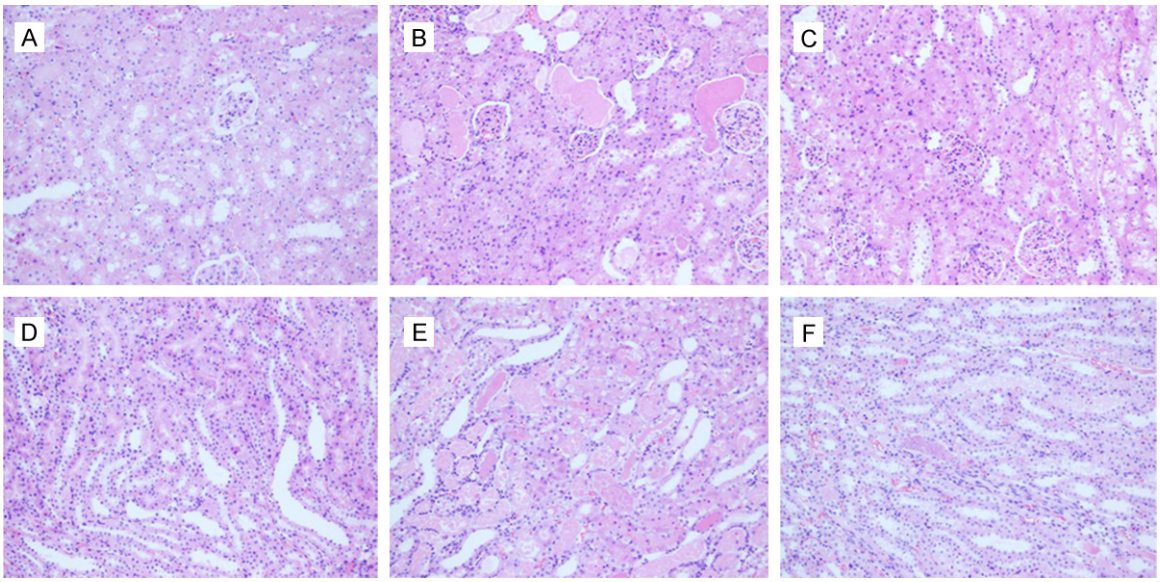

Figure 3. Renal tissue histological examination at 24 h after reperfusion. The renal sections were stained with HE and examined by light microscopy at 200 × magnification. Minimal pathological changes were observed in the cortex (A) and medulla (D) of the sham-operated rats. Severe lesions were detected in the cortex (B) and medulla (E) after IRI induction. Brush border loss, epithelial cell detachment from the basement membrane, tubular obstruction, intratubular casts, peritubular capillary congestion, and intraluminal necrotic debris were observed. However, the severity of tissue injury was significantly attenuated in the cortex (C) and medulla (F) of the kidneys subjected to IPC.

Table 1. Effects of IPC on renal morphological changes at various time points post reperfusion

	Sham group	IRI group	IPC group
04 h	0.13 ± 0.11	1.84± 0.35*	1.03 ± 0.32*,†
12 h	0.13 ± 0.11	3.09± 0.55*	2.03 ± 0.48*,†
24 h	0.11 ± 0.10	3.54± 0.37*	2.54 ± 0.46*,†
03 d	0.13 ± 0.11	2.41± 0.33*	1.15 ± 0.41*,†
07 d	0.13 ± 0.12	0.91± 0.35*	0.37 ± 0.25†

IRI, ischemia reperfusion injury; IPC, ischemic preconditioning. *$P < 0.05$ vs. Sham group; †$P < 0.05$ vs. IRI group.

into the tail veins 5 min before IPC. Group 5 was the IPC + L-NAME (N^G-nitro-L-arginine methylester; Sigma-Aldrich Inc., Steinheim, Germany) group, in which L-NAME was dissolved in 0.9% saline and intravenously administered at 5 mg/kg (i.v.) 5 min before IPC. The surgical and drug pretreatment procedures were conducted as previously described with slight modifications [13, 14, 21], and the detailed experimental protocol was presented in **Figure 1**.

Measurement of systemic hemodynamics

The systolic blood pressure (SBP), diastolic blood pressure (DBP), and heart rates (HR) of the conscious rats were noninvasively measured using a volume pressure recording sensor through the occlusion tail-cuff method (BP-2000; Visitech Systems Inc., Apex, NC, USA). In brief, the rats were placed in a holding device mounted on a thermostatically controlled warming plate with mini-cuffs fixed around the tails to detect the artery pulsations. The rats were allowed to acclimate to the cuffs for 10 min to 15 min before the recording session, and the data were recorded thrice per session for the calculation of an average value.

Measurement of renal hemodynamics

Renal artery blood flow (RABF) and renal vein blood flow (RVBF) were evaluated with Vevo 2100 system (Visual Sonics, Toronto, Canada), as previously described by Boesen et al. [17]. Briefly, rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and placed in the supine position on the electrocardiogram pad to constantly monitor HR and body temperature (maintained at 37°C). The left kidney was initially imaged in the two-dimensional long-axis view. Color flow and velocity time integral were then determined using pulse-wave Doppler. Three to five blood flow-velocity recordings were obtained from each rat, and the average was calculated as the individual data.

Table 2. Summary of renal and systemic hemodynamics at various time points post reperfusion

	Sham group	IRI group	IPC group
RABF (mm/s)			
04 h	742.1 ± 29.2	660.0 ± 19.3*	698.9 ± 19.2*,†
12 h	751.1 ± 15.3	591.2 ± 15.2*	679.8 ± 15.4*,†
24 h	745.8 ± 14.0	435.9 ± 16.6*	631.9 ± 15.7*,†
03 d	750.8 ± 18.3	630.2 ± 17.0*	699.8 ± 13.6*,†
07 d	740.7 ± 19.2	705.8 ± 21.5*	722.6 ± 17.8
RVBF (mm/s)			
04 h	124.9 ± 13.3	120.6 ± 16.6	130.8 ± 18.5
12 h	131.5 ± 18.0	121.2 ± 18.1	124.9 ± 23.4
24 h	122.7 ± 12.8	128.5 ± 14.9	125.1 ± 16.0
03 d	128.9 ± 17.3	126.2 ± 21.0	120.0 ± 16.4
07 d	125.9 ± 14.6	128.8 ± 20.1	121.3 ± 17.1
SBP (mm Hg)			
04 h	121.8 ± 12.5	115.3 ± 10.2	116.1 ± 10.1
12 h	118.9 ± 11.0	112.7 ± 11.2	115.8 ± 10.8
24 h	122.5 ± 11.5	117.3 ± 10.7	118.9 ± 11.9
03 d	118.4 ± 8.7	121.1 ± 13.2	122.7 ± 11.9
07 d	121.9 ± 10.4	124.3 ± 11.1	118.9 ± 11.8
DBP (mm Hg)			
04 h	74.8 ± 8.3	73.1 ± 7.6	77.4 ± 7.8
12 h	72.7 ± 7.7	78.2 ± 9.2	74.9 ± 8.4
24 h	76.2 ± 8.3	73.3 ± 7.7	70.4 ± 8.1
03 d	72.7 ± 6.9	76.9 ± 9.9	72.7 ± 10.2
07 d	70.9 ± 10.0	75.6 ± 10.0	73.8 ± 9.5
HR (beats/ min)			
04 h	355 ± 21	366 ± 19	360 ± 14
12 h	353 ± 12	364 ± 16	358 ± 21
24 h	350 ± 12	360 ± 16	358 ± 13
03 d	355 ± 16	354 ± 14	352 ± 15
07 d	357 ± 12	353 ± 11	355 ± 11

IRI, ischemia reperfusion injury; IPC, ischemic preconditioning; RABF, renal artery blood flow; RVBF, renal vein blood flow; SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rates. *$P < 0.05$ vs. Sham group; †$P < 0.05$ vs. IRI group.

Blood and kidney tissue preparation

A midline incision was made immediately after renal hemodynamics measurement (still under complete anesthesia), and 2 ml of blood was obtained from the abdominal inferior vena cava of each rat for further renal function analysis. The rats were sacrificed and transcardially perfused with 0.1 mol/L phosphate buffered saline (PBS; pH 7.4 and 4°C). The left kidney was immediately harvested and cut into two parts. One half was fixed in 4% paraformaldehyde (pH 7.4 and 4°C) for renal injury assay, and the other half was snap frozen in liquid nitrogen and stored at -80°C for Western blot analysis.

Renal function analysis

The blood samples were centrifuged at 3,500 rpm for 10 min, and the supernatants were collected to measure serum creatinine (SCr), cystatin C (Cys C), and blood urine nitrogen (BUN) levels using clinically automated analysis methods (Hitachi 7600-10; Hitachi High-Technologies Corp., Tokyo, Japan).

Histological examination

The kidney specimens were dehydrated in a graded ethanol series, embedded in paraffin, and sectioned to 5 μm thicknesses. The sections were deparaffinized with xylene, stained with hematoxylin and eosin (HE), and then microscopically assessed by an experienced pathologist, who was blinded to the experiment protocol. As described previously [14], renal tubulointerstitial injury was defined as tubular necrosis, tubular dilatation and/or atrophy, inflammatory cell infiltration, or cellular edema. Histopathologic scores of kidneys (HSK) ranged from 0 to 4, and high values represent severe damage.

Western blot analysis

The total kidney tissues were homogenized, and total proteins were extracted with a protein extraction kit (KeyGEN Biotechnology, Nanjing, China) for further analysis of the expression levels of endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS). Different samples of the same 50 μg of protein were loaded on each lane and subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The separated proteins were then transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA). Afterwards, the membranes were blocked with 5% skimmed milk in Tris-buffered saline-Tween 20 (TBST) for 2 hand incubated overnight at 4°C with primary antibodies against eNOS, iNOS, and β-actin (all above from Santa Cruz Biotechnology Inc., CA, USA). The membranes

were rinsed with TBST buffer thrice, incubated with horseradish peroxidase-conjugated secondary antibody (SuperSignal WestPico; Thermo Scientific Inc., MA, USA) for 2 h at room temperature, and developed with an enhanced chemiluminescence system (ECL kit; Thermo Scientific Inc., MA, USA). The signals were quantified by scanning densitometry using the Image J analysis system (NIH, MD, USA).

Measurement of nitric oxide content in kidney

As the stable end products of nitric oxide (NO) metabolites, nitrite and nitrate were measured to assess the NO levels in the kidney. The amount of nitrite and nitrate in renal homogenate was determined with a NO colorimetric assay kit (Jiancheng Biotechnology, Nanjing, China), as previously described [22]. The concentration of NO in the kidney tissue was expressed as μmol/g protein.

Statistical analysis

All data were expressed as mean ± standard deviation (SD). Statistical differences between groups were evaluated by one-way analysis of variance, followed by least-significant difference or Dunnett's C *post hoc* test when appropriate. All statistical analysis was performed with SPSS software (version 20.0; SPSS Institute, Chicago, IL, USA), and two-sided $P < 0.05$ was considered significant.

Results

General observations

A total of 136 Sprague-Dawley rats were initially included and underwent right-side nephrectomy. Six rats died of massive bleeding of the right renal hilum (n = 2), unsuccessful recovery from anesthesia (n = 2), and severe postoperative infection (n = 2) despite of the careful operation and close observation. Thus, the remaining 130 healthy and well-performing rats were randomly assigned into the five treatment groups and analyzed at various time points (4 h, 12 h, 24 h, 3 d, and 7 d) post reperfusion (Figure S1). No significant difference in body weight was observed among the five groups (Sham group: 298.3 ± 10.7 g, IRI group: 302.8 ± 10.2 g, IPC group: 299.2 ± 10.3 g, IPC + Veh group: 303.9 ± 13.1 g, and IPC + L-NAME group: 304.2 ± 9.6 g; $P > 0.05$), nor the subgroups at various time points post reperfusion (Table S1).

Effects of IPC on renal function

Figure 2 depicted that the renal function of the rats suffered from IRI significantly deteriorated, as indicated by the changes of BUN (**Figure 2A**), SCr (**Figure 2B**), and Cys C (**Figure 2C**). These renal function markers changed in a monophasic manner: significantly increased from 4 h, peaked at 24 h, and returned to the baseline values at 7 d. These changes were remarkably attenuated in the ischemic preconditioned rats starting from 4 h after reperfusion because Cys C levels decreased at 4 h (IPC vs. IRI: 0.15 ± 0.02 mg/L vs. 0.19 ± 0.04 mg/L; $P < 0.05$). The detailed parameters of renal function were summarized in Table S2.

Effects of IPC on renal morphology

The animals subjected to 45 min ischemia without IPC demonstrated the recognized features of sever acute tubular injury in the cortex (**Figure 3B**) and medulla (**Figure 3E**) compared with the Sham group (**Figure 3A**, **3D**). These features included tubular epithelial cell necrosis, tubular dilatation and/or atrophy, inflammatory cell infiltration, and cellular edema, which were less pronounced in the IPC group (**Figure 3C**, **3F**). Furthermore, HSK was also applied to quantify interstitial tubule damage, and the results were summarized in **Table 1**. The results were in agreement with the renal function parameters, especially serum Cys C levels (**Figure 2C**; Table S2).

Differential effects of IPC on renal and systemic hemodynamics

The changes of RBF (both RABF and RVBF) were evaluated with the aid of Doppler ultrasound method. As summarized in **Table 2** and **Figure 4**, the rats subjected to 45-min ischemia insult demonstrated significant deterioration of RABF. RABF started to decrease at 4 h post reperfusion, reached the bottom at 24 h (**Figure 4B**), and returned near the baseline values at 7 d (IRI vs. Sham groups: 705.8 ± 21.5 mm/s vs. 740.7 ± 19.2 mm/s). However, the decrease of RABF was significantly improved by IPC treatment (**Figure 4**; **Table 2**). In contrast to RABF, no significant difference of RVBF was detected among the three groups (**Figure 4D-F**, **4H**; **Table 2**). Furthermore, the systemic hemodynamic parameters were also measured, but no significant differences in SBP, DBP, or HR were observed among the three groups (**Table 2**).

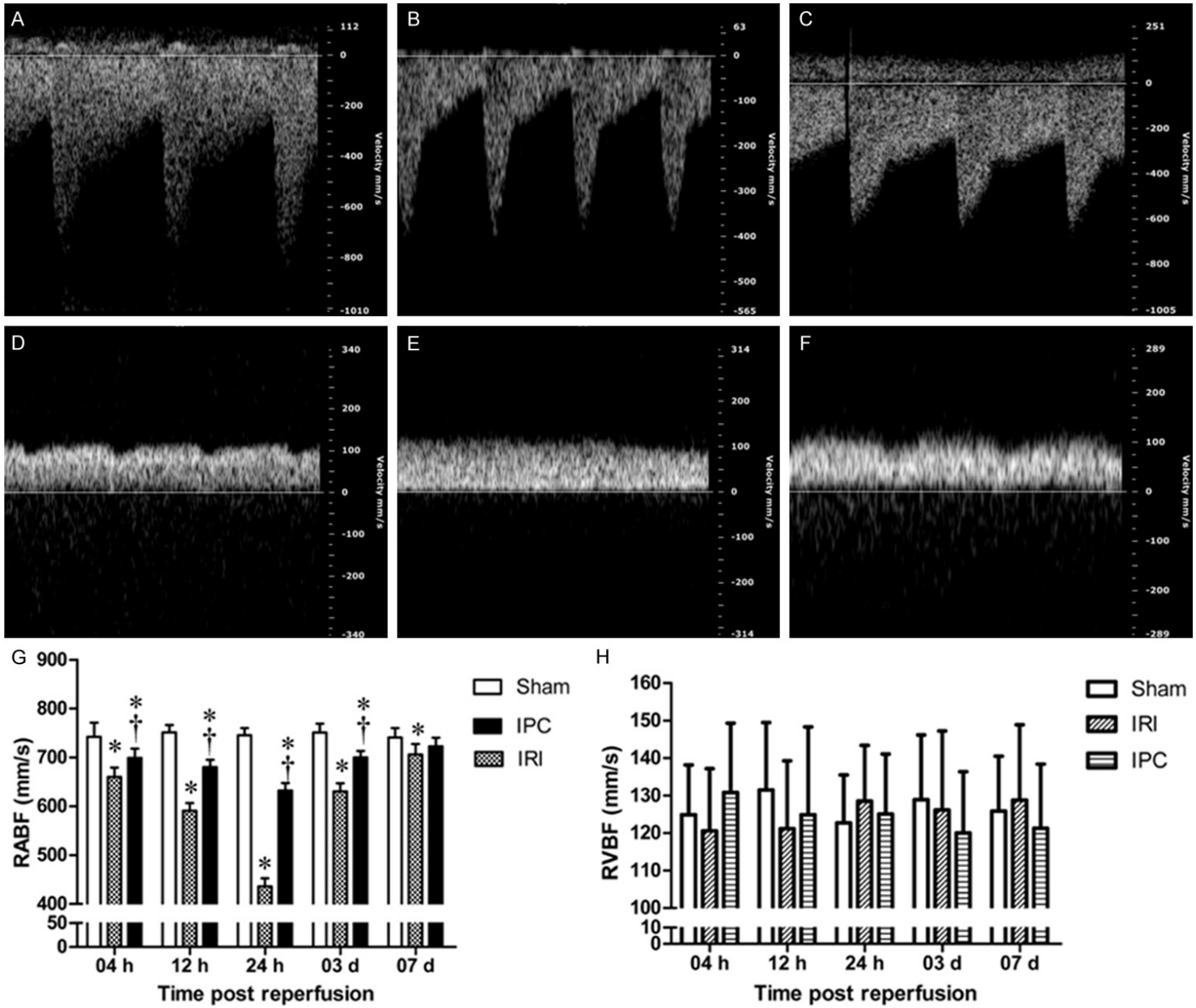
A
B
C
D
E
F
Velocity mm/s
G
RABF (mm/s)
900
800
700
600
500
400
50
0
Sham
IPC
IRI
04 h
12 h
24 h
03 d
07 d
Time post reperfusion
H
RVBF (mm/s)
160
150
140
130
120
110
100
10
0
Sham
IRI
IPC
04 h
12 h
24 h
03 d
07 d
Time post reperfusion

Figure 4. Effects of IPC on renal hemodynamic changes. The renal artery blood flow (RABF) and renal vein blood flow (RVBF) were measured with Doppler ultrasound. The RABF in the IRI group (B. ~430 mm/s) was reduced significantly at 24 h post-reperfusion compared with the Sham group (A. ~750 mm/s), whereas the IPC group (C. ~630 mm/s) showed remarkable improvement in RABF compared with the IRI group. However, no significant differences in RVBF were observed between the Sham (D. ~ 20 mm/s), IRI (E. ~130 mm/s), and IPC groups (F. ~125 mm/s) at 24 h after reperfusion. Time-dependent changes in RABF (G) and RVBF (H) in the different groups are presented. Columns represent mean ± SD. *significant difference vs. Sham group ($P < 0.05$); †significant difference vs. IRI group ($P < 0.05$).

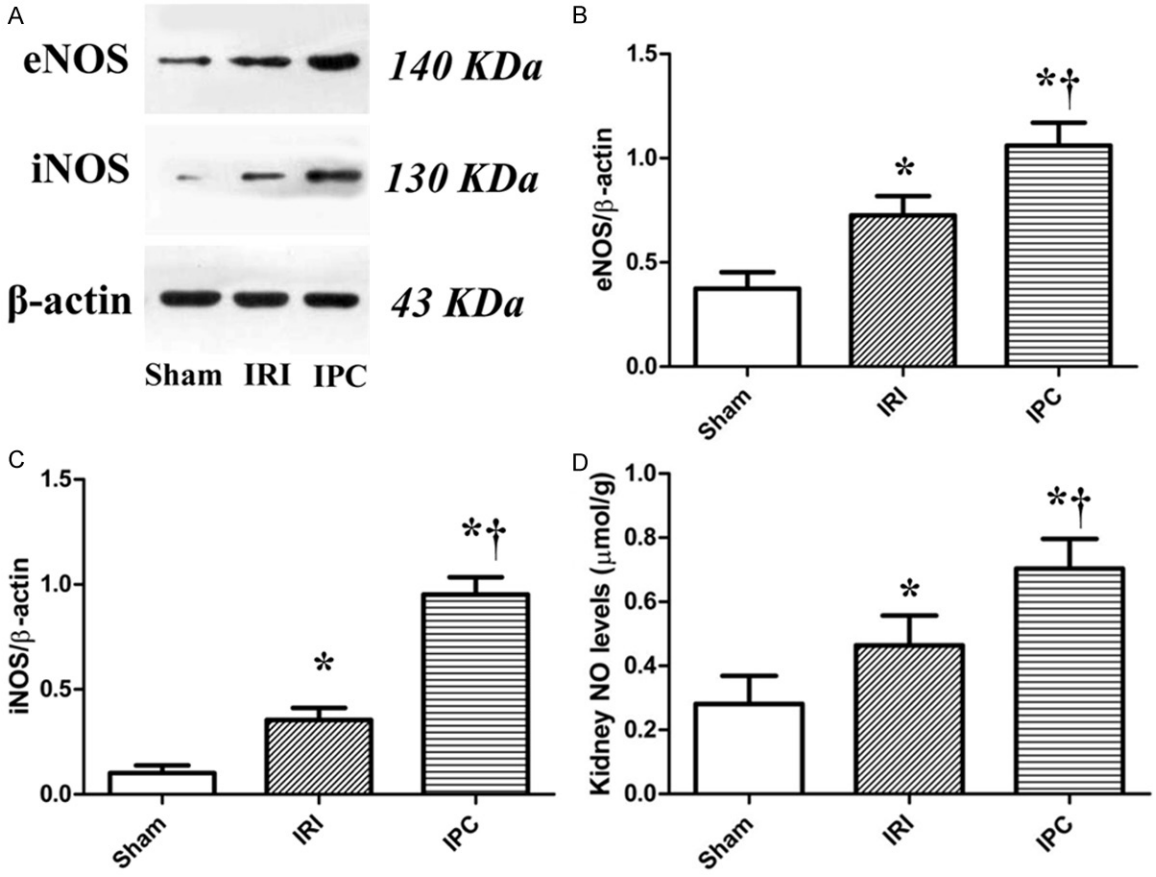

Figure 5. Effects of IPC on eNOS, iNOS, and NO expression levels at 24 h post reperfusion. (A) Representative Western blots show the effects of IPC on eNOS and iNOS expression levels. Densitometric analyses of the eNOS (B) and iNOS (C) activities were also conducted. (D) The NO content in kidney tissues. Columns represent mean ± SD. *Significant difference vs. Sham group ($P < 0.05$); †significant difference vs. IRI group ($P < 0.05$).

IPC-induced expression of eNOS, iNOS, and NO

Western blot analysis was performed to determine whether IPC induces eNOS and iNOS expression in the kidneys. **Figure 5** showed that eNOS (**Figure 5A**, **5B**) and iNOS (**Figure 5A**, **5C**) expression levels significantly increased in the kidneys of the rats in the IRI group at 24 h after reperfusion compared with the Sham group, and such increase was highly prominent in the IPC group ($P < 0.05$). Furthermore, the NO levels in the kidneys were measured (**Figure 5D**), and found that IPC could significantly increase the NO concentration in renal homogenate compared to the sham group (IPC vs. sham: 0.70 ± 0.09 μmol/g vs. 0.28 ± 0.08 μmol/g; $P < 0.05$) and the IRI group (IPC vs. IRI: 0.70 ± 0.09 μmol/g vs. 0.46 ± 0.10 μmol/g; $P < 0.05$).

Inhibition with L-NAME abolished the renoproctecion of IPC

To determine whether eNOS- and iNOS-mediated NO production conferred the renoprotective effects of IPC, rats were pretreated with L-NAME (5 mg/kg, i.v.) 5 min before IPC. The eNOS, iNOS, and NO expression levels in the kidneys were analyzed at 24 h after reperfusion. **Figure**

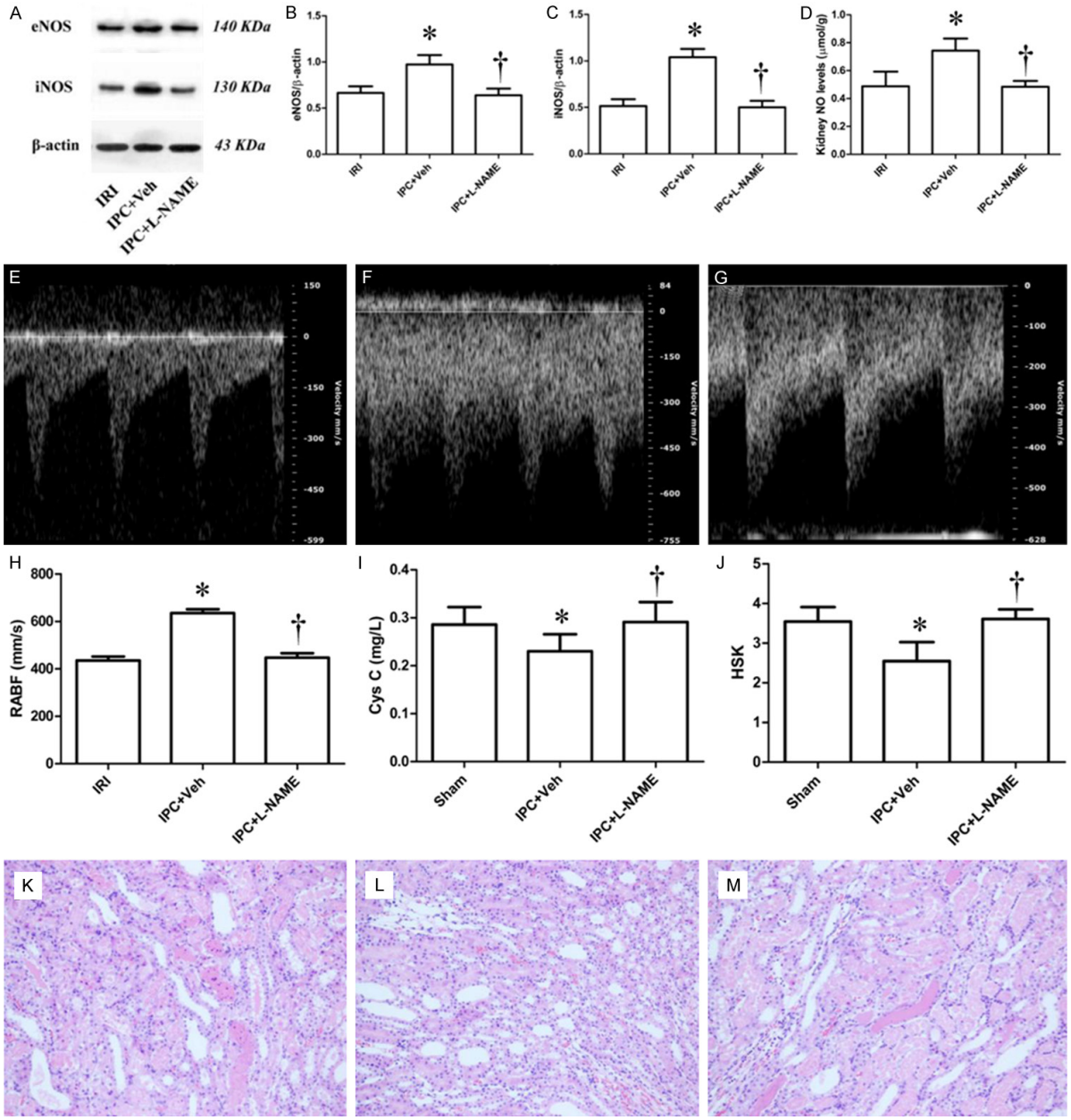
A
eNOS
140 KDa
iNOS
130 KDa
β-actin
43 KDa
IRI
IPC+Veh
IPC+L-NAME
B
eNOS/β-actin
C
iNOS/β-actin
D
Kidney NO levels (μmol/g)
E
F
G
Velocity mm/s
H
RABF (mm/s)
I
Cys C (mg/L)
J
HSK
Sham
K
L
M

Figure 6. Inhibition of eNOS/iNOS by L-NAME significantly attenuated IPC-mediated renoprotection. (A-C) The decreased expression levels of eNOS and iNOS were detected in the IPC + L-NAME group by Western blot analyses. (D) The concentrations of NO in the kidney tissues of different groups. The RABF values of the rats in the IRI (E), IPC + Veh (F), and IPC + L-NAME (G) groups were measured by Doppler ultrasound, and the quantification analysis is presented in (H, I) The serum Cys C levels at 24 h post reperfusion were evaluated. K to M. Representative images of renal tissue histological examination in the IRI (K), IPC + Veh (L), and IPC + L-NAME (M) groups, respectively. (J) HSKs in the three different groups. Columns represent mean ± SD. *significant difference vs. Sham group ($P < 0.05$); †significant difference vs. IRI group ($P < 0.05$).

6A-D showed that L-NAME significantly attenuated the IPC-induced increase in eNOS, iNOS, and NO expression levels. The substantial RABF improvement was remarkably reserved (IPC + L-NAME vs. IPC+ Veh: 447.6 ± 18.8 vs. 635.4 mm/s ± 16.1 mm/s, $P < 0.05$; **Figure 6E-H**), and the beneficial effects of IPC against renal IRI was abolished as proven by the significantly increased serum Cys C levels compared to the IPC + vehicle group (**Figure 6I**). Similar results were confirmed by renal histological examination (**Figure 6J-M**).

Discussion

The current study provides evidence that IPC contributes to the early restoration of RABF through inducing eNOS- and iNOS-mediated NO production, which subsequently attenuates renal dysfunction and histological damage caused by IRI (Figure S2). A recent meta-analysis of animal studies have confirmed that IPC could significantly improve SCr and BUN levels and renal histological damage following IRI [12], which are consistent with the results of our previous studies [14, 15] and the present study. In the current study, we adopted Cys C as an additional marker apart from SCr and BUN to measure renal function, which is an endogenous marker of glomerular filtration rate (GFR) and more sensitive than SCr in the early detection of reduced GFR [23, 24]. Serum Cys C could reflect histological damage more accurately, as Cys C and HSK rather than SCr and BUN, distinguished the attenuation effects of IPC against renal IRI at the very beginning time point (4 h) post reperfusion.

Renal microcirculation disturbance and impaired renal vascular activity that occurs after renal reperfusion are key factors to the development of renal IRI, which could sustain RABF decrease and hamper the full recovery of renal IRI [18, 19]. IPC has been reported to attenuate microcirculation dysfunction through inducing the recruitment and homing of EPCs and overexpression of angiogenic factors [14, 15, 20]; however, the influence of IPC on renal hemodynamics remains far from complete [25, 26]. In the study reported by Ogawa et al, the effects of IPC on renal and systemic hemodynamics were investigated for 60 min post reperfusion with fewer parameters (RABF and mean artery blood pressure). The short study interval and few parameters had limited the capacity to unravel the exact effects of IPC. Furthermore, the underlying mechanisms were not explored [25]. Hence, we stringently designed this study to comprehensively investigate its impact on systemic and renal hemodynamics and the potential underlying mechanisms. In the current study, the Doppler ultrasound method was applied and demonstrated that IPC significantly alleviated the RABF decrease starting from 4 h after reperfusion without significantly affecting RVBF and systemic hemodynamic parameters (SBP, DBP, and HR). To the best of our knowledge, this is the first study to comprehensively examine the potential influence of IPC on renal and systemic hemodynamics for 7 d, and the findings of the present study could help the smooth translation of IPC in the clinical practice.

The possible mechanisms that contributed to the selective vasodilatation of renal artery and improvement of RABF were further explored. The eNOS-/iNOS-mediated NO production has been reputed as a potent regulator of vascular smooth muscle tone and organ perfusion [27-29]. In agreement of these evidence, higher eNOS, iNOS, and NO expression levels were detected in the ischemic preconditioned kidneys, and RABF improvement was negated by the pharmacological inhibition using L-NAME, which is a nonselective NO synthase inhibitor of both eNOS and iNOS. The reversal of the beneficial effects provided by IPC was also observed, thereby suggesting that eNOS and iNOS were involved in IPC-induced renal protection. The eNOS- and/or iNOS-mediated NO production exerts a key function in triggering IPC phenom-

enon in different organs via its antioxidant, anti-apoptotic, and anti-inflammatory properties [4, 21, 30, 31]. The renoprotective effects conferred by IPC were negated by the pharmacological inhibition of eNOS and/or iNOS; and the effects were not observed in eNOS and iNOS knockout mice [32, 33]. Taken together, the upregulating eNOS- and iNOS-mediated NO production through IPC substantially alleviated RABF impairment and preserved injured renal function and morphology.

Even though the results obtained were promising, our study still has limitations. First, the indirect tail-cuff and Doppler ultrasound methods were applied, which could mimic the clinical settings without invasive interventions. However, the pulmonary artery catheter positioned in a branch of the pulmonary artery can continuously and accurately monitor cardiac output, core temperature, and central venous pressure apart from SBP, DBP, and HR [34]. As for evaluating RABF, a perivascular ultrasonic flow probe was utilized in some studies, which could monitor the real-time changes in RABF [18]. Second, the effect of IPC on renal IRI was observed for only 7 d post reperfusion without longer follow-up; and the satisfactory long-term outcomes have been observed by Reutzel-Selke et al. when follow-up treatment was conducted for 12 weeks [35]. Third, the detailed mechanisms that regulate eNOS and iNOS expression levels through IPC and the differential effects of eNOS and iNOS remain unknown and need to be studied in future experiments.

In summary, IPC contributes to the early restoration of RABF, probably through eNOS- and iNOS-mediated NO production, thereby alleviating IRI-induced renal dysfunction and histological damage. Further studies with different measuring methods and long-term follow-up are warranted to validate our results.

Acknowledgements

This study was supported by grants from the National Natural Science Foundation of China (81070597 and 81370853), Science and Education Development Program of the Jiangsu Province Health Board (LJ201107), Six Talent Peaks of the Jiangsu Province Health Bureau (2011-WS-093), and the Research and Innovation Program for Graduates of Jiangsu Province (CXZZ13_0583).

Disclosure of conflict of interest

None.

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References

[1] Munshi R, Hsu C and Himmelfarb J. Advances in understanding ischemic acute kidney injury. BMC Med 2011; 9: 11.

[2] Leung KC, Tonelli M and James MT. Chronic kidney disease following acute kidney injury-risk and outcomes. Nat Rev Nephrol 2013; 9: 77-85.

[3] Bonventre JV and Yang L. Cellular pathophysiology of ischemic acute kidney injury. J Clin Invest 2011; 121: 4210-4221.

[4] Eltzschig HK and Eckle T. Ischemia and reperfusion--from mechanism to translation. Nat Med 2011; 17: 1391-1401.

[5] Zhang J, Yao Y, Xiao F, Lan X, Yu C, Zhang Y, Jiang C, Yang J, Pei G, Li Y, Rong S, Hu S, Li J and Xu G. Administration of dexamethasone protects mice against ischemia/reperfusion induced renal injury by suppressing PI3K/AKT signaling. Int J Clin Exp Pathol 2013; 6: 2366-2375.

[6] Zhao Z, Guan R, Song S, Zhang M, Liu F, Guo M, Guo W, Yu Q, Zhang L and Wang Q. Sinomenine protects mice against ischemia reperfusion induced renal injury by attenuating inflammatory response and tubular cell apoptosis. Int J Clin Exp Pathol 2013; 6: 1702-1712.

[7] McCafferty K, Byrne C and Yaqoob MM. Ischaemic conditioning strategies for the nephrologist: a promise lost in translation? Nephrol Dial Transplant 2014; 29: 1827-40.

[8] Murry CE, Jennings RB and Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. Circulation 1986; 74: 1124-1136.

[9] Ruiz-Meana M, Nunez E, Miro-Casas E, Martinez-Acedo P, Barba I, Rodriguez-Sinovas A, Inserte J, Fernandez-Sanz C, Hernando V, Vazquez J and Garcia-Dorado D. Ischemic preconditioning protects cardiomyocyte mitochondria through mechanisms independent of cytosol. J Mol Cell Cardiol 2014; 68: 79-88.

[10] Lin WY, Chang YC, Ho CJ and Huang CC. Ischemic preconditioning reduces neurovascular damage after hypoxia-ischemia via the cellular inhibitor of apoptosis 1 in neonatal brain. Stroke 2013; 44: 162-169.

[11] O'Neill S, Leuschner S, McNally SJ, Garden OJ, Wigmore SJ and Harrison EM. Meta-analysis of ischaemic preconditioning for liver resections. Br J Surg 2013; 100: 1689-1700.
[12] Wever KE, Menting TP, Rovers M, van der Vliet JA, Rongen GA, Masereeuw R, Ritskes-Hoitinga M, Hooijmans CR and Warle M. Ischemic preconditioning in the animal kidney, a systematic review and meta-analysis. PLoS One 2012; 7: e32296.
[13] Jia RP, Xie JJ, Luo FY and Zhu JG. Ischemic preconditioning improves rat kidney allograft function after ischemia/reperfusion injury: the role of tumor necrosis factor-alpha. Transplant Proc 2008; 40: 3316-3320.
[14] Liu H, Wu R, Jia RP, Zhong B, Zhu JG, Yu P, Zhao Y, Ge YZ and Wu JP. Ischemic preconditioning increases endothelial progenitor cell number to attenuate partial nephrectomy-induced ischemia/reperfusion injury. PLoS One 2013; 8: e55389.
[15] Bo CJ, Chen B, Jia RP, Zhu JG, Cao P, Liu H, Wu R, Ge YZ and Wu JP. Effects of ischemic preconditioning in the late phase on homing of endothelial progenitor cells in renal ischemia/reperfusion injury. Transplant Proc 2013; 45: 511-516.
[16] Snoeijs MG, Vink H, Voesten N, Christiaans MH, Daemen JW, Peppelenbosch AG, Tordoir JH, Peutz-Kootstra CJ, Buurman WA, Schurink GW and van Heurn LW. Acute ischemic injury to the renal microvasculature in human kidney transplantation. Am J Physiol Renal Physiol 2010; 299: F1134-1140.
[17] Boesen EI, Crislip GR and Sullivan JC. Use of ultrasound to assess renal reperfusion and P-selectin expression following unilateral renal ischemia. Am J Physiol Renal Physiol 2012; 303: F1333-1340.
[18] Pechman KR, De Miguel C, Lund H, Leonard EC, Basile DP and Mattson DL. Recovery from renal ischemia-reperfusion injury is associated with altered renal hemodynamics, blunted pressure natriuresis, and sodium-sensitive hypertension. Am J Physiol Regul Integr Comp Physiol 2009; 297: R1358-1363.
[19] Regner KR and Roman RJ. Role of medullary blood flow in the pathogenesis of renal ischemia-reperfusion injury. Curr Opin Nephrol Hypertens 2012; 21: 33-38.
[20] Patschan D, Krupincza K, Patschan S, Zhang Z, Hamby C and Goligorsky MS. Dynamics of mobilization and homing of endothelial progenitor cells after acute renal ischemia: modulation by ischemic preconditioning. Am J Physiol Renal Physiol 2006; 291: F176-185.
[21] Mahfoudh-Boussaid A, Zaouali MA, Hadj-Ayed K, Miled AH, Saidane-Mosbahi D, Rosello-Catafau J and Ben Abdennebi H. Ischemic preconditioning reduces endoplasmic reticulum stress and upregulates hypoxia inducible factor-1alpha in ischemic kidney: the role of nitric oxide. J Biomed Sci 2012; 19: 7.
[22] He K, Chen X, Han C, Xu L, Zhang J, Zhang M and Xia Q. Lipopolysaccharide-induced cross-tolerance against renal ischemia-reperfusion injury is mediated by hypoxia-inducible factor-2alpha-regulated nitric oxide production. Kidney Int 2014; 85: 276-288.
[23] Slocum JL, Heung M and Pennathur S. Marking renal injury: can we move beyond serum creatinine? Transl Res 2012; 159: 277-289.
[24] Xie HG, Wang SK, Cao CC and Harpur E. Qualified kidney biomarkers and their potential significance in drug safety evaluation and prediction. Pharmacol Ther 2013; 137: 100-107.
[25] Ogawa T, Mimura Y, Hiki N, Kanauchi H and Kaminishi M. Ischaemic preconditioning ameliorates functional disturbance and impaired renal perfusion in rat ischaemia-reperfused kidneys. Clin Exp Pharmacol Physiol 2000; 27: 997-1001.
[26] Ogawa T, Mimura Y and Kaminishi M. Renal denervation abolishes the protective effects of ischaemic preconditioning on function and haemodynamics in ischaemia-reperfused rat kidneys. Acta Physiol Scand 2002; 174: 291-297.
[27] Toda N and Toda H. Nitric oxide-mediated blood flow regulation as affected by smoking and nicotine. Eur J Pharmacol 2010; 649: 1-13.
[28] Forstermann U and Sessa WC. Nitric oxide synthases: regulation and function. Eur Heart J 2012; 33: 829-837, 837a-837d.
[29] Rochette L, Lorin J, Zeller M, Guilland JC, Lorgis L, Cottin Y and Vergely C. Nitric oxide synthase inhibition and oxidative stress in cardiovascular diseases: possible therapeutic targets? Pharmacol Ther 2013; 140: 239-257.
[30] Tsutsui H, Tanaka R, Yamagata M, Yukimura T, Ohkita M and Matsumura Y. Protective effect of ischemic preconditioning on ischemia/reperfusion-induced acute kidney injury through sympathetic nervous system in rats. Eur J Pharmacol 2013; 718: 206-212.
[31] Ambros JT, Herrero-Fresneda I, Borau OG and Boira JM. Ischemic preconditioning in solid organ transplantation: from experimental to clinics. Transpl Int 2007; 20: 219-229.
[32] Yamasowa H, Shimizu S, Inoue T, Takaoka M and Matsumura Y. Endothelial nitric oxide contributes to the renal protective effects of ischemic preconditioning. J Pharmacol Exp Ther 2005; 312: 153-159.
[33] Joo JD, Kim M, D'Agati VD and Lee HT. Ischemic preconditioning provides both acute and de-

layed protection against renal ischemia and reperfusion injury in mice. J Am Soc Nephrol 2006; 17: 3115-3123.

[34] Versteilen AM, Korstjens IJ, Musters RJ, Groeneveld AB and Sipkema P. Rho kinase regulates renal blood flow by modulating eNOS activity in ischemia-reperfusion of the rat kidney. Am J Physiol Renal Physiol 2006; 291: F606-611.

[35] Reutzel-Selke A, Pratschke J, Martins PN, Denecke C, Jurisch A, Kotsch K, Pascher A, Neuhaus P and Tullius SG. Ischemic preconditioning produces systemic protective and adoptively transferable effects. Kidney Int 2008; 74: 622-630.

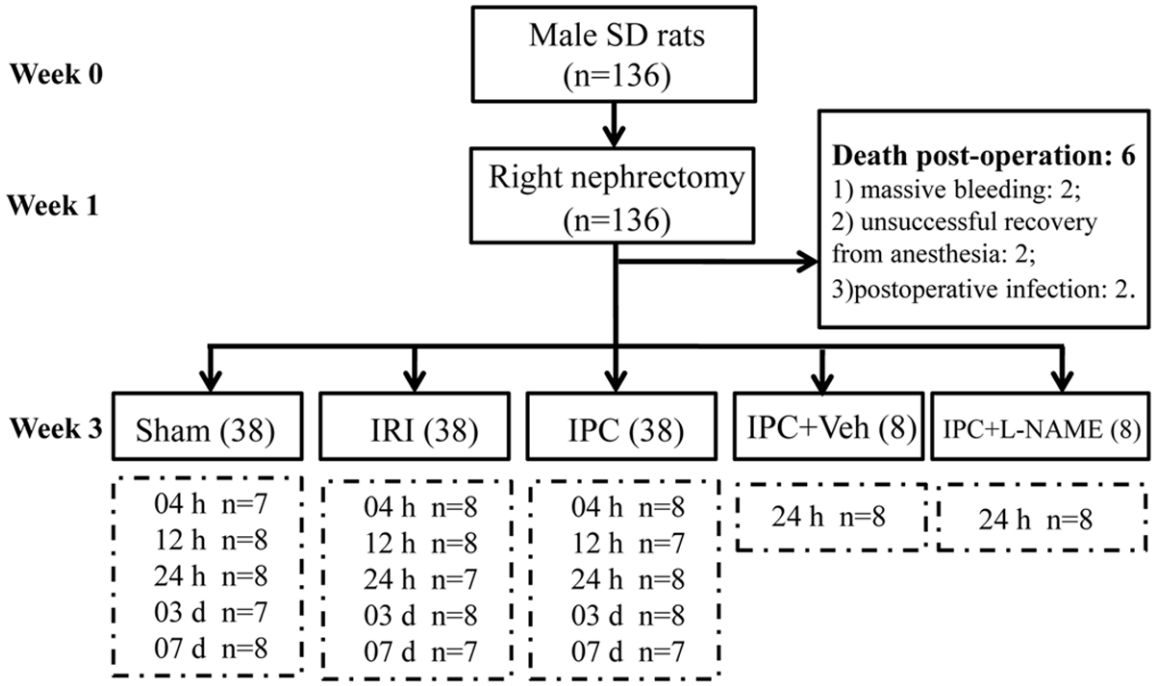

Figure S1. Flow chart of study design. SD, Sprague–Dawley; IRI, ischemia reperfusion injury; IPC, ischemic preconditioning; Veh, vehicle (0.9% saline); L-NAME, NG-nitro-L-arginine methylester (a nonselective NO synthase inhibitor).

Table S1. Body weight of rats included in the current study

	Sham group		IRI group		IPC group		IPC+Veh group		IPC+L-NAME group	
	N[a]	Weight (g)	N[a]	Weight (g)	N[a]	Weight (g)	N[a]	Weight (g)	N[a]	Weight (g)
TOTAL	38	298.3 ± 10.7	38	302.8 ± 10.2	38	299.2 ± 10.3	8	303.9 ± 13.1	8	304.2 ± 9.6
Subgroup										
04 h	7	297.7 ± 8.3	8	303.2 ± 11.5	8	300.7 ± 8.3	NA	NA	NA	NA
12 h	8	292.0 ± 10.0	8	302.7 ± 10.3	7	299.5 ± 11.2	NA	NA	NA	NA
24 h	8	303.5 ± 10.4	7	300.7 ± 10.7	8	293.3 ± 9.5	8	303.9 ± 13.1	8	304.2 ± 9.6
03 d	7	295.5 ± 11.6	8	303.7 ± 8.7	8	305.2 ± 9.3				
07 d	8	302.2 ± 11.2	7	303.3 ± 12.2	7	297.2 ± 11.6				

IRI, ischemia reperfusion injury; IPC, ischemic preconditioning; Veh, vehicle; L-NAME, N^G-nitro-L-arginine methylester; NA, not available. [a]Number of SD rats included.

Table S2. Effects of IPC on renal function changes at various time points post reperfusion

	Sham group	IRI group	IPC group
BUN (mmol/L)			
04 h	6.07 ± 1.50	12.22 ± 2.38*	10.38 ± 1.34*
12 h	6.61 ± 1.46	19.94 ± 4.46*	15.01 ± 3.09*,†
24 h	6.10 ± 2.18	30.29 ± 4.52*	20.76 ±5.18*,†
03 d	6.43 ± 2.12	22.15 ± 3.95*	8.36 ± 2.41†
07 d	6.49± 2.05	8.81 ± 2.64	6.63 ± 2.07
SCr (μmol/L)			
04 h	39.4 ± 8.5	57.2 ± 11.0*	59.9 ± 11.0*
12 h	43.5 ± 6.4	75.9 ± 11.1*	63.1 ± 9.9*,†
24 h	39.0 ± 7.6	101.9 ± 14.9*	80.6 ± 13.5*,†
03 d	41.9 ± 8.7	74.2 ± 12.2*	51.6 ± 10.3†
07 d	38.2 ± 7.2	48.2 ± 11.7	42.5 ± 8.3
Cys C (mg/L)			
04 h	0.10±0.03	0.19 ± 0.04*	0.15 ± 0.02*,†
12 h	0.12 ± 0.02	0.22 ± 0.03*	0.18 ± 0.03*,†
24 h	0.09 ± 0.03	0.29 ± 0.04*	0.23 ± 0.03*,†
03 d	0.11 ± 0.03	0.20 ± 0.03*	0.13 ± 0.02†
07 d	0.10 ± 0.03	0.16 ± 0.05*	0.13 ± 0.05

IRI, ischemia reperfusion injury; IPC, ischemic preconditioning; BUN, blood urine nitrogen; SCr, serum creatinine; Cys C, cystatin C. *< 0.05 vs. Sham group; †<0.05 vs. IRI group.

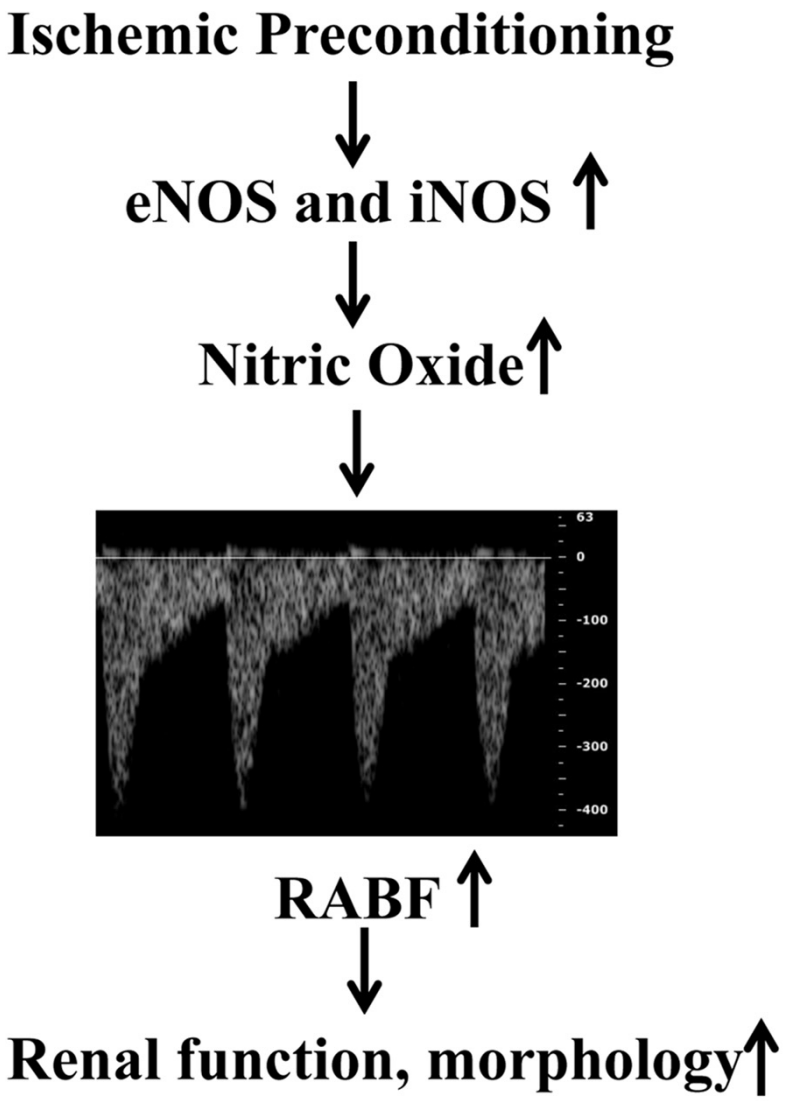

Figure S2. A model illustrating the role of eNOS-/iNOS-mediated NO production in the improvement of renal artery blood flow and renoprotection of IPC.