ELSEVIER

Effects of Ischemic Preconditioning in the Late Phase on Homing of Endothelial Progenitor Cells in Renal Ischemia/Reperfusion Injury

C.-J. Bo, B. Chen, R.-P. Jia, J.-G. Zhu, P. Cao, H. Liu, R. Wu, Y.-Z. Ge, and J.-P. Wu

ABSTRACT

Objective. The aim of this study was to determine whether the mobilization and recruitment of endothelial progenitor cells (EPCs) contribute to the protection of kidneys from ischemia/reperfusion (I/R) injury after ischemic preconditioning (IPC) during the late phase.

Methods. Seventy-five male Sprague-Dawley rats were divided into the following groups: sham-operated (group A; n = 25), ischemia/reperfusion hosts that underwent 45 minutes of left renal artery ischemia (group B; n = 25), and ischemic preconditioning-treated group (group C; n = 25). Group C underwent 3 cycles of 5 minutes of occlusion and 5 minutes of reperfusion followed by 24 hours of reperfusion before the following 45 minutes of occlusion. Serum samples were collected and renal tissues harvested for histological examination terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay, immunohistochemical staining, and Western blot analysis to determine the expression levels of CD34, VEGFR-2 (Vascular Endothelial Growth Factor Receptor 2)/flk-1, vascular endothelial growth factor (VEGF), and stromal cell–derived factor-1α (SDF-1α).

Results. Compared with group B, the levels of blood urea nitrogen (BUN), serum creatinine (Scr) and acute tubulointerstitial injury at 24 hours after operation were significantly reduced in group C. At 72 hours, tubular epithelial cell apoptosis was also decreased (17.6 ± 4.45 vs 63.8 ± 6.10; $P < .01$). CD34+ and flk-1+ cells that mostly accumulated in the medullopapillary parenchyma were significantly increased at 72 hours ($P < .05$). Expression levels of VEGF and SDF-1α were also significantly higher in group C ($P < .05$).

Conclusion. The present work suggested that IPC protected kidneys from IR injury in the later phase through enhanced mobilization and recruitment of EPCs. VEGF and SDF-1α may play important roles in this protective effect.

ACUTE renal failure (ARF) is a common, severe clinical problem for which effective therapy is currently lacking. In daily clinical practice ischemia/reperfusion (I/R) injury is the most frequent cause of ARF. Although it may cause various pathological conditions, ARF mostly affects the function and structure of tubular epithelial cells. Microvascular endothelial cell dysfunction (ECD) in peritubular capillaries inhibits postischemic renal reperfusion, thereby prolonging kidney malfunction.[1,2] Ischemic preconditioning (IPC), a well-known phenomenon involving one or more brief episodes of ischemia, protects various tissues from subsequent I/R injury.[3–5] The protective effects after IPC include 2 distinct phases: the early phase develops rapidly from the time of the initial ischemic insult, lasting 2 to 3 hours; and the later phase, which begins 12 to 24 hours after the initial insult, persisting for several days.[6–8] Our previous studies have shown that IPC protects the kidney from

From the Department of Urology and Transplantation (C.-J.B., R.-P.J., J.-G.Z., P.C., H.L., R.W., Y.-Z.G., J.-P.W.), Nanjing First Hospital Affiliated to Nanjing Medical University, Nanjing, and Department of Urology (B.C.), Southeast University, Nanjing, China.

C.-J.B. and B.C. contributed equally to the present work and each is considered first author.

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Address reprint requests to Rui-Peng Jia, PhD, Department of Urology and Transplantation, Changle Road, Qinhuai District No. 68, Nanjing First Hospital Affiliated to Nanjing Medical University, Nanjing 210006, China.

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Table 1. Effects of IPC on BUN mM Levels After Reperfusion

Group	3 h (n = 5)	24 h (n = 5)	3 d (n = 5)	7 d (n = 5)	14 d (n = 5)
A	5.23 ± 0.43	5.69 ± 0.56	5.91 ± 0.81	5.57 ± 0.44	5.88 ± 0.65
B	9.24 ± 1.46*	20.70 ± 2.05*	14.52 ± 1.84*	9.47 ± 1.01*	6.62 ± 0.57*
C	6.91 ± 1.25†	13.07 ± 1.09*†	9.07 ± 1.15*†	6.36 ± 0.64†	5.92 ± 0.78

Abbreviations: group A, sham operation; group B, I/R treatment; group C, IPC treatment.
*$P < .05$ vs group A.
†$P < .05$ vs group B.

renal I/R injury through inhibition of tumor necrosis factor (TNF)-alpha and its feedback signaling on NF-κB pathways, but the mechanism remains unclear, especially with respect to late IPC.[9,10] Interestingly, recent investigations have observed that after IPC endothelial progenitor cells (EPCs) mobilize to ischemic organs.[11,12] Mobilization of EPCs from their niches may be due to ischemic stress generating specific cell factors. EPCs can be efficiently delivered to ischemic tissues, preserving or restoring organs by participating in vasculogenesis. We investigated the relationship between EPCs and late IPC. A renal I/R injury model was used to determine whether IPC contributed to the recruitment of mobilized EPCs and whether IPC enhanced the release of protective cytokines, including vascular endothelial growth factor (VEGF) and stromal cell–derived factor-1α (SDF-1α).

MATERIALS AND METHODS

Rat Model and Surgical Procedure

All protocols were approved by our Institutional Animal Care and Use Committee. Seventy-five male Sprague-Dawley rats, weighing 230–270 g and provided by our Experimental Animal Center, were maintained under pathogen-free conditions with free access to food and water. They were anesthetized with sodium pentobarbital (40 mg/kg intraperitoneally) and placed on a warming table to maintain a rectal temperature of 37°C. Via a midline laparotomy we removed the right kidney and separated the left renal artery that was subjected to ischemia and reperfusion. The rats were divided into 3 groups: Group A, sham-operated controls that only underwent the surgical procedure (without the clamping of the renal artery); group B, I/R animals that had their arteries occluded with a nontraumatic vascular clamp for 45 minutes while the kidney was kept warm and moist during the observation period; group C, animals that underwent the IPC protocol of 3 cycles of 5 minutes of occlusion and 5 minutes of reperfusion. The other procedures were the same as in group B at 24 hours after IPC. Subsequently, blood urea nitrogen (BUN), serum creatinine (Scr), kidney morphology, and apoptosis were observed at 3 hours, 24 hours, 72 hours, 7 days, and 14 days after reperfusion (n = 5 each). One half of each harvested kidney was fixed in 10% formalin. The other half flash-frozen in liquid nitrogen was stored at −80°C. Blood drawn from the inferior vena cava after reperfusion at each time was centrifuged at 2000*g* for 5 minutes for equal volumes of supernate to be stored at −80°C.

Serum Levels of BUN and Cr

Serum BUN and Cr levels were measured using clinical automated analysis (Hitachi 7020, Hitachi High-Technologies Corporation, Tokyo, Japan).

Histological Examination

Renal tissues fixed in 10% neutral buffered formalin for 24 hours and embedded in paraffin, were sectioned at 4 μm. After gradual deparaffinizing and hydration, they were examined using hematoxylin-eosin staining. Tissue sections were scored in blinded fashion, using a previously described semiquantitative scale designed to evaluate the degree of tubular necrosis. Tubulointerstitial injury was defined as tubular necrosis, tubular dilatation and/or atrophy, inflammatory cell infiltration, or cellular edema. Histopathologic scores of kidneys (HSK) were graded on a scale of 0–4 with higher values representing more severe damage: 0, normal kidney; 1, minimal necrosis (<25% involvement of the cortex or outermedulla); 2, mild necrosis (25%–50% involvement of the cortex or medulla); 3, moderate necrosis (50%–75% involvement of the cortex or medulla; and 4, severe necrosis (>75% involvement of the cortex or medulla).[9]

Terminal Deoxynucleotidyl Transferase dUTP Nick Endlabeling Assay

Terminal deoxynucleotidyl transferase dUTP nick endlabeling (TUNEL) staining was performed using the in situ Apoptosis Detection Kit (Roche, Basel, Switzerland), according to the manufacturer's instructions. The number of TUNEL-positive versus total cell nuclei were counted in 10 random high-power fields (×400) containing at least 500 cells to calculate an apoptotic index (AI). The percentages of TUNEL-positive to total nuclei were defined as the AI.

Estimation of the Recruitment of EPCs by Immunohistochemical Staining

For immunostaining 4 μm paraffinized sections were dewaxed and washed 3 times for 5 minutes each in PBST (PBS, pH 7.4, 0.05%

Table 2. Effects of IPC on Scr (μmol/L) Levels After Reperfusion

Group	3 h (n = 5)	24 h (n = 5)	3 d (n = 5)	7 d (n = 5)	14 d (n = 5)
A	50.2 ± 4.51	54.8 ± 5.56	50.6 ± 5.17	52.1 ± 5.83	48.9 ± 4.55
B	68.4 ± 7.09*	153.8 ± 17.34*	119.0 ± 14.40*	83.3 ± 8.44*	57.4 ± 6.35*
C	57.8 ± 6.57†	93.4 ± 6.47*†	77.4 ± 5.94*†	71.6 ± 5.90*†	52.8 ± 6.91

*$P < .05$ vs group A.
†$P < .05$ vs group B.