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Adenosine and Purine Nucleosides Prevent the Disruption of Mitochondrial Transmembrane Potential by Peroxynitrite in Rat Primary Astrocytes

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Previously, we have shown that astrocytes deprived of glucose became highly vulnerable to peroxynitrite, and adenosine and its metabolites attenuated the gliotoxicity *via* the preservation of cellular ATP level. Here, we found that adenosine and related metabolites prevented the disruption of mitochondrial transmembrane potential (MTP) in glucose-deprived rat primary astrocytes exposed to 3-morpholinosydnonimine (SIN-1), a peroxynitrite releasing agent. Exposure to glucose deprivation and SIN-1 (2 h) significantly disrupted MTP in astrocytes, and adenosine prevented it in dose-dependent manner with an EC_{50} of 5.08 μM. Adenosine also partially prevented the cell death by myxothiazol, a well-known inhibitor of mitochondrial respiration. Blockade of adenosine deamination or intracellular transport with erythro-9-(-hydroxy-3-nonyl)adenosine (EHNA) or S-(4-nitrobenzyl)-6-thioinosine (NBTI), respectively, completely reversed the protective effect of adenosine. Other purine nucleos(t)ides including inosine, guanosine, ATP, ADP, AMP, ITP, and GTP also showed similar protective effects. This study indicates that adenosine and related purine nucleos(t)ides may protect astrocytes from peroxynitrite-induced mitochondrial dysfunction.

Key words: Adenosine, Mitochondrial transmembrane potential (MTP), SIN-1, Peroxynitrite, Astrocyte, Glucose-deprivation

INTRODUCTION

Mitochondria have been suggested as a main target in reactive nitrogen species-mediated cell death occurring in the ischemia-reperfusion injury (Sims and Anderson, 2002; Heales and Bolanos, 2002). Peroxynitrite can easily alter the activity of the mitochondrial enzymes in the respiratory chain and cause opening of mitochondrial permeability transition (MPT) pores (Packer and Murphy, 1995; Lizasoain *et al.*, 1996; Gow *et al.*, 1998). We previously reported peroxynitrite-induced mitochondrial dysfunction (Choi *et al.*, 2000; Ju *et al.*, 2000) in cultured rat primary astrocytes. Mitochondrial oxidative phosphorylation, along with glycolysis, is essential for the maintenance of brain ATP levels (Beltran *et al.*, 2000), and depolarization of the mitochondrial transmembrane potential (MTP) would result in ATP depletion and subsequent cell death. Several studies have shown that mitochondrial dysfunction can be prevented by stimulation of intracellular ATP production *via* glycolysis (Mazzio and Soliman, 2003; Gonzalez-Polo *et al.*, 2003; Delgado-Esteban *et al.*, 2000; Jurkowitz *et al.*, 1998).

Purines including adenosine and ATP have been suggested to have neuroprotective effects against acute ischemia or chronic neurodegenerative insults (Bell *et al.*, 1998; Hagberg *et al.*, 1987) *via* the adenosine (A1 and A2) or purine (P2) receptor-dependent and -independent mechanisms (Michel *et al.*, 1999; Jurkowitz *et al.*, 1998; Haun *et al.*, 1996). Several nucleos(t)ides including adenosine were suggested to preserve intracellular ATP level by providing ribose-1-phosphate, which could be eventually converted to glycolytic intermediates (Jurkowitz *et al.*, 1998). Previously, we have reported that adenosine and related purine nucleos(t)ides protect rat primary astrocytes from peroxynitrite and glucose deprivation-induced toxicity *via* the preservation of cellular ATP level (Shin *et al.*, 2002).

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In this study, we investigated whether intracellular ATP preservation by adenosine and related purine nucleos(t)ides protected rat primary astrocytes from peroxynitrite and glucose deprivation-induced disruption of MTP.

MATERIALS AND METHODS

Materials

Tetramethylrhodamine, ethyl ester, perchlorate (TMRE) and 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimideazolocarbocyanine iodide (JC-1) were purchased from Molecular Probes (Eugene, OR). 3-Morpholinosydnonimine (SIN-1) was obtained from Calbiochem (La Jolla, CA). Adenosine deaminase inhibitor erythro-9-(-hydroxy-3-nonyl)adenosine (EHNA) was a product of RBI (Natick, MA) and adenosine transport inhibitor *S*-(4-nitrobenzyl)-6-thioinosine (NBTI) was obtained from Sigma (St. Louis, MO). All other chemicals were purchased from Sigma (St. Louis, MO).

Rat primary astrocyte culture

Rat primary astrocytes were cultured from the prefrontal cortices of 2- to 4-day-old Sprague-Dawley rat pups as previously described (Shin *et al.*, 2002). Glucose deprivation was achieved by repeated rinsing and incubation with glucose-free Dulbecco's modified Eagle's medium (DMEM) that was not supplemented with fetal bovine serum (FBS), which interfered with the lactate dehydrogenase (LDH) assay. To investigate the effect of adenosine treatment on peroxynitrite-potentiated death of cells, the cells were treated with 50 μM of SIN-1 known as peroxynitrite releasing reagent during the glucose deprivation period, and adenosine was added during the same period.

Measurement of cell death

Cell death was assessed by measuring LDH release into the medium at various time points after glucose deprivation. The LDH amount corresponding to complete glial damage/death was measured in sister cultures treated with 0.1% Triton X-100 for 30 min at 37°C. Basal LDH levels (generally less than 3% of total LDH release) were determined in sister cultures subjected to sham wash with 5 μM glucose containing DMEM and subtracted from the levels in experimental conditions to yield the LDH signal specific to experimental injury.

Mesurement of ATP contents

The level of intracellular ATP was measured by the methods previously described (Shin *et al.*, 2002). Briefly, cells were lysed with 10% trichloroacetic acid (TCA) and sonicated for 15 sec on ice. The lysates were added with 2 mM EDTA and 2 mg/mL BSA. After centrifugation, the supernatant was neutralized with 4 M KOH and the ATP content was determined using luminescence detection kit (Molecular Probe, Eugene, OR).

Fluorescence imaging of mitochondrial transmembrane potential (MTP)

To evaluate the changes in MTP in rat primary astrocytes, cells were loaded with TMRE (100 nM) for 20 min at the end of treatment. TMRE fluorescence at the excitation wavelength of 568 nm and emission wavelength of 590 nm in cells was measured with a microscope (Leica, Germany) equipped with a confocal attachment (TCS NT system). To minimize photobleaching and other free radical dye reactions, laser intensity and exposure time were minimized. Several fields per sample were selected randomly by phase-contrast optics, and then fluorescence images were captured using MetaMorph imaging and analysis software. The average mitochondrial TMRE pixel intensity for each cell was then determined. As a positive control for the complete mitochondrial depolarization, the potent mitochondrial inhibitor, carbonyl cyanide 4-trifluoromethoxyphenylhydrazone (FCCP, 5 μM) was applied at the end of the experiment. Alternatively, MTP was also assessed with JC-1 as described previously (Choi *et al.*, 2000). Cells were incubated for 30 min at 37°C with 1 μg/mL of JC-1, which was prepared in culture media and exposed to various condition. Depolarization of MTP was assessed by measuring the fluorescence intensity at excitation wavelength of 485 nm and emission wavelength of 530 nm (for JC-1 monomer fluorescence) and 590 nm (for JC-1 aggregate fluorescence) using a fluorescence microplate reader (Tecan, Austria). At each emission wavelength, autofluorescence (i.e., fluorescence of cells not loaded with JC-1) was subtracted from the JC-1 fluorescence. Data were expressed as the ratios of JC-1 aggregate fluorescence to monomer fluorescence (F_{590}/F_{530}).

Statistical analysis

Data are expressed as the mean ± standard error of means (S.E.M.) and analyzed for statistical significance by using one way analysis of variance (ANOVA) followed by Neuman-Keuls test as a *post-hoc* test and a P value <0.05 was considered significant.

RESULTS

Although glucose deprivation or SIN-1 treatment alone had no effect on cell viability, simultaneous treatment of astrocytes with SIN-1 (50 μM) and glucose deprivation induced more than 80% of total intracellular LDH release into the extracellular medium at 8 h. We previously reported that massive ATP depletion was observed at 4 h ahead of LDH release (Shin *et al.*, 2002). Co-incubation with 50 μM adenosine during glucose deprivation period

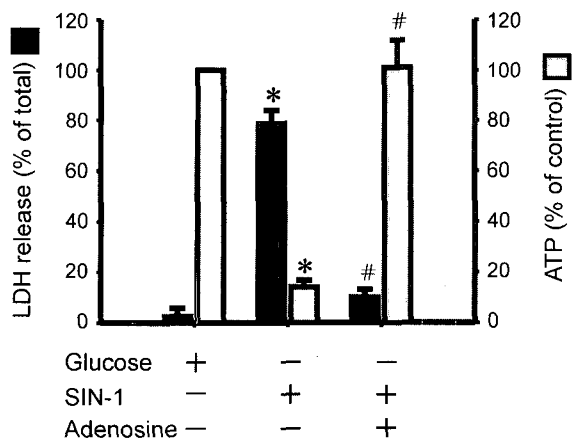

Fig. 1. Adenosine blocked the LDH release and ATP depletion in SIN-1/glucose deprivation-treated rat primary astrocytes. Cells were deprived of glucose in the presence of SIN-1 (50 μM) and adenosine (50 μM). LDH release (black bar) and cellular ATP content (gray bar) were determined at 8 and 4 h, respectively. Each data indicates the mean ± S.E.M. (n=4). *Significant difference from control cells (G+) (*:P <0.05). #Significant difference from glucose-deprived (GD)/SIN-1-treated cells (# : P < 0.05).

markedly inhibited ATP depletion and LDH release (Fig. 1). To investigate whether the protective effect of adenosine is mediated *via* the inhibition of MTP disruption, we determined the mitochondrial potential by two different ways. MTP loss was assessed using the fluorescent probe, TMRE or another probe, JC-1 at 2 h after treatment. As shown in Fig. 2A, MTP assessed by JC-1 was significantly decreased as early as 2 h and complete MTP disruption was observed at 4 h after treatment of SIN-1 and glucose deprivation, which was consistent with our previous report (Ju *et al.*, 2000). A severe disruption of MTP was observed only in the glucose-deprived and SIN-1-treated astrocytes (Fig. 2B) which is comparable to that observed after the treatment of a potent mitochondrial uncoupler, FCCP (5 μM, Fig. 2B). Under same experimental conditions, adenosine blocked MTP disruption in dose-dependent manner (Fig. 2C), and EC_{50} value was 5.08 μM which was close to the values of 16.3 μM and 8.45 μM for the inhibition of ATP depletion and LDH release, respectively, as shown in our recent report (Shin *et al.*, 2002).

Myxothiazol known as a specific inhibitor of mitochondrial respiration, could induce cell death *via* inhibition of mitochondrial function (Bal-Price and Brown, 2001). We investigated here whether adenosine prevent the cell death induced by myxothiazol. As shown in Fig. 3, 24 h treatment with myxothiazol (2~10 μM) induced more than 50% of LDH release, and co-treatment with adenosine (50 μM) significantly reduced the cell death (46.2±1.9 and 20.9±3% reduction as compared to the 2 and 5 μM myxothiazol-treated groups, respectively). However, adenosine did not prevent cell death induced by 10 μM myxothiazol. Myxothiazol inhibits the mitochondrial function reversibly or irreversibly depending on the extent of time or dose of exposure (Bal-Price *et al.*, 2001). Adenosine-mediated protection shown in only low doses of myxothiazol-treated groups suggests that adenosine could be protective against reversible dysfunction of mitochondria.

To investigate whether the protective effect of adenosine is due to its receptors, an agonist (RPIA) or antagonists

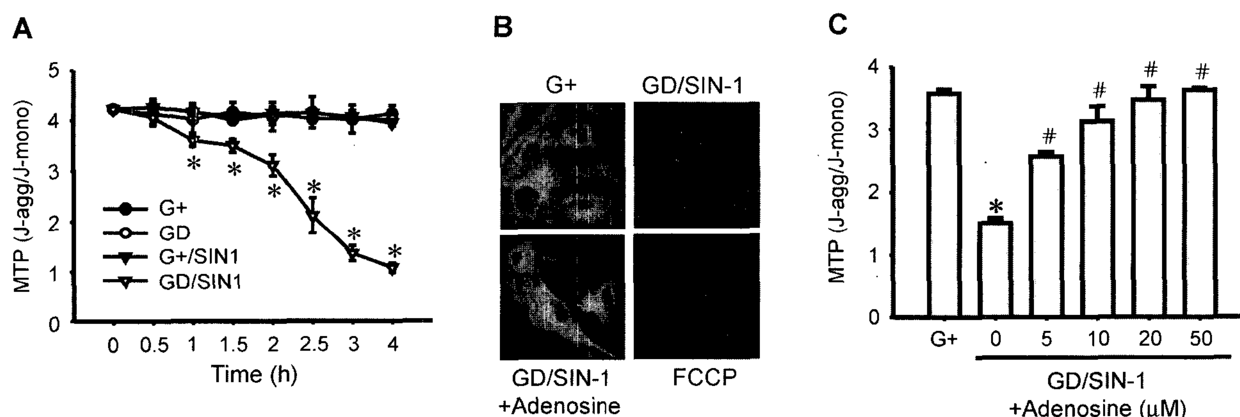

Fig. 2. Adenosine blocked MTP loss in SIN-1/glucose deprivation-treated rat primary astrocytes. (A) Cells were incubated with JC-1 (1 mg/mL) for 30 min, and then deprived of glucose in the absence or presence of SIN-1 (50 μM). The ratio of fluorescence intensity (JC-1 aggregate/JC-1 monomer) was determined at the indicated time points. (B) Cells were deprived of glucose in the presence of SIN-1 (50 μM) and adenosine (50 μM). Two hours later, cells were incubated with TMRE (100 nM) for 20 min, and the fluorescence intensity was determined using a confocal microscope. (C) Cells were incubated with JC-1 (1 μg/mL) for 30 min, and then deprived of glucose in the presence of SIN-1 (50 μM) and various concentrations of adenosine. Two hours later, the ratio of fluorescence intensity (JC-1 aggregate/JC-1 monomer) was determined. Each data indicates the mean ± S.E.M. (n=4). *Significant difference from control cells (G+) (*: P < 0.05). #Significant difference from glucose-deprived (GD)/SIN-1-treated cells (# : P < 0.05).

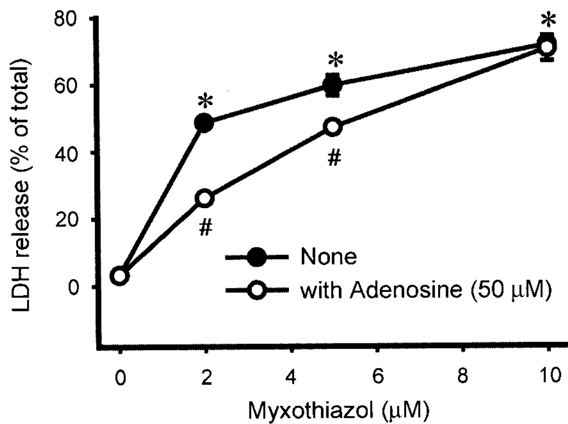

Fig. 3. Protective effect of adenosine against cell death induced by myxothiazol, a specific mitochondrial inhibitor. Rat primary astrocytes were treated by various concentrations of myxothiazol in the presence and absence of 50 μM adenosine for 24 h. The amounts of LDH released into the media were determined at the end of incubation. Each data indicates the mean ± S.E.M. (n=4). *Significant difference from control (untreated) (*:P < 0.05). #Significant difference from myxothiazol-treated cells (#: P < 0.05).

(DMPX for adenosine receptor A2 and DPCPX for adenosine receptor A1) of adenosine receptor were used. All drugs used in this study were ineffective (Fig. 4), which indicates the protective effect of adenosine seems to be receptor-independent. Many researchers including us proposed that preservation of cellular energy by adenosine is due to the ribose 1-phosphate moiety produced during cellular metabolism (Haun *et al.*, 1996; Jurkowitz *et al.*, 1998; Shin *et al.*, 2002). To provide ribose 1-phosphate moiety, adenosine should be transported into the cell and also be deaminated. Therefore, we examined the effect of adenosine deaminase inhibitor and adenosine transport inhibitor. Adenosine deaminase inhibitor EHNA (50 μM) or adenosine transport inhibitor NBTI (50 μM) completely reversed the effect of adenosine (Fig. 4). Since adenosine transported into the cell could be converted to inosine and thereby be used as substrate for glycolysis, we tested whether inosine and related metabolites known as precursor for glycolytic ATP production prevent MTP disruption induced by SIN-1/glucose deprivation. Purine nucleos(t)ides including inosine, guanosine, AMP, ADP, ITP, and GTP at a concentration of 50 μM were added at the same time of SIN-1/glucose deprivation. These nucleos(t)ides significantly inhibited MTP disruption induced by SIN-1/glucose deprivation (Fig. 5). These results are in good agreement with our previous reports that only purine nucleos(t)ides protect rat primary astrocytes from ATP depletion and cell death (Shin *et al.*, 2002).

DISCUSSION

We previously reported that peroxynitrite induced cell death in glucose-deprived rat primary astrocytes (Shin *et al.*, 2002; Choi *et al.*, 2000; Ju *et al.*, 2000). The peroxynitrite-induced cell death was dependent on the disruption of MTP (Choi *et al.*, 2000; Ju *et al.*, 2000). In this study, we showed that adenosine and purine nucleotides protected glucose-deprived rat primary astrocytes from peroxynitrite-induced cell death by maintaining MTP.

Mitochondrial oxidative phosphorylation is essential for

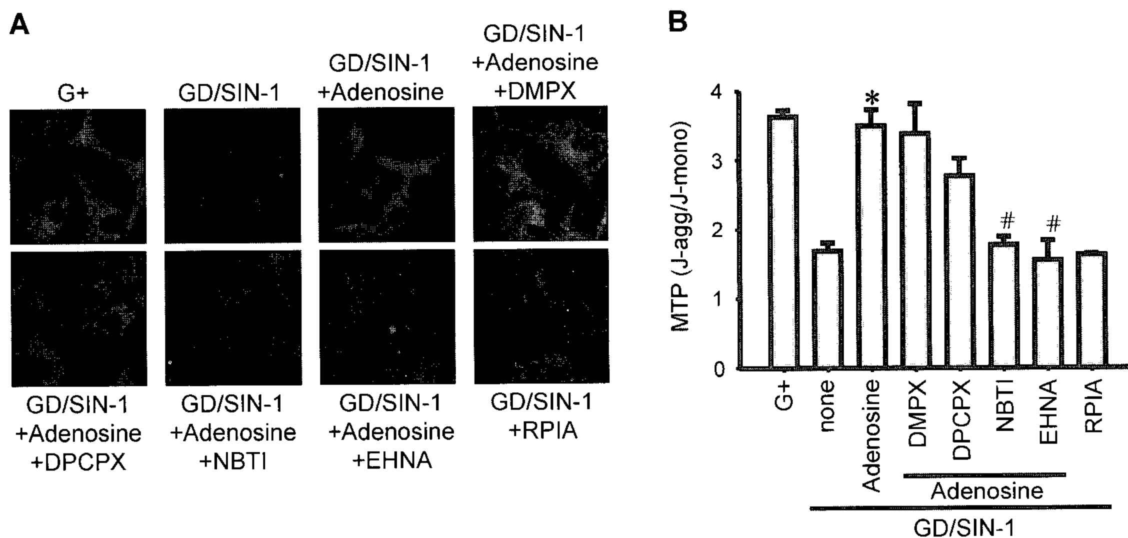

Fig. 4. Effect of adenosine transporter inhibitor or adenosine deaminase inhibitor on the protective effect of adenosine in rat primary astrocytes. (A) Cells were exposed to SIN-1 (50 μM) in the absence of glucose (control) with adenosine (50 μM), and adenosine deaminase inhibitor EHNA (50 μM) or adenosine transport inhibitor NBTI (50 μM) were co-treated with adenosine. Two hours later, MTP was assessed using by the probe TMRE (A) or JC-1 (B). Each bar indicates the mean ± S.E.M. (n=4). *Significant difference from glucose-deprived (GD)/SIN-1-treated group (second bar)(*: P < 0.05). #Significant difference from adenosine-treated group (third bar) (#: P < 0.05).

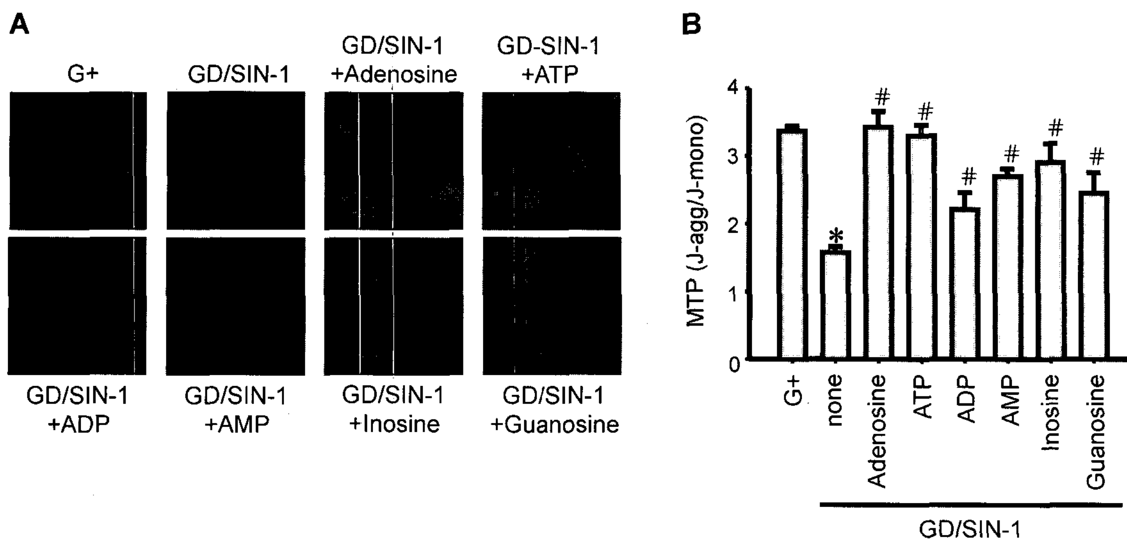

Fig. 5. Effect of several purine nucleot(s)ides on SIN-1 and glucose deprivation induced MTP disruption in rat primary astrocytes. Rat primary astrocytes were exposed to SIN-1 in the absence of glucose with various purine nucleot(s)ides (50 μM). 2 h later, MTP was assessed using by the probe TMRE (A) or JC-1 (B). Each bar indicates the mean ± S.E.M. (n=4). *Significant difference from control (G+) (*: $P < 0.05$). #Significant difference from glucose-deprived (GD)/SIN-1-treated group (none, second bar) (#: $P < 0.05$).

the brain energy supply (Erecinska and Silver, 1989), and loss of mitochondrial function is one of the causes for the cell death. Maintenance of cellular ATP levels is important for the proper functioning of mitochondria. In this study, a significant protection by adenosine on the mitochondrial dysfunction in SIN-1/gulcose deprivation-exposed cultured rat primary astrocytes was observed. SIN-1/glucose deprivation caused mitochondrial dysfunction in cultured astrocytes as measured by MTP loss, which was blocked by adenosine in dose- and time-dependent manners. And adenosine also prevented mitochondrial dysfunction induced by myxothiazol. We previously demonstrated that adenosine protected cultured astrocytes from peroxynitrite /glucose deprivation-induced death *via* preservation of cellular ATP level (Shin *et al.*, 2002). Therefore, it is reasonable to assume that the recovery of cellular ATP level by adenosine is responsible for the maintenance of mitochondrial function. Several reports are in good agreement with our findings. NO-mediated decrease in cellular ATP levels due to the inhibition of glycolysis caused the collapse of mitochondrial membrane potential (MMP), which indicated the importance of glycolytic ATP in the maintenance of MMP (Beltran *et al.*, 2000). Glucose supplementation is enough to protect neurons against the death induced by 1-methyl-4-phenylpyridinium ion (MPP^+), a neurotoxin impairing mitochondrial function (Gonzalez-Polo *et al.*, 2003; Mazzio and Soliman, 2003). This protective effect against MPP^+-mediated mitochondrial damage is due to the energy production *via* glycolytic pathway. These results implicate if the cellular energy level sustains, intracellular homeostatic mechanisms involving maintenance of MTP operate normally.

The neuroprotective effects of adenosine have been reported to be both receptor-dependent and -independent in various neurotoxic paradigms (Kobayashi *et al.*, 1998; Haun *et al.*, 1996, Jurkowitz *et al.*, 1998). In the present study, adenosine deaminase inhibitor EHNA completely inhibited the action of adenosine, and adenosine transport inhibitor NBTI also inhibited the protective effect of adenosine, which implicates that adenosine must enter the cell and de-aminated to exert its protective effect. In combined glucose-oxygen deprivation model of toxicity in astroglial culture, Haun *et al.* (1996) reported that the protective effect of adenosine is inhibited by nucleoside transport inhibitor dipyridamole. Our findings implicate that the protective effect of adenosine is mediated in adenosine receptor-independent fashion.

It has been suggested that the protective effect of adenosine and other nucleosides is related to intracellular metabolism of adenosine (Jurkowitz *et al.*, 1998; Shin *et al.*, 2002). Indeed, we have found that other purine nucleos(t)ides including inosine, guanosine, ATP, ADP, and AMP were also effective to inhibit MTP disruption of SIN-1/glucose deprivation-exposed cultured rat primary astrocytes. Indeed, there have been some reports that above nucleos(t)ides preserve intracellular ATP level by providing ribose-1-phosphate, which could be eventually converted to glycolytic intermediates (Jurkowitz *et al.*, 1998; Litsky *et al.*, 1999).

In conclusion, we have shown that the preservation of cellular ATP by adenosine treatment attenuates MTP disruption in SIN-1/glucose deprivation-exposed cultured

rat primary astrocytes. Because mitochondrial dysfunction is one of toxic mechanisms in brain ischemia, these findings may provide useful evidence for the protective effect of exogenous adenosine or pharmacological reagents able to control extracellular adenosine levels in ischemia where deterioration of energy metabolism is observed.

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