Preconditioning and Adenosine Protect Human Proximal Tubule Cells in an In Vitro Model of Ischemic Injury

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Abstract. Renal ischemic reperfusion injury results in unacceptably high mortality and morbidity during the perioperative period. It has been recently demonstrated that ischemic preconditioning or adenosine receptor modulations attenuate renal ischemic reperfusion injury in vivo. An in vitro model of ischemic renal injury was used in cultured human proximal tubule (HK-2) cells to further elucidate the protective signaling cascades against renal ischemic reperfusion injury. ATP depletion preconditioning (1 h of antimycin A and 2-deoxyglucose treatment followed by 1 h of recovery), adenosine, an A₁ adenosine receptor selective agonist, or an A₂a adenosine receptor selective agonist significantly attenuated subsequent severe ATP depletion injury of HK-2 cells. In contrast, an adenosine receptor antagonist failed to prevent protection induced by ATP depletion preconditioning. Cytoprotection by ATP depletion preconditioning or A₁ adenosine receptor activation was prevented by inhibitors of extracellular signal-regulated mitogen-activated kinases, protein kinase C, and tyrosine kinases. The A₁ and A₂a adenosine receptor-mediated cytoprotection were also dependent on G<sub>io</sub> proteins and PKA activation, respectively. It is concluded that ATP depletion preconditioning and A₁ and A₂a adenosine receptor activation protect HK-2 cells against severe ATP depletion injury via distinct signaling pathways.

The A₁ and A₂a adenosine receptors serve to protect against ischemic reperfusion injury in many organ systems, including the heart, brain, liver, and kidney (1–9). We have recently demonstrated that pre- or post-ischemic activation of renal A₁ and A₂a adenosine receptors, respectively, protected renal function against ischemic reperfusion injury in vivo (3–5). The mechanism of pre-ischemic A₁ adenosine receptor-mediated renal protection in vivo involved protein kinase C (PKC) and pertussis toxin–sensitive G-proteins (G<sub>io</sub>), whereas post-ischemic A₂a adenosine receptor–mediated protection was through protein kinase A (PKA) activation via cAMP.

Ischemic preconditioning, defined as multiple cycles of brief ischemia and reperfusion before a prolonged ischemic insult, was first described in cardiac tissue and subsequently demonstrated in skeletal muscle (10), brain (11), and liver (6). In these organs, ischemic preconditioning is mediated by activation of adenosine receptors. Ischemic preconditioning in cardiac and cerebral tissues also involves extracellular signal-regulated mitogen-activated kinases (12). We have recently demonstrated renal protective effects of ischemic preconditioning in vivo via mechanisms involving G<sub>io</sub> and PKC (3). However, in contrast to cardiac, cerebral, and hepatic models, renal ischemic preconditioning in vivo was not blocked by adenosine receptor antagonism.

In vivo models pose limitations in the further elucidation of the signaling cascades mediating cytoprotection by adenosine receptor or ischemic preconditioning. Therefore, we used a pure population of human renal proximal tubular (HK-2) cells to study the signaling cascades directly. HK-2 cells are immortalized adult human proximal tubular cells transfected with E6/E7 genes of a human papilloma virus (HPV 16 [13,14]). Transfection with HPV16 has been shown to immortalize epithelial cells of diverse origin without significantly altering their phenotype or function. HK-2 cells have been shown to retain the phenotypic expression and functional characteristics of human proximal tubules (13,14). Many studies have utilized HK-2 cells to study in vitro renal physiology and pathology (15–17).

There are few studies of in vitro renal cell protection with adenosine receptor modulations or with ischemic preconditioning. Massive depletion of intracellular ATP and glucose and a large increase in intracellular calcium are the hallmark intracellular changes in cells undergoing lethal ischemic insult (18,19). We have used an in vitro model of anoxia/ischemia, causing severe ATP depletion by using the combination of a mitochondrial respiration inhibitor (antimycin A), a non-metabolizable glucose analog (2-deoxyglucose), and a calcium ionophore (A23187) in human renal cells to mimic the ischemic phase of renal ischemic reperfusion injury (15). Our goal was to extend our in vivo findings into an in vitro model to further elucidate the signal transduction pathways of renal protection induced by ischemic preconditioning or adenosine receptor modulations. We hypothesized that, as we observed in vivo, A₁ adenosine receptor activation or ATP depletion pre-
conditioning would protect against severe ATP depletion injury in human renal proximal tubule cells.

**Materials and Methods**

**HK-2 Cell Culture**

HK-2 cells (immortalized human proximal tubular cell line; American Type Culture Collection, Manassas, VA) were grown and passaged in 75-cm² cell culture flasks containing culture medium (keratinocyte serum-free medium plus 5 ng/ml epidermal growth factor and 40 mg/ml bovine pituitary extract) and antibiotics (100 U/ml penicillin G, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B) at 37°C in a 100% humidified atmosphere of 5% CO₂-95% air.

**Induction of Severe ATP Depletion Injury with ATP/Glucose Depletion and Calcium Inophore in HK-2 Cells**

When HK-2 cells were confluent, the culture medium was replaced with Hank’s balanced salt solution (HBSS; containing 1.3 mM Ca²⁺ and 0.8 mM Mg²⁺). In preliminary experiments, we determined that simply depleting ATP and glucose (with 10 µM antimycin A, a complex III inhibitor of mitochondrial electron transport, and 10 mM 2-deoxyglucose, a non-metabolizable isomer of glucose, respectively) failed to produce significant human proximal tubular (HK-2) cell death within 2 h. Therefore, rapid HK-2 cell injury was induced using a method of ATP and glucose-depletion plus 1 to 5 µM calcium ionophore (A23187) for 1 to 3 h. This model of ATP depletion injury in HK-2 cells has been widely used and extensively characterized (13,20–23).

A confluent monolayer of HK-2 cells grown in 6- or 24-well plates was incubated with antimycin A, 2-deoxyglucose, and 1, 2, or 5 µM calcium ionophore for 1 to 3 h (severe ATP depletion injury). The 2-h time point and 2 µM calcium ionophore were chosen for the subsequent studies, as this time and dose of calcium ionophore reproducibly induced moderate cellular injury. Some wells were pretreated (e.g., adenosine or adenosine receptor agonists) before severe ATP depletion injury. Other cells were preconditioned with 1 h of moderate ATP depletion (approximately 40% of baseline) using 10 µM antimycin A and 10 mM 2-deoxyglucose and then allowed to recover for 1 h before severe ATP depletion injury. In further experiments, inhibitors of ERK₁/₂, PKC, tyrosine kinase, or G₁/o were given before receptor agonists or preconditioning and then severe ATP depletion injury was induced. The drugs used and duration of pretreatments for selective inhibitors of signal transduction intermediates and adenosine receptor agonists and antagonist are listed in Table 1.

**Measurement of Cell Viability and Cell Death**

After severe ATP depletion injury, cell viability assays were performed using trypan blue dye exclusion. After the treatment protocols (e.g., injury ± adenosine receptor agonists), cells were trypsinized and stained with 0.4% trypan blue dye for 5 min. Preliminary studies demonstrated that trypsinization alone had no measurable effect on cell viability (data not shown). The proportion of nonviable cells (unable to exclude trypan blue) was counted using a hemocytometer and expressed as a percent of the total number of cells.

Lactate dehydrogenase (LDH) released into the media was also measured as a marker of cellular injury using a commercially available colorimetric method (Sigma, St. Louis, MO). In some experiments, LDH released into the media was expressed as the percent of total cellular LDH measured after lysing the cells with 1% Triton-X. Otherwise, LDH release after the various treatment protocols (e.g., ATP and glucose depletion plus calcium ionophore mediated severe ATP depletion injury ± adenosine receptor agonists) was expressed as the percent of LDH release by severe ATP depletion injury alone. n = 1 denotes average values of LDH released or trypan blue uptake obtained from duplicate wells in a single plate.

**Measurement of Intracellular ATP Content**

Intracellular ATP content in HK-2 cells (per well in a 24-well plate) after various combinations of metabolic inhibitors were determined using a commercially available quantitative enzymatic method at 340 nm (Sigma).

**Immunoblotting of Activated Form of ERK**

We measured ERK₁/₂ activation in HK-2 cells following ATP/glucose depletion and calcium ionophore injury by immunoblotting with antibodies to the phosphorylated forms of ERK₁/₂ (pERK₁/₂; Santa-Cruz Biotechnologies, Santa Cruz, CA) as described previously (2).

**Table 1.** Selective adenosine receptor agonists and antagonist and selective inhibitors of signaling intermediates utilized

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration and Incubation Time</th>
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<tbody>
<tr>
<td>Adenosine</td>
<td>100 µM for 30 min</td>
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<tr>
<td>Selective A₁ AR agonist</td>
<td>1 nM-10 µM of R-N⁶-phenyl-isopropyladenosine (R-PIA) for 30 min</td>
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<tr>
<td>Selective A₂a AR agonist</td>
<td>1 nM-10 µM of 4-((N-ethyl-5'-carbamoyadenos-2-yl)-aminoethyl)-phenylpropionic acid (CGS-21680) for 30 min</td>
</tr>
<tr>
<td>Selective A₃ AR agonist</td>
<td>1 nM-10 µM of N⁶-(3-iodobenzyl)-N-methyl-5'-carbamoyladenosenos (IB-MECA) for 30 min</td>
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<tr>
<td>Nonselective AR antagonist</td>
<td>100 µM of 8-phenyltheophylline for 30 min</td>
</tr>
<tr>
<td>Inhibitor of G₁/o</td>
<td>100 ng/ml pertussis toxin for 14 h</td>
</tr>
<tr>
<td>PKC inhibitor</td>
<td>100 nM of GF-109203X for 30 min</td>
</tr>
<tr>
<td>Tyrosine kinase inhibitor</td>
<td>20 µM of genistein for 30 min</td>
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<tr>
<td>ERK₁/₂ inhibitor</td>
<td>50 µM of PD-98059 for 30 min</td>
</tr>
<tr>
<td>PKA antagonist</td>
<td>100 µM of Rp-Isomer, Adenosine 3',5'-cyclic monophosphorothioate (Rp-cAMPS) for 30 min</td>
</tr>
<tr>
<td>PKA agonist</td>
<td>100 µM of Sp-Isomer, Adenosine 3',5'-cyclic monophosphorothioate (Sp-cAMPS) for 30 min</td>
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</table>
Protein Determination

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

Statistical and Data Analyses

The data were analyzed with t test when comparing means between two groups or with one-way ANOVA plus Dunnett post hoc multiple comparison test to compare mean values across multiple treatment groups.

Materials

Adenosine was dissolved in saline. All other drugs were dissolved first in DMSO and then were diluted in water such that the final concentration of DMSO in each experimental condition was <0.01%. Solutions were made daily. Unless specified, all chemicals were obtained from the Sigma Chemical Company.

Results

Depletion of Intracellular ATP by Antimycin A, 2-Deoxyglucose, and Calcium Ionophore

We used an in vitro model of ischemic injury using a combination of ATP and glucose depletion (antimycin A and 2-deoxyglucose) superimposed with calcium overload (calcium ionophore [A23187]) in HK-2 cells (severe ATP depletion injury) (15,20). This method of cellular injury produced significant ATP depletion, as intracellular ATP levels incrementally decreased with 2 h of 10 μM antimycin A (6.2 ± 0.2 μmol/dl per well, n = 6, P < 0.01 versus controls; 8.6 ± 0.9 μmol/dl per well, n = 6), 10 μM antimycin A + 10 mM 2-deoxyglucose (3.5 ± 0.6 μmol/dl per well, n = 6, P < 0.01 versus controls, P < 0.01 versus antimycin A only group), and 10 μM antimycin A + 10 μM 2-deoxyglucose + 2 μM calcium ionophore (1.8 ± 0.6 μmol/dl per well, n = 6, P < 0.01 versus controls, P < 0.01 versus antimycin A only group) treatments. ATP depletion preconditioning (8.1 ± 1.1 μmol/dl per well, n = 3, P < 0.05 versus severe ATP depletion injury) and pretreatment with 100 μM adenosine (7.0 ± 1.7 μmol/dl per well, n = 3, P < 0.05 versus severe ATP depletion injury), 10 μM R-PIA (6.6 ± 2.0 μmol/dl per well, n = 3, P < 0.05 versus severe ATP depletion injury), or 10 μM CGS-21680 (5.0 ± 1.8 μmol/dl per well, n = 3, P < 0.05 versus severe ATP depletion injury) prevented the severe decrease in intracellular ATP levels after injury.

Calcium Ionophore/ATP Depletion Injury Kills HK-2 Cells

As described by others (15), our pilot studies have demonstrated that simply depleting ATP and glucose without calcium ionophore treatment failed to kill HK-2 cells rapidly, as indicated by LDH release (<10% total cellular LDH released after 2 h). Therefore, we added a calcium ionophore (A23187) to facilitate HK-2 cell death, as a rapid and massive rise in intracellular calcium occurs in ischemic renal cell injury (18,19). Figure 1 shows that this method of calcium ionophore/ATP depletion injures HK-2 cells in a dose-dependent (Figure 1A; n = 3) and time-dependent (Figure 1B; n = 3) manner.

HK-2 cell injury was quantified by measuring percent LDH released into the cell culture media and by the percent of trypan blue dye exclusion. The 2-h time point was chosen for subsequent studies because cell death plateaus at this time point, and 2 μM calcium ionophore was chosen because this dose induced moderate (approximately 60%) cellular injury.

ATP Depletion Preconditioning and Adenosine Protect against Subsequent Calcium Ionophore/ATP Depletion Injury

Figure 2 shows that 1 h of moderate ATP depletion by pretreatments with 10 μM antimycin A and 10 mM 2-deoxyglucose followed by 1 h of recovery in normal cell culture medium (ATP depletion preconditioning) significantly protected against more severe ATP depletion injury induced by a combination of mitochondrial oxidative and glycolytic inhibitors (10 μM antimycin A and 10 mM 2-deoxyglucose, respectively) plus 2 μM calcium ionophore. After ATP depletion preconditioning, significantly less LDH (53.0 ± 3.1% of severe ATP depletion injury alone group, n = 6, P < 0.05, Figure 2A) was released into the cell culture media and more

Figure 1. (A) Severe ATP depletion (calcium ionophore plus ATP and glucose depletion) injures human proximal tubule (HK-2) cells in a calcium ionophore dose-dependent manner (2-h incubation, n = 3). (B) The injury plateaus at 2 h of incubation (5 μM calcium ionophore data shown, n = 3). Cell injuries were quantified by measuring percent of total cellular LDH released into the culture media after specified incubation period and dose. * P < 0.05 versus vehicle-treated control group (n = 3). Error bars represent 1 SEM.
produced cell death. With 100 μM 3, the significantly protects against severe ATP depletion injury in combination with ATP depletion preconditioning (AD PC, n = 6, Figure 2B). In addition, we show that the combination of ATP depletion preconditioning plus adenosine receptor activation failed to demonstrate improved protection beyond either maneuver alone (LDH = 62.2 ± 6.1% of severe ATP depletion injury alone group, n = 3, P < 0.05, Figure 2A). ATP depletion preconditioning is not mediated by adenosine receptors as 100 μM 8-PT (a nonselective adenosine receptor antagonist) failed to block the cytoprotection by ATP depletion preconditioning (LDH = 63.1 ± 9.2% of severe ATP depletion injury alone group, n = 3).

A<sub>1</sub> and A<sub>2a</sub> Adenosine Receptors Are Involved in Adenosine-Mediated Protection against Calcium Ionophore/ATP Depletion Injury

In HK-2 cells, adenosine-mediated protection against severe ATP depletion injury involves the A<sub>1</sub> and A<sub>2a</sub> adenosine receptors, as the A<sub>1</sub> adenosine receptor agonist R-PIA (Figures 2 and 3A) and the A<sub>2a</sub> adenosine receptor agonist CGS-21680 (Figures 2 and 3B) pretreatment provided dose-dependent protection against severe ATP depletion injury. The LDH release in HK-2 cells pretreated with 10 μM R-PIA and 10 μM CGS-21680 were 64.5 ± 2.5% (n = 9) and 63.1 ± 3.8% (n = 6) of the severe ATP depletion injury alone group, respectively. The trypan blue uptake of cells treated with 10 μM R-PIA (17.0 ± 4.5% of total cells, n = 6) and 10 μM CGS-21680 (12.5 ± 2.3% of total cells, n = 6) were also significantly reduced when compared with the severe ATP depletion injury alone group (33.4 ± 5.5% of total cells, n = 6). The A<sub>3</sub> adenosine receptor agonist IB-MECA (10 μM) failed to protect against severe ATP depletion injury (LDH release of 96.3 ± 2.9% of severe ATP depletion injury alone group, n = 6, Figure 3A; trypan blue uptake of 32.5 ± 5.1% of total cells, n = 6, Figure 2B).

Signaling Pathways of ATP Depletion Preconditioning and A<sub>1</sub> Adenosine Receptor-Mediated Protection

We used specific inhibitors of G<sub>i/o</sub> proteins (pertussis toxin), tyrosine kinases (genistein), MEK-1 (PD98059 to inhibit ERK1/2 activation), and PKC (GF-109203X [Bisindolylmaleimide I]) to determine the involvement of these signaling intermediates in ATP depletion preconditioning and in A<sub>1</sub>- and A<sub>2a</sub>-adenosine receptor-mediated protection against subsequent severe ATP depletion injury. We first demonstrated that these inhibitors alone or with severe ATP depletion injury had no effect on LDH released or trypan blue uptake in HK-2 cells (data not shown). Pretreatment with pertussis toxin (100 ng/ml for 14 h, n = 6), PD98059 (50 μM for 30 min, n = 3), genistein (20 μM for 30 min, n = 3), or GF-109203X (100 nM for 30 min, n = 6) blocked A<sub>1</sub> adenosine receptor-mediated protection from severe ATP depletion injury conferred by 10 μM R-PIA (LDH = 97.9 ± 3.1%, 97.3 ± 5.7%, 105 ± 6.6%, and 98.3 ± 4.9% of severe ATP depletion injury alone group, respectively; Figure 4A). In addition, PD98059 (n = 3), genistein (n = 3), or GF-109203X (n = 6) blocked the protection conferred by ATP depletion preconditioning to subse-

![Figure 2](image-url)

**Figure 2.** ATP depletion preconditioning (AD PC, n = 6), 100 μM adenosine (ADO, n = 6), 10 μM A<sub>1</sub> adenosine receptor-selective agonist (PIA, n = 6), and 10 μM A<sub>2a</sub> adenosine receptor-selective agonist (CGS, n = 6) pretreatment protect against severe ATP depletion (calcium ionophore/ATP depletion) injury in HK-2 cells (CAD, n = 6). 10 μM A<sub>1</sub> adenosine receptor-selective agonist (IB, n = 6) had no effect. Combination of ATP depletion preconditioning and adenosine (ADO-CAD, n = 3) had no protective effect when compared with either ATP depletion preconditioning or adenosine pretreatment. Cell injuries were quantified by measuring LDH released into the culture media (A) or by the percent of trypan blue exclusion (B) from cells that underwent ATP depletion preconditioning (1 h of ATP depletion with antimycin A and 2-deoxyglucose before 1 h of recovery) or pretreated with adenosine, PIA, CGS, IB, or vehicle for 30 min, and subsequently injured with 10 μM antimycin A, 10 mM 2-deoxyglucose, and 2 μM calcium ionophore (CAD, n = 6) for 2 h. * P < 0.05 versus severe ATP depletion injury group (CAD). Error bars represent 1 SEM.

...into the cell culture media, and more cells excluded trypan blue dye (19.2 ± 2.6% trypan blue-positive cells, n = 6, P < 0.05, Figure 2B). One hour of ATP depletion preconditioning itself resulted in 10.7 ± 1.4% of the cells taking up trypan blue dye (n = 6, P < 0.05 versus severe ATP depletion injury alone group).

Figure 2 also shows that adenosine pretreatment for 30 min significantly protects against severe ATP depletion injury induced cell death. With 100 μM adenosine pretreatment, significantly less LDH (68.0 ± 2.5% of severe ATP depletion injury alone group, n = 6, P < 0.05, Figure 2A) was released...
quent severe ATP depletion injury (LDH = 97.3 ± 5.7%, 99.2 ± 14.0%, and 98.3 ± 4.9% of severe ATP depletion injury alone group, respectively; Figure 4A). In contrast, the nonselective adenosine receptor antagonist (100 nM 8-PT, LDH = 56.0 ± 4.7% of severe ATP depletion injury alone group, n = 6) or pertussis toxin (100 ng/ml, LDH = 65.3 ± 3.3% of severe ATP depletion injury alone group, n = 3) failed to block protection conferred by ATP depletion preconditioning. Additionally, pertussis toxin, genistein, PD98059 or GF-109203X failed to block the A2a adenosine receptor-mediated protection against severe ATP depletion injury conferred by 10 nM CGS-21680 (data not shown). Taken together, these results suggest that (1) A1 adenosine-mediated protection involves G_{i/o} proteins, MEK-1 → ERK, tyrosine kinases, and PKC; (2) ATP depletion preconditioning involves MEK-1 → ERK, tyrosine kinases, and PKC but not adenosine receptors or G_{i/o} proteins; and (3) A2a adenosine receptor-mediated protection does not involve G_{i/o} proteins, MEK-1, tyrosine kinases, or PKC.

A2a Adenosine Receptors Protect via cAMP → Protein Kinase A (PKA)

The A2a adenosine receptor-mediated cytoprotection against severe ATP depletion injury involves PKA because the PKA antagonist Rp-cAMPS inhibited the A2a adenosine receptor agonist-induced protection against severe ATP depletion injury (100 nM, 30-min pretreatment, LDH = 87.2 ± 3.9% of severe ATP depletion injury alone group, n = 6, P < 0.05 versus 10 nM CGS-21680 treated group, Figure 4B). Rp-cAMPS itself

![Figure 3](image3.png)

**Figure 3.** The A1 adenosine receptor agonist (PIA; panel A; n = 6) and the A2a adenosine receptor agonist (CGS; panel B; n = 6) attenuate severe ATP depletion-mediated HK-2 cell injury (CAD; n = 6) in dose-dependent manners. Cell injuries were quantified by measuring LDH released into the culture media from cells treated with 1 nM to 10 μM PIA or 0.01 to 10 μM CGS or vehicle for 30 min before the addition of 10 μM antimycin A, 10 mM 2-deoxyglucose, 2 μM and calcium ionophore (CAD, n = 6) for 2 h. * P < 0.05 versus severe ATP depletion injury group (CAD). Error bars represent 1 SEM.

![Figure 4](image4.png)

**Figure 4.** The A1 adenosine receptor-mediated protection against severe ATP depletion-mediated HK-2 cell injury is mediated by pertussis toxin-sensitive G-proteins, ERK, protein kinase C, and tyrosine kinases (panel A, n = 6), whereas the A2a adenosine receptor-mediated protection involves PKA activation by cAMP (panel B, n = 6). ATP depletion preconditioning (AD PC) is mediated by ERK, protein kinase C, and tyrosine kinases (panel A, n, n = 6). Cell injuries were quantified by measuring LDH released into the culture media from cells pretreated with an inhibitor (of G_{i/o} ERK, PKC, tyrosine kinase, or PKA) 30 min before PIA or CGS treatment, which were applied 30 min before calcium ionophore/ATP depletion-mediated HK-2 cell injury (CAD, n = 6). PIA and CGS, 10 μM A1 and A2a adenosine receptor selective agonist, respectively (30-min pretreatment); PTX, pertussis toxin (100 ng/ml, 14-h pretreatment); GF, protein kinase C antagonist (GF-109203X [Bisindolylmaleimide I], 100 nM, 30-min pretreatment); Sp, protein kinase A agonist (Sp-cAMPS, 100 μM, 30-min pretreatment); Rp, protein kinase A antagonist (Rp-cAMPS, 100 μM, 30-min pretreatment). * P < 0.05 versus severe ATP depletion injury group (CAD); $ P < 0.05 versus PI A+C AD group; ^ P < 0.05 versus AD PC+C AD group; % P < 0.05 versus CGS+C AD group. Error bars represent 1 SEM.
had no effect on severe ATP depletion injury (data not shown). Moreover, the PKA agonist Sp-cAMPS mimicked the protection provided by the A2a adenosine receptor agonist (100 μM, 30-min pretreatment, LDH = 75.0 ± 3.9% of severe ATP depletion injury alone group, n = 3).

Role of ERK1/2 in Calcium Ionophore/ATP Depletion Injury

We have recently demonstrated that activation of A1 adenosine receptors in HK-2 cells results in phosphorylation (activation) of ERK1/2 via G and tyrosine kinase (24). We provide further evidence that ERK1/2 activation is required for protection against severe ATP depletion injury with immunoblotting approaches (Figure 5). Under basal conditions, HK-2 cells express large amounts of p-ERK1/2, and the 42-kD p-ERK3 is the predominant subtype of activated ERK. This p-ERK1/2 expression is significantly abolished with severe ATP depletion injury. One hour of ATP depletion preconditioning (1 h of antimycin A and 2-deoxyglucose followed by 1 h of recovery in normal cell culture media) prevented the loss of activated ERK1/2 after severe ATP depletion injury. Moreover, A1 adenosine receptor activation, but not A2a adenosine receptor activation, significantly preserved ERK1/2 activity after severe ATP depletion injury (Figure 5). Therefore, improved preservation of ERK1/2 activity correlates with improved cellular survival when HK-2 cells are subjected to severe ATP depletion injury.

Discussion

The major new findings of the present study are that either ATP depletion preconditioning or adenosine receptor activation in vitro protects against cell death induced by subsequent calcium ionophore/ATP depletion-induced injury in human renal proximal tubule (HK-2) cells. Protection afforded by ATP depletion preconditioning involves extracellular signal-regulated kinases 1 and 2 (ERK1/2), protein kinase C (PKC), and tyrosine kinase, but it is independent of adenosine receptor or G protein activation. Moreover, both A1 and A2a adenosine receptor agonists protected HK-2 cells against the direct cytotoxic effects of severe ATP depletion injury (non-receptor-mediated cytotoxicity) via distinct receptor-mediated cellular mechanisms. A2a adenosine receptor-mediated protection involves the cAMP → protein kinase A (PKA) pathway, whereas A1 adenosine receptor-mediated protection involves G, PKC, tyrosine kinase, and ERK1/2: signaling intermediates frequently implicated in protection from ischemic reperfusion injury in the myocardium (25) and in the kidney (3–5, 26). Cytoprotection by ATP depletion preconditioning and AR activation is associated with significantly improved cellular ATP levels. These in vitro findings complement our previous in vivo studies in which differential adenosine receptor modulations protected against global renal ischemic reperfusion injury (3–5). In addition, as we observed in vivo, preconditioning-mediated protection was independent of adenosine receptor activation in vitro.

Profound intracellular ATP depletion and a fall in tissue oxygen content with a concomitant rise in intracellular calcium are the hallmark features of renal ischemic-reperfusion injury (18, 19). Intracellular calcium levels rise after anoxia and ATP depletion, and this rise in calcium contributes further to the necrotic cellular death. In this study, we produced ATP/glucose depletion-calcium ionophore–mediated HK-2 cell death as an in vitro model of ischemic or ATP depletion injury. This injury was achieved by a combination of metabolic inhibitors (antimycin A and 2-deoxyglucose) and a calcium ionophore (A23187). Addition of a calcium ionophore has been employed frequently to induce lethal ATP depletion injury in HK-2 cells (13, 20–23), as the metabolic inhibitors alone fail to produce rapid cellular death and measurable LDH release. Iwata et al. (15) have demonstrated that the combination of ATP and glucose depletion with antimycin A and 2-deoxyglucose, respectively, produces approximately 90% ATP depletion but fails to rapidly kill HK-2 cells. Epithelial cells, such as HK-2 cells, are glycolytic and can utilize amino acids via gluconeogenesis to produce glucose and, therefore, are not susceptible to rapid hypoxic/anoxic cell death (personal communication.
with Dr. Richard Zager, University of Washington, July 1999). Moreover, true anoxia by removing \(O_2\) from the cell culture environment is often impractical to achieve. We demonstrate that cytoprotection by ATP depletion preconditioning and adenosine receptor activation is associated with significantly higher cellular ATP levels.

Currently, there are four subtypes of identified adenosine receptors (\(A_1\), \(A_{2a}\), \(A_{2b}\), and \(A_3\) adenosine receptors) that mediate extracellular action of adenosine in the kidney (27). Adenosine also acts intracellularly to increase the level of \(S\)-adenosylhomocysteine (SAH) by inhibiting SAH hydrolase (28). We have recently verified the presence of all four subtypes of adenosine receptors and demonstrated several key signaling intermediates in HK-2 cells (24). Adenosine has cytoprotective effects in several cell types including renal cells (2–5). Adenosine receptor activation, specifically the \(A_1\) and \(A_{2a}\) subtypes, attenuates several factors responsible for generating ischemic-reperfusion injury (29). \(A_1\) adenosine receptor activation attenuates ischemic-reperfusion injury when given before the ischemic insult in cerebral (9,30), cardiac (29), and renal (3) cells. Conversely, post-ischemic \(A_{2a}\) adenosine receptor activation also protects against tissue injury by attenuating the reperfusion phase of injury process in pulmonary (31), cardiac (7), and renal (5,32) cells.

Activation of ERK\(_{1/2}\) by ATP depletion preconditioning or the \(A_1\) adenosine receptor agonist R-PIA potently protected against subsequent severe ATP depletion injury in our study. This is consistent with the finding that preservation of ERK\(_{1/2}\) activity correlates with improved cellular survival after ATP depletion injury (12). In mouse renal proximal tubules, cell survival after ischemic-reperfusion injury is dependent on ERK\(_{1/2}\) activation (26). In murine inner collecting ducts, ERK activation correlates with markedly reduced cell loss after ATP depletion (33). Additionally, an important role of ERK\(_{1/2}\) activation has been implicated in cardiac and cerebral ischemic preconditioning (12). In a recent study of neuronal ischemic preconditioning, oxygen-glucose deprivation preconditioning (analogous to our ATP depletion preconditioning) was neuroprotective via mechanisms involving p21ras and ERK\(_{1/2}\) (34). Similar to our study, their preconditioning stimulus produced robust ERK\(_{1/2}\) activation, and this activation was required for protection against subsequent and more severe ischemic/ATP depletion injury. The mechanism by which ERK\(_{1/2}\) activation leads to cytoprotection in both \(A_1\) adenosine receptor activation and ATP depletion preconditioning is not known but may involve ERK\(_{1/2}\)-s pro-survival/anti-apoptotic pathways and/or new gene expression and new protein synthesis. ERK activation may also increase the cellular repair capacity of renal epithelial cells (26).

We also demonstrate in this study that \(A_{2a}\) adenosine receptor activation protected against severe ATP depletion injury in HK-2 cells via cyclic AMP-dependent signaling pathways that are distinct from \(A_1\) adenosine receptor activation-mediated cytoprotection. We and others have previously demonstrated that \(A_{2a}\) adenosine receptor activation protects against renal ischemic-reperfusion injury in vivo (35) and that the \(A_{2a}\) adenosine receptor is the receptor subtype frequently implicated in the attenuation of reperfusion injury (36). Stimulation of \(A_{2a}\) adenosine receptors, including those present in renal tubule cells and vasculature, classically result in increased cellular cAMP to activate PKA (37). It has been suggested that increased intracellular cAMP protects against renal reoxygenation-oxidant injury in both in vivo (38) and in vitro (39,40) models. The mechanism of cAMP’s protection against severe ATP depletion injury is not clear. It does not involve ERK\(_{1/2}\) as we have previously demonstrated that \(A_{2a}\) adenosine receptors failed to activate ERK\(_{1/2}\) (24), and the ERK inhibitor failed to prevent \(A_{2a}\) adenosine receptor-mediated protection against severe ATP depletion injury in this study. The adenosine \(A_{2a}\) agonist (10 \(\mu\)M CGS-21680) failed to preserve p-ERK\(_{1/2}\) expression after severe ATP depletion-induced injury indicating that \(A_{2a}\) receptor activation mediates protection from severe ATP depletion injury by a different mechanism than the protection mediated by adenosine \(A_1\) receptor activation (which is p-ERK\(_{1/2}\)-dependent).

The present in vitro study illustrates remarkable similarities to our previous in vivo findings (4): (1) both \(A_1\) and \(A_{2a}\) adenosine receptor activations produce cytoprotection against severe ATP depletion-induced injury; (2) ATP depletion preconditioning, an in vitro model of renal ischemic preconditioning, is mediated via G\(_{11o}\), tyrosine kinase, and ERK\(_{1/2}\); and (3) protection afforded by preconditioning is independent of adenosine receptor activation. Moreover, we showed that the cytoprotection observed with ATP depletion preconditioning and adenosine receptor activation is not additive; that is, combination of ATP depletion preconditioning and adenosine receptor failed to demonstrate improved protection from either maneuver alone. Consistent with our in vivo and current in vitro studies, previous studies in the heart illustrate that transient glucose deprivation confers a preconditioning-like protection against subsequent ischemic reperfusion injury (41) via mechanisms involving PKC.

In summary, this is the first report of in vitro protective effects of adenosine receptors and ATP depletion preconditioning against subsequent severe ATP depletion-induced injury in human proximal tubule cells. We have systematically deciphered the signaling pathways of \(A_1\) and \(A_{2a}\) adenosine receptor-mediated renal cytoprotection as well as protection by prior ATP depletion preconditioning. As we observed with in vivo ischemic preconditioning, in vitro ATP depletion preconditioning confers cytoprotection via adenosine receptor-independent pathways.

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