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A_{2a} Adenosine Receptor Mediates HepG2 Cell Apoptosis by Downregulating Bcl-X_L Expression and Upregulating Bid Expression

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ABSTRACT

Extracellular adenosine induced apoptosis in HepG2 cells, a human hepatoma cell line, by tuning apoptosis-mediator gene transcription. The present study aimed at identifying the responsible adenosine receptor and clarifying the signaling pathway underlying adenosine-induced HepG2 cell apoptosis. Adenosine and CGS21680, an A_{2a} adenosine receptor agonist, induced HepG2 cell apoptosis, and the effect was inhibited by DMPX, an A_{2a} adenosine receptor antagonist, or by knocking-down A_{2a} adenosine receptors. Adenosine reduced expression of Bcl-X_L mRNA and protein but otherwise increased expression of the Bid mRNA and protein in HepG2 cells, and those effects were also prevented by knocking-down A_{2a} adenosine receptors. Adenosine caused disruption of mitochondrial membrane potentials and stimulated cytochrome c efflux from the mitochondria in HepG2 cells. Adenosine activated caspases-3 and -9 in HepG2 cells, which was significantly inhibited by knocking-down A_{2a} adenosine receptors. The results of the present study indicate that extracellular adenosine downregulates Bcl-X_L expression and upregulates Bid expression, thereby disrupting mitochondrial membrane potentials to allow cytochrome c efflux from the mitochondria, and then causing activation of caspase-9 and the effector caspase-3, as mediated via A_{2a} adenosine receptors. J. Cell. Biochem. 113: 1766–1775, 2012.

KEY WORDS: A_{2a} ADENOSINE RECEPTOR; Bcl-X_L; BID; HepG2 CELL; APOPTOSIS

Apoptosis is induced through a wide-range of pathways. Accumulating evidence has shown that extracellular adenosine induces apoptosis in a variety of cancer cells via diverse signaling pathways. For adenosine-induced intrinsic apoptotic pathway, intracellularly transported adenosine through adenosine transporters is converted to AMP by adenosine kinase, thereby activating AMP-activated protein kinase (AMPK) to induce apoptosis in GT3-TKB human lung cancer cells and HuH-7 human hepatoma cells [Saitoh et al., 2004; Yang et al., 2011b]. Intracellularly transported adenosine followed by conversion to AMP, alternatively, produces a signal to downregulate expression of c-Fas-associated death domain protein (FADD)-like interleukin-1β-

Abbreviations used: AMPK, AMP-activated protein kinase; FADD, c-Fas-associated death domain protein; FLIP, FADD-like interleukin-1β-converting enzyme inhibitory protein; IAP, inhibitor of apoptosis protein; AMID, apoptosis-inducing factor-homologous mitochondrion-associated inducer of death; TNF, tumor necrosis factor; TRADD, TNF receptor 1-associated death domain protein; TRAIL-R2, TNF-related apoptosis inducing ligand receptor 2; RIPK1, receptor-interacting protein kinase 1; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; FITC, fluorescein isothiocyanate; A_{2a}R siRNA, siRNA to silence A_{2a} adenosine receptor-targeted gene; NC siRNA, negative control siRNA; PKA, protein kinase A; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; SDS, sodium dodecyl sulfate; SDS–PAGE, SDS–polyacrylamide gel electrophoresis; RT-PCR, reverse transcription-polymerase chain reaction.

Kunihiro Tamura and Takeshi Kanno contributed equally to this work.

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converting enzyme inhibitory protein (c-FLIP) to neutralize caspase-8 inhibition due to c-FLIP, resulting in the activation of caspase-8 and the effector caspase-3, responsible for HuH-7 cell apoptosis [Yang et al., 2007]. Intracellularly transported adenosine could also activate caspase-3 in HuH-7 cells by neutralizing caspase-3 inhibition due to inhibitor of apoptosis protein (IAP) as a result of decreased IAP2 expression and reduced IAP activity in response to increased DIABLO expression and DIABLO release from damaged mitochondria, regardless of caspase-9 activation [Yang et al., 2010b]. Moreover, adenosine induces HuH-7 cell apoptosis in a caspase-independent manner: adenosine upregulates expression of apoptosis-inducing factor-homologous mitochondrion-associated inducer of death (AMID) to cause DNA fragmentation [Yang et al., 2011a].

For adenosine-induced extrinsic apoptotic pathway, adenosine receptors, such as A_1, A_{2a}, A_{2b}, and A_3 receptors mediate apoptosis. Adenosine induces apoptosis in CW2 human colonic cancer cells by activating caspases-3, -8, and -9 via adenosine A_1 receptors linked to G_i protein [Saito et al., 2010]. Adenosine, on the other hand, induces apoptosis in RCR-1 astrocytoma cells both via an intrinsic pathway relevant to AMPK-dependent caspase-3/-9 activation and an adenosine A_1 receptor-mediated extrinsic pathway [Sai et al., 2006]. Adenosine A_2 receptors linked to G_s protein mediates apoptosis in glioma cells, myeloid leukemia cells, and mammary carcinoma cells [Pratt and Martin, 1975; Vintermyr et al., 1993; Boe et al., 1995]. Adenosine induces apoptosis in Caco-2 human colonic cancer cells by activating caspase-9/-3 via A_{2a} adenosine receptors [Yasuda et al., 2009], but conversely, A_{2a} adenosine receptors exert their anti-apoptotic action on $CD4^+$ T lymphocytes, injured lung cells, PC12 cells, and neutrophils [Walker et al., 1997; Huang et al., 2001; Rivo et al., 2007; Himer et al., 2010]. Activation of A_3 adenosine receptors linked to G_i or G_q protein induces apoptosis in human lung cancer cells, breast cancer cells, hepatocellular carcinoma cells, and thyroid cancer cells [Panjehpour and Karami-Tehrani, 2007; Bar-Yehuda et al., 2008; Kim et al., 2008; Morello et al., 2009].

In our earlier study, extracellular adenosine induced apoptosis in HepG2 cells, a human hepatoma cell line and upregulated expression of mRNAs for tumor necrosis factor (TNF), TNF receptor 1-associated death domain protein (TRADD), TNF-related apoptosis inducing ligand receptor 2 (TRAIL-R2), receptor-interacting protein kinase 1 (RIPK1), and FADD [Yang et al., 2010a]. This indicates that adenosine induces HepG2 cell apoptosis by activating caspase-8 through a TNFR1/TRADD/RIP1/FADD pathway and the effector caspases-3. A study shows that adenosine induces HepG2 cell apoptosis by activating caspase-3, but not caspases-8 and -9, without perturbing mitochondrial membrane potentials [Wu et al., 2006]. The adenosine effect is inhibited by the adenosine transporter inhibitor dipyridamole, while it is not affected by inhibitors of adenosine receptors [Wu et al., 2006]. This suggests that adenosine induces HepG2 cell apoptosis through a mitochondrial-independent intrinsic pathway rather than an adenosine receptor-mediated extrinsic pathway. Little is known about the pathway for adenosine-induced HepG2 cell apoptosis via an adenosine receptor.

The present study focused upon this pathway and investigated the relevant signaling pathway. We show here that extracellular adenosine downregulated Bcl-X_L expression but otherwise upregulated Bid expression via A_{2a} adenosine receptors, thereby disrupting mitochondrial membrane potentials to allow cytochrome c release from the mitochondria into the cytosol, responsible for caspase-9 activation.

MATERIALS AND METHODS

CELL CULTURE

HepG2 cells, obtained from RIKEN cell bank (Ibaraki, Japan), were cultured in Dulbecco's modified Eagles medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin (final concentration, 100 U/ml), and streptomycin (final concentration, 0.1 mg/ml), in a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

ASSAY OF CELL VIABILITY

Cell viability was assayed by the method using 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) as described previously [Yang et al., 2010a].

TUNEL STAINING

TUNEL staining was performed to detect in situ DNA fragmentation as a marker of apoptosis using an In Situ Apoptosis Detection Kit (Takara Bio, Otsu, Japan). Briefly, fixed and permeabilized HepG2 cells were reacted with terminal deoxynucleotidyl transferase and fluorescein isothiocyanate (FITC)-deoxyuridine triphosphate for 90 min at 37°C. FITC signals were visualized with a confocal scanning laser microscope (LSM 510; Carl Zeiss Co., Ltd., Oberkochen, Germany).

CONSTRUCTION AND TRANSFECTION OF siRNA

The siRNA to silence A_{2a} adenosine receptor-targeted gene (A_{2a}R siRNA) and the negative control siRNA (NC siRNA) were obtained from Ambion (Austin, TX). The sequences of siRNA used to silence the human A_{2a} receptor gene were 5′-GACGGGAACUCCACGAA-GATT-3′ and 5′-UCUUCGUGGAGUUCCCGUCTT-3′. The nonspecific siRNA with the scrambled sequence, the same GC content and nucleic acid composition was used as a negative control (NC siRNA).

The A_{2a}R siRNA and the NC siRNA were reverse-transfected into HepG2 cells using a Lipofectamine reagent (Invitrogen, Carlsbad, CA). Cells were used for experiments 48 h after transfection.

IN SITU PKA ASSAY

PKA activity in HepG2 cells was assayed by the method described previously [Fujikawa et al., 2008]. HepG2 cells were treated with adenosine (3 mM) in the presence and absence of H-89 (1 μM) at 37°C for 5 min in an extracellular solution (137 mM NaCl, 5.4 mM KCl, 10 mM $MgCl_2$, 5 mM EGTA, 0.3 mM Na_2HPO_4, 0.4 mM K_2HPO_4, and 20 mM HEPES, pH 7.2). Then, cells were incubated in the extracellular solution containing 100 μg/ml digitonin, 50 mM glycerol 2-phosphate, 400 μM ATP, and 200 μM Kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly), a synthetic substrate peptide for PKA (Calbiochem, San Diego, CA) at 30°C for 5 min. The supernatants were collected and boiled at 100°C for 5 min to terminate the reaction. An aliquot of the solution (20 μl) was loaded onto a

reverse-phase HPLC system (LC-10ATvp; Shimadzu Co., Kyoto, Japan). A substrate peptide peak and a new product peak were detected at an absorbance of 214 nm (SPD-10Avp UV–VIS detector; Shimadzu). It was confirmed that each peak corresponds to nonphosphorylated and phosphorylated substrate peptide in an analysis by MALDI-TOF MS (Voyager DE-STR; PE Biosystems, Inc., Foster City, CA). Molecular weights were calibrated from the two standard spectrums, bradykinin and neurotensin. Areas for nonphosphorylated and phosphorylated PKA substrate peptide were measured (total area corresponds to the concentration of PKA substrate peptide used here), and the amount of phosphorylated substrate peptide was calculated. Phosphorylated substrate peptide (pmol/min/cell protein weight) was used as an index of PKA activity.

WESTERN BLOTTING

HepG2 cells were lysed in an ice-cold mitochondrial buffer (210 mM mannitol, 70 mM sucrose, 1 mM EDTA, and 10 mM HEPES, pH 7.5) containing 1% (v/v) protease inhibitor cocktail, and then centrifuged at 3,000 rpm for 5 min at 4°C. The supernatant was further centrifuged at 11,000 rpm for 15 min at 4°C, and the pellet and supernatant were used as mitochondria- and cytosol-enriched components. To confirm that each component is successfully separated, Western blotting was carried out using an anti-prohibitin antibody (Abcam, Cambridge, UK), a mitochondrial marker.

For Western blotting, samples were lysed with 1% (w/v) sodium dodecyl sulfate (SDS). Proteins were separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) using a TGX gel (BioRad, Hercules, CA) and then transferred to polyvinylidene difluoride membranes. Blotting membranes were blocked with TBS-T (150 mM NaCl, 0.1% Tween 20, and 20 mM Tris, pH 7.5) containing 5% (w/v) bovine serum albumin and subsequently incubated with an anti-Bid antibody (Abcam, Cambridge, MA), an anti-Bad antibody (IMGENEX, San Diego, CA), an anti-Bax antibody (Millipore, Temecula, CA), an anti-Bcl-2 antibody (Pierce, Rockford, IL), an anti-Bcl-X_L antibody (Invitrogen), an anti-A_1 receptor antibody (Oncogene, Cambridge, MA), an anti-A_{2a} receptor antibody (Oncogene), an anti-A_{2b} receptor antibody (Santa Cruz Biotechnology, Santa Cruz, CA), an anti-A_3 receptor antibody (Santa Cruz), an anti-cytochrome c antibody (Chemicon, Temecula, CA), or an anti-β-actin antibody (Sigma, St. Louis, MO). After washing, membranes were reacted with a horseradish peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG antibody. Immunoreactivity was detected with an ECL kit (GE Healthcare, Piscataway, NJ) and visualized using a chemiluminescence detection system (GE Healthcare). Protein concentrations for each sample were determined with a BCA protein assay kit (Pierce).

REAL-TIME RT-PCR

Total RNAs of HepG2 cells treated with adenosine (3 mM) were purified by an acid/guanidine/thiocyanate/chloroform extraction method using the Sepasol-RNA I Super kit (Nacalai, Kyoto, Japan). After purification, total RNAs were treated with RNase-free DNase I (2 U) at 37°C for 30 min to remove genomic DNAs, and 10 μg of RNAs was resuspended in water. Then, random primers, dNTP, 10× RT buffer, and Multiscribe Reverse Transcriptase were added to an RNA solution and incubated at 25°C for 10 min followed by 37°C for 120 min to synthesize the first-strand cDNA. Real-time RT-PCR was performed using a SYBR Green Realtime PCR Master Mix (Takara Bio) and the Applied Biosystems 7900 real-time PCR detection system (ABI, Foster City, CA). Thermal cycling conditions were as follows: first step, 94°C for 4 min; the ensuing 40 cycles, 94°C for 1 s, 65°C for 15 s, and 72°C for 30 s. The expression level of each mRNA was normalized by that of GAPDH mRNA. Primers used for real-time RT-PCR are shown in Table I.

ASSAY OF MITOCHONDRIAL MEMBRANE POTENTIALS

Mitochondrial membrane potentials were measured using a DePsipher^{TM} kit. HepG2 cells were untreated and treated with adenosine (3 mM) for 24 h, and cells were incubated in a DePsipher^{TM} solution at 37°C for 20 min. Then, cells were washed with 1 ml of a reaction buffer containing a stabilizer solution. The fluorescent signals were observed with a laser scanning microscopes (LSM 510) equipped with an epifluorescence device using a fluorescein long-pass filter (fluorescein and rhodamine) at an absorbance of 590 nm for red aggregations and at an absorbance of 530 nm for green aggregations.

ENZYMATIC ASSAY OF CASPASES-3, -8, AND -9

Caspase activity was measured using a caspase fluorometric assay kit (Ac-Asp-Glu-Val-Asp-MCA for a caspase-3 substrate peptide; Ac-Ile-Glu-Thr-Asp-MCA for a caspase-8 substrate peptide; and Ac-Leu-Glu-His-Asp-MCA for a caspase-9 substrate peptide) as previously described [Yasuda et al., 2009]. Briefly, HepG2 cells were harvested before and after treatment with adenosine (3 mM), and then centrifuged at 3,000 rpm for 5 min at 4°C. The pellet was incubated on ice in cell lysis buffer for 10 min, and reacted with the fluorescently labeled tetrapeptide at 37°C for 2 h. The fluorescence was measured at an excitation of wavelength of 400 nm and an emission wavelength of 505 nm with a fluorometer (Fluorescence Spectrometer, F-4500, Hitachi, Japan).

STATISTICAL ANALYSIS

Statistical analysis was carried out using unpaired *t*-test and Dunnett's test.

TABLE I. Primers Used for Real-Time RT-PCR

PCR primers	Oligonucleotide sequence
Bcl-2	Sense: 5′-GAACTGGGGGAGGATTGTGGCC-3′
	Anti-sense: 5′-TCGACGTTTTGCCTGAAGACTGTTAA-3′
Bcl-X_L	Sense: 5′-TGGAATTCATGTCTCAGAGCAACCGGGAGC-3′
	Anti-sense: 5′-CAGAATTCTCATTTCCGACTGAAGAGTGAGC-3′
Bad	Sense: 5′-CTGGGGCTGTGGAGATCCGGAGTCGCC-3′
	Anti-sense: 5′-TCACTGGGAGGGGGCGGAGCTTCCCC-3′
Bax	Sense: 5′-CGGACCCGGCGAGAGGC-3′
	Anti-sense: 5′-TCAGCTTCTTGGTGGACGCATCC-3′
Bid	Sense: 5′-TAAAAGATGAAAGAGCAGTTGGACTTTTTAAAA-3′
	Anti-sense: 5′-TGAACAATATAGAGTTTGTTTTTCCTTTCTGATGA-3′
GAPDH	Sense: 5′-GACTTCAACAGCGACACCCACTCC-3′
	Anti-sense: 5′-AGGTCCACCACCCTGTTGCTGTAG-3′

RESULTS

EXTRACELLULAR ADENOSINE INDUCES HepG2 CELL APOPTOSIS VIA A_{2A} ADENOSINE RECEPTORS

We have earlier found that extracellular adenosine reduced HepG2 cell viability in a concentration (0.1–20 mM)- and treatment time (24–72 h)-dependent manner, the extent reaching the peak at 3 mM for each treatment time [Yang et al., 2010a]. The present study was conducted to understand the pathway underlying adenosine-induced HepG2 cell apoptosis, and therefore, 3 mM of adenosine was chosen and used throughout experiments. Treatment with extracellular adenosine (3 mM) for 24 h reduced HepG2 cell viability to approximately 40% of untreated control cells (Fig. 1A). The adenosine effect was partially inhibited by 8-cyclopentyltheophylline (8-CPT, 10 μM), an antagonist of adenosine A_1 receptor, 3,7-dimethyl-1-propargylxanthine (DMPX, 10 μM), an antagonist of A_{2a} adenosine receptor, MRS1706 (50 nM), an antagonist of adenosine A_{2b} receptor, MRS1191 (10 μM), an antagonist of adenosine A_3 receptor, with the most potent inhibition for DMPX (Fig. 1A). Treatment with adenosine (3 mM) for 48 h reduced HepG2 cell viability to nearly 20% of untreated control cells (Fig. 1B). Notably, the cell death was significantly attenuated only by DMPX, although 8-CPT, MRS1706, or MRS1191 exhibited no effect (Fig. 1B), suggesting more prominent role played by the A_{2a} adenosine receptors in adenosine-induced HepG2 cell death.

To see whether adenosine-induced reduction in HepG2 cell viability is due to apoptosis, we carried out TUNEL staining. For cells untreated with adenosine, TUNEL-positive cells were within 5% of total cells (Fig. 2A,B). In contrast, approximately 70% was positive to TUNEL for cells treated with adenosine (3 mM) for 24 h, and the adenosine effect significantly inhibited by DMPX (10 μM; Fig. 2A,B). This indicates that adenosine induces HepG2 cell apoptosis via A_{2a} adenosine receptors.

Like adenosine, the A_{2a} adenosine receptor agonist, 2-*p*-(2-carboxyethyl)phenethylamino-5′-*N*-ethylcarboxamidoadenosine hydrochloride (CGS21680), reduced HepG2 cell viability in a treatment time (24–48 h)- and concentration (0.3–3 μM)-dependent manner (Fig. 3), supporting the notion that adenosine-induced

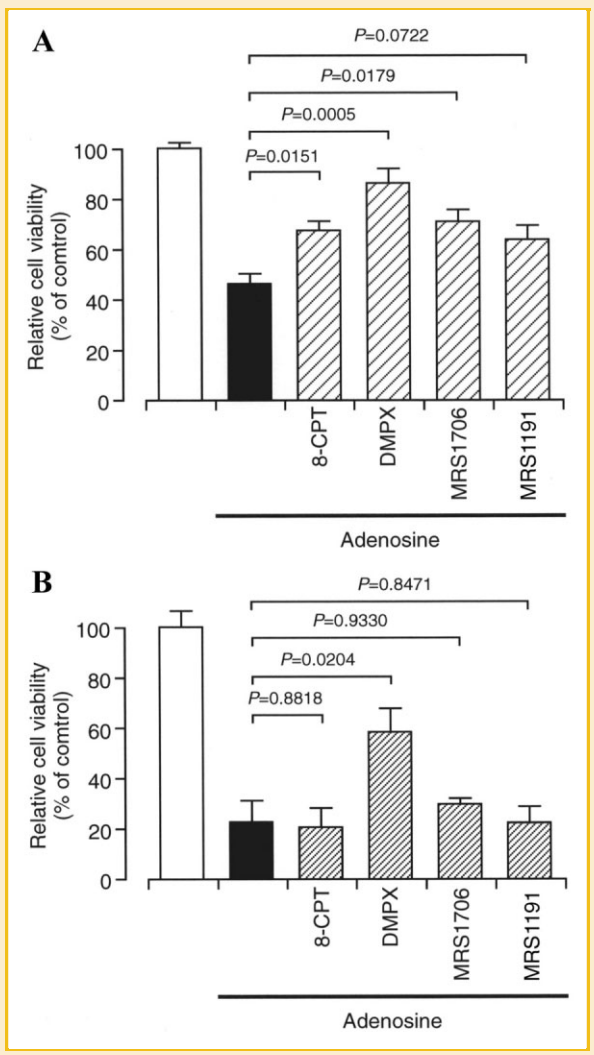

Fig. 1. Effect of adenosine receptor antagonists on adenosine-induced HepG2 cell death. Cells were treated with adenosine (3 mM) in the presence and absence of 8-CPT (10 μM), DMPX (10 μM), MRS1706 (50 nM), or MRS1191 (10 μM) for 24 h (A) and 48 h (B), and then MTT assay was carried out. In the graphs, each column represents the mean (±SEM) percentage of basal cell viabilities (MTT intensities before adenosine treatment in the absence of antagonists, n = 8 independent experiments). *P*-values, Dunnett's test.

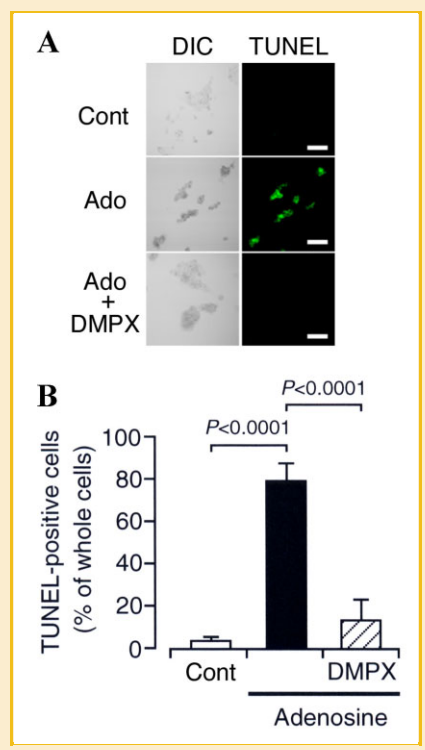

Fig. 2. Adenosine-induced HepG2 cell apoptosis. TUNEL staining was carried out in cells untreated (Cont) and treated with adenosine (Ado, 3 mM) in the presence and absence of DMPX (10 μM). A: DIC, differential interference contrast. Bars, 100 μm. B: TUNEL-positive cells were counted in the area (0.4 mm × 0.4 mm) selected at random. In the graph, each column represents the mean (±SEM) TUNEL-positive cell percentage of whole cells (n = 4 independent experiments). *P*-values, Dunnett's test.

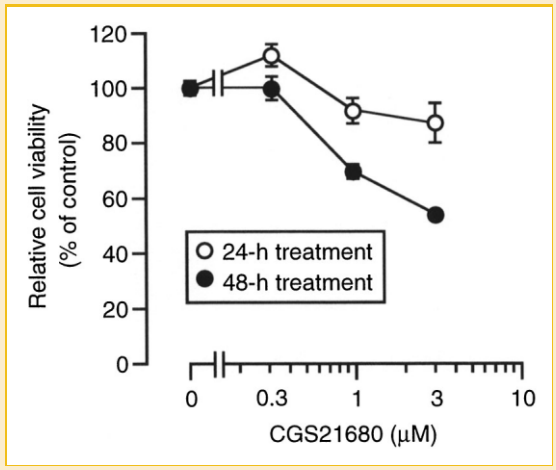

Fig. 3. CGS21680-induced HepG2 cell death. Cells were treated with CGS21680 at concentrations as indicated for 24–48 h followed by MTT assay. In the graphs, each point represents the mean (±SEM) percentage of basal cell viabilities (MTT intensities before CGS21680 treatment, n = 4 independent experiments).

HepG2 cell apoptosis occurs via A_{2a} adenosine receptors.

To obtain further evidence for this, the $A_{2a}R$ siRNA was constructed. For cells transfected with the $A_{2a}R$ siRNA, expression of A_{2a} adenosine receptor protein was clearly suppressed as compared with the expression for cells transfected with the NC siRNA, while expression of adenosine A_1, A_{2b}, and A_3 receptors was not affected (Fig. 4A). This confirms A_{2a} adenosine receptor knock-down for cells transfected with the $A_{2a}R$ siRNA. A_{2a} adenosine receptor is linked to G_s protein involving activation of adenylate cyclase to produce cAMP from ATP, causing PKA activation. In the in situ PKA assay, adenosine (3 mM) activated PKA in HepG2 cells, and the PKA activation was prevented by DMPX (10 μM) or H-89 (1 μM), an inhibitor of PKA (Fig. 4B). In addition, adenosine (3 mM)-induced PKA activation was not obtained with cells transfected with the $A_{2a}R$ siRNA (Fig. 4B). This implies shutdown of A_{2a} adenosine receptor signaling for cells transfected with the $A_{2a}R$ siRNA. HepG2 cell death induced by 24-h treatment with adenosine (3 mM) was significantly attenuated by knocking-down A_{2a} adenosine receptors (Fig. 5). A_{2a} adenosine receptor, thus, appears to contribute to adenosine-induced HepG2 cell apoptosis.

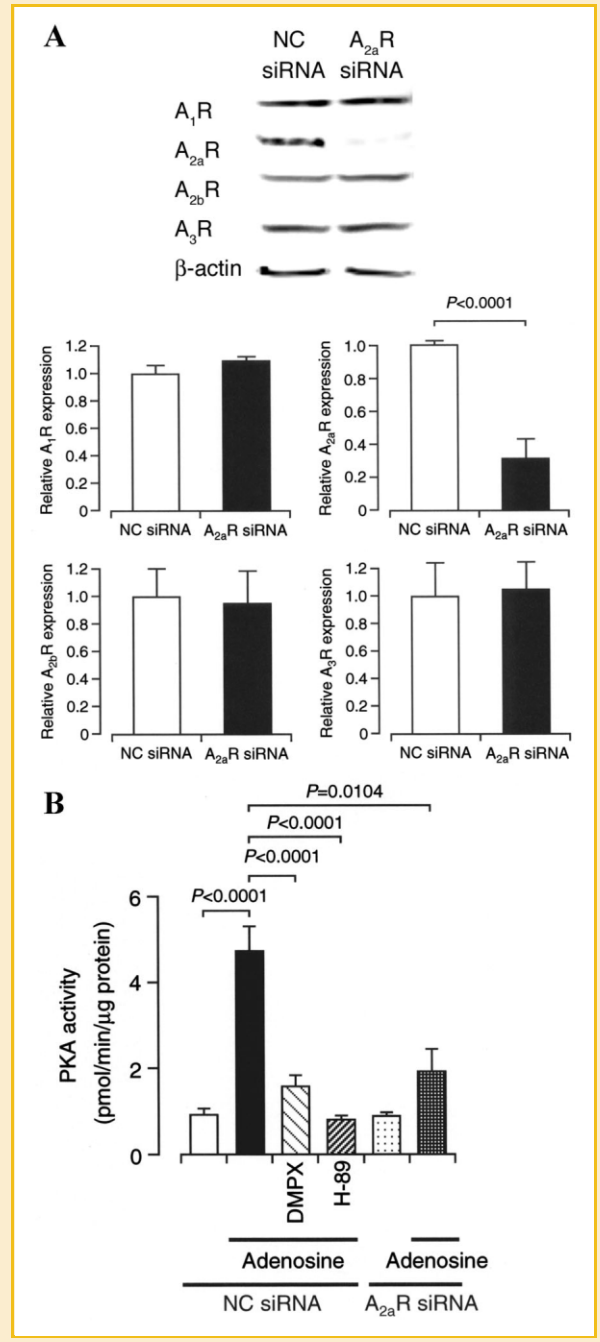

Fig. 4. Knocking-down of A_{2a} adenosine receptors. HepG2 cells were transfected with the NC siRNA or the $A_{2a}R$ siRNA. A: Western blotting using antibodies against A_1, A_{2a}, A_{2b}, and A_3 adenosine receptors was carried out 48 h after transfection. In the graphs, each column represents the mean (±SEM) intensity of each adenosine receptor protein normalized by β-actin intensity (n = 4 independent experiments). *P*-value, unpaired *t*-test. B: In situ PKA assay was carried out 48 h after transfection in the presence and absence of DMPX (10 μM) or H-89 (1 μM). In the graph, each column represents the mean (±SEM) PKA activity (pmol/min/μg protein, n = 6 independent experiments). *P*-values, Dunnett's test.

EXTRACELLULAR ADENOSINE DOWNREGULATES THE Bcl-X_L mRNA AND PROTEIN BUT UPREGULATES THE BID mRNA AND PROTEIN VIA A_{2A} ADENOSINE RECEPTORS

We next probed expression of mRNAs and proteins for the Bcl-2 family, such as Bcl-2, Bcl-X_L, Bad, Bax, and Bid in HepG2 cells. In the real-time RT-PCR analysis, adenosine (3 mM) significantly decreased expression of the Bcl-X_L mRNA at 12-h treatment followed by a little increase at 24-h treatment (Fig. 6A). A significant increase in the expression of the Bid mRNA in HepG2 cells was found at 6-h treatment with adenosine (3 mM), being evident 24-h treatment (Fig. 6A). Up- and down-expression of the Bad mRNA was found with adenosine treatment for 6–24 h, without significant

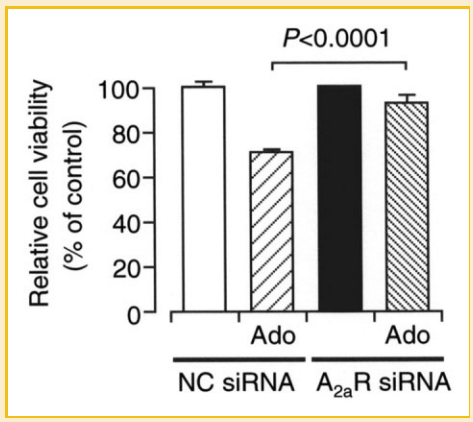

Fig. 5. Effect of A_{2a} adenosine receptor knocking-down on adenosine-induced HepG2 cell death. Cells transfected with the NC siRNA or the A_{2a}R siRNA were untreated and treated with adenosine (Ado, 3 mM), and then cell viability was assayed. Each column represents the mean (±SEM) percentage of basal cell viabilities (MTT intensities before adenosine treatment, n = 8 independent experiments). *P*-values, Dunnett's test.

difference (Fig. 6A). Adenosine (3 mM) not significantly decreased expression of the Bax mRNA to a similar level throughout 6- and 24-h treatment (Fig. 6A).

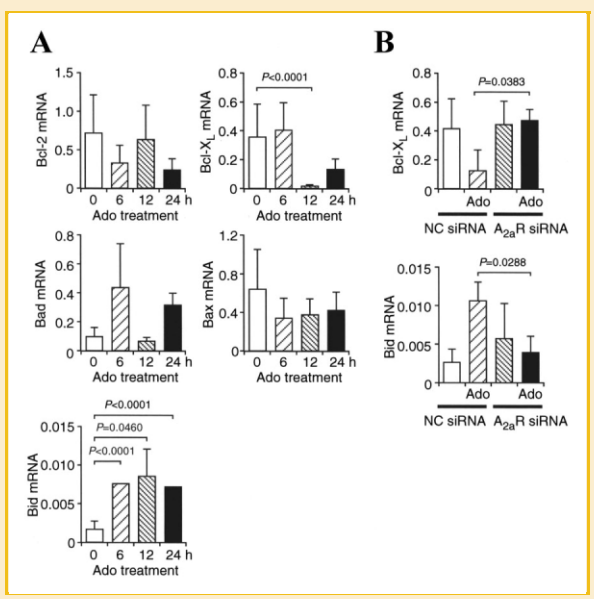

Fig. 6. Effects of adenosine on expression of the mRNAs for Bcl-2, Bcl-X_L, Bad, Bax, and Bid in HepG2 cells. A: Real-time RT-PCR was carried out in cells treated with adenosine (Ado, 3 mM) for periods of time as indicated. In the graphs, each column represents the mean (±SEM) expression for each mRNA normalized by the expression level of the GAPDH mRNA (n = 8 independent experiments). B: Real-time RT-PCR was carried out in cells transfected with the NC siRNA or the A_{2a}R siRNA before and after 24-h treatment with adenosine (Ado, 3 mM). In the graphs, each column represents the mean (±SEM) expression for the Bcl-X_L and Bid mRNAs normalized by the expression level of the GAPDH mRNA (n = 8 independent experiments). *P*-values, Dunnett's test.

In the Western blot analysis, adenosine (3 mM) significantly decreased expression of Bcl-X_L protein at 12- and 24-h treatment (Fig. 7A). Adenosine (3 mM), on the other hand, significantly increased expression of full-length Bid protein at periods of treatment time from 6 to 24 h, without affecting expression of truncated Bid (Fig. 7A). In contrast, adenosine had no effect on expression of Bcl-2, Bad, and Bax proteins (Fig. 7A). Adenosine-induced downregulation and upregulation of the mRNA and protein for Bcl-X_L and Bid, respectively, were neutralized by knocking-down A_{2a} adenosine receptors (Figs. 6B and 7B). Collectively, these results suggest that adenosine regulates transcription for Bcl-X_L and Bid via A_{2a} adenosine receptors.

EXTRACELLULAR ADENOSINE DISRUPT MITOCHONDRIAL MEMBRANE POTENTIALS AND STIMULATES CYTOCHROME C EFFLUX FROM THE MITOCHONDRIA

Bcl-X_L and Bcl-2 exert their protective action on the mitochondria, and therefore, reduced Bcl-X_L expression might cause disruption of mitochondrial membrane potentials. Increased Bid expression, alternatively, might be a factor for perturbation of mitochondrial membrane potentials [Eskes et al., 2000; Landshamer et al., 2008]. We, therefore, monitored mitochondrial membrane potentials in HepG2 cells using DePsipher™. DePsipher™, a mitochondrial activity marker, is detected as an orange-red fluorescence at an absorbance of 590 nm for normal mitochondrial membrane potentials, but in case of the disruption the dye turns into green fluorescence at an absorbance of 530 nm. For untreated cells, the mitochondria exhibited orange-red fluorescent signals alone without green fluorescent signals (Fig. 8). In contrast, 24-h treatment with adenosine (3 mM) accumulated green fluorescent signals with weaker orange-red fluorescent signal (Fig. 8). This suggests that adenosine disrupts mitochondrial membrane potentials.

To examine intracellular cytochrome c distribution, HepG2 cells were separated into the cytosolic and mitochondrial components. It was confirmed that the mitochondrial components are reactive to an anti-prohibitin antibody, a mitochondrial marker, but that no immunoreactive signal is obtained with the cytosolic components (Fig. 9A). Adenosine (3 mM) increased cytosolic cytochrome c along decreased mitochondrial cytochrome c in a treatment time (6–24 h)-dependent manner (Fig. 9B). Taken together, these results suggest that adenosine disrupts mitochondrial membrane potentials, allowing cytochrome c efflux from the mitochondria into the cytosol, possibly as a result from downregulation of Bcl-X_L and upregulation of Bid.

If adenosine disrupts mitochondrial membrane potentials and stimulates cytochrome c efflux, then adenosine should activate caspase-9 in HepG2 cells. To address this point, we monitored activity of caspases-3, -8, and -9. Expectedly, adenosine (3 mM) activated caspase-9, and the activation was clearly inhibited by knocking-down A_{2a} adenosine receptors (Fig. 10). Similarly, adenosine (3 mM) activated caspase-3, which was significantly attenuated by knocking-down A_{2a} adenosine receptors (Fig. 10). Adenosine (3 mM) also activated caspase-8, but the activation was not inhibited by knocking-down A_{2a} adenosine receptors (Fig. 10).

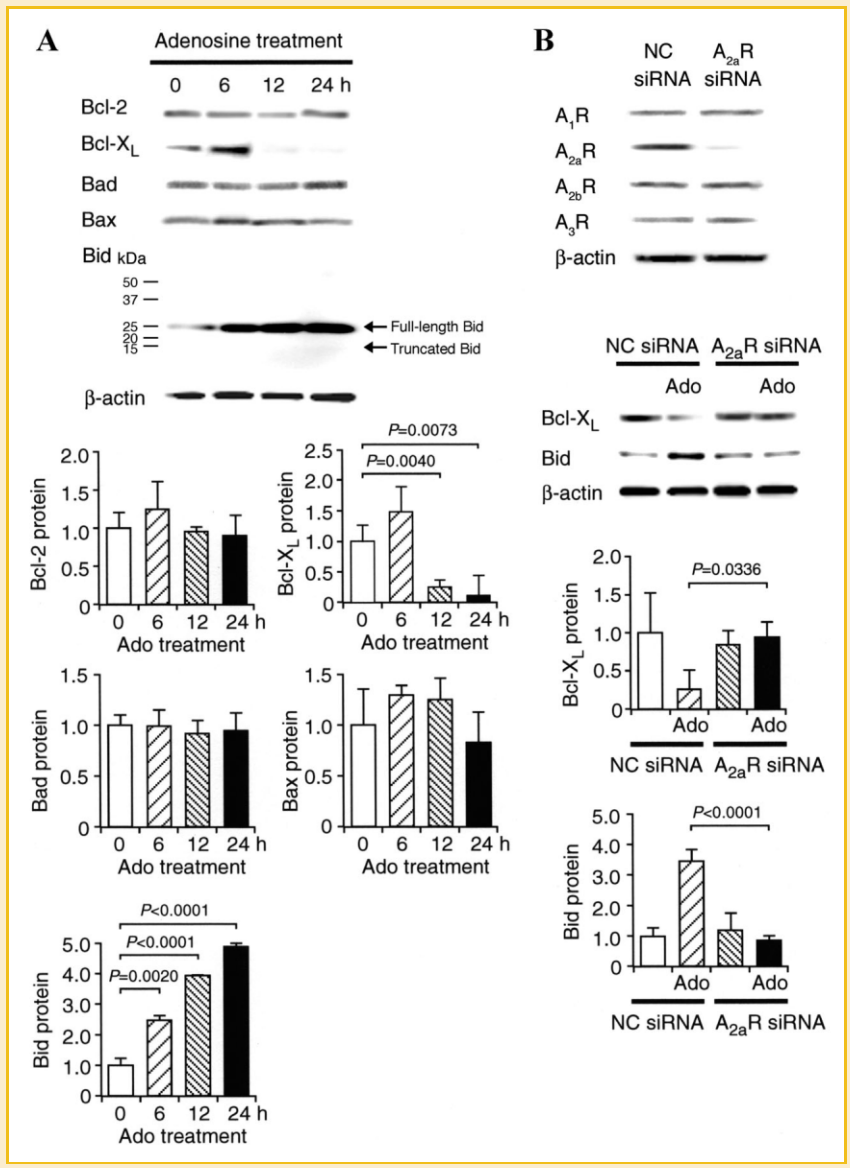

Fig. 7. Effects of adenosine on expression of proteins for Bcl-2, Bcl-X_L, Bad, Bax, and Bid in HepG2 cells. A: Western blotting was carried out in cells treated with adenosine (Ado, 3 mM) for periods of time as indicated using antibodies against Bcl-2, Bcl-X_L, Bad, Bax, and Bid. In the graphs, each column represents the mean (±SEM) ratio against basal expression levels (before treatment with adenosine) for each protein normalized by the expression level of β-actin (n = 4 independent experiments). Note that full-length Bid is found at 25 kDa but that truncated Bid at 15 kDa is not detectable. B: Western blotting was carried out in cells transfected with the NC siRNA or the A_{2a}R siRNA before and after 24-h treatment with adenosine (Ado, 3 mM). Note that protein expression of A_{2a} adenosine receptor, but not A_1, A_{2b}, and A_3 adenosine receptors, are clearly suppressed for cells transfected with the A_{2a}R siRNA, confirming knock-down of A_{2a} adenosine receptor in samples used here. In the graphs, each column represents the mean (±SEM) ratio against basal expression levels (before treatment with adenosine) for Bcl-X_L and Bid normalized by the expression level of β-actin (n = 4 independent experiments). *P*-values, Dunnett's test.

Overall, these results indicate that adenosine activates caspase-9 followed by caspase-3 in HepG2 cells via A_{2a} adenosine receptors.

DISCUSSION

In the present study, extracellular adenosine and the A_{2a} adenosine receptor agonist CGS21680 induce HepG2 cell apoptosis, and the effect was partially prevented by adenosine receptor antagonists, dominantly the A_{2a} adenosine receptor antagonist DMPX, or by knocking-down A_{2a} adenosine receptors. This accounts for the implication of A_{2a} adenosine receptor/its signaling cascades in adenosine-induced HepG2 cell apoptosis.

Extracellular adenosine downregulated expression of the Bcl-X_L mRNA and protein, but otherwise upregulated expression of the Bid mRNA and protein in HepG2 cells, without affecting expression of the mRNA and protein for Bcl-2, Bad, or Bax. Notably, the adenosine effect on expression of Bcl-X_L and Bid was inhibited by knocking-

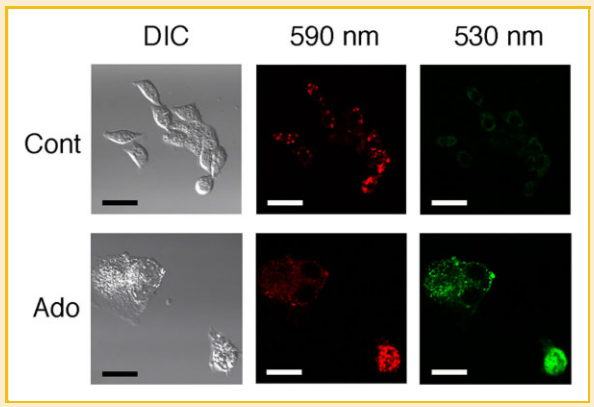

Fig. 8. Effect of adenosine on mitochondrial membrane potentials in HepG2 cells. Mitochondrial membrane potentials were monitored before (Cont) and after 24-h treatment with adenosine (Ado, 3 mM) using a DePsipher™ kit. DIC, differential interference contrast. Bars, 10 μm. Note that a similar effect was obtained from four independent experiments.

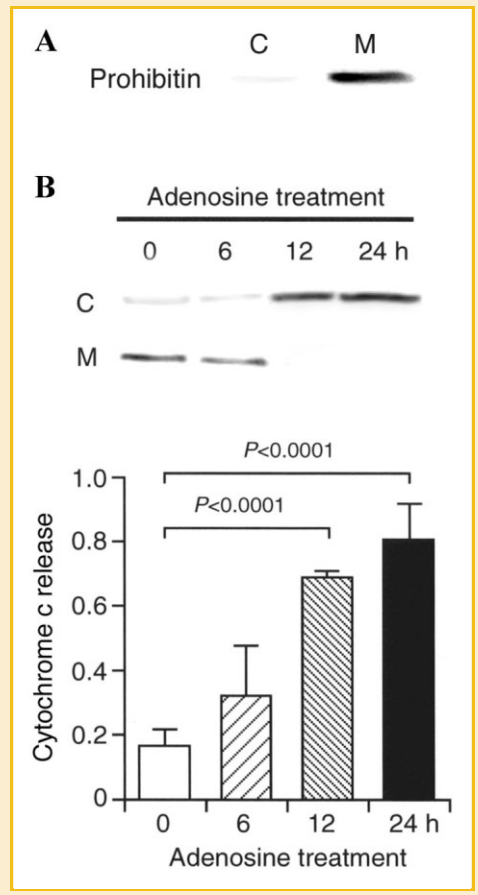

Fig. 9. Effect of adenosine on cytochrome c efflux from the mitochondria in HepG2 cells. A: Cells were lysed and separated into the cytosolic (C) and mitochondrial components (M), followed by Western blotting using an anti-prohibitin antibody. Note that a similar effect was obtained from four independent experiments. B: After treatment with adenosine (3 mM) for periods of time as indicated, Western blotting was carried out in the cytosolic (C) and mitochondrial components (M) using an anti-cytochrome c antibody. In the graphs, each column represents the mean (±SEM) cytochrome c release (cytosolic cytochrome c/total cytochrome c, n = 4 independent experiments). *P*-values, Dunnett's test.

down A_{2a} adenosine receptors. This implies that adenosine regulates transcription for Bcl-X_L and Bid via an A_{2a} receptor signaling pathway. A_{2a} adenosine receptors are linked to G_s protein involving adenylate cyclase activation to generate cAMP and activate PKA. How cAMP/PKA regulates transcription for Bcl-X_L and Bid in HepG2 cells remains to be explored.

Bcl-X_L engages mitochondrial protection as well as Bcl-2. Bid accumulation in the mitochondria might perturb the mitochondria [Eskes et al., 2000; Landshamer et al., 2008]. Reduced Bcl-X_L expression and increased Bid expression, therefore, could cause mitochondrial damage. Indeed, extracellular adenosine disrupted mitochondrial membrane potentials in HepG2 cells. Moreover, extracellular adenosine increased cytosolic localization of cytochrome c along decreased mitochondrial localization in HepG2 cells, indicating that adenosine perturbs mitochondrial membrane potentials and in turn, stimulates cytochrome c efflux from the mitochondria into the cytosol.

Activated caspase-8 produces truncated Bid through proteolysis of Bid, and truncated Bid forms a Bax/Bak complex at the mitochondrial membrane allowing cytocrome c efflux from the mitochondria [Wang et al., 1996; Gross et al., 1999; Rudner et al., 2005]. In spite of caspase-8 activation, adenosine here did not produce truncated Bid in HepG2 cells. This excludes the possibility for truncated Bid-mediated mitochondrial damage causing cytochrome c efflux from the mitochondria. Lines of evidence, alternatively, have shown that full-length Bid could still impair the mitochondria by interacting with Bax, to induce apoptosis [Valentijn and Gilmore, 2004; Ward et al., 2006; König et al., 2007; Pei et al., 2007]. Adenosine-induced upregulation of full-length Bid via A_{2a} adenosine receptors, accordingly, is likely to still participate in perturbation of mitochondrial membrane potentials and the ensuing apoptotic events in HepG2 cells.

Cytosolic cytochrome c forms an oligomeric complex with dATP, Apaf-1, and pro-caspase-9 and formation of this complex results in the activation of caspase-9, which then activates the effector caspase-3 [Li et al., 1997; Earnshaw et al., 1999; Zou et al., 1999]. Adenosine activated caspases-3, -8, and -9 in HepG2 cells. The activation of caspases-3 and -9 was significantly suppressed by knocking-down A_{2a} adenosine receptors, although the caspase-8 activation was not affected. This indicates that A_{2a} adenosine receptor mediates activation of caspase-9 and the effector caspase-3 in concert with mitochondrial damage and the ensuing cytochrome c efflux from the mitochondria. This also indicates that adenosine activates caspase-8 via a pathway independent of A_{2a} adenosine receptor.

In conclusion, the results of the present study show that extracellular adenosine downregulates Bcl-X_L expression and upregulates Bid expression via A_{2a} adenosine receptors, thereby disrupting mitochondrial membrane potentials to allow cytochrome c efflux from the mitochondria into the cytosol, responsible for activation of caspase-9 and the effector caspase-3. This may

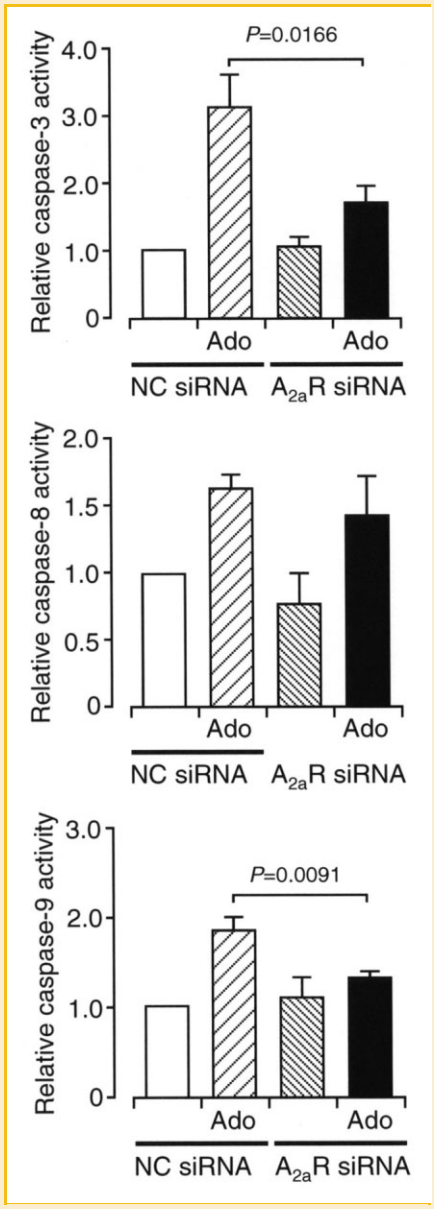

Fig. 10. Effects of adenosine on caspases-3, -8, and -9 activation in HepG2 cells. Activity of caspases-3, -8, and -9 was assayed in cells transfected with the NC siRNA or the $A_{2a}R$ siRNA before and after 24-h treatment with adenosine (Ado, 3 mM). In the graphs, each column represents the mean (±SEM) ratio against basal caspase activities (before treatment with adenosine, n = 4 independent experiments). *P*-values, Dunnett's test.

represent further insight into the pathway underlying extracellular adenosine-induced HepG2 cell apoptosis.

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