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Attenuation of renal ischemia–reperfusion injury by postconditioning involves adenosine receptor and protein kinase C activation

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Summary

Significant organ injury occurs after transplantation and reflow (i.e., reperfusion injury). Postconditioning (PoC), consisting of alternating periods of reperfusion and re-occlusion at onset of reperfusion, attenuates reperfusion injury in organs including heart and brain. We tested whether PoC attenuates renal ischemia–reperfusion (I/R) injury in the kidney by activating adenosine receptors (AR) and protein kinase C (PKC). The single kidney rat I/R model was used. Groups: (1) sham: time-matched surgical protocol only. In all others, the left renal artery (RA) was occluded for 45 min and reperfused for 24 h. (2) Control: I/R with no intervention at R. All antagonists were administered 5 min before reperfusion. (3) PoC: I/R + four cycles of 45 s of R and 45 s of re-occlusion before full R. (4) PoC + ARi: PoC plus the AR antagonist 8-ρ-(sulfophenyl) theophylline (8-SPT). (5) PoC + PKCi: PoC plus the PKC antagonist chelerythrine (Che). In shams, plasma blood urea nitrogen (BUN mg/dl) at 24 h averaged 23.2 ± 5.3 and creatinine (Cr mg/dl) averaged 1.28 ± 0.2. PoC reduced BUN (87.2 ± 10 in Control vs. 38.8 ± 9, $P = 0.001$) and Cr (4.2 ± 0.6 in Control vs. 1.5 ± 0.2, $P < 0.001$). 8-SPT and Che reversed renal protection indices after PoC. I/R increased apoptosis, which was reduced by PoC, which was reversed by 8-SPT and Che. Postconditioning attenuates renal I/R injury by adenosine receptor activation and PKC signaling.

Introduction

Renal reperfusion injury occurs after re-establishing blood flow to the ischemic or malperfused kidney in various disease conditions, such as transplants, renal artery stenosis, embolic disease, and descending aortic repair [1]. Reperfusion injury in the kidney is expressed as acute renal dysfunction, acute tubular necrosis, and apoptosis [2]. Indeed, acute renal failure is a predictor of long-term graft viability. These deleterious effects are triggered or mediated by a complex response to ischemia–reperfusion involving damage-associated molecular pattern molecules (DAMPs), including oxygen radical species, cytokines, and chemokines. On a cellular level, reperfusion injury is associated with oxidant-induced damage secondary to increased generation of reactive oxygen species and a decrease in endogenous anti-oxidant reserve. In addition, reperfusion is associated with a distinct inflammatory response characterized by an increase in inflammatory cytokine production and neutrophil accumulation in the reperfused tissue [1,3]. Both oxidants and pro-inflammatory mediators contribute to cell death expressed as necrosis and apoptosis. Both necrosis and apoptosis contribute to postischemic renal failure [4].

A large body of research has emerged focusing on innate tissue protective strategies that attenuate the deleterious effects of reperfusion injury [5]. For example, ischemic preconditioning (IPC), which was first described in 1986 by Murry *et al.* [6] in heart, decreased infarction size when applied before coronary occlusion. The inherent tissue protection of IPC has been applied to clinical situations where the onset of ischemia is predictable, including cardiac surgery and organ transplantation [7]. In the kidney, IPC has been reported to attenuate postischemic injury, including histologically apparent acute tubular necrosis (ATN) and renal dysfunction, using blood urea nitrogen and creatinine clearance as surrogate markers of function [8,9]. Subsequent studies demonstrated that renal preconditioning was triggered by ligands such as adenosine that stimulate G-protein coupled receptors, which then activate molecular mediators, such as the serine threonine protein kinase C (PKC). PKC, in turn, activates end effectors such as mitochondrial ATP-sensitive potassium channels and maintains the mitochondrial permeability transition pore in a closed state, which is a pivotal mechanism that ultimately determines cell salvage, or pursuit of a necrotic or an apoptotic pathway to cell death [10].

Although preconditioning consistently reduces postischemic injury in many organs, its application requires foreknowledge of the ischemic event, which is difficult to predict in most circumstances, with the exception of surgery and transplantation. Recently, postconditioning, defined as a series of brief alternating periods of arterial reperfusion and re-occlusions applied at the onset of reperfusion, has emerged as a clinically promising innate cardioprotective maneuver [11]. First reported in 2003 by Zhao *et al.* [12], postconditioning reduced infarct size and attenuated vascular endothelial cell dysfunction in *in vivo* myocardium. Unlike preconditioning, postconditioning can be applied at the onset of reperfusion in the previously ischemic tissue or organ, which is more clinically applicable. The cardioprotective potential of postconditioning has subsequently been confirmed in different acute and chronic animal models of myocardial infarction [11,13] (reviewed in [5]). In the heart, the postconditioning stimulus involves enhanced release of the endogenous cardioprotective molecule adenosine and interaction with its cognate receptors, as blockade of adenosine receptor interaction abrogates the cardioprotection of postconditioning [14]. However, there are few studies showing that postconditioning protects the kidney from reperfusion injury [15]. Liu *et al.* [16] reported in a rat model of kidney ischemia–reperfusion that postconditioning reduced renal dysfunction and apoptosis, and increased nitric oxide ($NO^{\cdot}$) synthase activity and NO release. Chen *et al.* [17] showed that postconditioning inhibited apoptosis via increased phosphorylation of the reperfusion injury survival kinases (RISK) ERK1/2 and Akt, which were shown to be associated with tissue protection [18]. This study, however, did not determine whether the increased phosphorylation of ERK1/2 and Akt was functionally linked to the observed renal protection. Furthermore, Serviddio *et al.* [19] reported that postconditioning reduced reactive oxygen species generation by kidney mitochondria. However, the involvement of adenosine and PKC as mediators of postconditioning has not been investigated in the kidney. Accordingly, this study tested the hypothesis that postconditioning attenuates postischemic renal dysfunction, cellular apoptosis, and tubular necrosis by mechanisms that are dependent on stimulation of adenosine receptors and PKC signaling.

Materials and methods

Chemicals

Adenosine receptor blocker 8-ρ-(sulfophenyl) theophylline (8-SPT, 5 mg/kg) was purchased from Sigma-Aldrich, St Louis, MO, USA. The PKC inhibitor, chelerytherine (Che, 10 mg/kg), was purchased from Santa Cruz Biothechnology, Santa Cruz, CA, USA. 8-SPT was dissolved in 0.9% saline and adminstered intravenously at a dose of 5 mg/kg. Chelerytherine was dissolved in 200 μl of dimethylsulfoxide per sample and also administered intravenously at a dose of 10 mg/kg.

In vivo surgical procedure

Investigations conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institute of Health, and all animals were handled in compliance with the guidelines approved by the Institutional Animal Care and Use Committees of Emory University. On the day of the experiment, male Sprague–Dawley rats (282–401 g) were anesthetized with pentobarbital sodium (30 mg/kg) administered intraperitonealy. The depth of anesthesia was monitored continuously, and additional boluses of anesthesia were given if required. A heating pad was placed under the surgical field to ensure a constant body temprature between 36.5 and 37.5 °C, as measured by a rectal temperature probe. The animal was prepped using topical betadiene and ethanol to the abdominal and neck regions, and draped in sterile fashion. The external jugular vein was cannulated for intravenous drug delivery. A midline incision was made and the bowel was eviscerated and mobilized to visualize the right kidney. A right nephrectomy was performed by isolating the renal pedicle, double ligating the vessels and ureter at the kidney hilum using 3-0 silk suture, and excising the kidney. After nephrectomy, the left renal pedicle was

isolated. A small padded vascular bulldog clamp was placed around the left renal artery and vein at the level of the hilum for a total of 45 min of reversible occlusion. Reperfusion was accomplished by releasing the clamp at the end of the ischemia time.

The rats were divided into seven groups (Fig. 1): (1) A sham group ($n = 8$) underwent the surgical protocol without other interventions. All remaining rats underwent renal arteriovenous occlusion; (2) Control ($n = 8$): reperfusion was achieved by removing the clamp on the renal arteriovenous pedicle, with no other intervention; (3) Postconditioning (PoC, $n = 8$): The postconditioning algorithm of four cycles of 45-s reperfusion (R) followed by 45-s re-occlusion was initiated at the onset of reperfusion; (4) ARi ($n = 5$): the subtype nonselective adenosine receptor (AR) antagonist 8-SPT, 5 mg/kg, was administered 5 min before R via the external jugular vein without postconditioning; (5) PoC + AR antagonist ($n = 6$): 8-SPT 5 mg/kg was administered via the external jugular vein 5 min before the PoC algorithm; (6) PKCi alone ($n = 5$): the PKC isoform nonselective PKC inhibitor chelerythrine (Che, 10 mg/kg) alone was administered intravenously 5 min before R via the external jugular vein; and (7) PoC + PKCi ($n = 6$): Che, 10 mg/kg, was administered intravenously via the external jugular vein 5 min before application of the postconditioning algorithm. After renal reperfusion was achieved, the abdominal region was examined for hemostasis, and the abdomen was closed using a 3-0 vicryl running stitch for muscular layer approximation and 4-0 nylon for skin layer approximation. The animals were placed in their cage to recover for 24 h, with free access to food and water after consciousness was achieved.

After 24 h, the animal was taken back to the operating room and re-anesthetized with pentobarital sodium (30 mg/kg, IP). The incision sutures were opened and the abdomen was entered. The bowel was retracted to expose the kidney and left renal vein. Blood (4–5 ml) was collected for analysis of blood urea nitrogen (BUN) and creatinine (Cr), and the animal was euthanized. The left kidney was harvested postmortem.

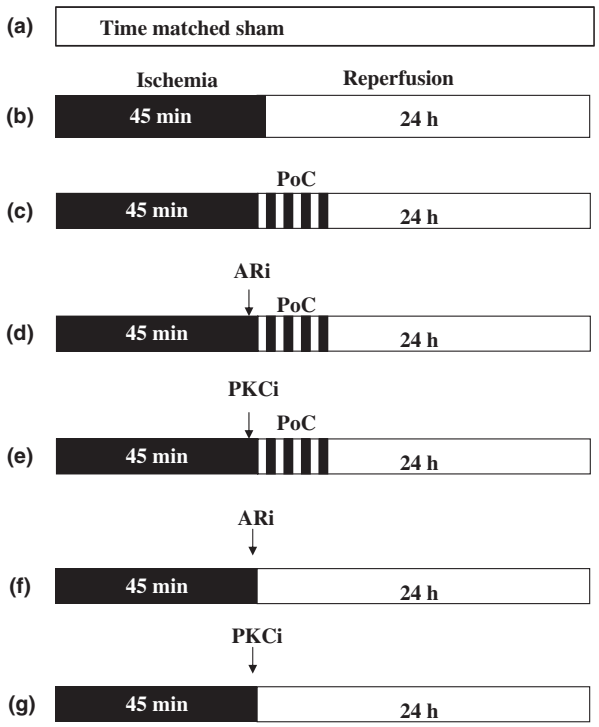

Figure 1 A schematic drawing of the experimental protocol 1 represents Sham, 2 represents I/R Control group, 3 shows I followed by PoC, which consisted of four cycles with alternating 45 s of I and 45 s R, followed by 24 h of complete R. Down arrow (↓) indicates i.v. administration of antagonists, 8-SPT in 4 and Che in 5, 5 min prior to R. Drug-alone groups are represented in groups 6 (8-SPT) and 7 (Che).

Determination of blood urea nitrogen (BUN) and plasma creatinine (Cr) levels

Plasma BUN was determined in conjunction with other variables, such as sodium, potassium, hemoglobin, and hematocrit, using Stat Profile M (Nova Biomedical, Waltham, MA, USA). BUN was reported as mg/dl. For plasma Cr, blood was centrifuged and the plasma was isolated. Using a spectrophotometer (spectraMax 250; Molecular Devices Co., Sunnyvale, CA, USA) at constant temperature of 37 °C, the plasma was mixed with Infiniti™ Creatinine Liquid Stable Reagent (Thermo Electron, Pittsburgh, PA, USA) at a ratio of 30 µl of plasma to 300 µl of reagent, and absorances were recorded electronically using Soft Max Pro 4.6 (Molecular Devices Co.). The excitation and emission wavelengths were 500 and 550 nm, respectively, and absorbance readings were taken at 1 min and 3 min of each run. Plasma Cr values were derived by converting optical density to mg/dl using a standard curve constructed with known standards.

TUNEL staining for apoptotic cells

In a blinded fashion, apoptotic cells were identified using an in situ cell death detection kit (Boehringer Mannheim, Ridgefield, CT, USA) and TUNEL assay. Tissue was fixed in paraformaldehyde and sections were cut and affixed to slides. The slides were incubated with 50 µl of TUNEL reaction mixture containing TdT, which catalyzes polymerization of nucleotides of single to free 3′-OH DNA ends, for 60 min at 37 °C. Converter-AP (50 µl) was then

added to the slides for 30 min, followed by incubation in 100 μl of substrate solution for an additional 10–15 min. The slides were mounted with glass cover slips and analyzed under a light microscope. Cell death expressed as a percentage of total cells identified by hematoxylin and eosin staining was measured. Five sections from each of the four experiments from the sham, PoC group, I/R Control group, PoC + ARi, and PoC + PKCi were analyzed.

Hematoxylin and eosin stain for morphologic features

Cross-sections (7-μ thick) of the kidney were obtained and affixed to slides, which underwent standard hematoxylin and eosin staining sequentially. Five sections of four experiments from the sham and five sections from six experiments from PoC group and I/R Control group were analyzed and quantified by a blinded pathologist. The following histologic features of tubular necrosis were observed and scored: interstitial edema, epithelial flattening, loss of nuclei, fragmented luminal cells/casts, and eosinophilic proteinaceous acellular casts. These five histomorphologic parameters for ATN were blindly observed, graded separately, and combined for severity scoring using a grading scale of 1 for no injury to 4 for severe injury.

Statistical analysis

All values are expressed as mean ± standard error of the mean (SEM). A one-way analysis of variance (ANOVA) was used to determine group differences in BUN, Cr, and apoptosis data. If the ANOVA was significant, multiple comparisons were carried out using the Student–Newman–Keuls *post hoc* test to locate the sources of differences. A P value <0.05 was considered significant.

Results

Function and levels of plasma BUN and Cr

Plasma BUN (Fig. 2) and Cr (Fig. 3) after 24 h of reperfusion in the Sham group averaged 23.2 ± 5.3 mg/dl and 1.2 ± 0.2 mg/dl, respectively. Compared with sham, BUN and Cr values in the Control group were fourfold greater (BUN 87.2 ± 10.0 mg/dl, $P = 0.001$; Cr 4.2 ± 0.6 mg/dl, $P = 0.001$) after 45 min of occlusion and 24 h of R. In the PoC group, the plasma BUN (38.8 ± 9.0 mg/dl) and plasma Cr (1.5 ± 0.2 mg/dl) values were significantly lower than those in Controls and were comparable with those in the Sham group ($P > 0.5$ vs. Sham). These data suggest that PoC applied at the onset of R significantly reduced postischemic renal dysfunction.

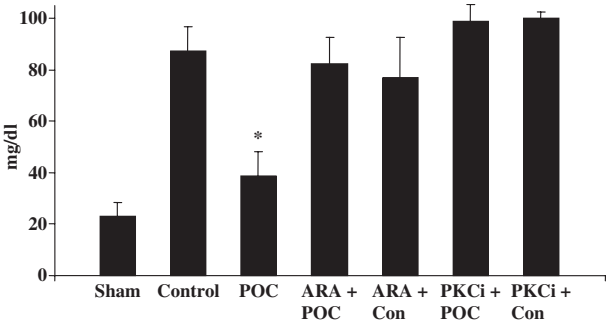

Figure 2 Blood urea nitrogen (BUN) values presented in mg/dl. The average values of blood urea nitrogen among the different groups. Error bars are SEM. There was no statistical significance between the PoC and Sham group. $^{*}P < 0.05$ Control vs. PoC. There was no statistical significance among the Control I/R group and the drug-alone groups (ARA + Con and PKCi + Con) or the PoC + drug groups.

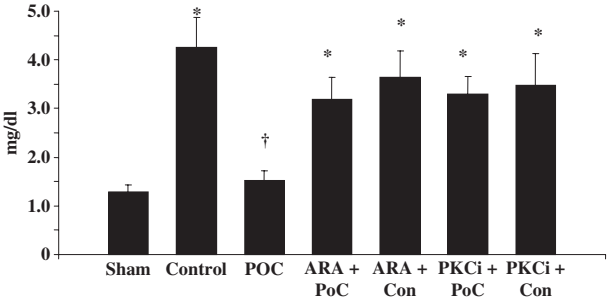

Figure 3 Creatinine values presented in mg/dl. The average values of plasma creatinine are shown for the seven groups. Error bars are SEM. There was no statistical significance comparing sham Cr averages with PoC values. $^{*}P < 0.05$ group vs. PoC; $^{\dagger}P < 0.05$ PoC vs. I/R Control.

Adenosine receptor inhibitor 8-SPT on renal postconditioning

8-SPT alone did not significantly affect either BUN (77.0 ± 15.0 mg/dl) or Cr (3.2 ± 0.4 mg/dl) values compared with those in the Control group. 8-SPT given 5 min before the PoC algorithm reversed the reductions in blood BUN (82.1 ± 10.3 mg/dl, Fig. 2) and plasma Cr (3.4 ± 0.5 mg/dl, Fig. 3) values observed in the PoC group. These values of blood BUN and plasma Cr were comparable with those in the Control group ($P > 0.05$). These data suggest that adenosine receptor-mediated effects are involved in renal protection by postconditioning.

PKC inhibitor Che on renal postconditioning

Che given intravenously 5 min prior to R in the absence of postconditioning did not affect either blood BUN

(100.0 ± 2.1 mg/dl, Fig. 2) or plasma Cr (3.4 ± 0.6 mg/dl, Fig. 3) values relative to those in the Control group. However, Che administered prior to PoC reversed the reductions in blood BUN (99.0 ± 6.5 mg/dl) and plasma Cr (3.3 ± 0.4 mg/dl) values observed with PoC. This suggests that PoC is dependent on PKC signaling.

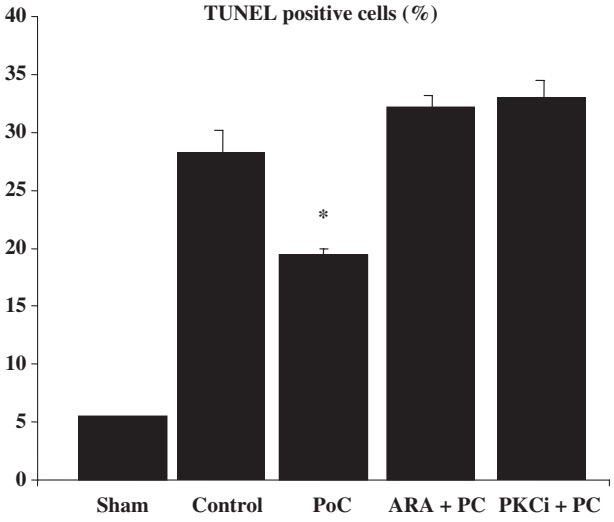

Figure 4 Percentage of TUNEL-positive cells per high power field. Values are mean ± SEM for five individual experiments in each group. The PoC group demonstrates fewer apoptotic cells compared with Control, $^{*}P < 0.05$ PoC vs. Control I/R. Addition of AR antagonist 8-SPT or the PKC inhibitor Che to PoC resulted in a reversal of PoC protection to values comparable with that of Control.

Apoptosis and TUNEL staining

In the Sham group, 5% of the total cells per high power field were TUNEL positive (Fig. 4). There was a sixfold increase in the number of TUNEL-positive cells (28.9%) in the Control group after I/R injury (Figs 4 and 5a). The number of TUNEL-positive cells was significantly reduced by PoC (Figs 4 and 5b). This reduction in TUNEL-positive cells by PoC was abolished with the adenosine receptor antagonist 8-SPT (Fig. 5c)and the PKC antagonist Che (Fig. 5d). These data suggest that PoC reduced the percentage of apoptotic cells observed on TUNEL staining by mechanisms that involve adenosine receptor stimulation and activation of PKC.

Changes in renal morphology

Morphologic features at the corticomedullary junction were visualized in five sections from four kidneys in each group stained by hematoxylin and eosin (Fig. 6). PoC preserved the morphologic integrity of tubular structural and tubular brush border with less evidence of acute tubular necrosis compared with that in the Control group. In addition, there was also less cast formation. Morphologic preservation by PoC was reversed by both 8-SPT and Che. We were further able to quantify the severity of ATN using the five categories described in the method section. These five histomorphologic parameters for ATN were blindly examined and graded separately as well as combined (total score) in Fig. 7. One-way ANOVA showed no statistically significant differences among all the groups for fragmented luminal cells/casts. There was a

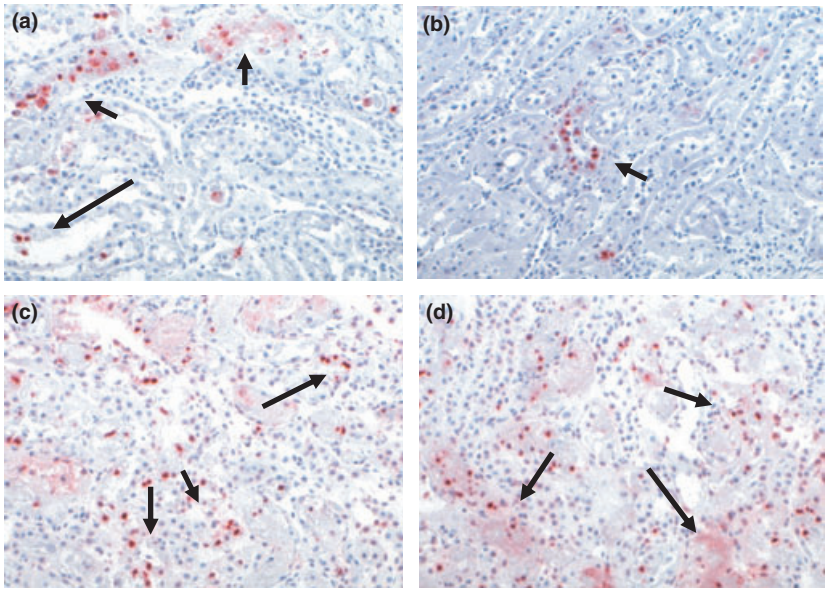

Figure 5 Photomicrograph of apoptotic cells identified by TUNEL staining. (a) Control I/R; (b) PoC; (c) ARi + Poc; (d) PKCi + PoC; PoC significantly reduced the number of TUNEL-positive cells (red staining indicated by arrows) relative to that of Control I/R. Addition of PKCi and ARi to PoC eliminated protection.

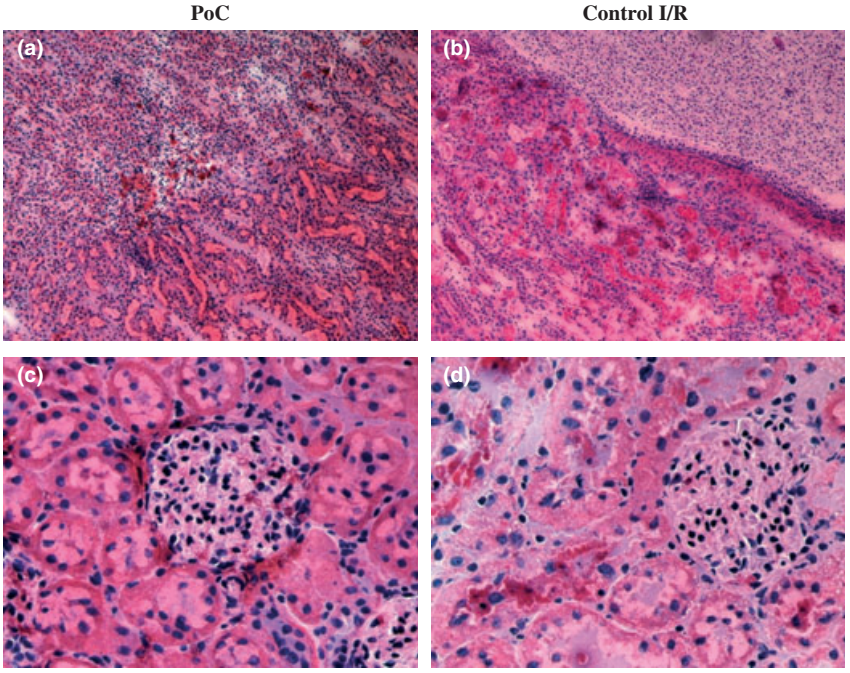

Figure 6 Hematoxylin and eosin staining of the superficial medulla. (a) and (c) represent lower (magnification ×100) and higher (×400) magnification of PoC kidneys; (b) and (d) represent lower and higher magnifications of Control I/R kidneys, respectively. Notice the preservation of the tubular structure in (a) compared with the I/R group in (b) in which the kidney shows remarkable tubular necrosis and the presence of casts. (c) in higher magnification demonstrates the preservation of proximal tubular structure and the brush border in the postconditioned kidney compared with the loss of the epithelial lining, shedding of the cells, and cast formation, indicating necrosis in a Control kidney in (d).

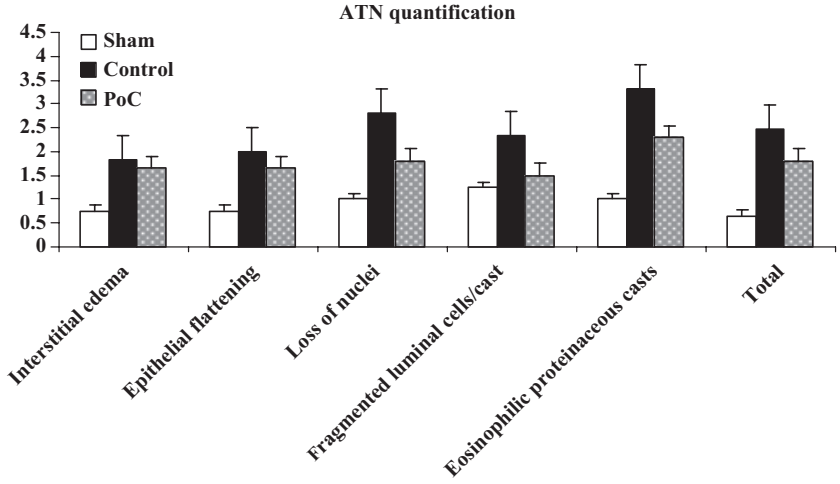

Figure 7 Quantification of the tubular necrosis observed with H&E staining. The following histologic features of tubular ischemia/necrosis were observed: interstitial edema, epithelial flattening, loss of nuclei, fragmented luminal cells/casts, and eosinophilic proteinaceous acellular casts. These five histomorphologic parameters for ATN were blindly observed, graded separately, and combined for severity scoring. One-way ANOVA showed no statistical differences among the groups for fragmented luminal cells/casts. There was a statistically significant difference between the Sham and Con I/R group in rest of the categories ($P < 0.05$), with statistical significance achieved only between PoC and Con I/R groups in eosinophilic proteinaceous acellular casts and a trend toward significance in the other categories.

statistically significant difference between the Sham and Con I/R group for all the other categories ($P < 0.05$). Statistical significance was achieved only between PoC and Con I/R groups in eosinophilic proteinaceous acellular cast categories, and a trend toward significance in the other categories.

Discussion

Postconditioning was first reported as a cardioprotective strategy in the heart in 2003 by Zhao *et al.* [12]. Although many studies have confirmed the cardioprotective effects of postconditioning in the heart (reviewed in Vinten-

Johansen [5]) and brain [20] (reviewed in Zhao [21]), few studies have shown that the brief mechanical maneuver is effective in attenuating postischemic injury in the kidney [15,16]. The results of this study confirm that 45 min of renal ischemia followed by 24 h of reperfusion causes significant renal dysfunction, which is in agreement with studies by Lee *et al.* [9,22,23], and renal morphologic injury, which is in agreement with studies reported by Padanilam *et al.* [2] and Lee *et al.* [24]. Furthermore, a brief postconditioning algorithm lasting a total of 6 min applied at the onset of reperfusion attenuated renal dysfunction (blood BUN and plasma Cr levels), decreased apoptosis identified by TUNEL staining, and decreased acute tubular necrosis after 24 h of reperfusion. The renoprotective effects of postconditioning were abrogated by blocking adenosine receptors and PKC at the time of reperfusion, suggesting that the mechanism(s) of postconditioning in the kidney involve(s) activation of both adenosine receptors and the PKC signaling pathway. These data suggest that similar pathways are engaged by postconditioning in the kidney and the heart. The study also implies that the first minutes of reperfusion are critical in the pathogenesis of reperfusion injury in the kidney as they are in the heart. [25]. Postconditioning's protection may be derived from adenosine's potent anti-inflammatory effect, such as inhibition of neutrophil function and tissue cytokine release.

In this study, the choice of the postconditioning algorithm of 45 s each of occlusion and reperfusion was somewhat arbitrary. Szwarc *et al.* [15] used three cycles of 30-s reperfusion alternating with re-occlusion, whereas Liu *et al.* [16] used six cycles of 10-s reperfusion and re-occlusion in a unilaterally nephrectomized rat model similar to that used in this study. Like preconditioning, the protective effects of postconditioning depend to some extent on the duration of the ischemic or reperfusion cycles [26] as well as on the number of cycles applied, [25,27] although both the duration of each cycle and number of cycles are species dependent. The duration and number of cycles are different between rats, pigs, dogs, and humans. In the rat, Kin *et al.* [25] have reported similar reductions in infarct size in the heart after applying a 3- or 6-cycle postconditioning algorithm. However, Iliodromitis *et al.* [27] showed that eight cycles of 30-s reperfusion–re-occlusion cycles were necessary in the anesthetized pig to achieve a reduction in myocardial infarct size. The reason for this dependency on the number and duration of postconditioning cycles is not clear, but some have suggested that the shorter cycles are effective in tissues with higher metabolic rates, or in smaller animal species [26] in which ATP breakdown and release of adenosine are more rapid during the occlusion phase of postconditioning. In the kidney, the proximal tubule cells are the most metabolically active cells, but they have a lower metabolic rate and, hence, slower ATP utilization than that of the cardiomyocytes [28,29]. A slower metabolic rate would ostensibly translate into a slower rate of adenosine production from ATP degradation during the occlusion phase of the postconditioning algorithm, and a slower buildup of endogenous adenosine would require more time in the kidney than in the heart [30]. The optimal postconditioning algorithm is yet to be defined in both the kidney and the heart. However, other mechanisms not related to adenosine production, for example, realkalinization of tissue pH [31] and attenuated activation of the sodium-hydrogen exchanger, may be relevant to the effective periodicity and number of postconditioning cycles.

The involvement of adenosine in the renoprotection of postconditioning requires that adenosine is present during the early reperfusion phase. As in the heart [32], the kidney generates interstitial adenosine concentrations of less than 1 μM under normoxic conditions, which are increased up to sixfold during hypoxia/anoxia [33] or renal artery occlusion in canines and rats [30,34]. Adenosine levels normalize to baseline levels within 10 min of reperfusion in the rat because of washout and rapid degradation by adenosine deaminase. Even in the normoxic state, the interstitial levels of adenosine are sufficient to stimulate G-protein coupled adenosinergic receptors [35]. In addition, both A_1 and A_{2A} receptors are present in the rat kidney [36]. These data support the presence of sufficient adenosine to exert G-protein coupled adenosinergic receptor-mediated effects at the time that postconditioning is applied. Although activation of the A_{2A} receptor, and not the A_1 receptor, has been implicated in myocardial postconditioning [14], the activated receptor subtype in renal postconditioning has not been determined as of yet.

Accordingly, this study suggested that adenosine receptor activation at reperfusion was functionally involved in protection of the kidney by postconditioning. Both exogenous and endogenous adenosine are protective in many organs when administered after ischemia or after reperfusion [1,37]. Endogenous adenosine has been shown to be involved in postconditioning in the heart [14], and in remote postconditioning using renal ischemia as the postconditioning stimulus to reduce myocardial infarct size [38]. In studies performed in the isolated perfused rat heart, postconditioning decreased the rate of adenosine washout during the first few minutes of reperfusion, and ostensibly increased the retention time of endogenously released adenosine. This increased retention of adenosine, particularly in the intravascular space, would enhance receptor activation, and has been linked to tissue protection [39]. Although intravascular adenosine was not measured in this study, the protection observed in the

kidney is consistent with adenosine-mediated effects observed in the heart, i.e., a reduction in tissue damage, infarct size, and apoptosis. A role for adenosine in postconditioning is also supported by blockade of renoprotection by the nonsubtype selective antagonist 8-SPT. Adenosine blockers, such as theophylline, by themselves have been known to attenuate postischemic renal dysfunction and morphologic defects. However, in this study, 8-SPT alone showed no change in any end point, in agreement with a report from Sugino *et al.* [40]. Hence, unlike ischemic preconditioning in which endogenous and exogenous adenosine were presumed to exert protection during ischemia [40,41], renal protection by postconditioning involves activation of adenosinergic receptors by endogenous adenosine during the early moments of reperfusion. The hypothesis that postconditioning involves adenosinergic receptor stimulation is consistent with the observations that the kidney is protected by administration of exogenous adenosine [9]. This is also consistent with the observation of the 18-fold rise of adenosine concentration in the renal interstitium with clamping of the renal artery made by Li *et al.* [42]. The mechanical trapping of adenosine during postconditioning may act to prevent adenosine washout, causing the lingering of enough substrate to activate the adenosinergic receptors.

The protection by postconditioning has been associated with a reduction in the inflammatory response to reperfusion. Accordingly, the pro-inflammatory cytokines TNFα and interleukin-8 were reduced by postconditioning [43]. In addition, myocardial [12,25] and neutrophil [44] superoxide production is significantly reduced in postconditioned hearts after ischemia–reperfusion. It is interesting that both adenosine and mechanical postconditioning demonstrate similar anti-inflammatory attributes. However, we did not observe an accumulation of neutrophils in the vascular spaces or renal parenchyma in the Control group in this study. This is in contrast to other studies in which an accumulation of neutrophils after reperfusion of the ischemic kidney was observed [45,46]. In the study by Takada *et al.* [45] in which the duration of renal occlusion was similar to that used in this study, the accumulation of neutrophils peaked at 6 h, and decreased to levels approaching that in time-matched shams after 24 h of reperfusion. However, we can neither rule out that neutrophils did not accumulate during reperfusion, nor can we confirm that postconditioning inhibited neutrophil functions via an adenosine-related mechanism. Adenosine, during reperfusion, does exert protection independent of neutrophils by attenuating cytokine release and oxidant generation by vascular endothelium.

This study showed that the nonselective PKC inhibitor chelerythrine blocked the renal protective effects of postconditioning. Chelerythrine targets the catalytic site of all PKC isoforms. Protein kinase C is purportedly a downstream target of adenosine activation, principally by the A_1 and the A_3 receptors. At least twelve PKC isoforms have been identified in renal tubules and glomeruli. Coupling of adenosine A_1 receptors to PKC via pertussis toxin-sensitive Gi/Go G-protein subunits has been demonstrated in renal tubules [47–49]. In cardiomyocytes, PKC is translocated from the cytosol to the membrane, where it inhibits opening of the mitochondrial permeability transition pore [50] potentially via activating mitochondrial ATP-sensitive potassium (K_{ATP}) channels. The opening of the mPTP is viewed as the final effector and switch between necrosis and apoptosis [51,52]. A link between adenosine receptor activation and PKC stimulation was demonstrated in the kidney by Lee and Emala [22], who reported that the PKC antagonist chelerythrine blocked the renal protective effects of adenosine. In the heart, Zatta and colleagues showed that postconditioning was dependent on protein kinase C activation [13]. The results of this study are consistent with the concept that PKC is a downstream target of adenosine receptor stimulation. Future studies are needed to identify the individual PKC isoform and whether PKC translocates from the cytosol to the mitochondrial membrane to exert its effects similar to that in the heart [13].

In conclusion, this study demonstrates that postconditioning applied during the early moments of reperfusion after renal artery occlusion attenuates renal dysfunction and morphologic injury. Endogenously released adenosine, through its interactions with G-protein coupled receptors during reperfusion, may act as a proximal trigger of the postconditioning response. The G-protein coupled signal transduces to PKC, although a direct link to adenosine and the potential intervening pathways has not been demonstrated. In addition, the studies to date on postconditioning have focused on prevention of injury. There has been no demonstration of an effect on reparative processes that may be engaged days to weeks after reperfusion has been established. Finally, experiments on the modulation by postconditioning of transcriptional regulators and protein biomarkers of reperfusion injury, including neutrophil adhesion or activation markers [53], may prove to be of great interest as an additional pathway of organ protection in transplantation.

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Authorship

SME and JVJ: designed research/study. SME and JAD: performed research/study. RAG: contributed important concepts and clinical translation. MM: performed histology analysis. CJM: collected data. ZQZ, SME and NPW: analyzed data. SME, JVJ and CJM: wrote the paper.

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