Adenosine attenuates oxidant injury in human proximal tubular cells via A₁ and A₂a adenosine receptors

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Received 26 June 2001; accepted in final form 16 November 2001

Lee, H. T., and Charles W. Emala. Adenosine attenuates oxidant injury in human proximal tubular cells via A₁ and A₂a adenosine receptors. Am J Physiol Renal Physiol 282: F844–F852, 2002. First published November 20, 2001; 10.1152/ajprenal.00195.2001.—We have recently demonstrated protection against renal ischemic-reperfusion injury in vivo by A₁- and A₂a-adenosine receptor (AR) modulations. To further elucidate the signaling cascades of AR-induced cytoprotection against reperfusion/oxidant-mediated injury, immortalized human proximal tubule (HK-2) cells were treated with H₂O₂. H₂O₂ caused dose- and time-dependent HK-2 cell death that was measured by lactate dehydrogenase release and trypan blue dye uptake. Adenosine protected against H₂O₂-induced HK-2 cell death by means of A₁- and A₂a-AR activation. A₁-AR-mediated protection involves pertussis toxin-sensitive G proteins and protein kinase C, whereas A₂a-AR-mediated protection involves protein kinase A activation by means of cAMP and activation of the cAMP response element binding protein. Moreover, protein kinase A activators (forskolin and Sp-isomer cAMP) also protected HK-2 cells against H₂O₂ injury. De novo gene transcription and protein synthesis are required for both A₁- and A₂a-AR-mediated cytoprotection as actinomycin D and cycloheximide, respectively, blocked cytoprotection. Chronic treatments with a nonselective AR agonist abolished the protection by adenosine. Moreover, chronic treatments with a nonselective AR antagonist increased the endogenous tolerance of HK-2 cells against H₂O₂. We concluded that A₁- and A₂a-AR activation protects HK-2 cells against H₂O₂-induced injury by means of distinct signaling pathways that require new gene transcription and new protein synthesis.

ACUTE RENAL FAILURE SECONDARY to ischemic-reperfusion (I/R) injury continues to be a significant clinical problem (22, 32, 45). The onset of acute renal failure implies a poor prognosis and is frequently complicated by many other life-threatening complications, including sepsis and multiorgan failure (3, 31, 32). In high-risk patients undergoing high-risk surgery, the mortality and morbidity rate from perioperative acute renal failure has changed little over the past 30 years (3, 13, 20, 45).

The A₁ and A₂a adenosine receptors (ARs) serve to protect against I/R injury in many organ systems, including the heart, brain, and kidney (16, 23–26, 36). We have recently demonstrated that pre- and postschematic activation of renal A₁- and A₂a-ARs, respectively, protected renal function against I/R injury in vivo (24–26). The in vivo mechanism of preischemic A₁-AR-mediated renal protection involves pertussis toxin-sensitive G proteins and protein kinase C (PKC), whereas postschematic A₂a-AR-mediated protection is through protein kinase A (PKA) activation by means of cAMP.

There are many limitations in using in vivo models of renal I/R injury to elucidate the detailed signaling cascades of AR-mediated renal cytoprotection. In vivo approaches must deal with multiple cell types within an organ and also with complex physiological control within an animal. In vitro studies with a pure population of a single cell type eliminate these complex external physiological influences and allow the direct study of signaling cascades.

The proximal tubules (especially, the straight, distal portion or S3 segment) located in the outer medulla of the kidney are the primary site of injury in renal ischemia and reperfusion (28, 48). Immortalized adult human proximal tubular cells (HK-2) have been transfected with E6/E7 genes of human papilloma virus type 16 (41). Transfection with human papilloma virus type 16 has been shown to immortalize epithelial cells of diverse origin without significantly altering their phenotypic characteristics. HK-2 cells have been shown to retain the phenotypic expression and functional characteristics of human proximal tubules (39, 41). Extensive studies have utilized HK-2 cells to study in vitro renal physiology and pathology (18, 19, 54). Presently, there are four subtypes of identified ARs (A₁, A₂a, A₂b, and A₃) (12). We have recently verified the presence of all four subtypes of ARs and demonstrated several key signaling intermediates in HK-2 cells (26a).

There are few studies of in vitro renal cell protection with AR modulations. Adenosine protected against hypoxia-reoxygenation injury in a porcine kidney cell line (LLC-PK₁) by means of an A₂a-AR→cAMP-medi-
ated mechanism (52). However, AR-mediated protection in a human renal cell line has not been reported to date. In this study, we injured HK-2 cells with H$_2$O$_2$. Oxygen free radicals with resultant oxidant tissue stress are a key mediator of renal reperfusion injury (15, 37). During reperfusion after ischemia, reactive oxygen species, such as superoxide anion, hydroxyl radical, and H$_2$O$_2$, are generated. These reactive oxygen species cause lipid peroxidation of the renal cell membrane, with a resultant intracellular calcium overload and subsequent necrotic cell death (37, 42, 43). We hypothesized that as we observed in vivo, A$_2$A-AR activation would protect against oxidant-mediated injury in human renal cells. We also aimed to determine the signaling pathways of AR-mediated protection against oxidant injury in HK-2 cells.

MATERIALS AND METHODS

HK-2 Cell Culture

HK-2 cells (American Type Culture Collection, Manassas, VA) were grown and passaged in 75-cm$^2$ cell culture flasks containing culture medium (keratinocyte serum-free medium + 5 ng/ml epidermal growth factor and 40 mg/ml bovine pituitary extract) and antibiotics (100 U/ml of penicillin G, 100 µg/ml of streptomycin, and 0.25 µg/ml of amphotericin B) at 37°C in a 100% humidified atmosphere of 5% CO$_2$-95% air. They were plated in 6- or 24-well plates when 80% confluent. Cells were plated in 6- or 24-well plates when 80% confluent and used in the experiments described in Specific Protocols when confluent.

Methods for Inducing Oxidant Injury: H$_2$O$_2$-Induced Injury for HK-2 Cells

Oxidant injury in HK-2 cells was induced with H$_2$O$_2$. After indicated pretreatments (e.g., with AR agonists or with vehicle), confluent monolayers of HK-2 cells grown in 6- or 24-well plates were treated with H$_2$O$_2$ diluted in serum-free media for 1–3 h.

Measurement of Cell Viability and Cell Death

Cell viability assays were performed by using the trypan blue dye exclusion method. After the treatment protocols (e.g., H$_2$O$_2$ + AR agonists), cells were trypsinized and stained with 0.4% trypan blue dye for 5 min. The proportion of cells remaining nonviable (unable to exclude trypan blue) was counted by using a hemocytometer. The number of cells remaining nonviable was expressed as a percentage of the total number of cells.

Lactate dehydrogenase (LDH) released into the media was also measured as a marker of cellular injury by using a commercially available colorimetric method (Sigma, St. Louis, MO). In some experiments, LDH released into the media was expressed as the percentage of total cellular LDH per well measured after the cells were lysed with 1% Triton X. Otherwise, LDH release after the various treatment protocols (e.g., H$_2$O$_2$ ± AR agonists) was expressed as the percentage of the LDH release by the H$_2$O$_2$ group within the same experiment.

Immunoblot Analyses

We measured activation of cAMP response element binding (CREB) proteins in HK-2 cells by immunoblotting with antibodies to the phosphorylated forms of CREB. The HK-2 cells in 6-well plates were washed twice with Hanks’ balanced salt solution and scraped with 100 µl of calcium-free Hanks’ balanced salt solution plus protease inhibitors (2 µg/ml leupeptin and 2 µg/ml aprotinin). Aliquots were used for protein assay, and the remainder were mixed with an equal volume of sample buffer (62.5 mM Tris, pH 6.8, 2% SDS, 10% glycerol, and 5% β-mercaptoethanol, final concentration). Twenty to forty micrograms of each sample were electrophoresed at room temperature through discontinuous 10% SDS-polyacrylamide gels at 80 V for 4 h and subjected to immunoblot analysis as described previously (17). The primary polyclonal antibody for phospho-CREB (New England Biolabs) was diluted 1:1,000 in Tris-buffered saline–0.1% Tween 20 containing 1% nonfat dry milk and 0.02% sodium azide. The secondary antibody (goat anti-rabbit IgG conjugated to horseradish peroxidase at 1:5,000 dilution) was detected with enhanced chemiluminescence immunoblotting detection reagents (Amersham), with subsequent exposure to autoradiography film. The intensities of the immunoblots were quantified with a scanner coupled to a personal computer with MacBas 2.2 software.

Up- and Downregulation of ARs in HK-2 Cells

We have previously demonstrated that treatment for 48 h with a nonselective AR antagonist, 8-phenyltheophylline (8-PT), upregulated A$_1$- and A$_2$A-ARs, whereas 48-h treatment with a nonselective AR agonist 5’-N-ethylcarboxamidoadenosine (NECA), downregulated all four subtypes of ARs (26a). When confluent, HK-2 cells grown in 6- or 24-well plates were incubated with 100 µM of nonselective AR antagonist 8-PT or 10 µM of nonselective agonist NECA for 48 h in complete cell culture media.

Protein Determination

Protein content was determined with the Pierce Chemical (Rockford, IL) bicinchoninic acid protein assay reagent with BSA as a standard.

Specific Protocols

We first determined the protective roles of adenosine and AR subtype-specific agonists against H$_2$O$_2$-mediated oxidant injury in HK-2 cells.

H$_2$O$_2$-induced injury. HK-2 cells in serum-free cell culture media were subjected to 1–3 h of 2, 5, and 10 mM H$_2$O$_2$. [The 1-h time point was chosen for the subsequent studies with 5 mM of H$_2$O$_2$ as this time and dose of H$_2$O$_2$-induced moderate (~50–60%) cellular injury.] Adenosine before H$_2$O$_2$-induced injury. HK-2 cells were incubated with 1–100 µM of adenosine for 30 min in serum-free cell culture media and then subjected to 1 h of 5 mM H$_2$O$_2$ injury.

Selective AR agonists before H$_2$O$_2$-induced injury. HK-2 cells were incubated with 1 nM of nonselective AR agonists against H$_2$O$_2$-mediated oxidant injury in HK-2 cells. H$_2$O$_2$-induced injury. HK-2 cells in serum-free cell culture media were subjected to 1–3 h of 2, 5, and 10 mM H$_2$O$_2$. [The 1-h time point was chosen for the subsequent studies with 5 mM of H$_2$O$_2$ as this time and dose of H$_2$O$_2$-induced moderate (~50–60%) cellular injury.] Adenosine before H$_2$O$_2$-induced injury. HK-2 cells were incubated with 1–100 µM of adenosine for 30 min in serum-free cell culture media and then subjected to 1 h of 5 mM H$_2$O$_2$ injury.

Selective AR agonists before H$_2$O$_2$-induced injury. HK-2 cells were incubated with 1 nM–10 µM of R-N’-phenyl-2-propylnitrobenzene (R-PNA), 4-[(N-ethyl-5’-carbomoyl)aminoethyl]-2-aminophenol (CPS, 215,500), or N’-[(2-aminoethyl)carbonyl]-N’-methyl-5-phenylcarboxylamide (IMCA, 215,500), highly selective A$_1$- and A$_2$A-AR agonists, respectively, for 30 min in serum-free cell culture media and then subjected to 1 h of 5 mM H$_2$O$_2$ injury.

Selective AR antagonists before adenosine. HK-2 cells were incubated with 1–10 µM of 1,3-dipropyl-8-cyclopentylxanthine, 8-(3-chlorostyryl)caffeine, or 9-chloro-2-(2-furyl)adenosine (9-[5’-carbamoyl]adenosine, highly selective A$_1$- and A$_2$A-AR agonists) for 30 min in serum-free cell culture media before 30 min of 100 µM adenosine followed by 1 h of 5 mM H$_2$O$_2$ injury.
We have previously described that PKC and pertussis toxin-sensitive G proteins (G_{i/o}) play important roles in signal transduction of A1-AR-mediated renal protection in vivo (24). To determine the potential roles of G_{i/o} and PKC in adenosine-mediated protection against oxidant injury, HK-2 cells were subjected to the following protocols.

**Adenosine or selective AR agonists + pertussis toxin before H_2O_2-induced injury.** HK-2 cells were pretreated with 100 ng/ml pertussis toxin for 14 h before adenosine or selective A_1-, A_2a-, or A_3-AR agonist treatment followed by 1 h of 5 mM H_2O_2 injury.

**PKC antagonist before adenosine or AR agonists.** HK-2 cells were incubated with 100 nM of GF-109203X, a highly selective PKC antagonist, for 30 min in serum-free cell culture media before adenosine or selective A_1-, A_2a-, or A_3-AR agonist treatment followed by 1 h of 5 mM H_2O_2 injury.

**A_2a-ARs Are Involved in Adenosine-Mediated Protection Against Oxidant Injury**

In HK-2 cells, adenosine-mediated protection against H_2O_2-mediated injury involves the A_1- and A_2a-ARs as A_1-AR agonist R-PIA (Figs. 3 and 4A) and A_2a-AR agonist CGS-21680 (Figs. 3 and 4B) pretreatment provided significant protection against H_2O_2-induced cell death. With 100 μM of adenosine pretreatment, significantly less LDH (66.7 ± 5.3% of 5 mM H_2O_2-alone treated group, n = 6, P < 0.05; Fig. 2) was released into the cell culture media and more cells excluded trypan blue dye (6.1 ± 0.9% trypan blue-positive cells vs. 14.8 ± 1.1% for 5 mM H_2O_2-alone-treated group, n = 6, P < 0.05; Fig. 3).

**Adenosine Protects Against H_2O_2 Injury**

Figures 2 and 3 show that adenosine pretreatment for 30 min significantly protects against H_2O_2-mediated cell death. With 100 μM of adenosine pretreatment, significantly less LDH (66.7 ± 5.3% of 5 mM H_2O_2-alone treated group, n = 6, P < 0.05; Fig. 2) was released into the cell culture media and more cells excluded trypan blue dye (6.1 ± 0.9% trypan blue-positive cells vs. 14.8 ± 1.1% for 5 mM H_2O_2-alone-treated group, n = 6, P < 0.05; Fig. 3).
injury. The LDH release in 10 μM R-PIA- and 10 μM CGS-21680-pretreated HK-2 cells was 78.6 ± 3.1% (n = 15, P < 0.05) and 67.7 ± 3.6% (n = 18, P < 0.05) of the H2O2-alone-treated group. Similar results were observed for trypan blue uptake when investigators were blinded to experimental conditions. The trypan blue uptake of R-PIA (9.3 ± 0.2%, n = 6)- and CGS-21680-treated (5.1 ± 0.5%, n = 6) cells was also significantly reduced compared with the H2O2-alone-treated group (14.8 ± 1.1%, n = 6, P < 0.05; Fig. 3). The A3-AR agonist IB-MECA (10 μM) failed to protect against oxidant injury (LDH release of 115.3 ± 5.4% of the H2O2-alone-treated group, n = 12).

A1-ARs Protect Via G i/o and PKC Pathway

We utilized specific inhibitors of G i/o proteins and PKC to determine the involvement of these signaling intermediates in A1- and A2a-AR-mediated protection against oxidant injury. Pertussis toxin treatment (100 ng/ml, 14 h) or pretreatment for 30 min with a PKC inhibitor (GF-109203X, 100 nM) blocked A1-AR agonist (10 μM R-PIA)-mediated protection against H2O2 (LDH = 105.6 ± 8.0%, n = 6, and LDH = 97.4 ± 3.6%, n = 6, of H2O2-alone-treated group, respectively; Fig. 5A). However, pertussis toxin and GF-109203X failed to block A2a-AR agonist (10 μM CGS-21680)-mediated protection against oxidant injury (LDH = 75.0 ± 6.4%, n = 6, and LDH = 79.9 ± 2.7%, n = 6, of H2O2-alone-treated group, respectively; Fig. 5A).

A2a-ARs Protect Via cAMP → PKA Pathway

We utilized specific inhibitors of G i/o proteins and PKC to determine the involvement of these signaling intermediates in A1- and A2a-AR-mediated protection against oxidant injury. Pertussis toxin treatment (100 ng/ml, 14 h) or pretreatment for 30 min with a PKC inhibitor (GF-109203X, 100 nM) blocked A1-AR agonist (10 μM R-PIA)-mediated protection against H2O2 (LDH = 105.6 ± 8.0%, n = 6, and LDH = 97.4 ± 3.6%, n = 6, of H2O2-alone-treated group, respectively; Fig. 5A). However, pertussis toxin and GF-109203X failed to block A2a-AR agonist (10 μM CGS-21680)-mediated protection against oxidant injury (LDH = 75.0 ± 6.4%, n = 6, and LDH = 79.9 ± 2.7%, n = 6, of H2O2-alone-treated group, respectively; Fig. 5A).
ADENOSINE ATTENUATES RENAL OXIDANT INJURY

We have previously demonstrated that treatment for 48 h with a nonselective AR antagonist (8-PT) upregulated \( A_1 \) (-1.9-fold) and \( A_{2a} \) (-1.5-fold) ARs, whereas 48-h treatment with a nonselective AR agonist (NECA) downregulated all four subtypes of ARs (26a). In this study, we demonstrate that chronic treatments with a nonselective AR antagonist conferred significant endogenous protection against oxidant injury (Fig. 6). After 48 h of treatment with 8-PT, 5 mM \( \text{H}_2\text{O}_2 \) killed significantly fewer HK-2 cells (LDH = 50.5 \pm 3.1% of \( \text{H}_2\text{O}_2 \)-alone group, \( n = 6 \)). Conversely, chronic NECA treatment significantly attenuated the protection by 100 \( \mu \text{M} \) adenosine pretreatment (LDH = 90.8 \pm 1.2%, \( n = 6 \)).

Role of CREB in Oxidant Injury

We have previously demonstrated the expression of both phosphorylated (activated) and nonphosphorylated forms of CREB in HK-2 cells (26a). Oxidant injury with \( \text{H}_2\text{O}_2 \) significantly decreased the phosphorylated (activated) form of CREB. The \( A_{2a} \)-AR agonist (CGS-21680) and Sp-CAMP rescued the decreased phosphorylation of CREB induced by \( \text{H}_2\text{O}_2 \). Activation of the CREB transcription factor correlates with cytoprotection by the \( A_{2a} \)-AR agonist and Sp-CAMP (Fig. 7).

Role of New Gene Transcription and Protein Synthesis in Adenosine-Induced Cytoprotection

Actinomycin D and cycloheximide blocked the protection by the \( A_1 \)-AR agonist (LDH = 98.9 \pm 7.5, \( n = 6 \), and 101.4 \pm 4.7, \( n = 4 \), respectively, of the \( \text{H}_2\text{O}_2 \)-alone-treated group).

A 1-AR-mediated protection against \( \text{H}_2\text{O}_2 \) injury is mediated by pertussis toxin (PTX)-sensitive G proteins and is protein kinase C dependent (\( n = 6 \); A), whereas the \( A_{2a} \)-AR-mediated protection involves protein kinase A (PKA) activation by cAMP (\( n = 6 \); B). Cell injuries were quantified by measuring LDH released into the culture media from cells pretreated with an inhibitor of Gi/o, protein kinase C dependent (\( n = 6 \); A), whereas the \( A_{2a} \)-AR-mediated protection involves protein kinase C (PKC) or PKA) for 30 min before R-PIA or CGS-21680, which were applied 30 min before treatment with 5 mM \( \text{H}_2\text{O}_2 \) for 1 h. PTX 100 \( \text{ng/ml} \), 14-h pretreatment; GF, GF-109203X PKC antagonist (100 \( \mu \text{M} \), 30-min pretreatment); Forsk, forskolin (10 \( \mu \text{M} \), 30-min pretreatment); IB-MECA, \( 6-(3\text{-iodobenzyl})\text{-methyl-5}'\text{-carbamoyladenosine. Error bars, SE. } *P < 0.05 \text{ vs. vehicle } + \text{H}_2\text{O}_2\)-treated group.

Upregulation of \( A_1 \) - and \( A_{2a} \)-ARs Modulates Protection Against \( \text{H}_2\text{O}_2 \)

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treated group), the A<sub>2a</sub>-AR (LDH = 97.3 ± 2.3, n = 6, and 104.6 ± 2.9, n = 3, respectively) agonist, and Sp-CAMP (LDH = 100.2 ± 2.6, n = 6, and 103.2 ± 5.8, n = 3, respectively), indicating that new transcription and protein synthesis, respectively, are required for A<sub>1</sub>- and A<sub>2a</sub>-AR-mediated protection against H<sub>2</sub>O<sub>2</sub> oxidant injury (Fig. 8).

**DISCUSSION**

Adenosine protects against H<sub>2</sub>O<sub>2</sub>–mediated oxidant injury in HK-2 cells by means of both A<sub>1</sub>- and A<sub>2a</sub>-AR activation. The A<sub>1</sub>-AR-mediated protection involves PKC by means of pertussis toxin-sensitive G proteins (G<sub>o</sub>/i), whereas the A<sub>2a</sub>-AR-mediated protection is by means of PKA activation by cAMP. Our findings complement our previous studies in which differential AR modulations protected against global renal I/R injury in vivo (24–26).

The detrimental effect of renal I/R injury that results in acute renal failure is a serious and unresolved clinical challenge (47). Surgical procedures involving the aorta and renal arteries (e.g., supra- and juxta renal abdominal aortic aneurysms and renal transplantation, in particular) display significant postoperative renal complications in the form of acute tubular necrosis and acute renal failure (3, 31, 32). Although ischemia can cause renal cell death and injury, significant renal tubular and vascular damage develop during the reperfusion period secondarily to oxygen free radical-mediated cellular injury (2, 34). Oxygen free radicals are considered to be important mediators of reperfusion injury, because reperfusion of a previously ischemic kidney results in robust generation of oxygen free radicals (34). H<sub>2</sub>O<sub>2</sub> is formed in mitochondria as a dismutation product of the superoxide radical (O<sub>2</sub><sup>−</sup>) under physiological conditions. However, under ischemic stress, there is proteolytic modification of xanthine dehydrogenase to xanthine oxidase, which effectively produces a profoundly increased burst of O<sub>2</sub><sup>−</sup> and H<sub>2</sub>O<sub>2</sub> when oxygen is reintroduced during reperfusion. H<sub>2</sub>O<sub>2</sub> and its far more toxic metabolite, the hydroxyl radical, contribute significantly to renal injury during reperfusion. Cytotoxic oxidant free radicals produce cellular lipid peroxidation, cytotoxic enzyme activation, impaired energy metabolism, protein oxidation, and a massive rise in intracellular Ca<sup>2+</sup> concentration (34, 43).

Renal cells with proximal tubular characteristics, including LLC-PK<sub>1</sub> (pig), NHK-C (human), and opossum kidney cells, are much more susceptible to oxidant-reperfusion injury than those with distal tubular characteristics (Madin-Darby canine kidney cells) (50, 51). In this study, we utilized H<sub>2</sub>O<sub>2</sub>–mediated oxidant injury as an in vitro model of reperfusion injury. In initial pilot studies, our attempt to produce in vitro hypoxia–reoxygenation injury by using a hypoxic chamber was unsuccessful. Incubation (24 h) in a 95% N<sub>2</sub>-5% CO<sub>2</sub> chamber followed by reoxygenation failed to produce significant HK-2 cell death measured by LDH release (26a). Epithelial cells such as HK-2 can utilize amino acid by means of gluconeogenesis to produce glucose and, therefore, are not susceptible to hypoxic cell death. Moreover, achieving true anoxia by removing O<sub>2</sub> from the cell culture environment is often im-

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**Fig. 7.** Cytoprotective effects of PKA activation measured by phospho-specific antibody. H<sub>2</sub>O<sub>2</sub> (5 mM) profoundly attenuated cAMP response element binding (CREB) phosphorylation (total n = 6). Catalase (Catal; 9,000 U/ml), A<sub>2a</sub>-AR agonist (CGS-21680, 10 μM, 30-min pretreatment), and Sp-cAMP (100 μM, 30-min pretreatment) rescued CREB phosphorylation. A<sub>1</sub>-AR agonist (R-PIA, 10 μM, 30-min pretreatment) had no effect on CREB activity. Representative immunoblot of HK-2 cell lysates with phosphospecific CREB (top) is also shown (n = 2 for control (Cont), H<sub>2</sub>O<sub>2</sub>, catalase groups and n = 3 for R-PIA and CGS-21680). Bands for Sp-cAMP are not shown. Error bars, SE. *P < 0.05 vs. vehicle-treated control group.

**Fig. 8.** Cytoprotection mediated by both A<sub>1</sub>- and A<sub>2a</sub> is dependent on new gene and protein synthesis. Cell injuries were quantified by measuring LDH released into the culture media after addition of 5 mM H<sub>2</sub>O<sub>2</sub> for 1 h and compared with the LDH released by the H<sub>2</sub>O<sub>2</sub>-alone group. R-PIA and CGS-21680 = 10 μM of A<sub>1</sub>- and A<sub>2a</sub>-AR-selective agonist, respectively (30-min pretreatment); Sp-cAMP = 100 μM, 30-min pretreatment; n = 6 for actinomycin D experiments, and n = 4 for cycloheximide experiments. Error bars, SE. *P < 0.05 vs. H<sub>2</sub>O<sub>2</sub>-alone group.
practical. Many in vivo and in vitro models of renal oxidant injury utilized exogenous application of oxygen free radicals such as H$_2$O$_2$ or t-butylhydroperoxide to mimic the free radical-mediated injury of reperfusion (29, 53).

The proximal tubules (especially, the straight, distal portion or S3 segment) located in the outer medulla of the kidney are the primary site of injury in renal ischemia and reperfusion (28, 48). This region is marginally oxygenated under normal physiological conditions, with a high basal metabolic demand (11, 28). Therefore, with hypoxic or ischemic insult, proximal tubules in the outer medullary zone suffer the most damage.

To show that HK-2 cells are a valid cellular model to study adenosine-mediated protection of renal proximal tubule cells, we have demonstrated that HK-2 cells express all four subtypes (A$_1$, A$_2a$, A$_2b$, and A$_3$) of ARs and display key signaling intermediates, including several PKC isoforms (a, b, and e), G proteins (G$_i$, G$_o$, and G$_q$), and mitogen-activated protein kinases (extracellular signal-regulated kinase (ERK)1/2, c-Jun NH$_2$-terminal kinase, and p38) (26a). We also verified that A$_1$- and A$_2$-ARs inhibit forskolin-stimulated adenyllyl cyclase activity and that A$_2$-ARs stimulate adenyllyl cyclase activity. In addition, chronic (48-h) antagonist (8-PT) and agonist (NECA) treatment led to up- and downregulation of various ARs and G protein subtypes.

Adenosine has cytoprotective effects in several cell types, including renal cells (5, 10). AR activation, specifically the A$_1$ and A$_2$ subtypes, attenuates several factors responsible for generating I/R injury (27, 38, 49). A$_1$-AR activation attenuates I/R injury when given before the ischemic insult in cerebral (38), cardiac (9, 49), and renal (25) cells and has been implicated to mediate ischemic preconditioning. Conversely, posts ischemic A$_2$-AR also protects against tissue injury by attenuating the reperfusion phase of the injury process in pulmonary (1), cardiac (7), and renal cells (26).

In this study, we demonstrated that both A$_1$- and A$_2$-AR agonists protected against the direct cytotoxic effects of H$_2$O$_2$ (nonreceptor-mediated cytotoxicity) by means of distinct receptor-mediated cellular mechanisms in HK-2 cells. We demonstrated in this study that A$_2$-ARs, by means of cAMP-dependent mechanisms, protected HK-2 cells against H$_2$O$_2$-mediated cell death. The A$_2$-AR-mediated protection from H$_2$O$_2$ injury was blocked by Rp-cAMP, an inhibitor of PKA. Additionally, agents that increase cAMP, isoproterenol and forskolin, or direct activation of PKA by Sp-cAMP also protected against H$_2$O$_2$-induced cell injury. These in vitro results agree with in vivo results previously described in which A$_2$-AR activation protected against renal I/R injury in vivo (26, 35). Stimulation of A$_2$-ARs, including those present in renal tubule cells and vasculature, results in increased cellular cAMP to activate PKA (4, 40). Our study agrees with previous studies that suggest that increased intracellular cAMP protects against renal reoxygenation-oxidant injury in vivo (6) and in vitro (21, 52). Our study also agrees with previous studies showing that agents that increase intracellular cAMP also attenuate reperfusion injury in the heart, lung, and kidney in vivo and in vitro (8, 44).

After oxidant injury, CREB activity decreased significantly. We have previously demonstrated in HK-2 cells that A$_2$-ARs activate CREB via cAMP- and PKA-dependent pathways (26a). Moreover, improved preservation of HK-2 CREB activity after A$_2$-AR activation or with Sp-cAMP correlates with improved cellular survival after H$_2$O$_2$-mediated injury. We propose that increased HK-2 cell survival and activation of CREB is one of the potential mechanistic links after A$_2$-AR activation by means of cAMP and PKA.

We also demonstrated that activation of A$_1$-ARs in HK-2 cells also protects against H$_2$O$_2$-induced cellular injury via signaling pathways involving PKC and G$_i/o$ proteins. This was shown by attenuating the protection induced by the A$_1$-AR agonist R-PIA with pertussis toxin and GF-109203X. These findings agree with our previous in vivo studies in which both G$_i/o$ and PKC were intermediates in A$_1$-AR-mediated protection of renal I/R injury (24). This study agrees with heart studies in which A$_1$-ARs are involved in protection against H$_2$O$_2$-induced oxidative injury in vivo and in vitro (33, 46) by modulation of the detrimental increases in intracellular calcium concentration and by means of activation of cardiomyocyte K$_{ATP}$ channels after H$_2$O$_2$ exposure (14, 46). After exposure to H$_2$O$_2$, intracellular ATP decreases significantly (~30% of baseline, data not shown), and the A$_1$-AR activation may attenuate this component of cellular injury. Therefore, whereas the A$_2$-ARs attenuate the free radical-mediated cellular injury (e.g., lipid peroxidation and membrane damage), the A$_1$-ARs may attenuate the cellular injury caused by a lethal drop in intracellular ATP.

In summary, the first report of an in vitro protective effect of adenosine in HK-2 cells by means of A$_1$- and A$_2$-AR activation. We have systematically deciphered the signal pathways of A$_1$- and A$_2$-AR-mediated renal cytoprotection. These findings have potentially profound clinical significance in the protection of the kidney in the perioperative care of patients subjected to renal ischemia.

This work was funded in part by intramural grant support from the Department of Anesthesiology, Columbia University College of Physicians and Surgeons, and by National Institute of Diabetes and Digestive and Kidney Diseases Grant RO-1-DK-58547.

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AJP-Renal Physiol • VOL 282 • MAY 2002 • www.ajprenal.org


