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A_1 Receptor-mediated Protection against Amyloid Beta-induced Injury in Human Neuroglioma Cells

Yong Woon Cho, Hyun Ju Jung, Yong Keun Kim, and Jae Suk Woo

Department of Physiology, Pusan National University School of Medicine, Busan 602-739, Korea

Adenosine has been reported to provide cytoprotection in the central nervous systems as well as myocardium by activating cell surface adenosine receptors. However, the exact target and mechanism of its action still remain controversial. The present study was performed to examine whether adenosine has a protective effect against Aβ-induced injury in neuroglial cells. The astrocyte-derived human neuroglioma cell line, A172 cells, and A$\beta_{25\sim35}$ were employed to produce an experimental Aβ-induced glial cell injury model. Adenosine significantly prevented Aβ-induced apoptotic cell death. Studies using various nucleotide receptor agonists and antagonists suggested that the protection was mediated by A_1 receptors. Adenosine attenuated Aβ-induced impairment in mitochondrial functional integrity as estimated by cellular ATP level and MTT reduction ability. In addition, adenosine prevented Aβ-induced mitochondrial permeability transition, release of cytochrome c into cytosol and subsequent activation of caspase-9. The protective effect of adenosine disappeared when cells were pretreated with 5-hydroxydecanoate, a selective blocker of the mitochondrial ATP-sensitive K^+ channel. In conclusion, therefore we suggest that adenosine exerts protective effect against Aβ-induced cell death of A172 cells, and that the underlying mechanism of the protection may be attributed to preservation of mitochondrial functional integrity through opening of the mitochondrial ATP-sensitive K^+ channels.

Key Words: Adenosine, A_1 receptor, Amyloid beta peptide, Glial cell, Apoptosis

INTRODUCTION

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by a progressive cognitive decline, resulting from selective neuronal dysfunction, synaptic loss, and neuronal cell death. It is neuropathologically characterized by the presence of neurofibrillary tangles and the accumulation of senile plaques (Selkoe, 1994). The plaques contain a dense core of amyloid beta peptide (Aβ), surrounded by dystrophic neurites and activated glial cells. Fibrillated Aβ is able to initiate free radical processes, which resulting in formation of reactive oxygen species, protein and lipid oxidation, and cellular dysfunction, leading to neuronal death (Kontush, 2001).

It is becoming clear from both in vivo and in vitro studies that glial cells as well as neurons play a crucial role in the pathogenesis of AD. Furthermore, astrocytes accumulate Aβ and give rise to astrocytic amyloid plaques in AD brains (Nagele et al, 2003). Several studies indicate that glial cells in brains of AD are affected more by apoptotic cell death than neurons (Lassmann et al, 1995; Smale et al, 1995).

Adenosine has been suggested to be an endogenous protective agent in cerebral ischemia. It is normally present in the extracellular fluid in most tissues of the body, including the brain, and its level increases dramatically following hypoxia or ischemia. Several reviews provide details of therapeutic potential of adenosine and its analogues in hypoxic/ischemic or reactive oxygen species (ROS)-induced brain injury (Phillis, 1990; Von Lubitz & Diemer, 1990; Rudolphi et al, 1992; Von Lubitz et al, 1995a; Liu et al, 1997).

The mechanism of adenosine-mediated protection has not yet been completely understood. Adenosine acts through three major classes of cell surface receptors, A_1 to A_3. Among them, A_1 receptor-mediated protection was suggested to be attributable to modulation of the ATP-sensitive K^+ channels in cell surface membranes. However, growing evidence indicates that the protective efficacy does not always correlate with opening of the plasmalemmal K^{ATP}, implying additional cellular sites of the drug action. The prime candidates include mitochondria, which also harbour an ATP-sensitive K^+ channel (Inoue et al, 1991). However, the exact site and action mechanism are still unknown.

The present study was performed to examine whether adenosine has a protective effect against Aβ-induced injury in neuroglial cells. Thus, A172 cells, a human neuroglioma cell line of astrocyte origin, were exposed to A$\beta_{25\sim35}$, a shortened fragment of the full length peptide (A$\beta_{1\sim40/42}$), and results showed that adenosine provides an effective protection against Aβ-induced injury in A172 cells.

Corresponding to: Jae Suk Woo, Department of Physiology, Pusan National University School of Medicine, 1-10, Ami-dong, Seo-gu, Busan 602-739, Korea. (Tel) 82-51-240-7713, (Fax) 82-51-246-2976, (E-mail) jswoo@pusan.ac.kr

METHODS

Chemicals

Purinergic agonists and antagonists including adenosine, N^6-cyclopentyl-adenosine(CPA), 5'(N-cyclopropyl)-carboxamido-adenosine (CPCA), (2-Deoxy-1-[6-(3-iodophenyl)- methyl] amino)-9Hpurin-9yl]-N-methyl- β-D-ribofuranuronamide (IB-MECA), 8-cyclopentyl-1,3-dipropyl-xanthine (DPCPX), 3,7-dimethyl-1-propargy-xanthine (DMPX), 9-chloro-2-(-2-furanyl)-5-[(phenyl-acetyl) amino] [1,2,4]-triazolo (1,5-c) quinazoline (MRS-1220), amyloid beta peptide (Aβ), diazoxide and 5-hydroxydecanoate were obtained from Sigma Chemical (St. Louis, MO). Pertussis and cholera toxin were obtained from Calbiochem (La Jolla, CA). Calcein and tetramethylrhodamine methyl ester (TMRM) were purchased from Molecular Probes (Eugene, OR, USA). Reagents and medium for cell culture were purchased from GIBCO-BRL (Grand Island, NY).

Cell culture

A172 cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and maintained by serial passages in 75-cm^2 culture flasks (Costar, Cambridge, MA, USA). Cells were grown in Dulbeccos modified Eagles medium containing 10% heat inactivated fetal bovine serum at 37°C in humidified 95% air/5% CO_2 incubator. When the cultures reached confluence, subculture was prepared using a 0.02% EDTA-0.05% trypsin solution.

Induction of Aβ-induced injury

Cells were seeded and grown on 24-well culture plates or collagen-coated cover slips depending on the purpose of the experiments. All the experiments were carried out 2 to 3 days postseeding before reaching confluency. After washout of culture media, cells were treated with 25 μM Aβ in serum-free media and incubated for indicated time periods in a CO_2 incubator. When the effect of adenosine was examined, cells were treated with indicated concentration of adenosine 10 min prior to the exposure to Aβ.

Analysis of apoptotic cell death

Aβ-induced apoptosis was analyzed by nuclear staining with Hoechst 33,258. After exposure to the experimental protocols, cells were fixed with 4% paraformaldehyde and stained with 10 μM Hoechst 33258. Cells with condensed nuclei were counted as apoptotic cells.

Measurement of ATP content

ATP levels in cells were measured with a luciferin-luciferase assay (Lyman & DeVincenzo, 1967). After an exposure to oxidant stress, the cells were solubilized with 500 μl of 0.5% Triton X-100, acidified with 100 μl of 0.6 M perchloric acid and placed on ice. Cell suspension was then diluted with 10 mM potassium glutamate buffer containing 4 mM $MgSO_4$ (pH 7.4), and 100 μl of 20 mg/ml luciferin-luciferase was added to 10 μl of the diluted sample. Light emission was recorded for 20 sec with a luminometer (MicroLumat LB96P, Berthold, Germany). Protein content was determined with a small aliquot of the cell sample.

Assay of MTT reduction

Intact mitochondria reduce 3-(4,5-dimethyl-2-thiazyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) to formazan (Morgan, 1998). Therefore, the ability of cells to form formazan from MTT is a good indicator of mitochondrial function. A total 5 μl of MTT was added to each well (final concentration was 62.5 μg/ml). After exposure to experimental protocols, the supernatant was removed, and the formazan crystals formed in viable cells were solubilized in DMSO. The concentration of formazan was determined by measuring the absorbance at 570 nm using a spectrophotometer.

Assay of mitochondrial permeability transition

To determine the changes in mitochondrial permeability and membrane potential, a double staining method with fluorescent dyes, calcein and tetramethylrhodamine methyl ester (TMRM) was used as described by Lemasters et al (1998). Intact mitochondria maintain negative membrane potential inside and accumulate the lipophilic cationic dye, TMRM, and fluoresce bright yellow. Since impermeable to inner mitochondrial membrane, calcein can not enter the mitochondria. However, mitochondrial dysfunction due to a mechanism, referred to as mitochondrial permeability transition (MPT), makes the mitochondria permeable to solutes and depolarize their membrane potential. Therefore, injured mitochondria lose TMRM and become permeable to and stained with calcein (green).

Detection of cytochrome c release

After exposure to experimental protocols, cells were washed twice with ice-cold phosphate-buffered saline at 4°C and resuspended in 1 ml of extraction buffer (50 mM PIPES, pH 7.0, 50 mM KCl, 5 mM $MgCl_2$, 5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin and 10 μg/ml pepstatin A). Cells were lysed by five cycles of freezing in liquid nitrogen and thawing at 37°C. After verifying that more than 90% of cells were lysed by microscopic examination, the lysates were centrifuged at 100,000 g for 1 hr at 4°C. The resulting supernatant, which consisted of the cytosol, was separated from the pellet containing the cellular membrane and organelles, and was analyzed for cytochrome c by Western blots using polyclonal anti-cytochrome c antibody.

Assay of caspase activity

A quantitative enzymatic activity assay of caspase-9 was carried out using the R&D (Minneapolis, Mn) colorimetric assay kit according to the manufacturer-provided instructions. After treatment with Aβ, cells were harvested for the assays. Cells were then washed with ice-cold PBS, lysed, centrifuged, and analyzed for total protein by a Bradford assay (Bradford, 1976). Samples containing 50 μg of total protein were assayed for caspase activity with Ac-DEVD as caspase-8 and 9-specific substrate. Absorbance was measured at 405 nm in a plate reader.

RESULTS

Adenosine-induced protection against Aβ-induced apoptosis

The effect of Aβ on the cell viability was examined by nuclear staining with Hoechst 33258 (Fig. 1). In the micrographs of Hoechst 33258-stained cells presented in Fig. 1A, apoptotic cells are distinguished from normal viable cells by their condensed nuclei (indicated by arrows). The time-dependent effect of Aβ is summarized in Fig. 1B. In the presence of 25 μM Aβ, the number of apoptotic cells increased in a time-dependent manner. After 48 hr exposure, 48.2$\pm$5.7% of the cells were counted as apoptotic. Fig. 1C depicts the effect of adenosine on Aβ-induced death. Adenosine significantly protected the cells against Aβ-induced cell death in a concentration-dependent manner. The concentration to yield 50% protection was 0.87 ± 10^{-3} M.

Effects of various adenosine receptor agonists and antagonists

To define the receptor subtypes involved in the adenosine-induced protection against Aβ-induced cell death, the effects of different adenosine agonists were determined. CPA (Moos et al, 1985), CPCA (Daly, 1982), and IB-MECA (Von Lubitz et al, 1995b) are known as relatively specific agonists for A_1, A_2, and A_3 receptors, respectively. Among those agonists tested in the present study, only CPA mimicked adenosine to protect Aβ-induced cell death (Fig. 2A) suggesting that the protection by adenosine against Aβ-induced injury in A172 cells is mediated by the A_1 receptors. This suggestion is supported by the findings obtained with various adenosine receptor antagonists depicted in Fig. 2B. The protective effect of adenosine was blocked significantly by DPCPX, a selective A_1 receptor antagonist (Haleen et al, 1987). In contrast, the A_2 receptor antagonist DMPX (Ukena et al, 1986) and the A_3 receptor antagonist MRS-1220 (Jacobson et al, 1997) were not effective.

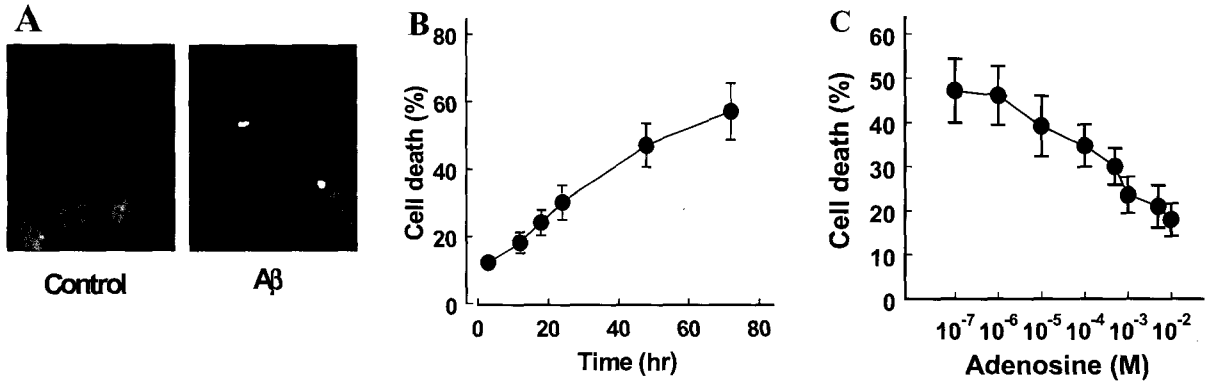

Fig. 1. Aβ-induced cell injury and its protection by adenosine. (A) Representative micrographs of Hoechst 33258-stained cells. Apoptotic cells are distinguished from normal viable cells by their condensed nuclei (indicated by arrows). (B) Time-dependent effect of Aβ. Cells were treated with Aβ (25 μM) for indicated time periods (C) concentration-dependent protection by adenosine against Aβ-induced cell death. Cells were treated with Aβ (25 μM) for 48 hr in the presence of indicated concentrations of adenosine and analyzed for apoptosis by Hoechst 33258 staining. Each point represents mean$\pm$S.E. of 4 experiments.

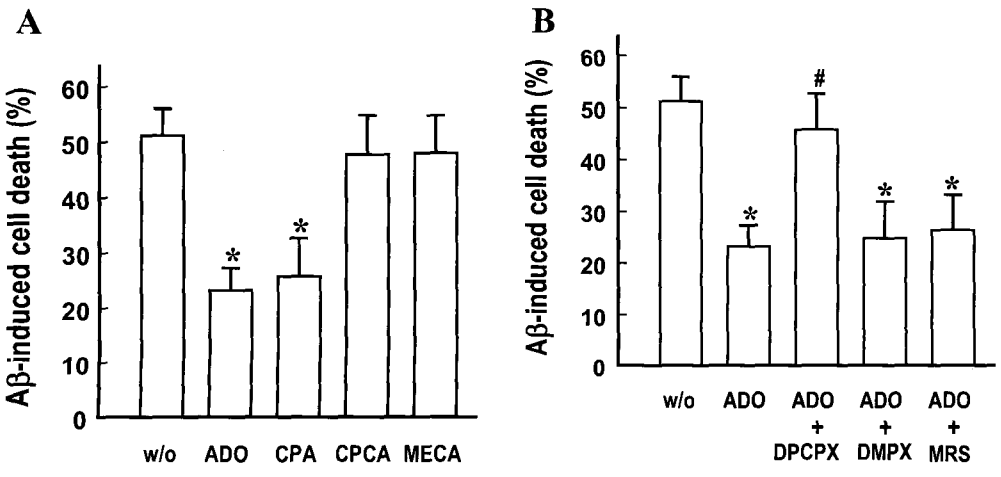

Fig. 2. Effects of different adenosine receptor agonists and antagonists on Aβ-induced cell death. (A) cells were treated with Aβ (25 μM) for 48 hr in the presence of 1 mM each of adenosine (ADO) and different adenosine receptor agonists, (B) cells were treated with Aβ (25 μM) for 48 hr in the presence of adenosine (ADO, 1 mM) with various adenosine receptor antagonists (0.1 mM each). Cells were analyzed for apoptosis by Hoechst 33258 staining. Each bar represents mean$\pm$S.E. of 4 experiments. *$p<0.01$ vs. Aβ alone (w/o), #$p<0.01$ vs Aβ + adenosine without antagonists.

Effect of pertussis and cholera toxin on the adenosine-induced protection

A_1 receptors couple to the pertussis toxin-sensitive G_i protein to modulate the activity of several effector proteins such as adenylate cyclase, phospholipase C and ATP-sensitive K^+ channels (Stiles, 1992). In contrast, A_2 receptor is known to interact with the G_s protein, which mediates activation of adenylyl cyclase. Therefore, the effects of pertussis and choleratoxin were examined. Cells were treated with pertussis or cholera toxin (0.1 μg/ml) for 3 hr before exposure to adenosine and Aβ. As shown in Fig. 3, the adsenosine-induced protection was blocked significantly by pertussis toxin treatment, whereas it was not affected by cholera toxin.

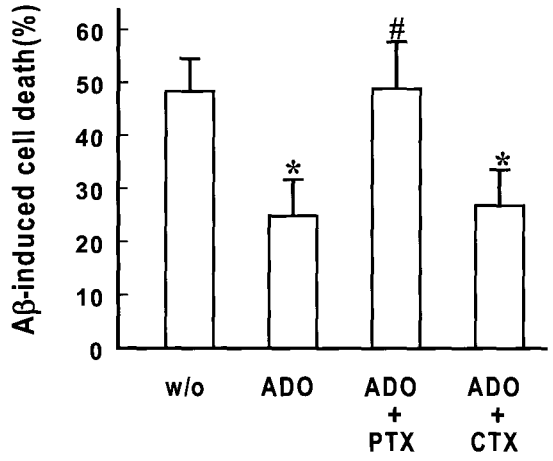

Fig. 3. Effects of pertussis- and cholera-toxin treatment on the adenosine-induced protection. Cells were pretreated for 3 hr with 0.1 g/ml of pertussis (PTX) or cholera toxin (CTX), and then exposed to Aβ (25 μM) and adenosine (ADO, 1 mM) for 48 hr. Cells were analyzed for apoptosis by Hoechst 33258 staining. Each bar represents mean±S.E. of 4 experiments. *$p<0.01$ vs. Aβ alone (w/o), #$p<0.01$ vs. without toxins.

Adenosine-induced protection of mitochondrial function

It is well known that, depending on the types of the stimuli, the mitochondria-dependent and/or-independent mechanisms are involved in the apoptotic cell death. To define if Aβ induces cell death through the mitochondria-dependent pathway, changes in mitochondrial function were determined. Time-dependent changes in intracellular ATP content and MTT reduction ability were determined as indexes of the mitochondrial functional integrity. Aβ decreased both the intracellular ATP content (Fig. 4A) and MTT reduction ability (Fig. 4B) in a time-dependent manner. After 48 hr exposure, intracellular ATP content and MTT reduction ability were reduced to 13.2±2.7% and 32.3± 4.9% of the control values, respectively. These changes were significantly attenuated by adenosine. Adenosine, at 1 mM concentration, attenuated the depletion of intracellular ATP content and deterioration of MTT reduction ability after 48 hr exposure to Aβ by 54.2±6.3 and 48.6±7.8%, respectively. These results strongly suggest that adenosine protects cells from mitochondrial injury induced by Aβ.

Aβ-induced mitochondrial permeability transition, cytochrome c release and caspase-9 activation, and their protection by adenosine

The effect of adenosine on Aβ-induced changes in mitochondrial permeability and cytochrome c release was examined by fluorescence imaging and immunoblot analysis, respectively. As shown in fluorescence micrographs of Fig. 5A, intact mitochondria of control cells accumulated TMRM. As a result, mitochondrial contours were visualized as bright yellow spots. In contrast, however, injured mitochondria of cells exposed to Aβ tended to lose TMRM and become permeable to calcein. As a result, it became hard to distinguish mitochondrial contours from cytosol in the micrographs. It suggests that exposure of cells to Aβ leads to disruption of the mitochondrial membrane potential and loss of the permeability barrier, which is referred to as mitochondrial permeability transition (MPT). Adenosine effectively prevented Aβ-induced changes in the nature of staining with TMRM and calcein, suggesting that it could

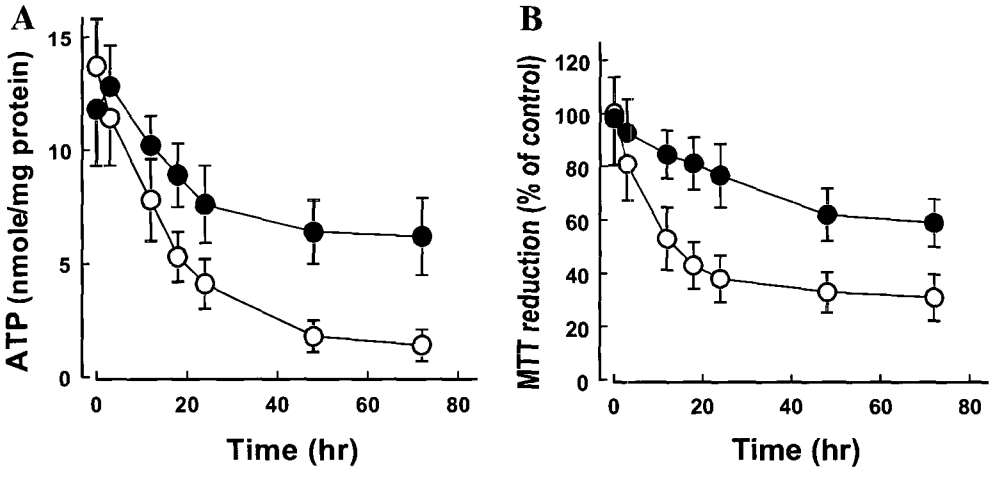

Fig. 4. Effect of adenosine on cellular ATP content and MTT reduction ability in Aβ-exposed cells. Cells were treated with A β (25 μM) for indicated time periods in the presence (closed circle) and absence (open circle) of adenosine (1 mM), and analyzed for intracellular ATP content (A) and MTT reduction ability (B). Each point represents mean±S.E. of 5 experiments.

attenuate the Aβ-induced MPT. Furthermore, as shown in Fig. 5B, it significantly prevented Aβ-induced release of cytochrome c into the cytosol.

Released cytochrome c activates caspase-9 cascades, which are critical for the execution phase of apoptosis (Lemasters et al, 1998). Aβ-induced changes in caspase-9 activity was examined in the presence and absence of adenosine. The result depicted in Fig. 5C shows the Aβ-induced increase of caspase-9 activity in a time-dependent manner. Adenosine significantly attenuated the Aβ-induced stimulation of caspase-9 activity.

Effect of K^+ channel modulators

To elucidate the possible role of mitochondrial ATP-sensitive K^+ channels in the adenosine-mediated protection against Aβ-induced cell death, the effects of modulators of mitochondrial ATP-sensitive K^+ channel were determined, and results are summarized in Fig. 6. Diazoxide, a selective opener of mitochondrial ATP-sensitive K^+ channels (Szewczyk & Marban, 1999), mimicked adenosine to produce protective effect against Aβ-induced cell death. In addition, the protective effects of adenosine and diazoxide were significantly blocked by 5-hydroxydecanoate, a selective blocker of mitochondrial ATP-sensitive K^+ channels (Szewczyk & Marban, 1999). These results strongly suggest that the protective effect of adenosine may be mediated via activation of mitochondrial ATP-sensitive K^+ channels.

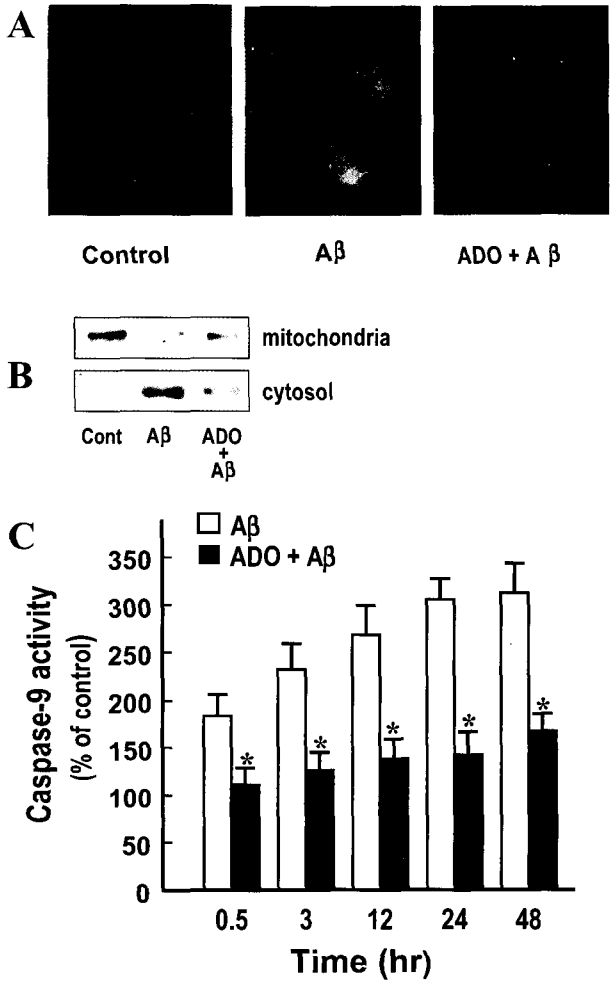

Fig. 5. Effect of adenosine on Aβ-induced mitochondrial permeability transition (MPT), cytochrome c release and caspase-9 activation. (A) Cells were treated with Aβ (25 μM) for 48 hr in the presence and absence of addenosine (ADO, 1 mM), and were then double-stained with fluorescent dyes, calcein and TMRM. (B) cytochrome c in the mitochondria and cytosol was determined by an immunoblot assay. (C) Cells were treated with Aβ (25 μM) for indicated time periods in the presence and absence of adenosine (1 mM), and analyzed for caspase-9 activity. Each bar represents mean$\pm$S.E. of 4 experiments. *$p<0.01$ vs. Aβ alone.

DISCUSSION

In the present study, a human astrocyte-derived glioma cell line, A172 cells, and A$\beta_{25\sim35}$ were used to produce an experimental model of Aβ-induced glial cell injury. A$\beta_{25\sim35}$ has been used in a number of studies and found to have many effects in common with full length A$\beta_{1\sim40/42}$ (Pike et al, 1995). The shortened fragment A$\beta_{25\sim35}$ contains the residues essential for aggregation and toxicity. A$\beta_{25\sim35}$ aggregates rapidly to form β-pleated sheet structure, similar aging procedures required for A$\beta_{1\sim40/42}$ aggregation. The toxicity of A$\beta_{25\sim35}$ is very similar to that of A$\beta_{1\sim42}$ on hippocampal neuronal cultures as assessed by percentage of cell loss (Pike et al, 1993).

The abundance and widespread distribution of activated astrocytes in AD brains suggests that they play a critical role in AD pathogenesis. Recently, Nagele et al (2003) revealed that astrocytes in AD brain accumulates Aβ. It is a consequence of the enhanced phagocytic activity of these cells through which Aβ is internalized and, like neurons, Aβ-burdened astrocytes can undergo lysis and give rise to astrocytic plaques. Moreover, loss of antioxidants, such as glutathione, due to astrocytic lysis contributes as a major cause leading to neuronal death (Abramov et al, 2003).

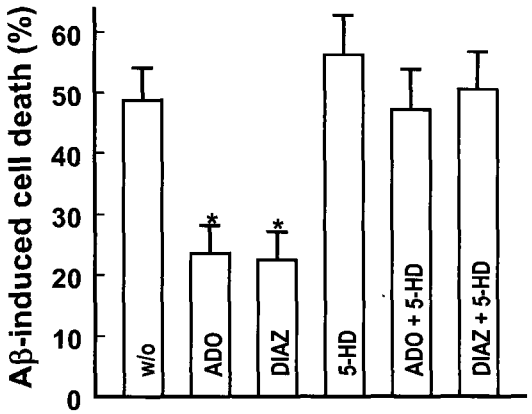

Fig. 6. Effects of adenosine, diazoxide, and 5-hydroxydecanoate on on Aβ-induced cell death. Cells were treated with Aβ (25 μM) for 48 hr in the presence of each one or combination of adenosine (ADO, 1 mM), diazoxide (DIAZ, 100 μM), and 5-hydroxy-decanoate (5-HD, 100 μM), and analyzed for apoptosis by Hoechst 33258 staining. Each bar represents mean$\pm$S.E. of 4 experiments. *$p<0.01$ vs. Aβ alone.

The present study provided a good evidence that the use of A172 cells and $A\beta_{25-35}$ as an experimental model reproduces the $A\beta$-induced glial cell injury. Along with apoptotic cell death, $A\beta$ led to deterioration of mitochondrial functional indexes, induction of MPT, release of cytochrome c and activation of caspase-9, suggesting that a great part of $A\beta$-induced apoptosis is mediated through a mitochondria-dependent mechanism.

In this study, adenosine provided an effective defense mechanism against $A\beta$-induced injury in A172 cells. In addition to the protection against $A\beta$-induced apoptosis, adenosine helped mitochondria preserve its functional and morphological integrity. Adenosine receptors (P_1 receptors) are divided into three major classes: $A_1 \sim A_3$ (Olah & Stiles, 1995) with two subclass of the A_2 receptor being designated "A" and "B". These receptors are members of the superfamily of G protein-coupled receptors. Among them, the A_1 receptor has been shown to interact with pertussis toxin-sensitive G proteins, particularly $G_{i1\sim3}$ and G_o (Olah & Stiles, 1995). In the present study, the findings from the experiments with selective adenosine receptor agonists and antagonists suggested that the A_1 receptors are responsible for the mediation of protection mechanism. The sensitivity to pertussis toxin of the adenosine-induced protection is consistent with the notion that A_1 receptor mediates the protection mechanism via G_i protein.

It has been suggested that $A\beta$-induced cell injury might be associated with deterioration of functional and structural integrity of the mitochondria. (Anandatheerthavarada et al, 2003; Bachurin et al, 2003). Disruption of the permeability barrier of the inner mitochondrial membrane, which is referred to as MPT, leads to release of cytochrome c through this MPT pore (Kroemer et al, 1998). Released cytochrome c activates caspase cascades, which are critical for the execution phase of apoptosis. The present study provided evidences to support this mitochondrial hypothesis. In cells treated with $A\beta$, mitochondrial functional indexes, such as cellular ATP content and MTT reduction ability rapidly declined. Double fluorescence imaging with calcein and TMRM demonstrated that MPT took place in these cells.

The results in this study strongly suggest that the adenosine-mediated protection against $A\beta$-induced cell death might be attributable to its beneficial effect to protect the functional and structural integrity of mitochondria against irreversible damages induced by $A\beta$. Adenosine helped mitochondria to preserve its ability to reduce MTT under $A\beta$-induced insult, and it delayed cellular ATP depletion. Furthermore, it significantly prevented $A\beta$-induced MPT, release of cytochrome c into cytosol and subsequent activation of caspase-9. Therefore, it is highly likely that these events would be accompanied by protection against apoptotic cell death.

A_1 receptors interact with at least three effector systems in the surface membrane (Olah & Stiles, 1995), namely, adenylate cyclase (inhibition), phospholipase C (activation), and potassium channels (activation). Among them, activation of ATP-sensitive K^+ channels on the surface membrane has been suggested to be a target for adenosine to mediate cell protection. Recently, Andoh et al (2006) demonstrated that activation of A_1 adenosine receptors promotes the opening of K_{ATP} channels in principal neurons of the substantia nigra by removing the blockade by ATP. Opening of ATP-sensitive K^+ channels upon activation of adenosine receptors hyperpolarizes neurons (Rudolphi et al, 1992), depresses neuronal firing rate and decreases neuronal metabolism, thereby promoting the preservation of ATP during ischemia. However, this hypothesis seems to be not reasonable to explain the effect of adenosine in non-excitable glial cells. Growing evidences indicate that the protective efficacy does not always correlate with opening of plasmalemmal ATP-sensitive K^+ channels, implying additional cellular sites of drug action. The prime candidates include mitochondria, which also harbour the ATP-sensitive K^+ channels.

In the present study, diazoxide, a selective opener of mitochondrial ATP-sensitive K^+ channels (Szewczyk & Marban, 1999), mimicked adenosine to protect $A\beta$-induced mitochondrial injury. In addition, the protecion by adenosine and diazoxide was effectively blocked by 5-hydroxydecanoate, a selective blocker of mitochondrial ATP-sensitive K^+ channels (Szewczyk & Marban, 1999). This result strongly suggests that mitochondrial ATP-sensitive K^+ channels are involved in the adenosine-mediated protection mechanism.

In conclusion, it is suggested that adenosine prevents functional and structural deterioration of mitochondria and provides a protection against $A\beta$-induced injury via opening of mitochondrial ATP-sensitive K^+ channels.

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