Acute and delayed renal protection against renal ischemia and reperfusion injury with A₁ adenosine receptors

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Joo JD, Kim M, Horst P, Kim J, D’Agati VD, Emala CW, Sr., Lee HT. Acute and delayed renal protection against renal ischemia and reperfusion injury with A₁ adenosine receptors. Am J Physiol Renal Physiol 293: F1847–F1857, 2007. First published October 10, 2007; doi:10.1152/ajprenal.00336.2007.—We showed previously that activation of A₁ adenosine receptors (AR) protects against renal ischemia-reperfusion (IR) injury in rats and mice. In the heart, transient A₁AR activation produces biphasic protective effects: acute protection wanes after several hours but protective effects return 24–72 h later (second window of protection). In this study, we determined whether A₁AR activation produces delayed renal protection and elucidated the mechanisms of acute and delayed renal protection. A₁AR wild-type mice were subjected to 30-min renal ischemia and 24 h of reperfusion to produce acute renal failure. Pretreatment with a selective A₁AR agonist 2-chloro-N⁶-cyclopentyl-adenosine (CCPA; 0.1 mg/kg bolus ip) either 15 min or 24 h before renal ischemia protected against renal IR injury and reduced renal cortical medullary necrosis, apoptosis, and inflammation. Transient A₁AR activation led to phosphorylation of extracellular signal-regulated protein kinase mitogen-activated protein kinase (ERK MAPK), Akt, and heat shock protein 27 (HSP27). Moreover, induction of HSP27 and Akt occurred with CCPA treatment. Inhibition of PKC with chelerythrine prevented acute but not delayed renal protection with A₁AR activation. Moreover, deletion of P3Kα or inhibition of Akt, but not inhibition of ERK, prevented delayed and acute renal protection with A₁AR activation. Inhibition of Gαq, with pertussis toxin obliterated both acute and delayed A₁AR-mediated renal protection. In contrast to renal protection with delayed ischemic preconditioning, nitric oxide synthase activity was not induced with delayed A₁AR-mediated renal protection. Therefore, transient activation of renal A₁AR led to acute as well as delayed protective effects against renal IR injury via distinct signaling pathways.

Acute renal failure; extracellular signal-regulated protein kinase/mitogen-activated protein kinase; heat shock protein 27; mice; protein kinase B/Akt

Acute renal failure (ARF) results frequently from renal ischemia and reperfusion (IR) injury and is a major contributor of morbidity and mortality during the perioperative period (3, 5, 6, 26, 47). Unfortunately, despite intense research focus, there is no effective therapy for ARF. We previously demonstrated that A₁ adenosine receptor (AR) activation immediately before renal ischemia protected against renal IR injury in rats and mice in vivo via pertussis toxin-sensitive G protein (Gi) and PKC pathways (27, 31). The A₁AR-mediated renal tubular protection can also be demonstrated in vitro to protect against necrosis and apoptosis in cultured proximal tubule cells (29, 30).

In the heart, A₁AR activation also protects against IR injury (36, 39). Interestingly, acute protective effects of transient A₁AR activation dissipate over several hours but reappear 24–72 h later (1, 2, 11). This “delayed A₁AR-mediated cardiac protection” or “second window of protection” involves K⁺,ATP channel activation and inducible nitric oxide synthase (iNOS) upregulation. In addition, we previously demonstrated that renal protection with ischemic preconditioning also shows a biphasic profile (acute and delayed) and that the mechanisms involve the PI3K–Akt pathway, heat shock protein 27 (HSP27), and iNOS induction but was independent of K⁺,ATP channel activation (23, 27). However, the delayed renal protection after transient A₁AR activation has never been examined in the kidney.

Activation of A₁ARs in many organs including the kidney initiates several cytoprotective kinase cascades including ERK MAPK, Akt, and PKC. In addition, upregulation of HSPs is thought to protect other intracellular proteins from denaturation and aggregation that occur in response to oxidative stress (12, 45). Of these HSPs, HSP27 has been implicated in mediating the cytoprotection in a variety of cell types (37, 41). We previously demonstrated in cultured renal proximal tubule cells that acute A₁AR activation phosphorylates HSP27, whereas chronic activation or overexpression of A₁ARs leads to upregulation of total HSP27 (32).

In this study, we further characterized the acute and delayed A₁AR-mediated renal protective effects and elucidated the mechanisms of renal protection in vivo. We tested the following three hypotheses utilizing a murine model of renal IR injury. We hypothesized that 1) transient A₁AR activation results in acute (immediate) as well as delayed (protection reappearing many hours later) renal protection against renal IR injury in mice, 2) acute A₁AR activation protects renal function via activation of preexisting cytoprotective kinases including ERK MAPK, Akt, and PKC, and 3) delayed renal protection with A₁AR occurs via induction of cytoprotective HSP27 and iNOS. Our results show that acute protection from A₁AR activation is dependent on PKC and Akt activation, whereas the delayed protection from A₁AR activation is dependent on Akt activation and induction of HSP27 without induction of iNOS activity.

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METHODS

Renal IR injury and A1AR activation. All protocols were approved by the Institutional Animal Care and Use Committee of Columbia University. Breeding pairs of noncongenic A1AR heterozygous mice were obtained from Dr. J. Schnermann (National Institute of Diabetes and Digestive and Kidney Diseases, Bethesda, MD) to generate A1AR wild-type (WT) and A1AR knockout (KO) mice as described previously (34, 46). P13K knockout mice (congenic on C57BL/6 background) were obtained from Dr. D. Wu (University of Connecticut Health Center) and genotyped as described (35). C57BL/6 mice were obtained from Harlan (Indianapolis, IN). iNOS gene-deleted (iNOS−/−) and C57BL/6J (iNOS+/+) male mice were purchased from Jackson Laboratory (Bar Harbor, ME; strain name: B6; 129P2-Nos2<sup>−/−</sup> mice; number: 002596). We initially utilized both noncongenic A1AR WT mice and congenic C57BL/6 mice to determine whether acute as well as delayed renal protection occurs after A1AR activation with 2-chloro-N<sup>6</sup>-cyclopentyladenosine (CCPA). For subsequent mechanistic signaling studies, we used C57BL/6 mice. Mice (20–25 g) were anesthetized with intraperitoneal pentobarbital sodium (50 mg/kg or to effect) and placed supine on a heating pad under a warming light to maintain body temperature between 36 and 38°C. In some mice, a carotid artery catheter was placed to measure systemic blood pressures. Additional pentobarbital sodium was given before renal ischemia. Animals were subjected to renal IR 15 min (acute protection), 4 and 24 h (delayed protection) after the CCPA injection. The mice in the 24-h group (delayed protection) were allowed to awaken, reanesthetized 24 h later, and subjected to left renal ischemia.

Assessment of renal function after IR injury. Renal function was assessed 1) by measurement of plasma creatinine 24 h after renal ischemia as described previously (31, 33, 34) and 2) by assessment of changes in renal outer medullary blood flow (ROMBF; near the corticomedullary junction) after renal ischemia as described previously (23). A needle flow probe (480-μm diameter; model TSD145) connected to a laser Doppler flowmeter (Biopac Systems, Goleta, CA) was used to measure the relative changes in ROMBF after renal ischemia. At the end of each experiment, the kidney was excised to confirm the position of the needle probe tips in the outer medullary area. Mice with incorrectly placed probes were excluded from the study.

Role of ERK and Akt in A1AR-mediated acute and delayed renal protection. To test the hypothesis that ERK MAPK and/or Akt participates in acute or delayed renal protection with A1AR activation, we pretreated the mice with PD98059 [an inhibitor of mitogen-activated protein kinase kinase (MEK1)] to inhibit ERK phosphorylation, 1 mg/kg ip] or wortmannin or LY294002 [selective inhibitors of phosphatidylinositol-3 kinase (PI3K) to inhibit Akt phosphorylation, 1 mg/kg ip] 15 min before CCPA injection. The dosages of PD98059, wortmannin, and LY294002 were selected on the basis of previous in vivo studies (23). In addition, we previously demonstrated that the dosage and method of administration of inhibitors that we used effectively blocked the phosphorylation of ERK and Akt in vivo, respectively (23). To further confirm the role of PI3K→Akt signaling in renal protection after A1AR activation, we also utilized P13K<sup>−/−</sup> mice and subjected them to renal IR either 15 min or 24 h after CCPA treatment. These mice lack the P13K subtype (P13Kγ) that is activated by G protein-coupled receptors (i.e., A<sub>1</sub>Rs). To further confirm the role of the A<sub>1</sub>AR in CCPA-mediated renal protection, we also subjected mice that lacked A<sub>1</sub>AR (A<sub>1</sub>AR KO mice) to renal IR either 15 min or 24 h after CCPA treatment.

Role of G<sub>11α</sub> and PKC in A1AR-mediated acute and delayed renal protection. For determination of the role of G<sub>11α</sub>, or PKC in acute or delayed renal protection with A1AR activation, mice were pretreated with pertussis toxin (25 μg/kg ip 48 h before) or with chelerythrine (5 mg/kg ip 15 min before), respectively, before CCPA injection (28, 31).

Role of iNOS in delayed A1AR-mediated renal protection. To test the hypothesis that iNOS is involved in mediating the cytoprotective effects of delayed A1AR-mediated renal protection, we used complementary approaches utilizing a pharmacological inhibitor of iNOS (L-N6-(1-iminoethyl) Lysine (L-NIL; 10 mg/kg ip, A.G. Scientific, San Diego, CA) and iNOS KO mice. C57BL/6 mice were treated with 0.1 mg/kg CCPA 24 h before renal ischemia. Fifteen minutes before renal ischemia, mice were injected with either vehicle or 10 mg/kg/L-NIL ip and subjected to 30-min renal ischemia. iNOS KO mice and their WT controls (C57BL/6J) were treated with 0.1 mg/kg CCPA and subjected to renal ischemia 24 h later.

Histological examinations to detect necrosis and apoptosis. For histological preparations, explanted kidneys were bisected along the long axis and were cut into three equal-sized slices. Kidney slices were obtained at 24 h from sham-operated mice, mice treated with vehicle plus IR injury, mice with acute CCPA treatment and IR injury, and mice with CCPA treatment and delayed IR injury and were fixed in 10% formalin solution overnight. Following automated dehydration through a graded alcohol series, transverse kidney slices were embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin-eosin (H&E). Morphological assessment was performed by an experienced renal pathologist who was unaware of the treatment which each animal had received. A grading scale of 0–4, as outlined by Jablonski et al. (21), was used to assess the degree of renal tubular necrosis after renal IR injury. Renal tubular apoptosis was assessed by counting the number of apoptotic bodies in proximal tubules in the outer stripe of the corticomedullary junction (expressed as mean number of apoptotic bodies per tubule). This area is the most severely injured area after renal IR injury. At least 25–30 tubules were counted per field and 6 fields were examined per slide.

Renal cortical NOS activity assay. We measured NOS activity in the renal cortices (including corticomedullary junction) of mice injected with vehicle or CCPA 24 h before with a commercially available kit (Calbiochem, EMD Biosciences, San Diego, CA). The assay is based on the enzymatic conversion of nitrate to nitrite by nitrate reductase, followed by the spectrophotometric quantification of nitrite levels using the Griess reagent.
tion reagents (Amersham, Piscataway, NJ), with subsequent exposure to a CCD camera coupled to a UVP Bio-imaging System (Upland, CA). The band intensities of the immunoblots were within the linear range of exposure for all experiments. For each experiment, the band intensities were normalized against the vehicle-treated group. For phospho-proteins (phospho-ERK, phospho-Akt, or phospho-HSP27), the band intensities were normalized against the total protein first then subsequently normalized against the vehicle-treated group.

**Immunohistochemistry for neutrophils.** Paraffin-embedded mouse kidney sections were deparaffinized in xylene and rehydrated through graded ethanolas to water. After being blocked with 10% normal horse serum/PBS solution, the slides were incubated overnight with primary antibody for neutrophils (MCA771G, Serotec, Raleigh, NC) at 4°C in a humidified chamber. Endogenous alkaline phosphatase activity was blocked with 1% levamisole. The primary antibody was localized by using the Vectastain ABC-Alkaline Phosphatase detection system using BCIP/NBT as substrate (Vector Laboratories, Burlingame, CA). Control serum IgG was used for negative isotype control experiments.

**Semiquantitative reverse transcription polymerase chain reaction for proinflammatory mRNAs, iNOS, and HSP27.** Four hours after renal IR injury, renal corticomedullary expression of mRNAs encoding proinflammatory chemokine macrophage inflammatory protein-2 (MIP-2), monocyte chemoattractant protein-1 (MCP-1), tumor necrosis factor-α (TNF-α), keratinocyte-derived cytokine (KC), iNOS, and HSP27 was determined using semiquantitative RT-PCR as described previously (34). Renal cortices were dissected, total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA) reagent, and RNA concentrations were determined with spectrophotometric readings at 260 nm. Primers were designed based on published GenBank sequences for mice (Table 1). Primer pairs were chosen to yield expected PCR products of 200–600 bp and to amplify a genomic region that spans one or two introns to eliminate the confounding effect of amplifying contaminating genomic DNA. RT-PCR was performed using the Access RT-PCR System (Promega), which is optimized to yield linear increases in the densitometric measurements of resulting bands with increasing PCR cycles (15–30 cycles). The starting amount of RNA was also varied to allow enzymatic completion of incomplete cDNAs. The PCR cycle number for each primer pair was optimized to yield linear increases in the densitometric measurements of resulting bands with an established number of PCR cycles. For each experiment, we also performed semiquantitative RT-PCR under conditions yielding linear results for GAPDH (15 cycles) to confirm equal RNA input. Five microliters of the RT-PCR product were analyzed on a 6% acrylamide gel stained with SYBR green (Invitrogen) for analysis with a UVP Bio-imaging System. Semiquantitative analysis of mRNA expression gene was accomplished by obtaining the ratio of the band density of the mRNA’s of interest to that of GAPDH (a housekeeping gene) from the same sample.

**Detection of DNA fragmentation by DNA laddering.** To assess for potential differences in DNA ladder (indicative of apoptosis), extracted DNA (Wizard, Promega) from dissected renal cortices taken at 24 h following vehicle plus IR, acute CCPA protection plus IR, and CCPA protection plus delayed IR was subjected to electrophoresis for 6 h at 70 V in a 2.0% agarose gel in Tris-acetate-EDTA buffer. Gels were stained with ethidium bromide and photographed under UV illumination. DNA ladder markers (100 bp) were used as a reference for the analysis of internucleosomal DNA fragmentation.

**Statistical analysis.** The data were analyzed with Student’s t-test when comparing means between two groups. One-way ANOVA plus Tukey’s post hoc multiple comparison test was used to compare mean values across multiple treatment groups. Two-way repeated-measures ANOVA was used to compare the differences in renal blood flow at varying time points. The ordinal values of the Jablonski scale were analyzed by the Kruskal-Wallis nonparametric test with Dunn posttest comparison between groups. In all cases, a probability statistic <0.05 was taken to indicate significance. All data are expressed throughout the text as means ± SE.

**Materials.** Unless otherwise specified, all chemicals were obtained from Sigma (St. Louis, MO), CCPA was first dissolved in DMSO and then diluted in saline such that the final DMSO concentration was 10%.

### RESULTS

**A1AR activation provided both acute and delayed renal protection against IR injury.** We utilized both a noncongenic strain of A1AR WT and KO mice (mixed C57BL/6 and SV129 background) as well as a congenic strain of C57BL/6 mice in the in vivo demonstration of early and delayed A1AR-mediated renal protection. For the mechanistic elucidation of signaling pathways of A1AR-mediated acute and delayed renal protection, we only utilized C57BL/6 mice. We determined in our preliminary studies that systolic blood pressure and heart rate were reduced by 9 and 12% after 0.1 mg/kg CCPA injection, respectively (n = 3). However, renal blood flow did not change significantly after intraperitoneal injection with 0.1 mg/kg CCPA. Thirty minutes of renal ischemia and 24-h reperfusion

<table>
<thead>
<tr>
<th>Primers</th>
<th>Species</th>
<th>Size, bp</th>
<th>Temp., °C</th>
<th>Sequence (5'-3')</th>
<th>PCR, Temp., °C/Cyc. #</th>
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<tbody>
<tr>
<td>HSP27</td>
<td>Mouse</td>
<td>409</td>
<td>69.3</td>
<td>AGGCCGCGCTTTGGATGCA</td>
<td>68/30</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>69.7</td>
<td>TGACCTCGACAGGCCTTC</td>
<td>69/24</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>71.4</td>
<td>TACCTGATTTGGGTTATGCTCC</td>
<td>65/26</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Mouse</td>
<td>290</td>
<td>69.8</td>
<td>CAGGCTTGTCTCTGAAAGGAC</td>
<td>60/26</td>
</tr>
<tr>
<td>KC</td>
<td>Mouse</td>
<td>203</td>
<td>69.4</td>
<td>CAATGAGTGCTTGGCTTGAGTG</td>
<td>60/26</td>
</tr>
<tr>
<td></td>
<td></td>
<td>203</td>
<td>65.5</td>
<td>CTTGCGGAGACCTTTTATGAC</td>
<td>60/22</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Mouse</td>
<td>312</td>
<td>59.5</td>
<td>ACGGCCTGCTACTCATTCAC</td>
<td>60/28</td>
</tr>
<tr>
<td>MIP-2</td>
<td>Mouse</td>
<td>282</td>
<td>62.5</td>
<td>TTGAGGTGGTTGTGGAAAAG</td>
<td>60/24</td>
</tr>
<tr>
<td>iNOS</td>
<td>Mouse</td>
<td>487</td>
<td>75.1</td>
<td>CTGTCGTGCTTGTTGATGAGGC</td>
<td>65/24</td>
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<td>GAPDH</td>
<td>Mouse</td>
<td>450</td>
<td>66.9</td>
<td>ACCACAGCTCATCGCATCAC</td>
<td>65/15</td>
</tr>
</tbody>
</table>

HSP27, heat shock protein 27; KC, keratinocyte-derived cytokine; MCP-1, monocyte chemoattractant protein-1; MIP-2, macrophage inflammatory protein-2; iNOS, inducible nitric oxide synthase.
resulted in a significant rise in serum creatinine (Cr; in mg/dl) in A1AR WT (Cr = 1.1 ± 0.1, n = 9), A1AR KO (Cr = 2.2 ± 0.3, n = 10), and C57BL/6 mice (Cr = 2.3 ± 0.2, n = 8; Table 2 and Fig. 1) compared with the sham-operated animals (Cr = 0.3 ± 0.1, n = 6, 0.4 ± 0.1, n = 4, and 0.3 ± 0.1, n = 6 for A1AR WT, A1AR KO, and C57BL/6 mice, respectively). As we published previously, acute A1AR activation with CCPA 15 min before renal ischemia provided powerful renal protection against renal IR injury in both A1AR WT mice (0.7 ± 0.1, n = 6) and in C57BL/6 mice (0.6 ± 0.1, n = 8). When the mice are subjected to renal ischemia 4 h after CCPA treatment, renal protective effects were lost (Cr in mg/dl = 1.42 ± 0.2, n = 4 for A1AR WT mice and 2.4 ± 0.2, n = 4 for C57BL/6 mice). However, when the mice are subjected to renal ischemia 24 h after CCPA, serum Cr were significantly lower in both A1AR WT mice (0.6 ± 0.1, n = 8) and in C57BL/6 mice (1.0 ± 0.2, n = 6). Mice lacking A1ARs were not protected with A1AR activation (with CCPA) when subjected to renal IR 15 min (2.2 ± 0.2, n = 4) or 24 h later (2.3 ± 0.1, n = 4).

**Acute and delayed A1AR-mediated renal protection improves ROMBF after renal IR injury.** Thirty minutes of renal ischemia and subsequent reperfusion led to a significant reduction in ROMBF at 60 min (62.6 ± 5.7% of preischemic value; n = 5; P < 0.01 vs. preischemic value; Fig. 2). Both acute (15 min before renal IR, n = 5) and delayed renal A1AR activation (24 h before renal IR, n = 5) led to better preserved ROMBF during early reperfusion after IR injury (with 30 min of renal IR; Fig. 2).

### Table 2. Plasma creatinine values in mice subjected to sham operation, renal IR, injected with 0.1 mg/kg CCPA 15 min before renal IR (acute IR after CCPA) or 24 h before renal IR (delayed IR after CCPA)

<table>
<thead>
<tr>
<th></th>
<th>Sham (n)</th>
<th>IR Acute CCPA (n)</th>
<th>IR Delayed CCPA (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle C57BL/6</td>
<td>0.3 ± 0.1</td>
<td>2.3 ± 0.2 (8)*</td>
<td>0.6 ± 0.1 (10)†</td>
</tr>
<tr>
<td>Wortmannin C57BL/6</td>
<td>0.4 ± 0.1</td>
<td>2.4 ± 0.2 (6)*</td>
<td>1.8 ± 0.1 (5)</td>
</tr>
<tr>
<td>LY294002 C57BL/6</td>
<td>0.4 ± 0.1</td>
<td>2.4 ± 0.4 (4)*</td>
<td>2.3 ± 0.3 (6)</td>
</tr>
<tr>
<td>PD98059 C57BL/6</td>
<td>0.4 ± 0.1</td>
<td>2.5 ± 0.2 (6)*</td>
<td>0.8 ± 0.1 (5)†</td>
</tr>
<tr>
<td>Chelerythrine C57BL/6</td>
<td>0.4 ± 0.2</td>
<td>2.6 ± 0.2 (5)*</td>
<td>1.9 ± 0.3 (6)</td>
</tr>
<tr>
<td>Pertussis Toxin C57BL/6</td>
<td>0.4 ± 0.2</td>
<td>2.6 ± 0.2 (4)*</td>
<td>2.8 ± 0.3 (4)</td>
</tr>
<tr>
<td>PI3K KO mice</td>
<td>0.3 ± 0.1</td>
<td>2.8 ± 0.2 (8)*</td>
<td>Not performed</td>
</tr>
<tr>
<td>iNOS KO mice</td>
<td>Not performed</td>
<td>Not performed</td>
<td>Not performed</td>
</tr>
<tr>
<td>A1AR WT mice</td>
<td>0.3 ± 0.1</td>
<td>1.1 ± 0.1 (9)*</td>
<td>0.7 ± 0.1 (6)†</td>
</tr>
<tr>
<td>A1AR KO mice</td>
<td>0.4 ± 0.1</td>
<td>2.2 ± 0.3 (10)*</td>
<td>2.2 ± 0.2 (4)</td>
</tr>
</tbody>
</table>

Values are means ± SE. Plasma creatinine values after inhibitor treatments are also shown. The number of experiments is indicated in parentheses. CCPA, 2-chlorocyclopentyladenosine; KO, knockout; WT, wild-type; L-NIL, 1-iminoethyllysine; IR, ischemia-reperfusion. *P < 0.05 vs. sham. †P < 0.05 vs. IR.
Signaling pathways of acute \( \text{A}_1 \text{AR} \) renal protection: role of PKC and PI3K\( \rightarrow \)Akt pathways. We probed the cytoprotective signaling pathways activated by acute \( \text{A}_1 \text{AR} \) activation. Acute \( \text{A}_1 \text{AR} \) activation resulted in rapid phosphorylation of ERK MAPK and Akt as well as phosphorylation of HSP27 in \( \text{A}_1 \text{AR} \) WT but not \( \text{A}_1 \text{AT} \) KO mice (Fig. 3). Total ERK, Akt as well as HSP27 expression did not change with acute \( \text{A}_1 \text{AR} \) activation. To determine whether ERK and/or Akt phosphorylation mediates the cytoprotective signaling of acute \( \text{A}_1 \text{AR} \) activation-mediated renal protection, C57BL/6 mice were pretreated with PD98059 (a MEK1 inhibitor), wortmanin (a PI3K inhibitor), or LY294002 (a PI3K inhibitor) before CCPA treatment. Inhibition of PI3K but not ERK prevented renal protection with acute \( \text{A}_1 \text{AR} \) activation (Table 2). Although the dose of PD98059 was effective in inhibiting MEK1 (23), inhibition of ERK phosphorylation did not prevent renal protection with acute \( \text{A}_1 \text{AR} \) activation. In addition, inhibition of PKC with chelerythrine also prevented acute renal protection with \( \text{A}_1 \text{AR} \) activation (Table 2). Inhibitors alone had no effect on renal function after IR injury (Table 2). To complement the pharmacological approaches of PI3K inhibition, we utilized PI3K\( \gamma \) KO mice and demonstrated that acute \( \text{A}_1 \text{AR} \)-mediated renal protection is abolished in these mice (Table 2).

Signaling pathways of delayed \( \text{A}_1 \text{AR} \)-mediated renal protection: role of HSP27 and PI3K\( \rightarrow \)Akt pathways. Twenty-four hours after activation of \( \text{A}_1 \text{AR} \) with CCPA, we observed...
increased protein expression of HSP27 along with increased form of phosphorylated HSP27 (Fig. 3). CCPA treatment 24 h before renal ischemia also resulted in increased renal HSP27 mRNA in renal cortices (6.6 ± 1.4-fold, n = 5) consistent with our hypothesis that HSP27 induction after A1AR activation is associated with the delayed renal protection. Surprisingly, total Akt as well as the phosphorylated form of Akt also increased 24 h after CCPA injection (Fig. 3). Since the induction and increased activity of iNOS mediate delayed renal ischemic preconditioning (23), we questioned whether induction of iNOS and increased NO release contribute to the cytoprotection observed 24 h after transient A1AR activation. We inhibited iNOS function with L-NIL before renal ischemia. Inhibition of iNOS function before renal ischemia in animals treated with CCPA 24 h before renal ischemia (delayed A1AR effects) failed to prevent the renal protection (Table 2). In addition, mice lacking iNOS were protected against renal injury when treated with CCPA 24 h before renal ischemia (Table 2) indicating that induction of iNOS is not responsible for delayed A1AR-mediated renal protection.

Acute and delayed renal protection with A1AR activation reduces renal necrosis. In Fig. 4, the renal protective effects of transient A1AR activation either 15 min or 24 h before renal IR are further supported by representative histological slides. Thirty minutes of renal ischemia followed by 24-h reperfusion resulted in significant renal injury as evidenced by severe tubular necrosis, medullary congestion and hemorrhage, and development of proteinaceous casts. Transient A1AR activation either 15 min or 24 h before renal IR preserved near normal morphology. The Jablonski scale histology grading scores are shown in Fig. 5. Thirty minutes of renal ischemia and 24 h of reperfusion resulted in severe acute tubular necrosis (grade = 2.2 ± 0.4, n = 8) in A1AR WT mice. Mice treated with CCPA 15 min (acute IR after CCPA, grade = 0.75 ± 0.3, n = 5) or 24 h (delayed IR after CCPA, grade = 1.2 ± 0.13, n = 6) before renal ischemia demonstrated significant protection of renal histology compared with the vehicle-treated sham-operated (grade = 0.3 ± 0.3, n = 6) group.

Acute and delayed renal protection with A1AR activation is associated with reduced neutrophil infiltration. Renal IR injury results in the recruitment of neutrophils, which accentuate renal injury. Figure 6 shows that 30 min of renal ischemia and 24 h of reperfusion result in renal interstitial neutrophil infiltration (A1AR WT IR) which was reduced in mice treated with 0.1 mg/kg CCPA either 15 min before (A1AR WT acute IR after CCPA) or 24 h before (A1AR WT delayed IR after CCPA) renal IR. A1AR KO mice showed increased neutrophil infiltration compared with the A1AR WT mice after IR injury. Intrarenal neutrophil infiltration was also quantified by manual counting of polymorphonuclear cells in H&E slides by a renal pathologist. Sham-operated A1AR WT mice did not exhibit detectable levels of neutrophils infiltrating the corticomedullary junction of the kidney. A1AR WT mice subjected to renal IR injury after receiving vehicle or 0.1 mg/kg CCPA either 15 min (acute IR after CCPA) or 24 h (delayed IR after CCPA) before renal IR. Severe tubular dilatation, tubular swelling and necrosis, medullary luminal congestion, and hemorrhage are present in the kidneys of mice subjected to IR and these changes are attenuated in animals subjected to IR injury after CCPA.

Acute and delayed renal protection with A1AR activation is associated with reduced mRNAs encoding proinflammatory proteins. We next measured mRNAs encoding proinflammatory chemokines MIP-2, MCP-1, TNF-α, and KC with semi-quantitative RT-PCR. Thirty minutes of ischemia and 4 h of reperfusion significantly increased these mRNA expressions in renal tubules (Fig. 7). Expressions of mRNA encoding proinflammatory molecules are reduced in renal proximal tubules of mice pretreated with either CCPA 15 min or 24 h before IR (Fig. 7).

Acute and delayed renal protection with A1AR activation is associated with reduced apoptosis. DNA isolated from renal tubules subjected to 30 min of renal ischemia and 24 h of
reperfusion showed profound laddering indicative of apoptosis (Fig. 8A). We showed previously that acute A1AR activation results in less renal tubular apoptosis (31, 34). We now show in Fig. 8 that delayed renal protection with CCPA reduced renal tubular apoptosis (DNA laddering). In addition, TUNEL staining also demonstrated reduced renal tubular apoptosis with acute and delayed renal protection with A1AR activation (Fig. 8B). Finally, the degree of renal tubular apoptosis was also quantified by counting the number of apoptotic bodies in proximal tubules in the corticomedullary area of the kidney (expressed as apoptotic bodies per tubule) on the H&E-stained sections. Twenty-five to 50 tubules/field were counted for each treatment group, and kidneys from 4 experiments were examined. Sham-operated A1AR WT mice exhibited no morphological evidence of apoptosis (0.02 ± 0.03 apoptotic bodies/tubule, n = 4). Renal IR injury increased the number of apoptotic bodies within the proximal tubules of A1AR WT mice (0.67 ± 0.12 apoptotic bodies/tubule, n = 8, P < 0.05 vs. sham operation). Pretreatment of mice with the A1AR agonist CCPA either 15 min (0.09 ± 0.04 apoptotic bodies/tubule, n = 4) or 24 h (0.13 ± 0.04 apoptotic bodies/tubule, n = 4) before IR injury caused a decrease in the number of apoptotic bodies at 24 h after IR.

**DISCUSSION**

The major findings of this study are that 1) transient A1AR activation produces acute and delayed renal protection against murine renal IR injury, 2) these biphasic protective effects are associated with reduced necrosis, inflammation, and apoptosis, 3) although both ERK MAPK and Akt phosphorylation occur in the kidney after A1AR activation, only the inhibition of Akt phosphorylation prevented renal protection with acute and delayed protection after A1AR activation, 4) inhibition of PKC abolished the acute but not delayed protective effects of A1AR activation, and 5) transient A1AR activation upregulates HSP27 in the kidney.

Renal IR injury results in significant impairment of renal function and is the leading cause of ARF in hospitalized patients (22). Activation of cell surface A1ARs produces cytoprotective effects against IR injury in many organ systems including the heart, kidney, and brain (17, 31, 34, 40, 42, 49). In cardiac protection with A1AR, the protective effects wane after several hours but reappear again 24–72 h later (defined as “delayed protective effects” or the “second window of protection”) (1, 2). We previously demonstrated that acute A1AR activation protected against renal IR injury in rats and mice (27, 31). Our current study demonstrates that mouse kidney cannot only be protected with acute A1AR activation but that A1AR activation also protects the murine kidney against renal IR injury occurring 24 h later (delayed A1AR-mediated renal protection). This delayed or second window of A1AR-mediated protection has never been previously described in the kidney.

Generation of extracellular renal adenosine and subsequent activation of A1AR produced renal protection to attenuate IR...
Injury (31, 34). In the kidney, extracellular adenosine is derived mainly via phosphohydrolysis of adenosine 5'-monophosphate (AMP) by ecto-5'-nucleotidase (CD73) (15). Moreover, extracellular ATP and ADP are rapidly hydrolyzed to adenosine due to the presence of ecto-nucleoside triphosphate diphosphohydrolases (ENTPDases) in the kidney (16). Indeed, extracellular adenosine generated by ENTPDase-1 (CD39)-dependent nucleotide phosphohydrolysis as well as ecto-5'-nucleotidase (CD73) serve to protect against renal IR injury as mice lacking CD39 or CD73 show increased renal injury after

**Fig. 7.** A: representative RT-PCR images for monocyte chemoattractive protein-1 (MCP-1), macrophage inflammatory protein-2 (MIP-2), ICAM-1, keratinocyte-derived cytokine (KC), and TNF-α mRNA expression in sham, IR, and mice treated with CCPA (0.1 mg/kg) 15 min (acute) or 24 h (delayed) before ischemia or sham operation. Representative of 3–5 separate experiments. GAPDH included to control for mRNA input. B: densitometric quantifications of relative band intensities normalized to GAPDH from RT-PCR reactions of proinflammatory mRNAs for MCP-1 (n = 4), MIP-2 (n = 5), TNF-α (n = 5), and KC (n = 3) from renal cortices (including outermedullary junction) of mice subjected to sham operation or IR after injection with vehicle or CCPA 15 min (acute) or 24 h (delayed) before renal ischemia. Means ± SE shown. The number of experiments is indicated in parentheses.
renal IR (15, 16). Therefore, endogenous intrarenal adenosine generation and activation of renal AR serve to protect the kidney against IR injury. These findings are analogous to our previous study that showed mice with genetic deletion of A1AR have worse renal injury, increased necrosis, and more apoptosis after IR (34).

The current study shares several similarities with our previous study elucidating the mechanisms of acute and delayed renal IPC. Similar to the mechanisms of acute ischemic preconditioning (IPC), inhibition of Akt, PKC, and Gi/o abolished the protective effects of acute A1AR-mediated renal protection and inhibition of ERK MAPK failed to prevent acute A1AR-mediated renal protection. Moreover, there was phosphorylation and induction of HSP27 with both IPC and A1AR-mediated acute and delayed renal protection. In contrast, unlike the mechanisms of delayed renal IPC, inhibition of Akt prevented delayed A1AR-mediated renal protection and induction of NOS was not involved in mediating delayed A1AR-induced renal protection. Also, in contrast with the mechanisms of delayed IPC, total as well as the phosphorylated form of Akt increased 24 h after CCPA injection.

We show that acute renal protection with A1AR activation is associated with rapid phosphorylation of the cytoprotective kinases ERK and Akt. Activation of ERK MAPK is strongly associated with enhanced protection against several forms of injury including necrosis and apoptosis (4, 13). The serine/threonine kinase Akt is an important component of cell survival pathways in many cell types (18, 19). In particular, Akt has diverse functions to counteract apoptosis including inhibition of mitochondrial cytochrome c and phosphorylation of several proapoptotic factors (e.g., bad, caspase 9, glycogen synthase kinase 3) (7, 24). Akt can also increase the activity of HSP27 in certain cell types (25, 43, 44).

However, we determined that only inhibition of the PI3K→Akt pathway blocked the renal protective effects of acute A1AR-mediated renal protection. Therefore, although ERK activation was observed, it is not responsible for the renal protective effects of acute A1AR-mediated renal protection. Our study is the first to implicate activation of the PI3K→Akt pathway in acute A1AR-mediated renal protection which is supported by two key findings: 1) inhibition of PI3K with wortmannin or LY294002 prevented the renal protection with acute A1AR activation, and 2) acute A1AR activation led to rapid phosphorylation of Akt. It is important to note that inhibition of the PI3K→Akt pathway with wortmannin or LY294002 has no effect on renal function of mice subjected to renal IR without CCPA. Further supporting a role of PI3K in acute and delayed A1AR-mediated renal protection is that mice lacking a subtype of PI3K that transduces G protein-coupled receptor signaling, PI3KH9253, were not protected against renal IR injury with CCPA. PI3KH9253 represents one of four isoforms of class I PI3K and PI3Kγ acts downstream of G protein-coupled receptors such as A1ARs.

Upregulation of NO with iNOS induction has been implicated in cardiac and neuronal protection induced by preconditioning (31). We show that A1AR activation with CCPA treatment (0.1 mg/kg) 15 min or 24 h before ischemia reduced TUNEL-positive cells. A1AR KO mice showed increased TUNEL-positive cells compared with A1AR WT mice. Sections were obtained 24 h after reperfusion. Representative of 5 similar experiments.
tioning and inhibition of iNOS prevents the protective effects of delayed renal IPC in the heart as well as in the kidney (20, 23, 48). NO plays an important role in maintaining normal renal physiological functions including tubular functions, regulating renal endocrine metabolism and the control of renal hemodynamics (14). Unlike our previous study where NOS induction was at least partially responsible for renal protection with delayed IPC, delayed A1AR-mediated renal protection is independent of NOS induction as delayed A1AR-mediated renal protection occurred with the pharmacological inhibitor of NO (L-NIL) and in iNOS KO mice.

We demonstrate in this study that A1AR activation produced increased phosphorylation of HSP27 that persisted for 24 h after agonist treatment. Therefore, phosphorylation of HSP27 may have contributed to the renal protective effect with both acute and delayed A1AR effects. Total HSP27 protein expression increased ~24 h after A1AR activation and this may have contributed to the renal protective effect. HSP27 is a powerful and ubiquitously cytoprotective HSP (38). Both phosphorylated and nonphosphorylated forms of HSP27 can reduce cellular injury against diverse forms of stress including renal injury. HSP27 stabilizes the actin cytoskeleton to preserve the renal architectural integrity after IR injury. HSP27 also prevents activation of several caspases and inhibits the release of cytochrome c from the mitochondria. It remains to be determined whether a direct link exists between HSP27 phosphorylation/induction and A1AR-mediated renal protection.

Thirty minutes of renal ischemia followed by 24 h of reperfusion resulted in significant functional (as evidenced by large increases in serum Cr) and morphological (necrosis and apoptosis) renal injury. We demonstrate in this study that transient A1AR activation produces both acute and delayed protective effects in the kidney including reduced renal cortical necrosis (Jablonski grading, Fig. 5) and as well as apoptosis (DNA laddering, Fig. 8, and quantification of apoptotic cells in renal corticomedullary junction) after IR injury. Renal apoptosis is an important component in the development of ARF after IR injury. Moreover, Daemen et al. (8–10) demonstrated that increases in apoptosis after renal IR injury were directly associated with increased renal inflammation. They also demonstrated that blocking apoptosis prevented renal inflammation after IR. In our previous study, we demonstrated that acute A1AR activation led to a reduction in apoptosis evidenced by reduced DNA laddering, TUNEL-positive cells, and visual counts of apoptotic bodies. We show in this study that transient A1AR activation 24 h before an ischemic insult also reduced renal tubular apoptosis (Fig. 8).

In our current study with mice as well as our previous study in rats (27), our regimen of PKC inhibition with chelerythrine attenuated the protective effects of acute A1AR activation indicating the efficacy of chelerythrine-mediated PKC inhibition in vivo. In contrast, the delayed A1AR renal protection (CCPA injection 24 h before renal ischemia) was neither prevented nor attenuated by PKC inhibition with chelerythrine. However, since it is difficult to fully ascertain the complex interactions of various drugs in vivo (e.g., half-life of chelerythrine vs. half-life of CCPA), we plan to obtain PKC knockout mice and confirm the PKC independence of delayed A1AR-mediated renal protection in vivo.

In summary, we demonstrate in this study that activation of A1AR produces both acute and delayed renal protection in mice. Acute A1AR-mediated protection is mediated via G\(_{\text{i/o}}\) PKC, and Akt phosphorylation, whereas delayed A1AR-mediated protection involves G\(_{\text{i/o}}\) and Akt activation. Additionally, roles for HSP27 phosphorylation and upregulation are strongly supported by our studies. Unlike the mechanisms involved in delayed A1AR-mediated protection in the heart and delayed renal IPC in the kidney, induction of NOS is not part of the mechanisms of delayed A1AR-mediated renal protection. Clinical manipulation of the signaling pathways of A1AR activation that mediate protection may lead to therapeutic improvements to prevent or reduce the incidence of ARF during the perioperative period.

GRANTS

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