A1 Adenosine Receptor Activation Inhibits Inflammation, Necrosis, and Apoptosis after Renal Ischemia-Reperfusion Injury in Mice

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Abstract. It was previously demonstrated that preischemic A1 adenosine receptor (AR) activation protects renal function after ischemia-reperfusion (IR) injury in rats. The role of the A1 AR in modulating inflammation, necrosis, and apoptosis in the kidney after IR renal injury was further characterized. C57BL/6 mice were subjected to 30 min of renal ischemia, with or without pretreatment with 1,3-dipropyl-8-cyclopentylxanthine or 2-chlorocyclopentyladenosine (selective A1 AR agonist and antagonist, respectively). Plasma creatinine levels and renal inflammation, necrosis, and apoptosis were compared 24 h after renal injury. C57BL/6 mice that had been pretreated with the A1 AR agonist demonstrated significantly improved renal function and reduced expression of inflammatory markers, necrosis, and apoptosis 24 h after IR injury. In contrast, C57BL/6 mice that had been pretreated with the A1 AR antagonist demonstrated significantly worsened renal function and increased expression of inflammatory markers, necrosis, and apoptosis. In conclusion, it was demonstrated that endogenous and exogenous preischemic activation of the A1 AR protects against IR injury in vivo, through mechanisms that reduce inflammation, necrosis, and apoptosis.

Acute renal failure (ARF) secondary to ischemia-reperfusion (IR) injury continues to be a significant perioperative problem. ARF is frequently complicated by many other life-threatening complications, including sepsis and multiorgan failure. The prognosis for ARF is poor (with mortality rates of approximately 50%) and has changed little in the past 40 yr (1–3). We previously demonstrated that pharmacologic adenosine receptor (AR) modulation significantly affects renal function after IR injury in rats (4–6). In particular, we demonstrated that preischemic activation of the A1 AR attenuated renal failure after IR injury in vivo (4). We also demonstrated the cytotoxic protective effects of A1 AR activation in cultured proximal tubule cells injured by H2O2 or severe ATP depletion (7,8).

Modulation of AR has been demonstrated to attenuate necrosis (9), inflammation (10,11), and apoptosis (12,13) after injury. Both apoptosis and necrosis contribute significantly to the pathogenesis of ARF after IR injury (14,15). Moreover, inflammatory renal injury is a significant component of necrotic renal cell death (16,17). It remains to be determined whether the renoprotective effect of preischemic A1 AR activation is associated with modulation of apoptosis, necrosis, and/or inflammation. Therefore, in this study, we aimed to extend our previous findings regarding the mechanisms of A1 AR effects on renal function after IR injury. We questioned whether the protective effects of A1 AR activation were mediated through a decrease in the inflammatory response in the kidney and whether necrotic or apoptotic cell death was attenuated. We hypothesized that preischemic activation or inhibition of A1 AR would decrease or increase levels of inflammation markers, respectively. Moreover, we hypothesized that necrosis and possibly apoptosis would be decreased or increased by activation or inhibition of A1 AR, respectively, before IR injury in mice.

Materials and Methods
Renal Injury Protocol
C57BL/6 (C57) mice (20 to 25 g; Harlan, Indianapolis, IN) were anesthetized with intraperitoneally administered pentobarbital (50 mg/kg or to effect) and placed supine on a heating pad under a warming light, for maintenance of body temperature between 36°C and 38°C. Additional pentobarbital was administered as needed, on the basis of responses to tail pinches. Bilateral flank incisions were made and, after right nephrectomy, the left kidney was subjected to 30 min of ischemia with a microaneurysm clip. This duration of ischemia was chosen on the basis of preliminary studies, to maximize the reproducibility of renal injury and to minimize mortality rates for these mice. Some C57 mice were pretreated with either 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) (1 mg/kg, administered intraperitoneally), a selective A1 AR antagonist, or 2-chlorocyclopentyladenosine (CCPA) (0.1 mg/kg, administered intraperitoneally), a selective A1 AR agonist, 15 min before renal ischemia, for determination of the role of A1 AR in renal IR injury.

A1 AR are classically thought to couple to second messenger pathways through pertussis toxin-sensitive G proteins (Gt), and we
previously demonstrated in rats that $A_1$ AR-mediated renoprotection requires $G_{i/o}$ (6). To confirm that the $A_1$ AR agonist CCPA couples through $G_{i/o}$, some C57 mice were pretreated with 25 $\mu$g/kg pertussis toxin, administered intraperitoneally, 48 h before CCPA treatment.

**Assessment of Renal Function after IR Injury**

Renal function was assessed by measuring plasma creatinine concentrations 24 h after renal ischemia, with a commercially available colorimetric method (Sigma Chemical Co., St. Louis, MO).

**Histologic Examinations to Detect Necrosis**

Explanted kidneys were bisected along the long axis and were fixed in 10% formalin solution overnight. After automated dehydration through a graded alcohol series, transverse kidney slices were embedded in paraffin, sectioned at 5 $\mu$m, and stained with hematoxylin and eosin. Morphologic assessment was performed by an experienced renal pathologist (Dr. Nasr), who was unaware of the treatment each animal had received. An established grading scale (scores of 0 to 4) for assessment of necrotic injury to the proximal tubules was used for the histopathologic assessment of IR-induced damage, as outlined by Jablonski et al. (18).

**Assessment of Renal Inflammation**

**Types of Assessments.** Renal inflammation after IR injury was determined with measurements of renal cortical myeloperoxidase (MPO) (a marker of leukocyte infiltration), measurements of neutrophil infiltration (with both hematoxylin and eosin staining and immunohistochemical analyses of neutrophils), and measurements of mRNA encoding markers of inflammation, including TNF-$\alpha$, IL-1$\beta$, and intercellular adhesion molecule 1 (ICAM-1).

**Renal Cortical MPO Assay.** MPO, an enzyme present in leukocytes, is a marker of tissue leukocyte infiltration (19). Twenty-four hours after renal ischemic injury, renal cortex (approximately 200 mg) was dissected and homogenized for 30 s in 2 ml of buffer A (50 mM potassium phosphate, pH 7.4) at 4°C. The samples were centrifuged for 15 min at 16,000 $\times g$ at 4°C, and the resultant pellet was resuspended in 2 ml of buffer A with 0.5% hexadecyltrimethyl ammonium bromide at 4°C. The samples were sonicated for 30 s and centrifuged at 16,000 $\times g$ for 15 min at 4°C. Fifty microliters of supernatant were mixed with 750 $\mu$l of 45 mM potassium phosphate buffer (pH 6.0) containing 0.167 mg/ml o-dianisidine and 0.3% H$_2$O$_2$. The remaining supernatant was used to determine protein concentrations. Absorbance (460 nm) was measured for 5 min, and MPO enzyme activity was expressed as the change in absorbance per minute per milligram of protein.

**Histologic Quantification of Neutrophil Infiltration.** Because posts ischemic infiltration of neutrophils contributes to the inflammatory process and injury in the kidney (17,20), neutrophil infiltration into the corticomedullary junction was measured. An experienced pathologist (Dr. Nasr) who was unaware of the treatment groups identified neutrophils on the basis of the localization of the cells and the morphologic features of the nuclei of the cells in light-microscopic assessments of hematoxylin/eosin-stained samples. Neutrophils were quantified in 75 randomly chosen microscopic fields (magnification, $\times$400) in the corticomedullary junction, and results were expressed as neutrophils counted per square millimeter.

**Immunohistochemical Detection of Neutrophils.** We also immunohistochemically detected renal neutrophil infiltration. Fixed mouse kidney sections (see above) were deparaffinized in xylene and rehydrated through a graded ethanol series to water. After blockage with 10% normal horse serum in PBS, the slides were stained for neutrophils in sequential incubations with rat anti-mouse neutrophil primary antibody (mAb 7/4, 1/60 dilution; Serotec, Raleigh, NC) for 30 min, horseradish peroxidase-conjugated rabbit anti-rat IgG (1/60 dilution) for 30 min, and diaminobenzidine reagent (Vector Laboratories, Burlingame, CA) for 10 min.

**Semiquantitative Reverse Transcription-PCR Assays of TNF-$\alpha$, IL-1$\beta$, and ICAM-1.** Twenty-four hours after renal ischemic injury, renal cortices were dissected and total RNA was extracted with Trizol reagent, according to the instructions provided by the manufacturer (Invitrogen, Carlsbad, CA). RNA concentrations were determined on the basis of spectrophotometric absorbance at 260 nm, and aliquots were subjected to electrophoresis on agarose gels for verification of equal loading and RNA quality. Semiquantitative reverse transcription (RT)-PCR was performed to analyze the expression of proinflammatory genes (TNF-$\alpha$, IL-1$\beta$, and ICAM-1). The PCR cycle number for each primer pair was first optimized to yield linear increases in the densitometric measurements for resulting bands with increasing PCR cycles (15 to 30 cycles). The starting amount of RNA was also optimized to yield linear increases in the densitometric measurements for resulting bands with the established number of PCR cycles. For each experiment, we also performed semiquantitative RT-PCR under conditions that yielded linear results for glyceraldehyde-3-phosphate dehydrogenase, to confirm equal RNA input. On the basis of these preliminary experiments, 0.5 to 1.0 $\mu$g of total RNA was used as the template for all RT-PCR assays. The numbers of PCR cycles that yielded linear results were 21, 24, 22, and 15 for ICAM-1,

### Table 1. Primer sequences

<table>
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<tr>
<th>Primer</th>
<th>Accession No., Mouse</th>
<th>Sequence (Sense/Antisense)</th>
<th>Product Size (bp)</th>
<th>Annealing Temperature ($^\circ$C)</th>
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<td>X02611</td>
<td>5’-TACTGAACCTCAGGGGATTTGCTCC-3’  5’-CAGGCTTTGCTCCCTGAAGAGAC-3’</td>
<td>290</td>
<td>65</td>
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<tr>
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<td>X52264</td>
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<td>60</td>
</tr>
<tr>
<td>IL-1$\beta$</td>
<td>NM_008361</td>
<td>5’-CTGAAAGCTCCTCCACCTC-3’  5’-TGTCCTGATCGACCTGGG-3’</td>
<td>292</td>
<td>56</td>
</tr>
<tr>
<td>GAPDH</td>
<td>M32599</td>
<td>5’-ACCACACGTCCATGGCCTACAC-3’  5’-CACCCACCTGTGTGCTGAGCC-3’</td>
<td>420</td>
<td>65</td>
</tr>
</tbody>
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*a ICAM-1, intercellular adhesion molecule-1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.*
TNF-α, IL-1β, and glyceraldehyde-3-phosphate dehydrogenase, respectively. Primers were designed on the basis of published GenBank sequences (Table 1). Primer pairs were chosen to yield expected PCR products of 200 to 600 bp and to amplify genomic regions spanning one or two introns, to eliminate the confounding effect of amplification of contaminating genomic DNA. Primers were purchased from Sigma Genosys (The Woodlands, TX). RT-PCR was performed with the Access RT-PCR system (Promega, Madison, WI), which is designed for a single-tube reaction for first-strand cDNA synthesis (48°C for 45 min) with avian myeloblastosis virus reverse transcriptase and subsequent PCR with T7 DNA polymerase. PCR cycles included denaturation at 94°C for 30 s, annealing at an optimized temperature (Table 1) for 1 min, and extension at 68°C for 1 min. All PCR were completed with a 7-min incubation at 68°C, to allow enzymatic completion of incomplete cDNA. The products were resolved on a 6% polyacrylamide gel and stained with Syber green (Roche, Indianapolis, IN), and the band intensities were quantified with a Fluor-S multi-imager (Bio-Rad, Hercules, CA).

Histologic Examinations to Detect Apoptosis

Apoptotic bodies in proximal tubules in the outer stripe of the corticomedullary junction were quantitated in hematoxylin/eosin-stained kidney sections (expressed as the mean number of apoptotic bodies per tubule). This area of the kidney is the most severely injured in renal IR injury. At least 25 to 30 tubules were counted in each field, and six fields were examined for each slide.

Apopotosis-Induced DNA Laddering

Renal apoptosis was also demonstrated by DNA laddering (intranucleosomal DNA fragmentation). Twenty-four hours after renal ischemic injury, renal cortices were dissected. The extracted DNA (Wizard; Promega) was subjected to electrophoresis at 70 V through a 2.0% agarose gel in Tris-acetate-EDTA buffer. The gel was stained with ethidium bromide and photographed with UV illumination, in a Bio-Rad Fluor-S multi-imager. DNA markers (100 bp) were added to each gel as a reference for the analysis of intranucleosomal DNA fragmentation (approximately 1800-bp fragments).

In Situ Terminal Deoxynucleotidyl Transferase Biotin-dUTP Nick End-Labeling Assays

We also used terminal deoxynucleotidyl transferase biotin-dUTP nick end-labeling (TUNEL) staining to detect DNA fragmentation in apoptosis. Fixed mouse kidney sections obtained 24 h after renal injury were deparaffinized in xylene and rehydrated through a graded ethanol series to water. In situ labeling of fragmented DNA was performed with TUNEL staining (green fluorescence) with a commercially available in situ cell death detection kit (Roche), according to the instructions provided by the manufacturer. For observation of the total number of cells in the field, kidney sections were also stained with propidium iodide (red fluorescence).

Statistical Analyses

The data were analyzed with one-way ANOVA plus the Dunnett post hoc multiple-comparison test, for comparisons of mean values among multiple treatment groups.

Reagents

Unless otherwise specified, all reagents were purchased from Sigma Chemical Co.

Protein Determination

Protein contents were determined with a bicinchoninic acid protein assay kit (Pierce Chemical Co., Rockford, IL), using BSA as a standard.

Results

Exogenous Blockade of A1 AR Increases Mortality Rates after IR Injury

All mice subjected to renal IR injury or pretreatment with CCPA before renal IR injury survived for 24 h. However, 40% of C57 mice that had been pretreated with DPCPX before IR injury died within 24 h after reperfusion.

Modulation of A1 AR Affects Renal Function after IR Injury

C57 mice that were subjected to 30 min of renal IR demonstrated a significant increase in creatinine values 24 h after injury, compared with C57 mice that underwent sham operations (anesthesia and laparotomy without renal ischemia) (IR injury: creatinine level, 2.5 ± 0.1 mg/dl, n = 4; sham: creatinine level, 0.3 ± 0.1 mg/dl, n = 3) (Figure 1). In contrast, C57 mice that had been pretreated with a selective A1 AR agonist (0.1 mg/kg CCPA) or with a selective A1 AR antagonist (1 mg/kg DPCPX) before renal ischemia demonstrated significantly improved (creatinine level, 0.6 ± 0.1 mg/dl, n = 8) or worsened (creatinine level, 3.4 ± 0.4 mg/dl, n = 6) renal function at 24 h, respectively. Renoprotection with the A1 AR agonist was mediated by the heterotrimeric G protein Gi.

**Figure 1.** Plasma creatinine levels at 24 h for sham-operated C57BL/6 (C57) control mice (n = 3), C57 mice subjected to 30 min of renal ischemia and 24 h of reperfusion [ischemia-reperfusion (IR) injury] (n = 4), C57 mice pretreated with 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) before IR injury (n = 6), C57 mice pretreated with 2-chlorocyclopentyladenosine (CCPA) before IR injury (n = 8), and C57 mice pretreated with pertussis toxin (PTX) 48 h before CCPA treatment and IR injury (n = 3). *P < 0.05 versus sham operation; #P < 0.05 versus IR; **P < 0.05 versus IR plus CCPA. Error bars, 1 SEM.
inasmuch as C57 mice that had been pretreated with pertussis toxin were no longer protected by pretreatment with CCPA (creatinine level at 24 h, 2.8 ± 0.3 mg/dl, n = 3).

Quantitative Assessments of Tubular Necrosis after A₁ AR Modulation and IR Injury Are Correlated with Renal Function

C57 mice subjected to IR injury demonstrated histologic evidence of necrosis, compared with sham-operated control mice (Figure 2, A and B). Mice that had been pretreated with the A₁ AR antagonist (DPCPX) before IR injury demonstrated even greater histologic evidence of necrosis (Figure 2C). In contrast, mice that had been pretreated with the A₁ AR agonist before IR injury exhibited less necrosis (Figure 2D), compared with mice subjected to IR injury alone. The results of quantitative assessments of renal tubular necrosis with the grading scale described by Jablonski et al. (18) are presented in Figure 3. Histologic grading at 24 h after 30 min of renal ischemia in C57 mice demonstrated mild/moderate acute tubular necrosis (grade of 2.8 ± 0.4, n = 8). Pretreatment of C57 mice with DPCPX before IR injury resulted in more severe tubular necrosis, as evidenced by medullary congestion, hemorrhage, and proteinaceous casts (grade of 4 ± 0, n = 4). In contrast, C57 mice that had been pretreated with the A₁ AR agonist CCPA before IR injury demonstrated significantly improved renal development of proteinaceous casts, which were accentuated by pretreatment with the A₁ adenosine receptor (AR) antagonist DPCPX and attenuated by pretreatment with the A₁ AR agonist CCPA. Hematoxylin and eosin staining. Magnification, ×200.

Figure 2. Representative light photomicrographs of the outer medulla of the kidney. (A) Sham-operated C57 mice. (B) C57 mice subjected to IR injury. (C) C57 mice pretreated with DPCPX before IR injury. (D) C57 mice pretreated with CCPA before IR injury. IR injury resulted in increased medullary congestion, hemorrhage, and the

Figure 3. Jablonski grading scale scores for the histologic appearance of acute tubular necrosis in sham-operated C57 mice, C57 mice subjected to IR, C57 mice pretreated with DPCPX before IR injury, and C57 mice pretreated with CCPA before IR injury (n = 4). *P < 0.05 versus sham operation; #P < 0.05 versus IR plus DPCPX. Error bars, 1 SEM.
morphologic features (grade of $0.7 \pm 0.7$, $n = 4$), compared with C57 mice subjected to IR only.

Modulation of $A_1$ AR Affects Renal Inflammation after IR Injury

Renal Cortical MPO Assay. Renal cortices isolated from C57 mice subjected to 24 h of reperfusion after 30 min of renal ischemia demonstrated increased MPO activity ($2.5 \pm 0.4 \Delta OD/min$ per mg protein, $n = 6$), compared with sham-operated control mice ($1.2 \pm 0.3 \Delta OD/min$ per mg protein, $n = 6$, $P < 0.05$) (Figure 4). C57 mice that had been pretreated with the $A_1$ AR antagonist DPCPX before renal IR demonstrated significantly higher MPO activity ($3.4 \pm 0.3 \Delta OD/min$ per mg protein, $P < 0.05$, $n = 6$), compared with C57 mice subjected to IR injury alone. In contrast, C57 mice that had been pretreated with the $A_1$ AR agonist CCPA before IR injury demonstrated significantly reduced MPO activity in renal cortices at 24 h ($1.5 \pm 0.1 \Delta OD/min$ per mg protein, $P < 0.05$, $n = 6$), compared with C57 mice subjected to IR injury alone.

RT-PCR Assays of Proinflammatory Genes. The expression of mRNA encoding proinflammatory proteins (ICAM-1, TNF-α, and IL-1β) was measured in total RNA isolated from renal cortices of C57 mice subjected to sham operations, renal IR injury, or pretreatment with an $A_1$ AR antagonist or agonist before IR injury. The quantitative accuracy of our RT-PCR technique was first established (see the Materials and Methods section). Images of representative Syber green-stained gels are presented in Figure 5A. Quantification of relative band intensities is presented in Figure 5B. C57 mice subjected to 30 min of renal IR injury demonstrated increased expression of mRNA encoding ICAM-1, IL-1β, and TNF-α in renal cortices at 24 h. The increase in mRNA encoding TNF-α and IL-1β was further enhanced by pretreatment of mice with the $A_1$ AR antagonist DPCPX before IR injury.

![Figure 4](image.png)

Figure 4. Myeloperoxidase (MPO) activity in renal cortices of C57 mice subjected to sham operation, IR injury, pretreatment with the $A_1$ AR antagonist DPCPX before IR injury, or pretreatment with the $A_1$ AR agonist CCPA before IR injury ($n = 6$). *$P < 0.05$ versus sham operation; *$P < 0.05$ versus IR. Error bars, 1 SEM.

![Figure 5](image.png)

Figure 5. (A) Representative images of semiquantitative reverse transcription (RT)-PCR of mRNA encoding IL-1β (top), TNF-α (bottom left), and intercellular adhesion molecule-1 (ICAM-1) (bottom right) in mouse renal cortices. Representative results are shown for sham-operated mice, mice subjected to IR, mice pretreated with DPCPX before IR injury, and mice pretreated with CCPA before IR injury. A 100-bp RNA size marker is shown for each gel. (B) Densitometric quantification of relative band intensities from RT-PCR assays for ICAM-1, IL-1β, and TNF-α ($n = 4$). *$P < 0.05$ versus sham operation; *$P < 0.05$ versus IR. Error bars, 1 SEM.
injury. In contrast, the increase in all three proinflammatory markers was attenuated in mice that had been pretreated with the A1 AR agonist CCPA before IR injury, compared with mice subjected to renal IR injury alone.

Immunohistochemical and Histologic Detection of Neutrophils. Figure 6 demonstrates representative immunohistochemical detection of neutrophil infiltration into the corticomedullary junction in sham-operated or IR-injured mice. Renal IR injury results in the recruitment of neutrophils, which accentuate renal injury. Sham-operated C57 mice did not exhibit detectable levels of neutrophils infiltrating the corticomedullary junction of the kidney (n = 4). C57 mice subjected to IR injury alone demonstrated an increase in neutrophil infiltration (45.5 ± 9.9 neutrophils/mm², n = 4) 24 h after IR injury, whereas mice that had been pretreated with DPCPX before IR injury exhibited an even greater increase (53.7 ± 4.0 neutrophils/mm², n = 4). Pretreatment of mice with the A1 AR agonist CCPA before IR injury reduced levels of neutrophil infiltration back to undetectable control levels (n = 4).

Modulation of A1 AR Affects the Severity of Apoptosis after IR Injury

DNA Laddering. Genomic DNA isolated from the kidneys of sham-operated control mice did not exhibit DNA laddering (n = 4). In contrast, laddering was present in DNA isolated from the kidneys of mice subjected to IR injury (Figure 7). DNA from mice that had been pretreated with the A1 AR antagonist (DPCPX) or agonist (CCPA) displayed increased or decreased fragmentation, respectively.

Assessment of Renal Tubular Apoptotic Bodies. The degree of renal tubular apoptosis was quantified by counting the number of apoptotic bodies in proximal tubules in the corticomedullary area of the kidney (expressed as apoptotic bodies per tubule). Twenty-five to 50 tubules/field were counted for each treatment group, and kidneys from four experiments were examined. Sham-operated C57 mice exhibited no morphologic evidence of apoptosis (0.02 ± 0.01 apoptotic bodies/tubule) (Figure 8). Renal IR injury increased the number of apoptotic bodies within the proximal tubules of C57 mice (0.67 ± 0.12 apoptotic bodies/tubule, P < 0.05 versus sham operation). Pretreatment of mice with the A1 AR agonist CCPA before IR injury caused a decrease in the number of apoptotic bodies at 24 h (0.09 ± 0.03 apoptotic bodies/tubule). In contrast, pretreatment of mice with the A1 AR antagonist DPCPX before IR injury led to an increase in the number of apoptotic bodies (1.07 ± 0.10 apoptotic bodies/tubule, P < 0.05 versus IR injury).

Figure 6. Representative photomicrographs of neutrophil accumulation in the renal cortices of sham-operated C57 mice (A), mice subjected to IR (B), mice pretreated with DPCPX before IR injury (C), and mice pretreated with CCPA before IR injury (D). Very few scattered neutrophils were observed in the renal cortices of sham-operated mice or mice that had been pretreated with the A1 AR agonist CCPA before IR injury. Mice subjected to IR injury alone exhibited large increases in neutrophil infiltration, and increases were even greater in mice that had been pretreated with the A1 AR antagonist DPCPX before IR injury. Quantification of the neutrophil infiltration is described in the text.
TUNEL Assay. We failed to detect TUNEL-positive cells in kidney sections (corticomedullary junction) from sham-operated mice (Figure 9A). Mice subjected to 30 min of renal ischemia and 24 h of reperfusion (Figure 9B) demonstrated few TUNEL-positive cells in the corticomedullary junction. Consistent with the results of visual inspection of apoptotic bodies, pretreatment of mice with the A1 AR antagonist or agonist displayed increased or decreased fragmentation, respectively. Each lane represents DNA from an individual animal. A 100-bp DNA size marker (M) was loaded in the first lane.

Discussion

The major findings of this study were that (1) exogenous activation of A1 AR before renal ischemia attenuated renal IR injury in C57 mice and was associated with decreased inflammation, necrosis, and apoptosis and (2) antagonism of A1 AR before renal ischemia exacerbated renal IR injury in C57 mice and was associated with increased inflammation, necrosis, and apoptosis. Therefore, both endogenous A1 AR activation and exogenous A1 AR activation play cytoprotective roles in the development of ARF after IR injury.

Preischemic A1 AR activation has been demonstrated to protect against IR injury in many organs, including the heart, kidney, and brain (7,21,22). In our previous studies, we demonstrated that preischemic A1 AR or postischemic A2a AR activation protected rats against renal IR injury (4–6), but a mechanism directly involving the kidney was not elucidated in those previous studies. This study in mice supports and extends our previous findings in rats (4), in that pretreatment with a highly selective A1 AR agonist (CCPA) protected renal function after IR injury, through a signaling pathway involving Gi/o, as indicated by the abolishment of CCPA-mediated renoprotection with pertussis toxin treatment. Moreover, we demonstrated for the first time that preischemic blockade of endogenous A1 AR exacerbated renal dysfunction after IR injury, suggesting a cytoprotective role of endogenous tonic A1 AR activation.

Our findings of renoprotection with A1 AR agonist (CCPA) pretreatment before IR injury and exacerbation of renal IR injury with A1 AR antagonist (DPCPX) pretreatment before IR injury contradict previous conclusions regarding the role of A1 AR in renoprotection. Several investigators reported that a nonselective AR antagonist (theophylline) or selective A1 AR antagonists (DPCPX or KW-3902) protected animals against ischemic and nephrotoxic ARF (23–26). The authors’ hypothesis was based on the renal hemodynamic effects of A1 AR stimulation (reduction of GFR, decreases in solute transport, and reduction of renal blood flow via afferent arteriolar constriction) and not on intrinsic effects in the kidney. Moreover, in those previous studies of IR injury, plasma creatinine levels...
Figure 9. Representative fluorescence photomicrographs of kidney sections from identical fields, demonstrating apoptotic nuclei [terminal deoxynucleotidyl transferase biotin-dUTP nick end-labeling (TUNEL) fluorescence staining] (left) or total nuclei (propidium iodide fluorescence staining) (right). (A) Sham-operated mice demonstrated no apoptotic nuclei. (B) Mice subjected to IR injury demonstrated occasional TUNEL-positive cells at 24 h. (C) Pretreatment of mice with the A1 AR antagonist DPCPX before IR injury led to an increase in the number of TUNEL-positive cells. (D) Pretreatment of mice with the A1 AR agonist CCPA before IR injury decreased the number of TUNEL-positive cells. Corticomedullary areas are shown. Magnification, ×400. Arrows, TUNEL-positive cells. Photomicrographs are representative of four independent experiments.
were not measured as indicators of renal function. Those studies demonstrated that AR antagonists reversed decreases in solute transport and stimulated increased diuresis in the presence of the acute reductions in renal function associated with ARF (23–26). At first glance, the effects of A1 AR activation seem to be detrimental to renal function, with reduced GFR and afferent cortical blood flow and impaired solute transport. However, in a closer examination of the renal effects of A1 AR, several renoprotective attributes are apparent, including a reduction in GFR, reduced renin release and sympathetic outflow, and decreased active solute transport, all of which would reduce renal oxygen consumption during the metabolically vulnerable periods of ischemic and nephrotoxic renal injury.

We previously demonstrated in rats that A3 AR activation with N6-(3-iodobenzyl)-N-methyl-5′-carbamoyladenosine and inhibition with MRS-1191 worsened IR-induced renal failure and protected rats against IR-induced renal failure, respectively (4). We also demonstrated that mice lacking A3 AR were endogenously protected against ischemic and myoglobinuric forms of ARF. Taken together, this study and our previous studies demonstrate that antagonism of the A3 AR subtype, instead of the previously proposed antagonism of the A1 AR subtype, produces renoprotective effects.

In addition to the demonstration of a role for endogenous A1 AR activation in protection from renal IR injury, a major focus of this study was elucidation of the mechanisms of A1 AR-mediated protection within the kidney cortex. Modulation of inflammatory responses after IR injury is an important component of renoprotection, because inflammation is a major component of cell death associated with renal injury. Significant necrosis occurs with IR injury and necrotic tissue initiates an inflammatory cascade, which causes further necrosis. MPO is an enzyme present in leukocytes and is a marker of leukocyte infiltration into the renal parenchyma (20). Several recent studies demonstrated that leukocyte infiltration into the kidney after IR injury contributes significantly to the pathogenesis of ARF (17,27,28). An influx of leukocytes, including polymorphonuclear neutrophils, macrophages, and lymphocytes (19), during reperfusion initiates a cascade of proinflammatory events involving cytokine/chemokine liberation and free radical-mediated tubular damage. Sublethally damaged proximal tubular epithelial cells release chemokines and cytokines and have the capacity to upregulate adhesion molecules (e.g., ICAM-1) to facilitate leukocyte infiltration and adhesion (20,29). Early renal tissue TNF-α expression contributes to neutrophil infiltration in IR injury, and TNF-α plays a key role in increasing the expression of adhesion molecules (e.g., ICAM-1) after injury (20). In this study, we demonstrated that A1 AR activation or blockade was associated with reduced or enhanced inflammatory responses, respectively, after renal IR injury in mice. Increased levels of markers of inflammation (MPO activity, neutrophil infiltration, and expression of proinflammatory mRNA, including ICAM-1, TNF-α, and IL-1β) in renal cortex were demonstrated after IR injury, with attenuation or exacerbation with A1 AR agonism or antagonism, respectively.

We also demonstrated modulation of renal cortical apoptosis with A1 AR activation or antagonism. Renal apoptosis is an important factor in the development of ARF after IR injury. Moreover, Daemen et al. (16,30,31) demonstrated that increases in apoptosis after renal IR injury were directly associated with increased renal inflammation. Those authors also demonstrated that blockade of apoptosis prevented renal inflammation after IR. They hypothesized that a significant component of renal inflammation is induced by apoptosis. Insufficient local phagocytic capacity to clear apoptotic cells has been associated with subsequent necrosis, a process termed “secondary necrosis” (16). It is difficult to estimate the contribution of secondary necrosis to IR-induced inflammation in the in vivo model used in this study. However, we conclude from our data that preischemic A1 AR activation or blockade reduces or increases renal apoptosis, respectively, in addition to renal necrosis and inflammation, after IR injury.

In conclusion, we demonstrated that an A1 AR agonist and an antagonist protected animals against IR-mediated renal injury and worsened injury, respectively. Our study demonstrated that endogenous A1 AR serve as cytoprotective receptors. We further demonstrated that A1 AR modulation affects inflammation, necrosis, and apoptosis within the renal cortex after renal IR injury. These findings support the potential role of selective A1 AR agonists in protection against perioperative renal failure.

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References


