Ameliorative Effect of Adenosine on Hypoxia-Reoxygenation Injury in LLC-PK$_1$, a Porcine Kidney Cell Line

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ABSTRACT—We studied the effects of adenosine on injury caused by hypoxia and reoxygenation in LLC-PK$_1$ cells. Lactate dehydrogenase and γ-glutamyltranspeptidase were released from cells exposed to hypoxia for 6 hr and then reoxygenation for 1 hr. The addition of adenosine at 100 μM to the medium before hypoxia began significantly decreased enzyme leakage into medium during both hypoxia and reoxygenation. The adenosine A$_1$-receptor agonist, $R(-)$-N^6-(2-phenylisopropyl)adenosine (R-PIA), at the concentration of 100 μM, did not affect enzyme release, but the adenosine A$_2$-receptor agonist 2-p-[2-carboxyethyl]phenethyl-amino-5'-N-ethylcarboxamido-adenosine hydrochloride (CGS 21680) at the concentration of 100 nM, suppressed the injury caused by hypoxia and reoxygenation. There were decreases in cAMP contents and ATP levels in LLC-PK$_1$ cells injured by hypoxia and reoxygenation. Adenosine (100 μM) restored ATP levels in the cells during reoxygenation. With adenosine, the intracellular cAMP level was increased prominently during reoxygenation. These results suggest that adenosine protects LLC-PK$_1$ cells from injury caused by hypoxia and reoxygenation by increasing the intracellular cAMP level via adenosine A$_2$ receptor.

Keywords: Acute renal failure, Hypoxia and reoxygenation, Adenosine A$_2$ receptor, Cyclic AMP, LLC-PK$_1$ cell

Renal proximal tubules, especially their second segments (S$_2$), may function abnormally when reperfusion of an ischemic kidney causes acute renal failure (1–3). The development of in vitro methods using modern cell culture techniques has enabled us to understand the cellular mechanisms of ischemia-reperfusion injury under in vivo conditions. LLC-PK$_1$ cells, derived from pig kidney, have characteristics of proximal tubules, and MDCK cells, derived from canine kidney, have characteristics of distal tubular and collecting duct epithelia. Exposure of LLC-PK$_1$ cells to hypoxia and reoxygenation increases the leakage of lactate dehydrogenase (LDH) from the cells, but MDCK cells treated in the same way are not injured (4). Lipid peroxidation mediated by oxygen radicals causes reperfusion injury in ischemic kidneys (5). Injury by hypoxia and reoxygenation of LLC-PK$_1$ cells is suppressed by an antioxidant and free radical scavengers, suggesting that oxidative stress is involved in the injury in vitro as well as in vivo (4).

Adenosine administered helps to prevent injury during postischemic reperfusion of the heart, and this effect may be mediated by adenosine A$_1$ receptors (6, 7). In the kidney, adenosine releases renin, contributes to the production of erythropoietin, and decreases the GFR; afferent and efferent arterioles and tubules have adenosine receptors (8). An adenosine A$_1$-receptor antagonist attenuates the renal injury caused by cisplatin in rats (9). LLC-PK$_1$ cells express both adenosine A$_1$ and A$_2$ receptors (10). In this study, we examined the effects of adenosine and two receptor agonists on injury caused by hypoxia and reoxygenation using LLC-PK$_1$ cells.

MATERIALS AND METHODS

Chemicals

An adenosine A$_2$-receptor agonist, 2-p-[2-carboxyethyl]phenethyl-amino-5'-N-ethylcarboxamido-adenosine hydrochloride (CGS 21680) (11), and a phosphodiesterase inhibitor, 4-([3-butoxy-4-methoxyphenyl]methyl)-2-imidazolidinone (Ro 20-1724), were purchased from Research Biochemicals, Inc. (Natick, MA, USA). $R(-)$-N^6-(2-phenylisopropyl)adenosine (R-PIA), an adenosine

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A_1-receptor agonist (11, 12), Dulbecco's modified Eagle medium (DMEM), HEPES and $NaHCO_3$ were purchased from Sigma Chemical Co. (St. Louis, MO, USA). *l*-Glutamine and DMEM were from ICN Biomedicals, Inc. (Costa Mesa, CA, USA) and Nissui Pharmaceutical Co., Ltd. (Tokyo), respectively. All other chemicals used were of the highest purity available commercially.

Cell culture

LLC-PK$_1$ cells were obtained from the American Type Culture Collection (Rockville, MD, USA), and grown in DMEM containing 5.55 mM glucose, 5% fetal bovine serum and 10 mM HEPES. The cells were subcultured every 4 days.

Hypoxia and reoxygenation

We used cells that had grown into a confluent monolayer for the experiments with hypoxia and reoxygenation as described elsewhere (4). In brief, the medium was removed and the cells were washed two times with DMEM base, which lacks glucose and serum. The cells were exposed to hypoxic conditions in a GasPak Pouch^{TM} (Becton Dickinson and Co., Cockeysville, MD, USA) in which the oxygen concentration was less than 2%. After 6 hr of hypoxia, the cells were put in a humidified atmosphere of 95% air and 5% CO_2 for 1 hr in the DMEM base to which glucose was added at the beginning of reoxygenation. Adenosine-receptor agonists and phosphodiesterase inhibitor were added to the medium just before the begining of hypoxia.

Evaluation of cell injury

After 7 hr of treatment, the medium was centrifuged at 5℃ for 10 min at 1,500×g. The activities of LDH and γ-glutamyltranspeptidase (γ-GTP) released into the medium were assayed with commercial kits (Wako Pure Chemical Industries, Ltd., Osaka) and used as indices of cell injury.

One milliliter of ice-cold 2% trichloroacetic acid was added to the cells after centrifugation, and the cells were scraped and sonicated for 30 sec. The cell homogenate was centrifuged at 2,000×g for 15 min at 5℃. The supernatant was assayed for ATP by the luciferin-luciferase method of Kimmich et al. (13) with an ATP photometer (model 2000; JRB Inc., La Jolla, CA, USA). The sediment was assayed for protein by the method of Bradford (14).

Measurement of intracellular cyclic AMP

For the assay of intracellular cAMP, the cells were sonicated in ice-cold 6% perchloric acid for 30 sec. The cell homogenate was centrifuged at 1,500×g for 15 min at 5℃. The supernatant was neutralized with appropriate amounts of 81 mM KOH and 0.54 M K_2HPO_4 and frozen at −80℃ until they were assayed for cAMP with a commercial kit (Yamasa Corp., Tokyo).

Assay of antioxidation by adenosine

The antioxidative activity of 100 μM adenosine was tested in terms of its inhibition of the reduction of ferricytochrome C to ferrocytochrome C caused by superoxide anions produced in the reaction of hypoxanthine and xanthine oxidase. The reaction mixture contained 7 mM hypoxanthine, 9 mM cytochrome C and 2.2 units of xanthine oxidase. The formation of ferrocytochrome C was estimated by monitoring of the absorbance at 560 nm.

Statistical analyses

Results are expressed as means±S.D. ANOVA followed by Fisher's procedure was used to evaluate the statistical significance of differences.

RESULTS

Effects of adenosine on cell injury

LDH, a cytoplasmic enzyme, was released into the medium during the 6 hr of hypoxia and more was released during the hour of reoxygenation (Fig. 1). The addition of adenosine before hypoxia suppressed the
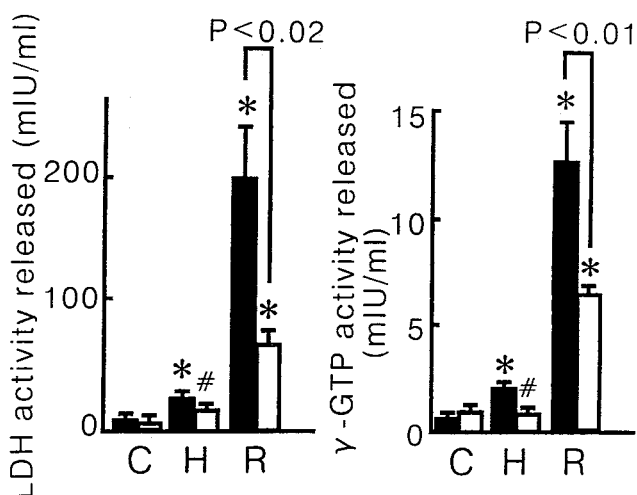

Fig. 1. Effects of adenosine on LDH and γ-GTP leakage from LLC-PK$_1$ cells exposed to 7 hr of normoxia (C), 6 hr of hypoxia (H) or 6 hr of hypoxia followed by 1 hr of reoxygenation (R). Closed columns show leakage of enzymes with no adenosine in the medium, and open columns show the leakage in the presence of 100 μM adenosine. Values are means±S.D. of at least three experiments. *P<0.01, compared with the corresponding "C" and #P<0.01, compared with the corresponding "H" without adenosine in the medium.

release of LDH during hypoxia. Adenosine helped to prevent more leakage of LDH during reoxygenation. The luminal surface of a LLC-PK₁ monolayer cultured on flat culture dishes was exposed directly to the medium. γ-GTP activity, which binds to the brush border membrane, was monitored to assess luminal membrane function. γ-GTP was also released from cells exposed to 6 hr of hypoxia and such release was accelerated by the reoxygenation. The release of γ-GTP during both treatments was also depressed with adenosine. ATP in the cells decreased during hypoxia and then returned to 80% of the control level during reoxygenation (Fig. 2). Adenosine had no effect on the decrease in the cellular ATP after hypoxia, but increased the rate for the return of the ATP level toward the base line during reoxygenation.

Radical scavenging by adenosine

Adenosine did not quench superoxide anion radicals generated in the reaction of hypoxanthine catalyzed by xanthine oxidase (data not shown).

Effects of adenosine receptor agonists on cell injury

R-PIA had no effect on LDH leakage from LLC-PK₁ cells exposed to hypoxia and reoxygenation (Fig. 3). However, after addition of CGS 21680 at the concentration of 100 nM, there was attenuation of cell injury as assessed by LDH leakage from the cells. CGS 21680 also lessened γ-GTP leakage induced by hypoxia and reoxygenation (data not shown). Neither of the adenosine-receptor agonists affected the activities of LDH and γ-GTP released from LLC-PK₁ cells cultured under normoxic condition (data not shown).

Effects of adenosine on intracellular cAMP

The intracellular cAMP level was decreased after hypoxia and reoxygenation (Fig. 4). We tried to delineate whether adenosine increases the intracellular cAMP level in the presence of a phosphodiesterase inhibitor, Ro 20-1724, in the medium. Adenosine remarkably increased cAMP level in the cells exposed to hypoxia and reoxygenation.

Fig. 3. Effect of adenosine-receptor agonists on LDH leakage from LLC-PK₁ cells exposed to hypoxia and then reoxygenation. Adenosine (ADO, 100 μM), an adenosine A₁-receptor agonist, $R(-)$-N^6-(2-phenylisopropyl)adenosine (R-PIA, 100 μM), or an A₂-receptor agonist (CGS 21680, 10 nM or 100 nM) was added to the medium just before hypoxia began. Values show means±S.D. of at least three experiments. *P<0.01, compared with results when no additions were made.
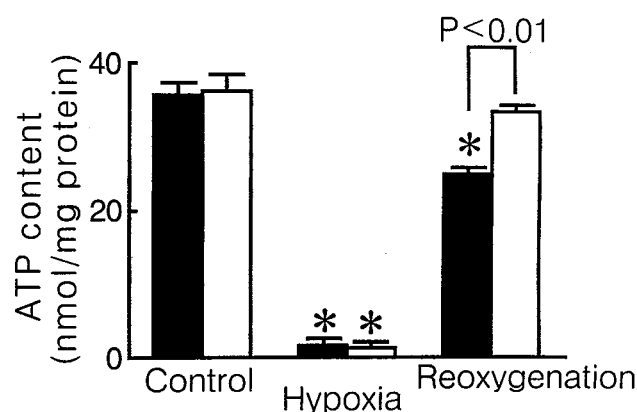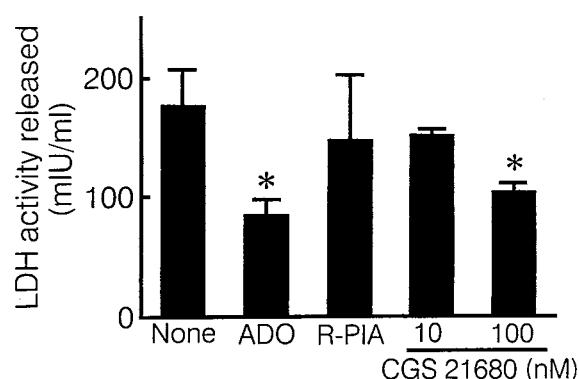

Fig. 2. Effects of hypoxia and reoxygenation with and without adenosine on the ATP concentration in LLC-PK₁ cells. Closed columns show ATP concentrations without adenosine, and open columns show ATP concentrations with 100 μM adenosine. Values are means±S.D. of at least three experiments. *P<0.01, compared with the corresponding control.
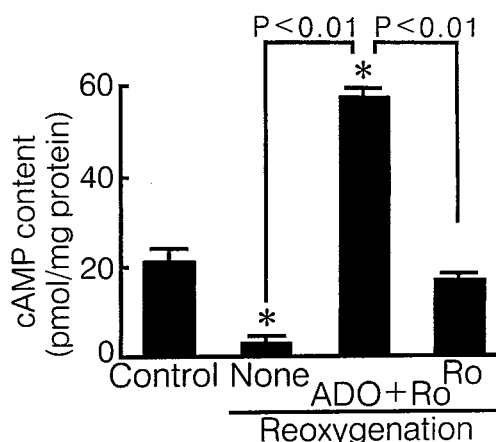

Fig. 4. Effects of hypoxia and reoxygenation with adenosine with or without a phosphodiesterase inhibitor on the cAMP concentration in LLC-PK₁ cells. The cells were exposed to 7 hr of normoxia (Control) or 6 hr of hypoxia followed by 1 hr of reoxygenation. Adenosine (ADO, 100 μM), the phosphodiesterase inhibitor 4-[(3-butoxy-4-methoxyphenyl)methyl]-2-imidazolidinone (Ro 20-1724, or Ro: 200 μM), or both were added to the medium just before hypoxia began. Values show means±S.D. of at least three experiments. *P<0.01, compared with the control.

DISCUSSION

Ischemia-followed by reperfusion of kidneys depletes ATP (15). Hypoxic cell injury causes in vitro decreases in the ATP level (4, 16) and the oxidative phosphorylation activity (17) in proximal tubular cells. ATP depletion, especially in proximal tubular cells which depend on oxidative phosphorylation for energy supply, leads to an intracellular disturbance of chloride and calcium in hypoxic injury of the cells (16, 17). The chloride channel blockers provide marked cytoprotection against the hypoxia-induced release of LDH, a marker for cellular injury, but do not associate with preservation of cell ATP content (16). Adenosine reduced the release of LDH and γ-GTP from hypoxic LLC-PK$_1$ cells. The reduction in both enzyme releases was not accompanied by protection against hypoxia-induced ATP decrease. Hypoxia-induced membrane damage as measured by the release of the enzymes LDH and γ-GTP from hypoxic tubule is a marker of advanced or late cellular injury as compared with ATP depletion in terms of onset in cellular injury. The pattern of cytoprotection by adenosine resembles the pattern of that by chloride channel blokers, suggesting that adenosine blocks a late event in the process of hypoxic injury, including LDH and γ-GTP release, but does not prevent early damage to cellular ATP content. In contrast to hypoxic injury, adenosine accelerates the return to the base line of the ATP concentration in isolated tubules during reoxygenation (18). In LLC-PK$_1$ cells during reoxygenation, adenosine had the same effect on the recovery of ATP level in the present study. In addition, adenosine decreased the cell injury taking place during reoxygenation, as assessed by the decreased leakage of LDH and γ-GTP. These results suggest that adenosine protects LLC-PK$_1$ cells from reoxygenation injury at least in part via the accelerated recovery of the ATP level. Antioxidants attenuate injury of LLC-PK$_1$ cells caused by reoxygenation (4). Some preliminary experiments in our laboratory have shown that when electron spin resonance (ESR) is used to detect free radicals in LLC-PK$_1$ cells, oxygen radicals are increased in the cells exposed to hypoxia followed by reoxygenation, and such an increase in oxygen radicals is lowered by adenosine. Recently, Yokoi et al. (19) have found that adenosine scavenges hydroxyl radicals at a concentration of 5 mM, fifty times the concentration used in our study. Adenosine itself at a concentration used in the present study had no scavenging action, so its effect on LLC-PK$_1$ cell injury probably does not arise from its being an antioxidant.

Adenosine receptors have been classified into subtypes A$_1$, A$_{2a}$, A$_{2b}$ and A$_3$ (20). The A$_1$ receptor participates in the attenuation by adenosine on cardiac injury caused by ischemia and reperfusion (6, 7). The A$_2$ receptor is involved in the suppression of the generation by human neutrophils of superoxide anions (21). The kidney expresses the genes of adenosine receptors (22) and adenosine receptors exist in the kidney (8). LLC-PK$_1$ cells express both adenosine A$_1$ and A$_2$ receptors, and the majority of stimulatory adenosine A$_2$ receptors are in the cell apical surfaces (10). R-PIA at the concentrations of 1 to 10 μM activates adenosine A$_1$ receptors (23, 24). R-PIA at up to 100 μM did not affect enzyme release from LLC-PK$_1$ cells exposed to hypoxia and reoxygenation, but CGS 21680 at 100 nM depressed cell injury caused by such treatment. These two results suggest that the ameliorative effect on such injury in LLC-PK$_1$ cells is probably mediated not by adenosine A$_1$ but by adenosine A$_2$ receptors.

Adenosine A$_2$ receptors stimulate adenylate cyclase activity in isolated tubules of the rabbit renal cortex (25). Our results indicate that adenosine increases cAMP level by activating adenosine A$_2$ receptors, and the increment of cAMP levels attenuates hypoxia and reoxygenation injury in LLC-PK$_1$ cells. N^6,O^2-dibutyryl cAMP, a cAMP derivative that can permeate cell membranes, attenuates the renal injury caused by ischemia and reperfusion in rats (26). Such injury is mediated by the generation of oxygen radicals that induce lipid peroxidation in rat kidneys (5). In LLC-PK$_1$ cells as well, reactive oxygen species participate in the cell injury caused by hypoxia and reoxygenation (4). Nosaka et al. (27) have found that an increase in intracellular cAMP inhibits the production of reactive oxygen metabolites in cultured rat mesangial cells.

In the present study, we demonstrated the ameliorative effect of adenosine at a concentration of 100 μM on hypoxia-reoxygenation injury in LLC-PK$_1$ cells. Such injury was significantly attenuated even by a much lower concentration of adenosine at 10 μM which achieved a concentration near to a clinical one in blood plasma (data not shown). A further study of the protective effect of adenosine on hypoxia and reoxygenation injury in LLC-PK$_1$ cells should be conducted to understand the in vivo effect of adenosine on acute renal failure induced by ischemia and reperfusion for its therapeutic application in the fields of clinical medicine.

In conclusion, adenosine attenuated the injury in LLC-PK$_1$ cells exposed to hypoxia and reoxygenation. That effect was accompanied by an increase in cAMP levels and by a rapid but slight return of the ATP levels to the base line during reoxygenation. The stimulation of adenosine A$_2$ receptors is the primary mechanism of the ameriolative effect of adenosine on cell injury during reoxygenation after hypoxia in LLC-PK$_1$ cells.

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