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Postconditioning ameliorates mitochondrial DNA damage and deletion after renal ischemic injury

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Keywords: mitochondrial DNA, mitochondrial K+ (K_{ATP}) channel, postconditioning, reactive oxygen species, renal protection

ORIGINAL ARTICLE

ABSTRACT

Background. Reactive oxygen species (ROS) play a major role in causing injury in ischemia-reperfusion (I/R). Mitochondrial DNA (mtDNA) is particularly vulnerable to oxidative damage. We propose that increased mitochondrial ROS production is likely to damage mtDNA, causing further injury to mitochondria, and postconditioning (POC) may ameliorate kidney I/R injury by mitigating mitochondrial damage.

Methods. Rats were divided into seven groups: (i) Sham-operated animals with an unconstricted renal artery; (ii) Sham + 5-hydroxydecanoate (5-HD); (iii) I/R; (iv) I/R + 5-HD; (v) POC; (vi) Sham POC and (vii) POC + 5-HD. Renal injury, oxidative DNA damage, mtDNA deletions, mitochondrial membrane potential (MMP) and expression of the ATP-sensitive K^+ (K_{ATP}) channel subunit Kir6.2 were evaluated.

Results. Following 1 h of reperfusion, animals in the I/R group exhibited increased ROS, oxidative mtDNA damage shown by 8-hydroxy-2-deoxyguanosine staining, multiple base pair deletions and decreased MMP. However, POC rats exhibited less ROS, oxidative mtDNA damage and deletions and improved MMP. After 2 days of reperfusion, serum creatinine was elevated in I/R rats and the number of TdT-mediated dUTP nick-end labeled-positive tubular cells was increased and was associated with activation of caspase-3. Therefore, POC prevented the deleterious effects of I/R injury. Furthermore, the expression of mitochondrial Kir6.2 was widely distributed in renal tubular epithelial cells in Sham and POC rats and was lower in I/R rats. All of the protective effects of POC were reversed by the K^+ (K_{ATP}) channel blocker 5-HD.

Conclusion. POC may attenuate I/R injury by reducing mitochondrial oxidative stress and mtDNA damage and sustaining MMP.

INTRODUCTION

Ischemia/reperfusion (I/R) injury in the kidney accounts for the majority of acute kidney injury and represents an important cause of morbidity and mortality of hospitalized patients [1, 2]. Kidney I/R injury is mainly caused by a large amount of reactive oxygen species (ROS) and reperfusion-induced inflammatory response, which lead to a combination of apoptosis and necrosis [3, 4]. It has been reported that ischemic preconditioning (IPC), a non-lethal period of ischemia, rendered the kidney refractory to subsequent and severe ischemic stress [5, 6]. However, the unpredictable occurrence of

ischemia and the controversial effects in large animal models limit the clinical application of IPC.

The protective effect of ischemic postconditioning (POC), which is defined as a series of brief alternating periods of arterial reperfusion and re-occlusion applied at the early phase of reperfusion, was originally documented by Zhao *et al.* [7] in a canine heart ischemia model. Recently, POC has been further studied in the brain, heart, liver and kidney [8–11]. Compared with IPC, POC has two major advantages: first, POC can be conducted after ischemia, which should improve the chances for helping patients and second, ischemia in solid organs is unpredictable, which limits the application of IPC. Although the POC strategy has been effectively applied to the experimental ischemic kidney in the rat and mongrel dog [8, 12], the mechanisms of POC are still unclear. Experimental data indicate that it may reduce ROS generation by the mitochondria and reduce lipid peroxidation and cellular apoptosis [13]. Our previous studies documented that excessive mitochondrial ROS production plays an important role in reperfusion injury by triggering mitochondrial DNA (mtDNA) injury even at 1 h after reperfusion [3]. Strikingly, agents that open the ATP-sensitive K^+ (K_{ATP}) channel have been found to be effective in preventing cardiac, neural and renal injury [3, 14–17]. We hypothesized that application of the POC strategy could attenuate renal I/R injury by dramatically preventing early-mitochondrial free radical generation during reperfusion and ameliorating mtDNA damage. We tested this hypothesis in rats subjected to severe kidney I/R injury.

METHODS

Reagents and antibodies

Pentobarbital sodium, 5-hydroxydecanoate (5-HD) and mitochondria isolation kits were purchased from Sigma-Aldrich (St Louis, MO, USA). 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide (JC-1), Amplex Red H_2O_2/peroxidase assay kit, dichlorodihydrofluorescein (CM-H_2DCFDA) and 4′,6-diamidino-2-phenylindole (DAPI) were purchased from Invitrogen (Carlsbad, CA, USA). Antibody against 8-hydroxy-2-deoxyguanosine (8-OHdG) was from JAICA (Shizuoka, Japan). Anti-nitrotyrosine antibody was from Invitrogen (Carlsbad, CA, USA). Anti-Kir6.2 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against the voltage-dependent anion channel (VDAC), cleaved caspase-3 and β-actin were from Cell Signaling Technology (Beverly, MA, USA). All the secondary antibodies were from Jackson ImmunoResearch (Pittsburgh, PA, USA).

Animals

Male Sprague-Dawley rats (SD rats, 8–10 weeks old; Changchun, China) were maintained in a pathogen-free facility at Jilin University in a manner that conformed to the *Guide for the Care and Use of Laboratory Animals* [U.S. National Institutes of Health, DHEW publication No. (NIH 85-23, 1996)] and cared for under a protocol approved by the Institutional Animal Care and Use Committee of Jilin University.

In vivo model of I/R

SD rats were placed on a homeothermic table to maintain the core body temperature at 37°C. Rats were anesthetized with an i.p. injection of 25 mg/kg pentobarbital sodium and underwent right nephrectomy followed by renal artery ischemia for 45 min. Serum creatinine (Cr) was measured 2 and 7 days following renal ischemia by the hospital laboratory, and kidneys were harvested and stored at −80°C until further analysis. Rats were divided into seven groups: (i) Sham-operated animals with an unconstricted renal artery; (ii) Sham + 5-HD group: Sham animals treated with 5 mg 5-HD/kg i.m. dissolved in saline; (iii) I/R kidneys were subjected to 45 min of ischemia followed by reperfusion; (iv) I/R + 5-HD: animals treated with 5 mg 5-HD/kg i.m. 5 min before ischemia; (v) POC kidneys were subjected to three cycles of 30 s of reperfusion followed by 30 s of ischemia immediately after 45 min of ischemia and 7 min short-time full reperfusion, then followed by reperfusion (Figure 1A); (vi) ischemia rats subjected to the sham POC protocol; (vii) 5-HD + POC group: animals treated with 5 mg 5-HD/kg i.m. 5 min before ischemia, then subjected to POC.

Preparation of mitochondria

Mitochondrial fractions were isolated from kidneys using mitochondria isolation kits according to the manufacturer's instructions. Briefly, 200 mg kidney tissue was homogenized in 5 mL extraction buffer (10 mM HEPES, pH 7.5, containing 200 mM mannitol, 70 mM sucrose and 1 mM EGTA) with a homogenizer powered by an overhead electric motor at 200 g, and the pestle was moved up and down 5–10 times to ensure the total homogenization. The homogenate was centrifuged at 800 *g* for 10 min to precipitate the nuclear fraction. The supernatant was then centrifuged at 7000 *g* for 10 min to yield the mitochondrial fraction. The mitochondrial pellet was suspended in 0.5 mL storage buffer (10 mM HEPES, pH 7.4, containing 250 mM sucrose, 1 mM ATP, 0.08 mM ADP, 5 mM sodium succinate, 2 mM K_2HPO_4 and 1 mM dithiothreitol). For assays, protein concentration was adjusted to 1.5–2.0 mg/mL. All procedures were carried out at 0–2°C.

Determination of mitochondrial membrane potential

Isolated kidney mitochondria were resuspended (final protein concentration = 100 μg/mL) in 1 mL of storage buffer in an attempt to mimic cytosolic and mitochondrial exposure conditions [18] but free of cytosolic factors that are generated during ischemic injury. Mitochondria were incubated with 1 μg/mL JC-1 for 10 min at 37°C according to the manufacturer's instructions. The electrical potential across the inner mitochondrial membrane ($\Delta\psi$) was detected by using a spectrofluorometer (Flexstation II; Sunnyvale, CA, USA) and monitoring the fluorescence of JC-1 at an excitation wavelength of 485 or 544 nm and an emission wavelength of 538 or 590 nm.

In situ detection of ROS production by dichlorodihydrofluorescein (CM-H_2DCFDA) staining

After 1 h or 2 days of reperfusion, rats were anesthetized with pentobarbital sodium and CM-H_2DCFDA (100 μg) was

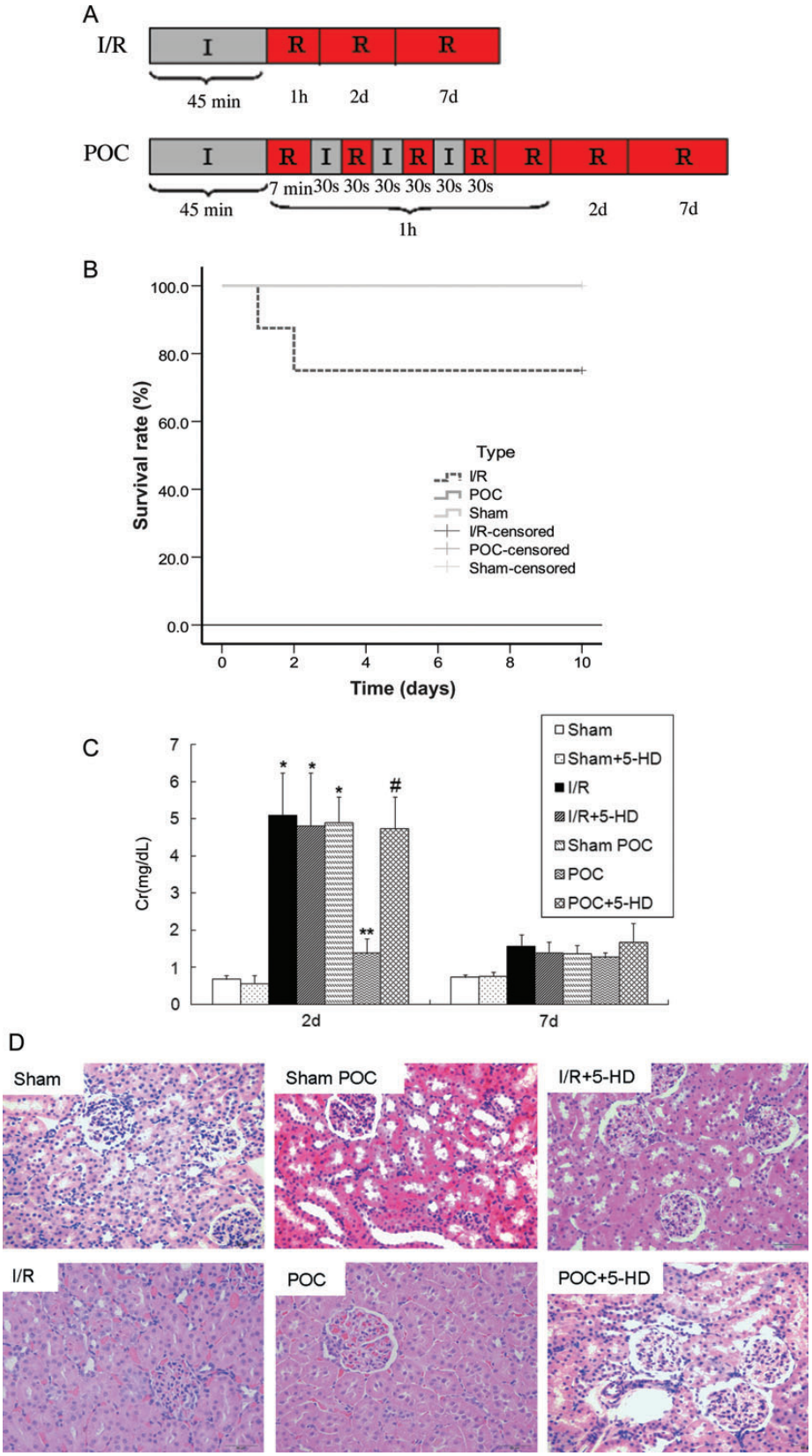

FIGURE 1: POC attenuates renal I/R injury. (**A**) Protocol for POC algorithm. (**B**) Survival rate. (**C**) Effect of POC on serum Cr. Serum Cr increased in the I/R, 5-HD + I/R and Sham POC groups compared with that of the Sham-operated group. However, POC treatment significantly decreased serum Cr, but this effect was reversed by 5-HD (mean ± SE; $n = 8$). *$P < 0.001$ versus Sham group, **$P < 0.01$ versus I/R group; #$P < 0.05$ versus POC group. (**D**) Histological evaluations of renal tissue stained with H&E. Original magnification ×40. Results are representative of eight animals in each group.

injected into the renal circulation as described elsewhere [19]. The kidney was harvested 45 min after CM-H_2DCFDA injection and fixed in 4% paraformaldehyde for 24 h. After treatment with 20% sucrose for 12 h, renal tissue was immediately frozen in liquid nitrogen, and cryostat sections (5 µm) were cut in a cabinet maintained at −20°C. The sections were placed on Star-Frost adhesive slides and air-dried for 3 min at room temperature. Sections were washed in PBS and then co-stained with DAPI for fluorescence microscopy analysis.

ROS release measurements

ROS production in isolated mitochondria was measured using the Amplex Red H_2O_2/peroxidase assay kit according to the manufacturer's instructions. Mitochondrial suspensions were incubated in the presence of 50 µM Amplex Red and 0.1 U/mL horseradish peroxidase, and fluorescence was monitored over time using a temperature-controlled (37°C) spectrofluorometer (Flexstation II; Sunnyvale, CA, USA) operating at excitation and emission wavelengths of 544 and 590 nm, respectively, with gentle continuous stirring.

Renal histopathology

Kidneys were excised and harvested 1 h or 2 days following 45 min of ischemia. Paraffin-embedded sections (4 µm) were stained with hematoxylin and eosin (H&E). Slides (4 µm) were prepared from paraffin-embedded blocks for 8-OHdG staining as described elsewhere [12]. The slides were incubated with anti-8-OHdG antibody (1:100) at 4°C overnight and stained with diaminobenzamide tetrahydrochloride (DAB) and counterstained with hematoxylin. Oxidative damage was further detected by using a specific mouse monoclonal antibody against nitrotyrosine (1:200). For caspase-3 staining, slides were incubated with anti-cleaved caspase-3 antibody (1:200). Apoptosis was assessed by the terminal deoxynucleotidyl-transferase-mediated dUTP nick-end labeling (TUNEL) Assay Kit (*In Situ* Cell Death Detection Kit; Roche, Basel, Switzerland) according to the manufacturer's instructions. Sections were also counterstained with hematoxylin to identify nuclei. The results of staining were analyzed and evaluated with the American Image-Pro Plus software (Media Cybernetics; Silver Spring, MD, USA). The percentage of positive cells with TUNEL staining in five 400× fields served as the index of apoptosis. For 8-OHdG and TUNEL double staining, 4 µm sections from frozen tissue were incubated with mouse anti-8-OHdG antibody (1:100) at room temperature for 1.5 h and then with Cy3-labeled donkey anti-mouse IgG (1:200) for 30 min, then followed by TUNEL staining. For Kir6.2 and VDAC staining, 4 µm sections from frozen tissue were incubated with goat anti-Kir6.2 antibody (1:200) and rabbit anti-VDAC antibody (1:200) at room temperature for 1.5 h and then with fluorescein isothiocyanate-labeled donkey anti-goat IgG (1:200) and Cy3-labeled donkey anti-rabbit IgG (1:200) for 30 min. Cell nuclei were stained blue with DAPI. Tissue sections were analyzed by fluorescence microscopy.

Western blot analysis

Cleaved caspase-3 antibody (1:1000) was used for western blotting to quantitate active caspase-3. Monoclonal antibody against β-actin (1:1000) was used as a control for equal protein loading. Kir6.2 antibody (1:1000) and VDAC antibody (1:1000) were used to quantitate Kir6.2 and VDAC expression in mitochondrial fractions, respectively. After reacting with the primary and horseradish peroxidase-conjugated secondary antibodies, protein bands were visualized by chemiluminescence (Bio-Rad; Hercules, CA, USA).

Detection of mtDNA deletion by polymerase chain reaction

Total mtDNA was extracted from the isolated mitochondria using the DNAeasy blood and tissue kit (Qiagen; Dusseldorf, Germany). mtDNA deletions were assessed as previously described [3]. Briefly, the primer sets for amplification of the common mtDNA deletion were 5′-TTTCTTCCCAAACC TTTCCT-3′ and 5′-AAGCCTGCTAGGATGCTTC-3′. The primer sets for control wild-type mtDNA were 5′-GGTTCT TACTTCAGGGGCCATC-3′ and 5′-GTGGAATTTTCTGA GGGTAGGC-3′. Sequence and numbering are based on the rat complete mitochondrial genome (GenBank accession no. AJ428514). PCR products were electrophoresed on 1.5% agarose gels and visualized with ethidium bromide staining.

Statistics

Values are means ± SEM of n independent experiments. Statistical significance was determined by ANOVA; $P < 0.05$ was considered significant.

RESULTS

Renal function after I/R

In survival experiments, two of eight rats in the I/R group died during the 12 days following I/R injury and right nephrectomy, but all animals in the POC group survived (Figure 1B). At 2 days after reperfusion, serum levels of Cr were significantly higher in I/R rats compared with Sham rats ($P < 0.001$), but were lower in POC rats compared with I/R rats ($P < 0.01$). However, 5-HD reversed the action of POC (Figure 1C). In all groups, Cr levels were closer to normal 7 days after reperfusion.

Histological changes

H&E staining of paraffin sections demonstrated no significant morphological changes in renal glomerular or tubular cells in the Sham group (Figure 1D). No pathological changes were detected in any of the groups at 1 h after reperfusion (data not shown). At 2 days, the I/R, 5-HD + I/R and Sham POC groups showed swelling of renal tubular epithelial cells and intraluminal necrotic cellular debris, vacuolar degeneration, luminal narrowing, interstitial congestion and edema, and formation of proteinaceous casts. POC attenuated these severe renal damages. In contrast, 5-HD antagonized renal protection of POC (Figure 1D).

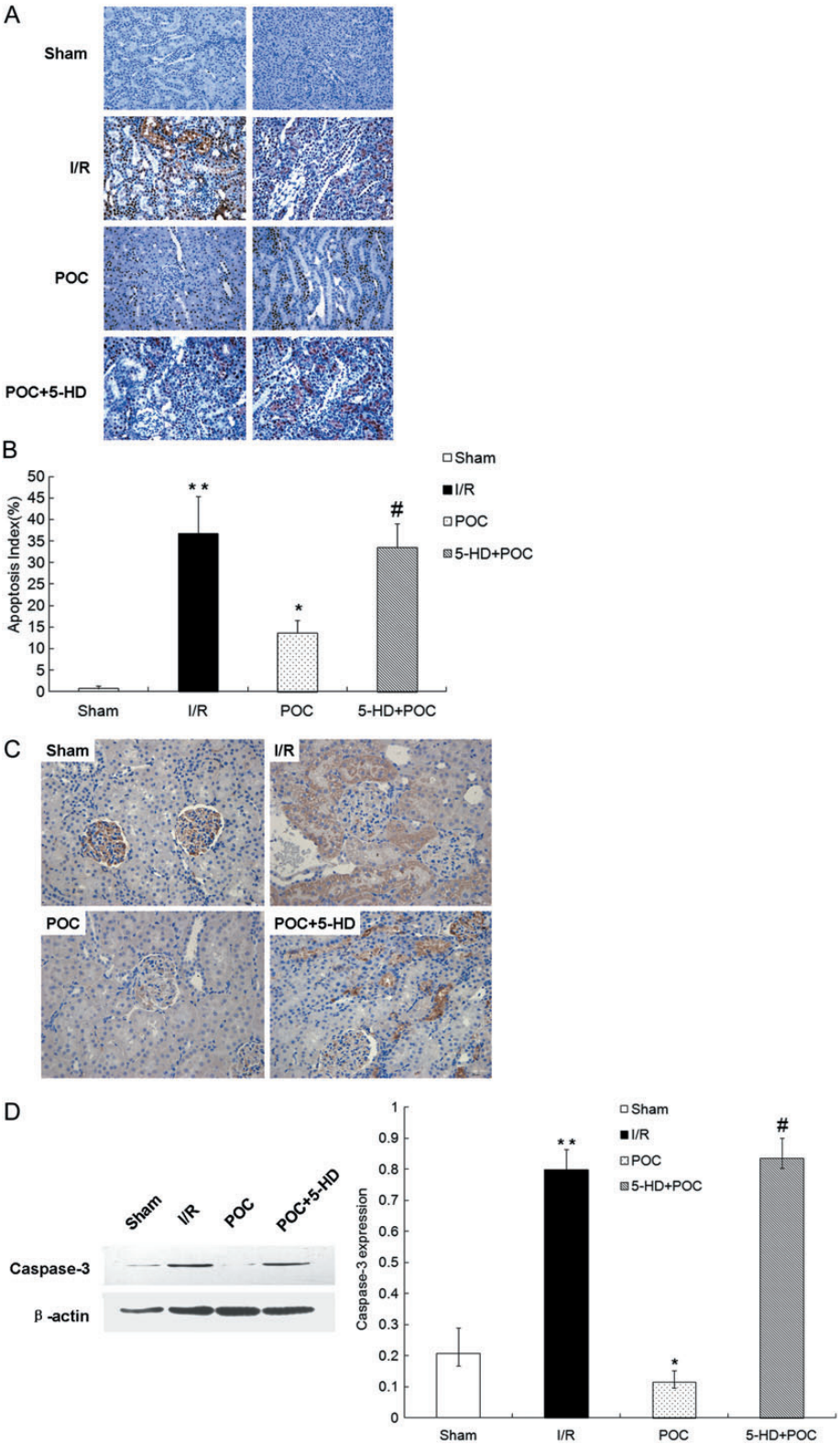

FIGURE 2: POC inhibits the activation of apoptosis in ischemic kidneys after 2 days of reperfusion. (**A**) Representative sections of nuclear DNA fragmentation staining performed by TdT-mediated dUTP nick-end labeling (TUNEL) with DAB; nuclei were counterstained with hematoxylin. Original magnification × 40. Scale bar, 50 μm. Results are representative of three animals in each group. (**B**) Quantitative analysis of the number of TUNEL-positive renal tubular epithelial cells. Data are presented as the mean ± SD. $^{**}P < 0.001$ versus Sham group, $^{*}P < 0.01$ versus I/R group; $^{\#}P < 0.05$ versus POC group. (**C**) Immunohistochemical staining for activated caspase-3. (**D**) Western blot analyses of activated caspase-3 expression. β-actin was used as a loading control. Expression of cleaved caspase-3 proteins was significantly increased in kidneys 2 days after I/R. POC treatment decreased cleaved caspase-3 expression but this was reversed by 5-HD. Representative data of three individual samples per group. $^{**}P < 0.01$ versus Sham group, $^{*}P < 0.01$ versus I/R group; $^{\#}P < 0.01$ versus POC group.

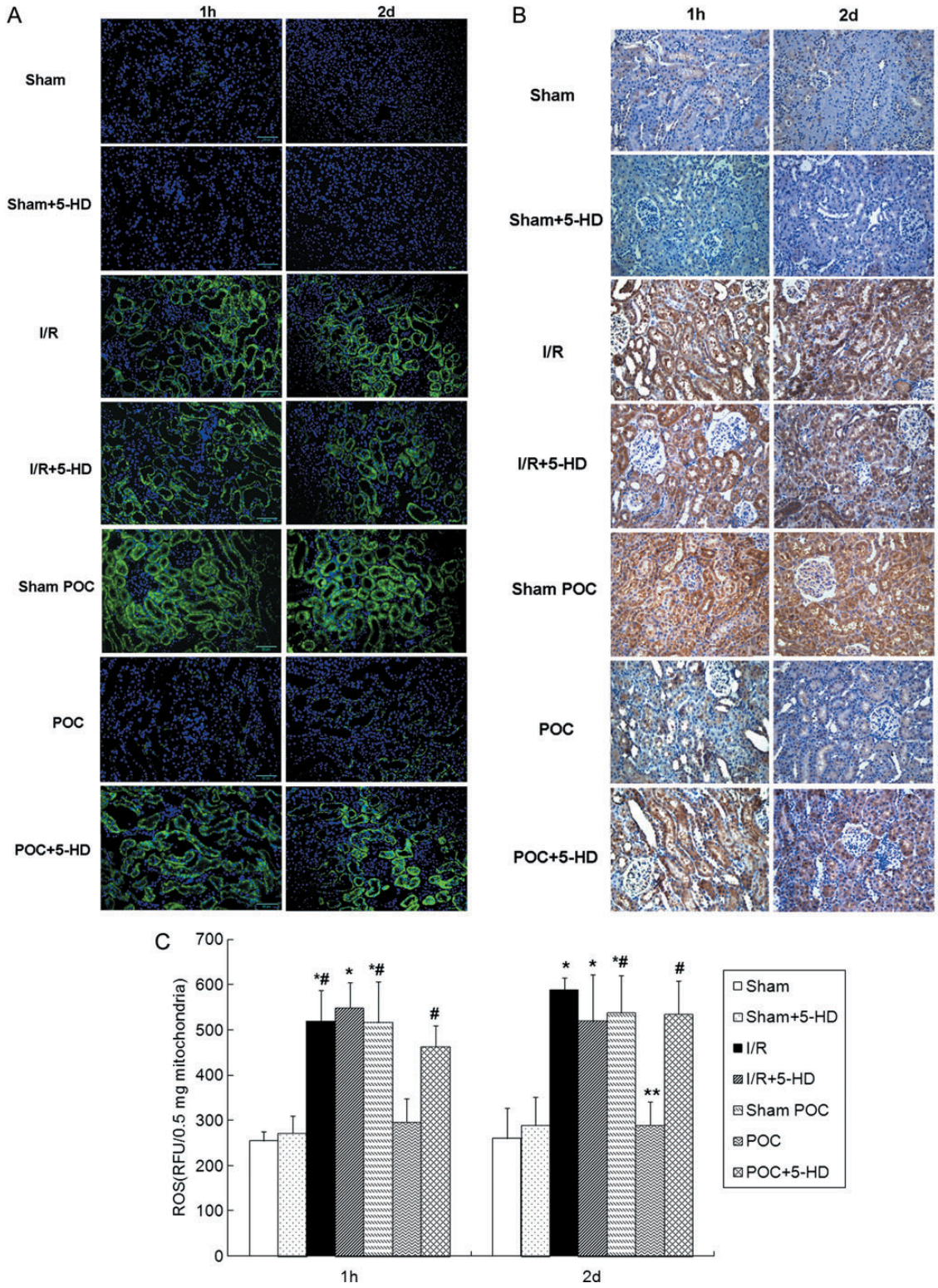

FIGURE 3: Free radical generation in ischemic kidneys after reperfusion. (**A**) Fluorescence microscopy detection of ROS generation by dichlorodihydrofluorescein (CM-H_2DCFDA). At 1 h and 2 days after reperfusion, a large number of tubular epithelial cells were strongly CM-H_2DCFDA positive; POC dramatically decreased ROS production in tubules. Glomeruli, interstitium and inflammatory cells reacted negatively to CM-H_2DCFDA. (**B**) Immunohistochemistry staining of nitrotyrosine. After 1 h and 2 days of reperfusion, kidney tissue sections obtained from I/R rats showed positive staining for nitrotyrosine mainly localized in tubular epithelial cells. POC reduced nitrotyrosine to levels found in Sham rats. Original magnification ×40. Renal tissue sections from 1 of 4 animals in each group are shown. (**C**) Effect of POC on mitochondrial ROS production. ROS increased in I/R, 5-HD + I/R and Sham POC groups compared with that of the Sham-operated group. However, POC treatment significantly decreased mitochondrial ROS, but this effect was reversed by 5-HD (mean ± SE; $n = 4$). At 1 h, *$P < 0.05$ versus Sham group, $^{\#}P < 0.05$ versus POC group; at 2 days, *$P < 0.05$ versus Sham group, $^{\#}P < 0.05$ versus POC group, **$P < 0.01$ versus I/R group.

Activation of apoptosis

TUNEL staining of kidney tissue sections revealed that few TUNEL-positive cells were present in kidneys 1 h after reperfusion (data not shown). However, TUNEL-positive tubular epithelial cells were plentiful 2 days after reperfusion, except in POC kidneys (Figure 2A). Similar to the Cr results, the proportion of TUNEL-positive cells was significantly lower in the POC kidneys compared with the I/R kidneys (Figure 2B). To determine the possible pathway of I/R injury, immunohistochemistry staining of activated caspase-3 was performed. Expression of cleaved caspase-3 protein was significantly increased in kidneys 2 days after I/R and in animals treated with 5-HD + POC, whereas cleaved caspase-3 expression was lower in the POC group (Figure 2C). This finding was further validated by western blotting. There was little expression of cleaved caspase-3 in POC renal tissue extracts compared with I/R and 5-HD + POC groups (Figure 2D).

Generation of free radicals

Few CM-H_2DCFDA-positive cells were present in tissue sections from Sham and 5-HD + Sham kidneys. As previously documented [3], I/R injury increased mitochondrial ROS production after reperfusion, as demonstrated by strong tubular epithelial cell staining (CM-H_2DCFDA fluorescence) of kidney tissue sections. POC dramatically decreased ROS production in tubules to nearly non-ischemic control levels at all-time periods (Figure 3A). Further, nitrotyrosine immunohistochemistry staining was performed to indicate peroxynitrite formation. Nitrotyrosine staining was strong in tubules in reperfusion kidneys except POC-treated animals (Figure 3B). Both CM-H_2DCFDA fluorescence and nitrotyrosine staining demonstrated that POC could reduce oxidative stress in I/R kidneys.

ROS production in isolated intact mitochondria was measured by the Amplex Red H_2O_2/peroxidase detection kit. After 1 h and 2 days of reperfusion, significantly increased levels of H_2O_2 in the mitochondrial fraction in I/R, 5-HD + I/R and Sham POC kidneys were detected compared with sham-operated kidneys (Figure 3C). Interestingly, POC treatment reduced the generation of H_2O_2 by the mitochondria to near levels in sham-operated controls, but this effect was blunted by the mitochondria-specific K_{ATP} channel blocker 5-HD (Figure 3C). These results indicate that I/R injury increased mitochondrial ROS production, and that POC treatment prevented the early and subacute effects by opening mitochondrial K_{ATP} channels.

Oxidative mtDNA damage and deletions

It is well accepted that mtDNA is more vulnerable to oxidative stress than nuclear DNA [20]. Oxidative stress can cause mtDNA damage, as indicated by 8-OHdG detection and PCR analysis showing mtDNA mutations or deletions [21]. In the present study, increased 8-OHdG production was detected at all-time points in the cytoplasm of tubular cells in ischemic kidneys by immunohistochemistry staining, while only a few 8-OHdG-positive cells were recognized in POC kidneys (Figure 4A). Staining for 8-OHdG, a biomarker of oxidative DNA damage, which stains nuclear DNA as well as mtDNA, was localized primarily in the cytoplasm, indicating that this oxidative adduct was mainly present in the mitochondria.

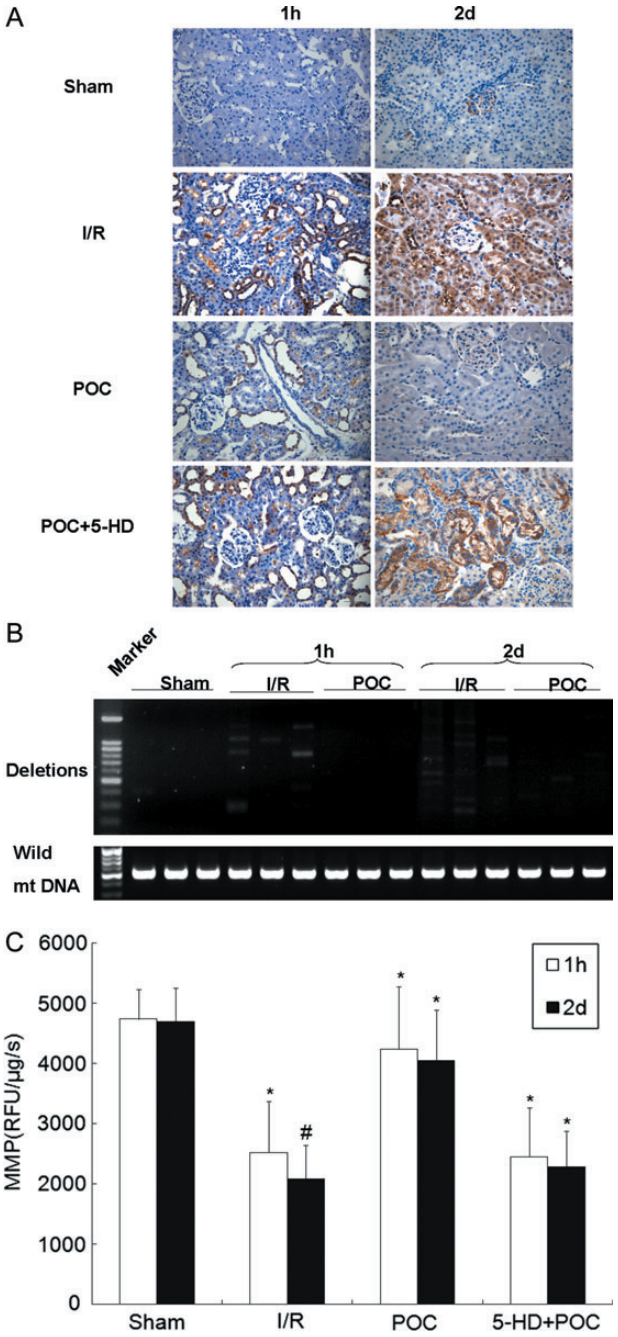

FIGURE 4: Protective effects of POC on the mitochondria in ischemic kidneys after reperfusion. (**A**) Immunohistochemical staining for 8-OHdG. Original magnification ×40. Data are representative of four animals in each group. (**B**) PCR analysis of mtDNA deletions. Template mtDNA from ischemic kidneys was amplified by 35 cycles using the primer pair between base pair 7835 and 13 129. PCR amplification showed multiple mtDNA deletions in mtDNA recovered from I/R kidneys 1 h and 2 days after reperfusion. However, POC attenuated mtDNA deletions. (**C**) MMP in freshly isolated kidney mitochondria was measured by using the JC-1 MMP detection Kit. MMP declined after 1 h and 2 days of reperfusion, but was maintained at high levels by POC. Values are means ± SEM of measurement from four samples. $^{*}P < 0.05$, $^{\#}P < 0.01$.

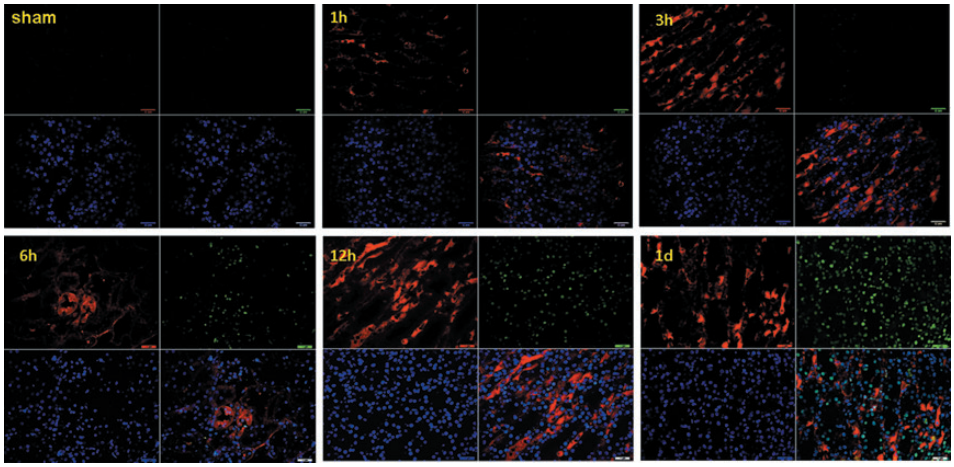

FIGURE 5: Immunofluorescence staining for 8-OHdG (red) and TUNEL (green) staining at serial time point in kidneys post-ischemia. 8-OHdG was detected in the cytoplasm of tubular epithelial cells 1 h post-ischemia, however, few TUNEL-positive cells were presented in kidneys 1 h after I/R. TUNEL-positive cells were detected 6 h after reperfusion and were plentiful 1 day after I/R. Original magnification ×40. Photomicrograph is representative of four animals in each group.

Template mtDNA from ischemic kidneys was amplified by 35 cycles of PCR using the primer pair between 7835 and 13 129 bp. PCR amplification showed multiple mtDNA deletions of 4,834 bp in ischemic kidneys 1 h and 2 days after reperfusion (Figure 4B). In contrast, only a few mtDNA deletions were detected in POC kidneys or in non-ischemic kidneys.

8-OHdG and TUNEL double staining

To clarify whether mtDNA damage occurred earlier or later than cell death and show the temporal relationship between mtDNA damage and cell death, we performed 8-OHdG and TUNEL double staining. At 1 h post-ischemia, 8-OHdG was detected in the cytoplasm of tubular epithelial cells but few TUNEL-positive cells were detected. A few TUNEL-positive cells were detected as early as 6 h post-ischemia (Figure 5). These results indicated that mtDNA damage likely occurs earlier than cell death.

Mitochondrial membrane potential analysis

We used a mitochondria isolation kit (Sigma), which enabled the preparation of isolated mitochondria containing intact inner and outer membranes [18, 22, 23]. Measurements of mitochondrial membrane potential (MMP) in freshly isolated mitochondria by using the fluorescent probe JC-1 revealed that after 1 h and 2 days of reperfusion, MMP was decreased in ischemic kidneys (Figure 4C). However, there was no significant difference in MMP between POC and Sham kidneys. Sustaining a strong MMP is essential for mitochondrial function and cell survival [24].

Expression of the mitochondrial K_{ATP} channel subunit Kir6.2

Previous studies have shown that Kir6.2, a subunit of the mitochondrial K_{ATP} channel, is localized to the mitochondria of renal tubular epithelial cells, smooth muscle cells and cardiomyocytes [25, 26]. To determine whether POC influenced mitochondrial K_{ATP} channels, subunit Kir6.2 was examined by immunofluorescence staining, using VDAC as an internal control. Immunofluorescence staining showed that Kir6.2 expression declined in ischemic kidneys after 2 days of reperfusion. However, POC sustained Kir6.2 expression and this effect was reversed by 5-HD (Figure 6A). Western blot analysis of isolated mitochondrial fractions confirmed that Kir6.2 expression relative to that of VDAC (Kir6.2/VDAC) was significantly increased in POC treatment of kidneys (Figure 6B).

DISCUSSION

The present studies demonstrated that I/R rats exhibited increased serum Cr, oxidative mtDNA damage (8-OHdG), caspase-3 activation, multiple mtDNA deletions, decreased MMP and severe renal injury. In contrast, POC resulted in less oxidative mtDNA damage and deletions and improved MMP. Furthermore, expression of mitochondrial ATP-dependent $K^+(K_{ATP})$ channel subunit Kir6.2 was increased in POC animals. Kir6.2 expression declined in I/R and POC + 5-HD animals 2 days after reperfusion.

The protective maneuver of POC reported by Zhao *et al.* [7] showed that three episodes of 30 s of reperfusion/30 s of ischemia conducted immediately after ischemia in the dog heart significantly attenuated reperfusion injury. However, in studies of other organs, in order to minimize the damage resulting from I/R, there are great variations in cycles and time of POC [27–30]. Some studies observed no protective effect with a delayed POC procedure, indicating that the optimal time for implementing POC might be at the moment of reperfusion [17]. However, Leconte *et al.* [31] reported that delayed POC still provided neuroprotection. These data indicated that the window of opportunity for POC was not unique but appeared to differ depending on the organ being studied. We found it possible to challenge this therapeutic time window of POC. First, a 7-min interval before POC in the current study

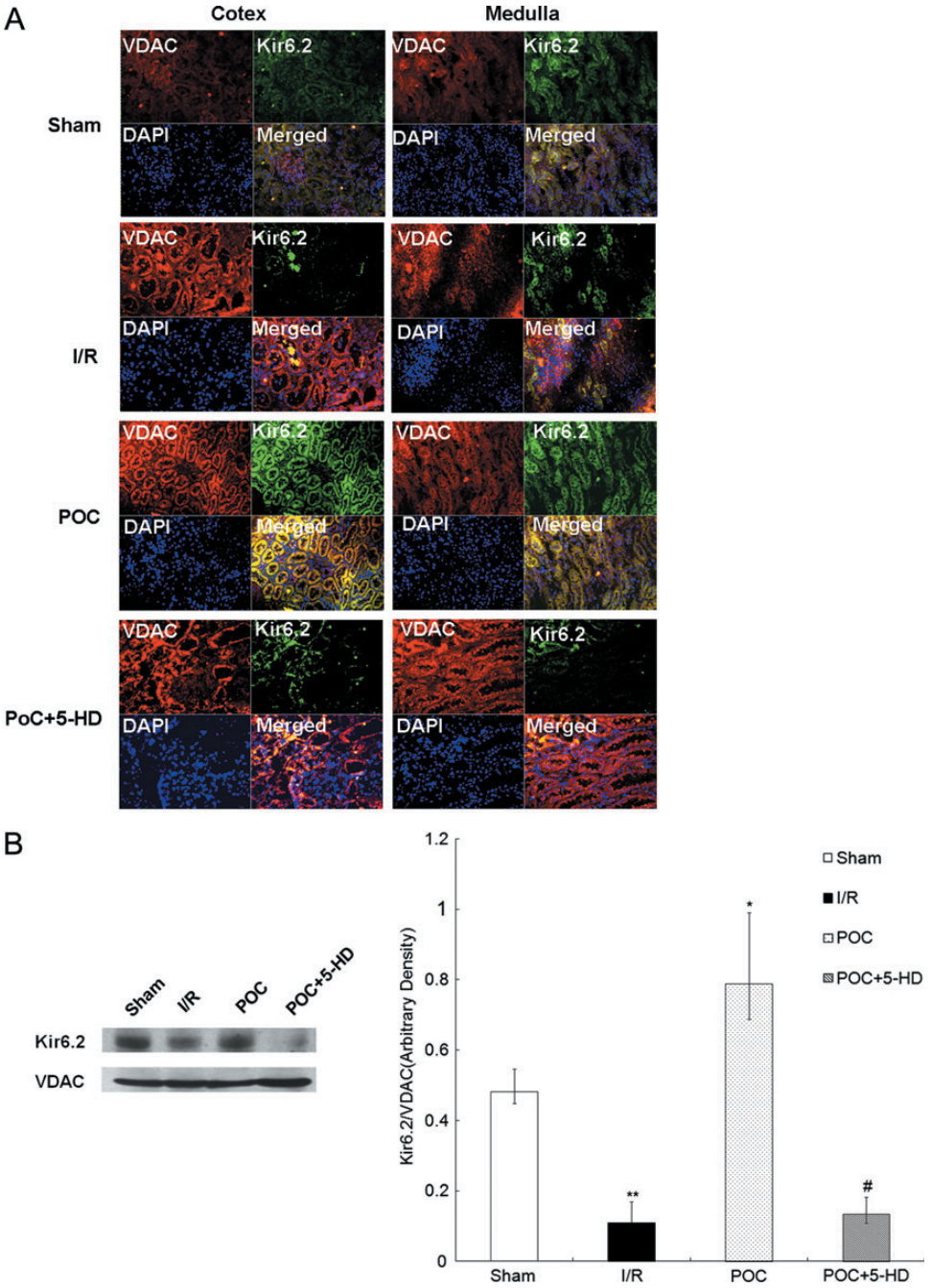

FIGURE 6: Expression of mitochondrial ATP-dependent potassium (K_{ATP}) channel subunit Kir6.2. (**A**) Immunofluorescent staining for Kir6.2. Kir6.2 was widely distributed in renal tubular epithelial cells in Sham-operated animals and increased in POC animals. Kir6.2 expression declined in I/R animals and 5-HD-treated POC animals 2 days after reperfusion. Results are representative of four animals from each group. (**B**) Western blot analysis of Kir6.2 protein expression in mitochondrial fractions. VDAC was used as an internal control. POC treatment sustained Kir6.2 expression but this effect was reversed by 5-HD. Each lane represents mitochondria extracted and pooled from four samples. $^{**}P < 0.001$ versus Sham group, $^{*}P < 0.001$ versus I/R group; $^{\#}P < 0.01$ versus POC group.

was enough for the kidney to get full reperfusion, as monitored microscopically; second, a short-time postponement of POC should facilitate the transition to the clinical setting.

POC has been considered as a new way to target mitochondria to reduce lethal reperfusion injury. In studies of the liver and brain, some evidence indicates that POC can modulate oxidative stress resulting from formation of ROS [32, 33]. ROS are generated from different sources, including NADPH oxidases, xanthine oxidase-hypoxanthine, inflammatory cells and mitochondria of parenchymal cells [34, 35]. We have confirmed that ROS, the initiator of all deleterious effects of reperfusion, were rapidly produced in the mitochondria of renal tubular cells after reperfusion, and POC reduced the generation of ROS by the mitochondria to lower levels as early as 1 h after reperfusion (Figure 3A). Furthermore, nitrotyrosine, a marker of nitrosative stress, was increased in renal tubular

epithelial cells after I/R. POC attenuated nitrotyrosine production (Figure 3B). ROS react with nitric oxide generating peroxynitrite, which may bind to protein residues such as tyrosine and yield highly cytotoxic nitrotyrosine [36, 37]. These results indicated that POC reduced generation of reactive free radicals such as ROS and their derivatives, as detected by H_2DCFDA and nitrotyrosine staining, respectively. Moreover, these results were further confirmed by biometric analysis of ROS production in isolated intact mitochondria, which was measured with the Amplex Red H_2O_2/peroxidase detection kit (Figure 3C). These changes may be considered as earlier signals of damage that occur prior to that indicated by overt histological analysis.

Excessive amounts of ROS cause damage to DNA, lipid and protein. mtDNA is more susceptible than nuclear DNA to increased oxidative stress because of the lack of histone protection and limited capacity of DNA repair systems [20, 38]. However, whether POC can protect mtDNA had not been previously investigated. In the current study, protection of mtDNA by POC was demonstrated by lower amounts of 8-OHdG and less mtDNA oxidative damage when compared with those in I/R rats (Figure 4A and B). To explain these findings, we propose that blocking production of free radicals in renal tubular epithelial cells by POC was associated with amelioration of all the parameters of mitochondrial injury during renal I/R. We found that the mtDNA deletions in the present study were similar to those reported in our previous work and other publications, and are flanked by two homologous repeats that span a region-encoding respiratory enzyme subunits for complexes I, IV and V. Progressive mtDNA injury induced by I/R could result in an unstable mitochondrial genome.

To determine whether mtDNA deletions influenced mitochondrial function, we measured MMP in freshly isolated mitochondria. MMP was significantly decreased after 1 h of reperfusion and was reduced to a low level at 2 days; however, MMP was sustained by POC (Figure 4C). Blocking abnormal generation of free radicals by POC subsequently decreased mutation of mtDNA and protected mitochondrial function, as demonstrated by MMP.

To clarify whether mtDNA damage is a consequence or a cause of renal injury, and to explain whether mtDNA damage occurred earlier or later than cell death, we performed 8-OHdG and TUNEL double staining at serial time points post-ischemia. As presented in Figure 5, mtDNA oxidative damage was observed 1 h post-ischemia, however, cell death was detected by TUNEL staining at 6 h post-ischemia. Thus, the temporal relationship between mtDNA damage and cell death was elucidated in the current study. Moreover, after 6 h post-ischemia, most 8-OHdG-positive cells were TUNEL-positive. Combined with mtDNA deletions detected by PCR at 1 h post-ischemia (Figure 4B), we speculate that mtDNA damage may be the cause of renal injury and may occur earlier than cell death.

We then speculated that the protective mechanisms of POC were related to mitochondrial K_{ATP} channels. To test this hypothesis, 5-HD, an ischemia-selective, mitochondrial K_{ATP} antagonist [39], was administered before ischemia. We chose 5-HD because it is accepted as a more specific mitochondrial K_{ATP} channel blocker than glibenclamide [40]. Opening of the K_{ATP} channel has been proposed to be associated with an uptake of potassium in the mitochondrial matrix, which could constitute a parallel potassium influx and attenuate Ca^{2+} overload. The reduction in mitochondrial Ca^{2+} uptake would prevent mitochondrial swelling and inhibit opening of the mitochondrial permeability transition pore during reperfusion [41]. Additionally, mitochondrial K_{ATP} channel activity effectively inhibits the development and release of ROS [42], the reactive molecules and possibly the initiator of all the deleterious effects of reperfusion. Mitochondrial K_{ATP} is normally closed in most conditions, but can be activated by diazoxide, a highly sensitive mitochondrial K_{ATP} opener, which is involved in cardioprotection [43]. Similarly, our previous work [3] showed that administration of diazoxide before ischemia played a pivotal role in renal protection.

In the current study, Kir6.2 expression declined in renal tubular epithelial cells 2 days after reperfusion, while POC resulted in significant up-regulation of Kir6.2 expression, which was completely antagonized by 5-HD (Figure 6). In accordance with these results, Zhang *et al.* [44] also found that POC prevented the decline in MMP in isolated I/R kidney epithelial cells and speculated that mitochondrial K_{ATP} channels play important roles in the protective mechanisms of POC in the kidney. However, our studies differed in both methods and timing. First, we measured MMP in freshly isolated mitochondria from kidney tissue at different time points. Second, we detected mitochondrial K_{ATP} channel Kir6.2 *in situ* by immunofluorescence staining and quantified Kir6.2 expression in isolated mitochondrial protein extracts by western blot. We found that 5-HD completely antagonized the effects of POC. Furthermore, we noted that 5-HD should be given before ischemia so that the mitochondrial K_{ATP} channels would be blocked when the POC algorithm was applied, thereby completely abolishing the favorable effects of POC. We speculate that opening of mitochondrial K_{ATP} channels might be one of the protective mechanisms of POC. First, POC mediated the activation of mitochondrial K^+ channels as indicated in the present and earlier studies [44, 45]. Conversely, blocking mitochondrial K_{ATP} channels blunted the kidney protection exerted by POC. Second, a number of studies concluded that activation of mitochondrial K_{ATP} channels confers protection against I/R injury, which has been shown not only by pharmacological means, using mitochondrial K_{ATP} channels activators and inhibitors, but also obtained by direct evidence of Kir6.2 gene transfection [43, 46, 47].

ROS generation, mtDNA damage and deletions and MMP can be considered as relatively early indicators for I/R injury and were detected prior to histological changes. We conclude that POC protects the kidney from I/R at a relatively early time by inhibiting the burst of ROS and by attenuating mtDNA damage and deletions. We further speculate that diminished mitochondrial damage produced by POC was responsible for the lower grade of kidney injuries, as detected by improved serum Cr values, decreased caspase-3 activation and a decreased number of TUNEL-positive cells. Moreover, opening of mitochondrial K_{ATP} channels by POC may play a pivotal

role in preventing oxidative stress and attenuating mtDNA damage in renal I/R injury. We conclude that POC may be a promising therapy for protection against I/R injury.

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CONFLICT OF INTEREST STATEMENT

None declared.

(See related article by Moradi and Wang. Renoprotective mechanisms of ischemic postconditioning in ischemia–reperfusion injury: improved mitochondrial function and integrity. *Nephrol Dial Transplant* 2013; 28: 2667–2669.)

ORIGINAL ARTICLE

REFERENCES

1. Chertow GM, Burdick E, Honour M *et al.* Acute kidney injury, mortality, length of stay, and costs in hospitalized patients. J Am Soc Nephrol 2005; 16: 3365–3370
2. Ishani A, Xue JL, Himmelfarb J *et al.* Acute kidney injury increases risk of ESRD among elderly. J Am Soc Nephrol 2009; 20: 223–228
3. Sun Z, Zhang X, Ito K *et al.* Amelioration of oxidative mitochondrial DNA damage and deletion after renal ischemic injury by the KATP channel opener diazoxide. Am J Physiol 2008; 294: F491–F498
4. Thadhani R, Pascual M, Bonventre JV. Acute renal failure. N Engl J Med 1996; 22: 1448–1460
5. Lee HT, Emala CW. Protective effects of renal ischemic preconditioning and adenosine pretreatment: role of A(1) and A(3) receptors. Am J Physiol 2000; 278: F380–F387
6. Murry CE, Jennings RB, Reimer KA. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. Circulation 1986; 74: 1124–1136
7. Zhao Z-Q, Corvera JS, Halkos ME *et al.* Inhibition of myocardial injury by ischemic postconditioning during reperfusion: comparison with ischemic preconditioning. Am J Physiol 2003; 285: H579–H588 (Erratum in: Am J Physiol 2004; 286: H477)
8. Jiang B, Liu X, Chen H *et al.* Ischemic postconditioning attenuates renal ischemic/reperfusion injury in mongrel dogs. Urology 2010; 76: 1519 e1–7
9. Staat P, Rioufol G, Piot C *et al.* Postconditioning the human heart. Circulation 2005; 112: 2143–2148
10. Sun K, Liu ZS, Sun Q. Role of mitochondria in cell apoptosis during hepatic ischemia-reperfusion injury and protective effect of ischemic postconditioning. World J Gastroenterol 2004; 10: 1934–1938
11. Wang JY, Shen J, Gao Q *et al.* Ischemic postconditioning protects against global cerebral ischemia/reperfusion-induced injury in rats. Stroke 2008; 39: 983–990
12. Eldaif SM, Deneve JA, Wang NP *et al.* Attenuation of renal ischemia-reperfusion injury by postconditioning involves adenosine receptor and protein kinase C activation. Transpl Int 2010; 23: 217–226
13. Yun Y, Duan WG, Chen P *et al.* Ischemic Postconditioning modifies renal oxidative stress and lipid peroxidation caused by ischemic reperfusion injury in rats. Transplant Proc 2009; 41: 3597–3602
14. Domoki F, Bari F, Nagy K *et al.* Diazoxide prevents mitochondrial swelling and Ca2+ accumulation in CA1 pyramidal cells after cerebral ischemia in newborn pigs. Brain Res 2004; 1019: 97–104
15. Miura T, Miki T. ATP-sensitive K^+ channel openers: old drugs with new clinical benefits for the heart. Curr Vasc Pharmacol 2003; 1: 251–258
16. Ren R, Zhang Y, Li B *et al.* Effect of β-amyloid (25–35) on mitochondrial function and expression of mitochondrial permeability transition pore proteins in rat hippocampal neurons. J Cell Biochem 2011; 112: 1450–1457
17. Robin E, Simerabet M, Hassoun SM *et al.* Postconditioning in focal cerebral ischemia: role of the mitochondrial ATP-dependent potassium channel. Brain Res 2011; 1375: 137–146
18. Reers M, Smith TW, Chen LB. J-aggregate formation of a carbocyanine as a quantitative fluorescent indicator of membrane potential. Biochemistry 1991; 30: 4480–4486
19. Doi K, Suzuki Y, Nakao A *et al.* Radical scavenger edaravone developed for clinical use ameliorates ischemia/reperfusion injury in rat kidney. Kidney Int 2004; 65: 1714–1723
20. Lum H, Roebuck KA. Oxidant stress and endothelial cell dysfunction. Am J Physiol 2001; 280: C719–C741
21. Nagakawa Y, Williams GM, Zheng Q *et al.* Oxidative mitochondrial DNA damage and deletion in hepatocytes of rejecting liver allografts in rats: role of TNF-α. Hepatology 2005; 42: 208–215
22. Gross A, Yin XM, Wang K *et al.* Caspase cleaved BID targets mitochondria and is required for cytochrome c release, while BCL-XL prevents this release but not tumor necrosis factor-R1/Fas death. J Biol Chem 1999; 274: 1156–1163
23. Zhang X, Tachibana S, Wang H *et al.* Interleukin-6 is an important mediator for mitochondrial DNA repair after alcoholic liver injury in mice. Hepatology 2010; 52: 2137–2147
24. Kulkarni GV, Lee W, Seth A *et al.* Role of mitochondrial membrane potential in concanavalin A-induced apoptosis in human fibroblasts. Exp Cell Res 1998; 245: 170–178
25. Flagg TP, Enkvetchakul D, Koster JC *et al.* Muscle KATP channels: recent insights to energy sensing and myoprotection. Physiol Rev 2010; 90: 799–829
26. Zhou M, He HJ, Suzuki R *et al.* Expression of ATP-sensitive K-channel subunit Kir6.1 in rat kidney. Eur J Histochem 2007; 51: 43–51
27. Huang H, Zhang L, Wang Y *et al.* Effect of ischemic post-conditioning on spinal cord ischemic-reperfusion injury in rabbits. Can J Anaesth 2007; 54: 42–48
28. Rehni AK, Singh N. Role of phosphoinositide 3-kinase in ischemic postconditioning-induced attenuation of cerebral ischemia-evoked behavioral deficits in mice. Pharmacol Rep 2007; 59: 192–198

29. Santos CH, Gomes OM, Pontes JC *et al.* The ischemic preconditioning and postconditioning effect on the intestinal mucosa of rats undergoing mesenteric ischemia/reperfusion process. Acta Cir Bras 2008; 23: 22–28
30. Teixeira AR, Molan NT, Kubrusly MS *et al.* Postconditioning ameliorates lipid peroxidation in liver ischemia reperfusion injury in rats. Acta Cir Bras 2009; 24: 52–56
31. Leconte C, Tixier E, Freret T *et al.* Delayed hypoxic postconditioning protects against cerebral ischemia in the mouse. Stroke 2009; 40: 3349–3355
32. Lin HC, Lee TK, Tsai CC *et al.* Ischemic postconditioning protects liver from ischemia-reperfusion injury by modulating mitochondrial permeability transition. Transplantation 2012; 93: 265–271
33. Sun J, Luan Q, Dong H *et al.* Inhibition of mitochondrial permeability transition pore opening contributes to the neuroprotective effects of ischemic postconditioning in rats. Brain Res 2012; 1436: 101–110
34. Akki A, Zhang M, Murdoch C *et al.* NADPH oxidase signaling and cardiac myocyte function. J Mol Cell Cardiol 2009; 47: 15–22
35. Lambeth JD. NOX enzymes and the biology of reactive oxygen. Nat Rev Immunol 2004; 4: 181–189
36. Viñas JL, Hotter G, Pi F *et al.* Role of peroxynitrite Role of peroxynitrite on cytoskeleton alterations and apoptosis in renal ischemia-reperfusion. Am J Physiol 2007; 292: F1673–F1680
37. Viñas JL, Sola A, Hotter G. Mitochondrial NOS upregulation during renal I/R causes apoptosis in a peroxynitrite-dependent manner. Kidney Int 2006; 69: 1403–1409
38. Stadtman ER, Levine RL. Free radical-mediated oxidation of free amino acids and amino acid residues in proteins. Amino Acids 2003; 25: 207–218
39. Auchampach JA, Grover GJ, Gross GJ. Blockade of ischaemic preconditioning in dogs by the novel ATP dependent potassium channel antagonist sodium 5-hydroxydecanoate. Cardiovasc Res 1992; 26: 1054–1062
40. Liu Y, Sato T, Seharaseyon J *et al.* Mitochondrial ATP-dependent potassium channels. Viable candidate effectors of ischemic preconditioning. Ann N Y Acad Sci 1999; 874: 27–37
41. Wu L, Shen F, Lin L *et al.* The neuroprotection conferred by activating the mitochondrial ATP-sensitive K^+ channel is mediated by inhibiting the mitochondrial permeability transition pore. Neurosci Lett 2006; 402: 184–189
42. Facundo HT, de Paula JG, Kowaltowski AJ. Mitochondrial ATP-sensitive K-channels are redox-sensitive pathways that control reactive oxygen species production. Free Radic Biol Med 2007; 42: 1039–1048
43. Garlid KD, Paucek P, Yarov-Yarovoy V *et al.* Cardioprotective effect of diazoxide and its interaction with mitochondrial ATP-sensitive K^+ channels. Possible mechanism of cardioprotection. Circ Res 1997; 81: 1072–1082
44. Zhang WL, Zhao YL, Liu XM *et al.* Protective role of mitochondrial K-ATP channel and mitochondrial membrane transport pore in rat kidney ischemic postconditioning. Chin Med J (Engl) 2011; 124: 2191–2195
45. Jin C, Wu J, Watanabe M *et al.* Mitochondrial K^+ channels are involved in ischemic postconditioning in rat hearts. J Physiol Sci 2012; 62: 325–332
46. Garlid KD, Dos Santos P, Xie ZJ *et al.* Mitochondrial potassium transport: the role of the mitochondrial ATP-sensitive K(+) channel in cardiac function and cardioprotection. Biochim Biophys Acta 2003; 1606: 1–21
47. Ljubkovic M, Marinovic J, Fuchs A *et al.* Targeted expression of Kir6.2 in mitochondria confers protection against hypoxic stress. J Physiol 2006; 577: 17–29

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