Adenosine Induced Apoptosis in BHK Cells via P1 Receptors and Equilibrative Nucleoside Transporters

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Adenosine, as a ubiquitous metabolite, mediates many physiological functions via activation of plasma membrane receptors. Mechanisms of most of its physiological roles have been studied extensively, but research on adenosine-induced apoptosis (AIA) has only started recently. In this study we demonstrate that adenosine dose-dependently triggered apoptosis of cultured baby hamster kidney (BHK) cells. Adenosine-induced apoptotic cell death was characterized by DNA laddering, changes in nuclear chromatin morphology and phosphatidylserine staining. Apoptosis was also quantified by flow cytometry. Results suggest the involvement of adenosine A_1 and A_3 receptors as well as equilibrative nucleoside transporters in apoptosis induced by adenosine. These results indicate a receptor-transporter co-signaling mechanism in AIA in BHK cells. The involvement of A_1 and A_3 receptors also implies a possible apoptotic pathway mediated by G protein-coupled receptors.

Keywords: Adenosine, Adenosine receptor, Apoptosis, BHK cells, Nucleoside transporter

Introduction

Adenosine (Ado) is a primordial signaling molecule that modulates physiological responses in all mammalian tissues,

Abbreviations: Ado, adenosine; DIP, dipyrindamole; DMPX, 3,7-dimethyl-1-propargylxanthine; DPCPX, 1,3-dipropyl-8-cyclopentylxanthine; EHNA, erythro-9-(2-hydroxy-3-nonyl) adenine; MRS-1220, 9-chloro-2-(2-furyl)-5-phenylacetylaminio-[1,2,4]-triazolo[1,5-c]quinazoline; NBMPR/NBTI: nitrobenzylmercaptapurine ribonucleoside or S-(4-nitrobenzyl)-6-thioinosine.

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many of which have been well studied and documented (Abbracchio and Williams 2001a, Abbracchio and Williams 2001b). However, less attention has been paid to the study of adenosine-induced apoptosis (AIA) (Chow *et al.*, 1997). While several researchers reported that adenosine or adenosine analogs have apoptotic effect on cells (Szondy 1994; Tanaka *et al.*, 1994; Wakade *et al.*, 1995; Szondy 1995; Abbracchio *et al.*, 1995; Shneyvays *et al.*, 1997; Ceruti *et al.*, 1997; Kohno *et al.*, 1998; Barbieri *et al.*, 1998; Rounds *et al.*, 1998; Peyot *et al.*, 2000; Schrier *et al.*, 2001; Di Iorio *et al.*, 2002; Koshiba *et al.*, 2002; Schrier *et al.*, 2002), anti-apoptotic effects of adenosine have also been reported (Walker *et al.*, 1997; Yao *et al.*, 1997). Both adenosine receptor-mediated pathways (Szondy 1994; Abbracchio *et al.*, 1995; Shneyvays *et al.*, 1997; Ceruti *et al.*, 1997; Walker *et al.*, 1997; Yao *et al.*, 1997; Kohno *et al.*, 1998; Barbieri *et al.*, 1998; Peyot *et al.*, 2000; Di Iorio *et al.*, 2002) and transporter mediated pathways (Tanaka *et al.*, 1994; Wakade *et al.*, 1995; Szondy 1995; Rounds *et al.*, 1998; Barbieri *et al.*, 1998; Schrier *et al.*, 2001; Di Iorio *et al.*, 2002; Koshiba *et al.*, 2002; Schrier *et al.*, 2002) were suggested. The results appear to indicate that the mechanism(s) of adenosine-induced apoptosis may be more complicated than expected, involving multiple pathways through which adenosine induces apoptosis under various conditions and in different type of cells.

Four adenosine receptors, termed A_1 , A_{2A} , A_{2B} and A_3 , have been cloned and characterized by pharmacological studies (Palmer *et al.*, 1995). All of the four subtype adenosine receptors belong to the family of G protein-coupled receptors (GPCRs). A_{2A} and A_{2B} receptors couple with G_{α_s} , whereas A_1 and A_3 receptors mainly couple with G_{α_i} and interact with phospholipase C (Ralevic *et al.*, 1998) by which most of adenosine's physiological functions are mediated (Abbracchio and Williams 2001a, Abbracchio and Williams 2001b). In addition to being mediated by receptors, extracellular adenosine is also a substrate for the membrane nucleoside transporters, through which adenosine can enter cells and be sequentially phosphorylated intracellularly to AMP, ADP and

ATP. This intracellular pathway is also responsible for some of adenosine's physiological functions (Cass *et al.*, 1998). Membrane nucleoside transporters are categorized into two groups on the basis of transport mechanisms (Griffith *et al.*, 1996). The equilibrative, or Na⁺-independent nucleoside transporters are "facilitators" and are driven solely by the concentration of nucleoside permeates. Na⁺-independent nucleoside transporters are further subdivided on the basis of sensitivity to NBMPR: *es* (equilibrative & sensitive) type and *ei* (equilibrative & insensitive) (Griffith *et al.*, 1996). Concentrative nucleoside transporters are classified into 6 subtypes according to permeate selectivity and sensitivity to NBMPR: *cif* (N1), *cit* (N2), *cib* (N3), *cit* (N4), *cs* (N5) and *csg* (N6) (Cass *et al.*, 1998).

In this study, we found that adenosine played different roles in cell death at different adenosine concentrations. In BHK cells, very low concentrations (2-5 μ M) of adenosine enhanced cell proliferation slightly while higher concentrations (10-1000 μ M) caused apoptosis, with medium concentrations (50-200 μ M) showing decreased apoptotic effects. Using selective adenosine receptor antagonists, we were able to confirm that A₁ and A₃ Ado receptors mediated apoptosis induced by low concentrations (20-50 μ M) of adenosine; whereas both *ei* type nucleoside transporter and A₁ and A₃ Ado receptors mediated adenosine-induced apoptosis at high adenosine concentrations (500-1000 μ M). This study is the first to demonstrate that adenosine's physiological function can be co-mediated by both adenosine receptors and nucleoside transporters. In addition, this study provides evidence for the possible involvement of GPCRs in apoptosis, for which there is yet no firm conclusion.

Materials and Methods

Culture of BHK cells BHK cells were maintained in α -MEM (Sigma M0894) supplemented with 10% FBS, 20 mM NaHCO₃, 5 mM HEPES and 100 U/ml penicillin at 37°C in a humid atmosphere of 5% CO₂/air. In this study, 24 h prior to any experiment, BHK cells were seeded on coverslips or in 75 cm² culture flasks at the density of 1×10^4 cells/cm². When adenosine was present in medium, 1% heat inhibited FBS_i (56°C, 2 h) was used instead of 10% FBS, 5 μ M EHNA was added to medium.

Double-staining of BHK cells with annexin V-FITC and propidium iodide (PI) Externalization of phosphatidylserine and condensed/fragmented chromatin were detected by an annexin V-FITC-Propidium Iodide double staining using an adaptation of the protocol outlined in the annexin V-FITC apoptosis detection kit (Pharmingen, BD Biosciences). Briefly, BHK cells seeded on coverslips in 6-well plates were incubated with 1 mM adenosine for 2 h or 24 h. At the end of incubation period, medium was replaced by fresh α -MEM, annexin V-FITC and PI were added for an additional 30-minute incubation. Coverslips were then placed upside down on a glass slide and immediately observed by fluorescence microscopy (Carl Zeiss LSM 510).

Analysis of internucleosomal DNA fragmentation: DNA laddering BHK cells were seeded in 6 culture flasks. 24 h after medium in each flask was replaced with α -MEM containing 0, 10, 20, 500, 1000 μ M adenosine or 10 μ M camptothecin, cells were incubated for another 24 h. DNA fragmentation was determined using an adaptation of a described technique (Liu *et al.*, 1996). Briefly, after shaking the flasks, weakly adherent and non-adherent cells were collected by centrifugation of the cell culture medium (200 g, 5 min). Adherent cells were trypsinised, harvested and kept separately. Cells were incubated at 37°C for 3 h in a lysis buffer consisting of, in mM, Tris-HCl (pH 8.0) 10, EDTA 5, and NaCl 100, as well as 0.5% SDS and 10 μ g/ml proteinase K (Boehringer) under agitation. This incubation was followed by dropwise addition of 5 M NaCl to a final concentration of 1 M and incubation at 4°C for 1 h. After centrifugation at 15,000 g for 30 min at 4°C, supernatants were recovered. DNA was extracted with an equal volume of 25 : 24 : 1 phenol/chloroform/isoamyl alcohol (vol : vol : vol) and precipitated in the presence of an equal volume of isopropanol at -20°C overnight. After centrifugation at 15,000 g for 10 min at 4°C, the pellets were washed in 75% ethanol, resuspended in water and digested with 1 mg/ml DNase-free RNase for 30 min at 37°C. DNA electrophoresis was carried out in 2% agarose gel containing 0.5 μ g/ml ethidium bromide. DNA fragments were visualized under UV light.

Assessment of apoptosis by flow cytometry using DNA fragment measurements BHK cells seeded in 6-well plates were treated with receptor antagonists or transporter inhibitors in the absence or presence of various concentrations of adenosine for 24 h. Cells in supernatant were collected by centrifugation (200 g, 5 min) and combined with adherent cells which were trypsinised and harvested. Cells were then washed with 5 ml PBS (pH 7.2), fixed by dropwise addition of ice cold 70% ethanol for no less than 2 h and passed through 0.44 mm filter to remove aggregates. Prior to flow cytometry, cells were centrifuged at 200 g for 5 min to remove ethanol and stained with 1ml PI/Triton X-100 staining solution with RNase A for 15 min at 37°C. Apoptotic cells were quantified using flow cytometry (Becton Dickinson FACS Vantage SE). Data were obtained from triplicates. 10,000 events were counted for each sample.

Results and Discussion

Induction of BHK cell apoptosis by adenosine Two hours after the induction of apoptosis with adenosine, BHK cells were double-stained with annexin V-FITC and PI. Phosphatidylserine was detected by annexin V-FITC (Fig. 1A) but no condensed or fragmented chromatin was detected by PI, indicating an early stage of apoptosis. Phosphatidylserine normally locates on the intracellular side of cell membrane in healthy cells. During the early stages of apoptosis, phosphatidylserine is known to flip over to the extracellular side of cell membranes. Thus exposed, it can be detected and visualized by annexin V-FITC (Martin *et al.*, 1995). The exposure of phosphatidylserine is regarded as a sign of early stage apoptosis (Homburg *et al.*, 1995; Rimon *et al.*, 1997). Induction of apoptosis by adenosine

Fig. 1. Induction of BHK cell apoptosis by adenosine. (A) Photomicrograph showing the exposure of phosphatidylserine in adenosine-treated BHK cells. BHK cells were incubated in α -MEM with 1 mM adenosine for 2 hours, double-stained with annexin V-FITC and PI, examined by laser scanning microscope LSM 510 (Carl Zeiss). Membrane phosphatidylserine was visualized by FITC, however no condensed or fragmented chromatin was detected, indicating an early stage of apoptosis. (B) Electrophoretic analysis of internucleosomal DNA fragmentation in adenosine-induced BHK cells. DNA was isolated from non-treated, adenosine-treated or camptothecin-treated BHK cells. M, 100-bp DNA ladder; lane 1-4, detached adenosine-treated BHK cells (lane 1, 10 μ M Ado; lane 2, 20 μ M Ado; lane 3, 500 μ M Ado; lane 4, 1000 μ M Ado); lane 5, adherent adenosine-treated BHK cells (1000 μ M); lane 6, BHK cells without treatment, lane 7, camptothecin-treated (10 μ M) BHK cells. (C) Photomicrograph showing the nuclear morphology of adenosine-treated BHK cells. BHK cells were incubated 1 mM adenosine for 24 h, double-stained with annexin V-FITC and PI. Both morphology and condensed or fragmented chromatin suggest a late stage of apoptosis.

was confirmed by the presence of internucleosomal DNA fragmentation of adenosine-treated BHK cells into multimers of 180 bp nucleosomal units (Fig. 1B). In adherent adenosine-treated BHK cells and control cells, no DNA fragmentation was detected (lane 5, 6). A 24-hour exposure of BHK cells to 10, 20, 500 or 1000 mM adenosine resulted in DNA fragmentation typical of apoptosis (lane 1, 2, 3 and 4 respectively). Together with the morphological changes observed in BHK cells after a 24-hour exposure to 1 mM adenosine (Fig. 1C), nucleus fragmentation with condensed chromatin detected by PI (Fig. 1C) suggests a late stage of apoptosis.

Dose-dependent BHK cell apoptosis induced by adenosine AIA in BHK cells, measured by detection of DNA fragmentation using flow cytometry, was shown to be strongly dependent on the extracellular adenosine concentration (Fig. 2). It should be noted that in the control cells without added adenosine, apoptosis remained at a low level ($\sim$ 5%). Treatment with low concentrations of adenosine (10–20 μ M) decreased cell viability significantly to 60–70%. Cell viability recovered with increasing concentrations of adenosine (50–200 μ M) while higher concentrations (500–1000 μ M) resulted in reduced viability (Fig. 2). These results show a biphasic apoptotic effect of adenosine in BHK cells.

Involvement of adenosine receptors in adenosine-induced apoptosis in BHK cells AIA in BHK cells might occur in one of the following ways: (a) binding to one or more of its receptors, (b) intracellularly after adenosine uptake by

Fig. 2. Biphasic apoptotic effect of adenosine in BHK cells. BHK cells were treated with adenosine for 24 h. Apoptotic cells (late stage) were counted by flow cytometer. Cell viability was calculated as the percentage of non-apoptotic cells. Data were obtained from triplicates, 10,000 events were counted for each sample.

nucleoside transporters or (c) a combination of both. To determine the possible adenosine receptor subtypes (if any) involved in AIA in BHK cells, all the four subtypes of adenosine receptors were investigated using selective receptor antagonists. The subtype specific adenosine receptor antagonists we used in this study were: DPCPX, an A_1 receptor antagonist; DMPX, a non-specific A_2 receptor antagonist and MRS 1220, an A_3 receptor antagonist. As shown in Fig. 3A, DPCPX (7.5 and 30 μ M) successfully blocked two thirds of the apoptosis induced by 20, 500 and

Fig. 3. (A) Effect of DPCPX on apoptosis induced by adenosine in BHK cells. BHK cells were treated with 20, 50, 500 and 1000 μM adenosine for 24 h, in the absence or presence of 1, 7.5 or 30 μM DPCPX. Apoptotic cells (late stage) were counted by flow cytometer. Cell viability was calculated as the percentage of non-apoptotic cells. Data were obtained from triplicates, 10,000 events were counted for each sample. (B) Effect of MRS-1220 on apoptosis induced by adenosine in BHK cells. BHK cells were treated with 20, 50, 500 and 1000 μM adenosine for 24 h, in the absence or presence of 1, 5 or 20 nM MRS-1220. Apoptotic cells (late stage) were counted by flow cytometer. Cell viability was calculated as the percentage of non-apoptotic cells. Data were obtained from triplicates, 10,000 events were counted for each sample.

1000 μM of adenosine. In contrast, the blocking of A_2 receptors by DMPX had no significant effect on AIA (data not shown). Fig. 3B shows that the A_3 receptor specific antagonist, MRS-1220 (5 and 20 nM) could block one half to two thirds of the apoptosis induced by both low and high concentrations of adenosine. These results suggest that AIA in BHK cells may involve mediation via A_1 and A_3 adenosine receptors, unlike the report by Merighi *et al.* (2002) who observed that A_{2A} and A_3 adenosine receptors appeared to be involved in the regulator of cell proliferation and survival in A375 cells. The expression of all four adenosine receptor subtypes (A_1 , A_{2A} , A_{2B} , A_3) in BHK cells was confirmed by immunocytochemistry (data not shown).

Fig. 4. (A) Effect of DIP on apoptosis induced by adenosine in BHK cells. BHK cells were treated with 20, 50, 500 and 1000 μM adenosine for 24 h, in the absence or presence of 1, 5 or 20 μM DIP. Apoptotic cells (late stage) were counted by flow cytometer. Cell viability was calculated as the percentage of non-apoptotic cells. Data were obtained from triplicates, 10,000 events were counted for each sample. (B) Effect of NBTI on apoptosis induced by adenosine in BHK cells. BHK cells were treated with 20, 50, 500 and 1000 μM adenosine for 24 h, in the absence or presence of 1, or 20 μM NBTI. Apoptotic cells (late stage) were counted by flow cytometer. Cell viability was calculated as the percentage of non-apoptotic cells. Data were obtained from triplicates, 10,000 events were counted for each sample.

Involvement of nucleoside transporters in adenosine-induced apoptosis in BHK cells In addition to the receptor-mediated mechanism, nucleoside transporter-mediated pathways were also investigated in this study. Two transporter inhibitors were employed to identify the involvement of *es* and/or *ei* type equilibrative nucleoside transporters. Dipyridamole is an inhibitor which can block both *es* and *ei* type adenosine transport, whereas NBTI is only effective against *es* type. Fig. 4A shows that dipyridamole could effectively protect BHK cells from apoptosis induced by high concentrations (500, 1000 μM) of adenosine but not by low concentrations (20, 50 μM). This suggests that in addition to the receptor-mediated pathway, nucleoside transporter-mediated pathway might also

take part in AIA in BHK cells at high adenosine concentrations (>500 μ M). NBTI could not block this nucleoside transporter-mediated pathway as significantly as dipyridamole (Fig. 4B), implying the involvement of *ei* type nucleoside transporter. All these results suggest that the mechanism of AIA at low concentrations of adenosine may be A_1 and A_3 receptor-mediated; while the mechanism of AIA at high concentrations of adenosine may be co-mediated by *ei* type nucleoside transporter and A_1 and A_3 receptors. This hypothesis was further confirmed by the use of propentophylline which is both an adenosine transport inhibitor and a non-selective adenosine receptor antagonist (Parkinson *et al.*, 1991; Parkinson *et al.*, 1993) (data not shown). In our study only the involvement of equilibrative nucleoside transporters in AIA in BHK cells was shown, that of concentrative nucleoside transporters *cit*, *cif* and *cib* is still uncertain (subtype *cs* and *csg* can be excluded as they are sensitive to NBTI).

Adenosine signaling mediated by receptors and uptake by nucleoside transporters has been extensively studied. The mechanisms of adenosine's physiological functions via receptors and nucleoside transporters are well documented. However, given the universal expression of adenosine receptors and nucleoside transporters in almost all mammalian cells, more complicated mechanisms can not be excluded. Our study provides a good reason to take into consideration the possibility of adenosine receptor-transporter co-signaling in the physiological micro-environment.

Membrane receptors are one of the most important functional cell membrane protein families. Currently cell surface receptors are classified into four classes: G protein-coupled receptors (GPCRs), ion-channel receptors, tyrosine kinase-linked receptors and receptor tyrosine kinases (RTKs) (Lodish *et al.*, 2000). The latter two types of receptors have been shown to play an important role in apoptosis. However, even though the involvement of GPCRs in apoptosis has been implied by many scientists, it is still to be confirmed and a model of GPCR-mediated apoptosis is yet to be established. Our study as well suggests a high possibility for the existence of GPCR-mediated apoptotic pathway(s).

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