CELLULAR MECHANISMS OF THE MACROPHAGE DEACTIVATING EFFECT OF ADENOSINE

Ph.D. Dissertation

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CONTENTS

1. ABSTRACT

Adenosine is released into the extracellular space from nerve terminals and cells subjected to ischemic stress. This nucleoside modulates a plethora of cellular functions via occupancy of specific receptors. Adenosine is also an important endogenous regulator of macrophage function, as it suppresses the production of a number of proinflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-12, macrophage inflammatory protein (MIP)-1α by these cells. On the other hand, adenosine increases the release of the anti-inflammatory cytokine IL-10 in bacterial lipopolysaccharide (LPS) stimulated macrophages. However, the mechanisms of these anti-inflammatory effects have not been well characterized. First, we hypothesized that adenosine may exert some of its anti-inflammatory effects by decreasing activation of the transcription factor nuclear factor κB (NF-κB), because gene expression of most of the proinflammatory cytokines inhibited by adenosine is dependent on NF-κB activation. Using LPS-stimulated RAW 264.7 macrophages, we found that adenosine as well as adenosine receptor agonists decreased the production of TNF-α, a typical NF-κB-regulated cytokine. This effect of adenosine was not due to an action on the process of TNF-α release, as adenosine suppressed also the intracellular levels of TNF-α. However, cDNA microarray analysis revealed that mRNA levels of neither TNF-α nor other cytokines were altered by adenosine in either LPS-activated or quiescent macrophages. In addition, although LPS induced expression of a number of other, non-cytokine genes, including the adenosine A_{2b} receptor, adenosine did not affect the expression of these genes. Furthermore, adenosine as well as adenosine receptor agonists failed to decrease LPS-induced NF-κB DNA binding, NF-κB promoter activity, p65 nuclear translocation and inhibitory κB (IκB) degradation.

We also hypothesized that adenosine would activate cyclic adenosine 5'-monophosphate (cAMP) response element binding protein (CREB) in macrophages since adenosine receptor stimulation has been associated with activation of the cAMP–protein kinase A system as well as of p38 mitogen-activated protein kinase (MAPK) and p42/44 MAPK, all of which can activate the CREB transcription factor system. Utilizing RAW

264.7 macrophages, we found that extracellular adenosine enhanced CREB transcriptional activity and increased phosphorylation of nuclear CREB. On the other hand, adenosine failed to alter CREB DNA binding. Adenosine stimulated both p38 and p42/44 MAPK activation. The p38 MAPK pathway inhibitor SB203580 but not the p42/44 MAPK pathway blocker PD98059 decreased adenosine-induced CREB activation indicating that p38 MAPK but not p42/44 MAPK is an upstream mediator of CREB activation. Thus, some of the immunomodulatory effects of adenosine in macrophages may be explained by its augmenting effect on CREB activation.

Taken together, our results suggest that the anti-inflammatory effects of adenosine are independent of NF-κB but some of the immunomodulatory effects in macrophages may be explained by its augmenting effect on CREB activation.

2. ABBREVIATIONS

ATP	adenosine 5'-triphosphate
AMP	adenosine 5'-monophosphate
cAMP	cyclic adenosine 5'-monophosphate
CCPA	2-chloro-N^6-cyclopentyladenosine
CGS-21680	2-p-(2-carboxyethyl) phenethylamino-5'-*N*-ethyl-carboxamidoadenosine
CHA	N^6-cyclohexyladenosine
CPA	N^6-cyclopentyladenosine
CSC	8-(3-chlorostyry)caffeine
2Cl-IB-MECA	2-chloro- N^6-(3-iodobenzyl)-adenosine-5'-N-methyluronamide
CREB	cAMP responsive element binding protein
DPCPX	8-cyclopentyl-1,3-dipropylxanthine
DMSO	dimethylsuphoxide
DNA	deoxyribonucleic acid
ELISA	enzyme-linked immunosorbent assay
EMSA	electromobility shift assay
IκB	inhibitory κB
IB-MECA	N^6-(3-iodobenzyl)-adenosine-5'-N-methyluronamide
IL	interleukin
LPS	bacterial lipopolysaccharide
MAPK	mitogen-activated protein kinase
MIP	macrophage inflammatory protein
MRS-1220	(9-chloro-2-(2-furanyl)-5-[(phenylacetyl)amino]-(1,2,4)-triazolo(1,5-c)quinazoline
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide
mRNA	messenger ribonucleic acid
NECA	5'-N-ethylcarboxamidoadenosine
NF	nuclear factor

NO	nitric oxide
PACPX	1,3-dipropyl-8-(2-amino-4-chlorophenyl)-xanthine
PCR	polymerase chain reaction
RIPA	radioimmunoprecipitation
RNA	ribonucleic acid
R-PIA	N^6-phenylisopropyladenosine
RT	reverse transcription
TNF	tumor necrosis factor
VEGF	vascular endothelial growth factor
ZM241385	4-[2-(7-amino-2-(2-furyl-(1,2,4)-triazolo(2,3-a)(1,3,5)triazin-5-ylamino)ethyl]phenol

3. INTRODUCTION

3. 1. Adenosine: an endogenous nucleoside with multiple functions

Adenosine is an endogenous purine nucleoside which following its release from cells or after being formed extracellularly travels to the cell membrane of surrounding cells where it binds specific cell surface structures that recognize it, termed adenosine receptors (Ralevic and Burnstock 1998; Fredholm et al. 2001). The concept of adenosine as an extracellular signaling molecule was established following the seminal study of Szentgyörgyi and his colleague demonstrating that adenosine extracted from heart muscle, has pronounced biological effects, including heart block and arterial dilatation (Drury and Szentgyörgyi, 1929). Since extracellular adenosine formation was later shown to occur in the hypoxic and ischemic heart, the hypothesis was proposed that adenosine served a protective function in the heart against the consequences of metabolically detrimental situations both by decreasing the metabolic demands of the myocardium and by increasing coronary blood flow (Ralevic and Burnstock, 1998). Subsequently, evidence was obtained for similar protective actions for extracellular adenosine in other cellular and organ systems, including the brain, kidney, skeletal muscle, and adipose tissue. Based on this evidence a unifying hypothesis for adenosine action was formulated by Newby and the term "retaliatory metabolite" was coined to describe adenosine's protective function (Newby, 1984). This hypothesis states that adenosine released in response to a wide range of stressful injurious stimuli, participates in an autoregulatory loop, the function of which is to protect organs from injury following the initiating stressful stimuli.

3. 2. Protective effects of adenosine on organ functions via two different mechanisms

Adenosine exerts its protective effects via basically two different mechanisms.

Firstly, adenosine decreases the energy demand of the tissue via a direct inhibitory effect on parenchimal cell function, exemplified by the negative inotropic effect of adenosine on the heart muscle or the attenuation of neuronal firing and neurotransmission in the central nervous system.

Secondly, adenosine indirectly protects the tissue by providing a more favorable environment for parenchimal cells, for which the best example is that adenosine augments nutrient availability by inducing vasodilation. More recent evidence indicates that adenosine helps to maintain tissue integrity following harmful insults by another major indirect route and that is through the modulation of immune system function.

However the immune response to tissue injury plays an essential role in preserving tissue homeostatis, an uncontrolled immune activation can inflict further damage on the affected tissues. It appears that the release of adenosine followed by its binding to adenosine receptors on immune cells represents a potent endogenous immunosuppressive pathway that can prevent this deleterious exuberance of the immune response to harmful external insults.

Although adenosine is constitutively present in the extracellular space at low concentrations, neurologic or metabolic stressful conditions dramatically increase its extracellular levels both by stimulating its formation and by maintaining its bioavailability via blockade of its cellular uptake and degradation. The predominant source of extracellular adenosine during systemic activation of the stress system is the sympathetic nervous system. Sperlágh and her coworkers recently demonstrated that stimulation of sympathetic nerve terminals in immune organs resulted in the release of not only the classical neurotransmitter norepinephrine but also of adenosine (Sperlágh et al., 2000).

Furthermore, immune organs subjected to metabolic stress represented by hypoxia or ischemia release adenosine originating from the immune cells themselves but not nerve terminals (Sperlágh et al., 2000). Finally, specific inflammatory stimuli, such as bacterial products are also capable of triggering adenosine release from immune cells (Sperlágh et al., 1998; Bodin and Burnstock, 1998). These in vitro data are in line with in vivo evidence demonstrating a dramatic increase in extracellular adenosine levels under conditions associated with neurologic and metabolic stress (Jabs at al., 1998; Schmidt et al., 1995; Martin et al., 2000).

For example, a recent study by Martin and his coworkers has documented that systemic adenosine levels reach 4 to 10 μM in patients with sepsis, a condition associated with both neurologic and metabolic stress, whereas adenosine concentrations in healthy

individuals are below 1 μM (Martin et al., 2000). This distinction becomes important in light of the recent findings that macrophage function is not affected at adenosine levels of 1 μM, however, ≥ 10 μM adenosine has strong immunosuppressive effects (Haskó et al., 1996; Haskó et al., 2000). Finally, although the above studies did not address the question of intraorgan adenosine levels, it has been demonstrated that adenosine concentrations can reach as high as 100 μM in inflammatory foci (Cronstein, 1994).

3. 3. Subtypes of adenosine receptors

Adenosine receptors have been subdivided according to molecular, biochemical, and pharmacological evidence into four subtypes. These are A_1, A_{2a}, A_{2b}, and A_3 receptors (Ralevic and Burnstock, 1998). While A_1 and A_3 receptors are linked to Gi/o and inhibit adenylate cyclase, A_{2A} and A_{2b} receptors are coupled to Gs proteins and increase adenylate cyclase activity and intracellular cAMP levels. These receptors also signal through members of the mitogen activated protein kinase family, such as p38 MAPK, p42/p44 MAPK (ERK 1/2), and c-jun terminal kinase, as well as various phospholipases, protein phosphatases, ion channels, and even small G proteins such as Ras and Rho (Table 1).

3. 4. Immune cells express adenosine receptors

Although most immune cell types are responsive to the regulatory effects of extracellular adenosine, monocytes/macrophages have recently emerged as prime targets of the immunomodulatory effects of adenosine (Haskó and Szabó, 1998; Haskó et al., 2002b). Macrophages are specialized phagocytic cells that play an important role in clearance of host cells undergoing apoptosis as well as of potentially harmful molecules, such as immune complexes. In addition, macrophages are crucial players in the defense against infection. These antigen-presenting cells are widely dispersed throughout the body, including at portals of entry to microorganisms. They participate in initial capture and processing of potential antigens and then in activation of specific T and B lymphocyte effector mechanisms. These activated cells in turn cooperate with activated macrophages to enhance destruction of intra- and extracellular pathogens.

Table 1.

Adenosine receptors and their characterization

	A_1	A_{2a}	A_{2b}	A_3
Second messengers	cAMP↓, Ca^{2+}↑, p38↑, p42/44↑	cAMP↑, p42/44↑	cAMP↑, p38↑	cAMP↓, Ca^{2+}↑
G protein-coupling	Gi/o	Gs	Gs, Gq	Gi, Gq
Affinity to adenosine	High	High	Low	Low
Selective agonists	CPA, CCPA	CGS-21680	?	IB-MECA, 2Cl-IB-MECA
Selective antagonists	DPCPX, PACPX	ZM241385, CSC	ALLOXAZINE	MRS-1220

Macrophages have been shown to express all four adenosine receptor subtypes (Sajjadi et al., 1996; Xaus et al., 1999; Khoa et al., 2001; Gessi et al., 2000; Mirabet et al., 1999; Gessi et al., 2001), however, the intracellular pathways activated by adenosine receptors in macrophages are unknown.

3. 5. Adenosine reprograms macrophages resulting in an immunosuppressed and anti-inflammatory phenotype

Adenosine affects almost all macrophage functions and these effects occur via occupancy of specific adenosine receptors. Adenosine decreases the production of a number of proinflammatory cytokines (Haskó et al., 1996; Haskó et al., 1998; Haskó et al., 2000; Haskó and Szabó, 1998) that both orchestrate inflammatory/immune functions of other cell types and act as autoregulators of macrophage function (Németh et al., 2003a; Németh et al., 2003b; Haskó et al., 2002b) (Figure 1).

Maybe the best example is TNF-α, a regulator of neutrophil, endothelial cell, and lymphocyte function, whose production has been shown to be under the inhibitory control of adenosine (Haskó and Szabó, 1998; Németh et al., 2003a). The adenosine

receptor subtypes mediating the inhibitory effect of adenosine on TNF-α production have been extensively studied. Early pharmacological studies implicated A_2 receptors as being responsible for most of the TNF-α suppression of adenosine (Reinstein et al., 1994; Bouma et al., 1994). Subsequent studies have demonstrated that A_3 receptors could also contribute to the adenosine suppression of TNF-α production (Haskó et al., 1996; Haskó et al., 1998; Sajjadi et al., 1996; Haskó et al., 2000).

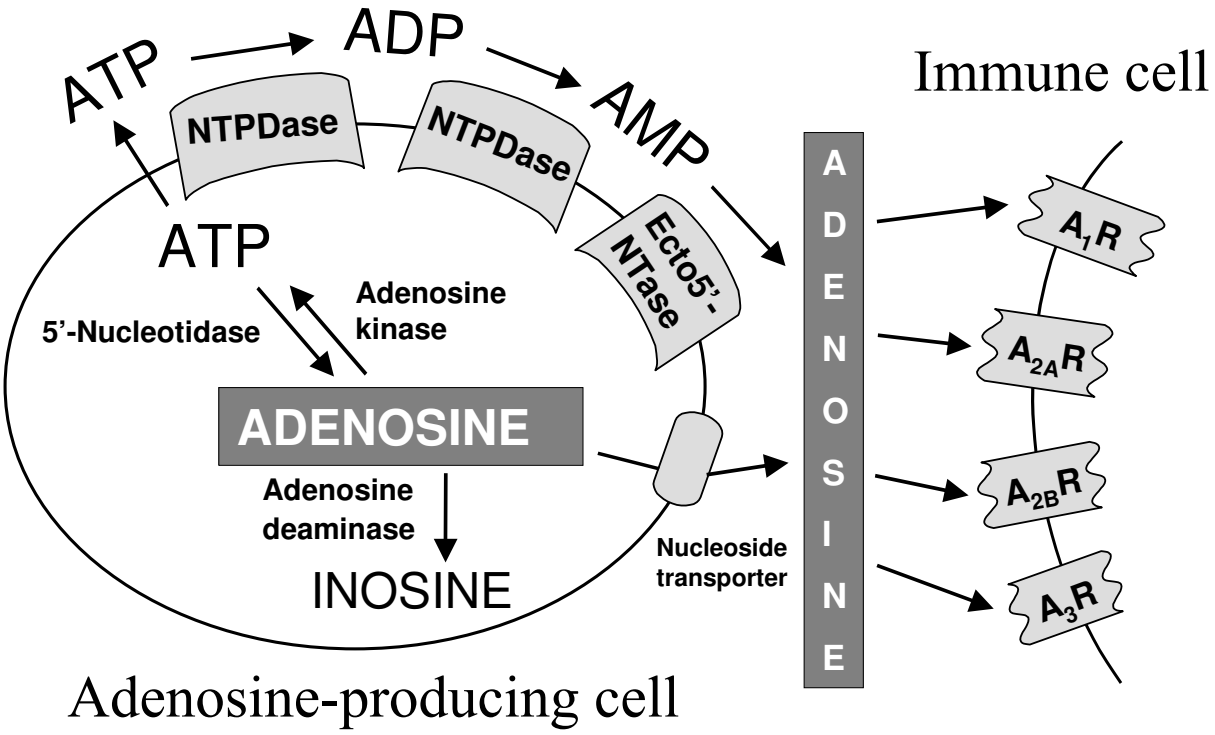


Figure 1.

Simplified summary of major pathways involved in adenosine metabolism.

Adenosine is formed from its precursor ATP in both the intracellular and extracellular spaces. The extracellular formation of adenosine is the result of an enzymatic cascade consisting of NTPDases and acto-5'-nucleotidase. Extracellular adenosine ligates its receptors, A_1R, $A_{2a}R$, $A_{2b}R$, A_3R, all of which are expressed on the surface of immune cells. Intracellular adenosine is shunted into the extracellular space through membrane nucleoside transporters. The adenosine kinase rephosphorylates adenosine to ATP while adenosine deaminase deaminates adenosine to inosine.

Recent studies using adenosine receptor knockout mice have confirmed that both A_{2a} (Haskó et al., 2000) and A_3 (Salvatore et al., 2000) receptors regulate macrophage TNF-α production. In addition, adenosine decreases the production of IL-12 by macrophages (Haskó et al., 2000; Khoa et al., 2001) and IL-12 is a pivotal factor in inducing a strong inflammatory response. Conversely, adenosine enhances the monocyte/macrophage ptroduction of IL-10, which is major anti-inflammatory cytokine (Haskó et al., 2000; Khoa et al., 2001; Le Moine et al., 1996).

Adenosine attenuates macrophage proinflammatory activity also by suppressing the production of both superoxide (Edwards et al., 1994) and nitric oxide (Xaus et al., 1999; Haskó et al., 1996) and both A_{2a} and A_{2b} receptors are involved in this suppression of free radical production by adenosine (Xaus et al., 1999; Haskó et al., 1996).

Taken together, adenosine has several actions on macrophages whose net result is the development of a deactivated, anti-inflammatory macrophage phenotype.

3. 6. Aims of the study

3. 6. 1. To investigate whether adenosine receptor stimulation exerts an inhibitory effect on TNF-α production by LPS-stimulated RAW 264.7 macrophages.

3. 6. 2. To examine whether adenosine has any effect on intracellular TNF-α levels in immune-stimulated RAW 264.7 macrophages.

3. 6. 3. To determine whether IL-10 production can be modulated by adenosine treatment in LPS-stimulated RAW 264.7 macrophages.

3. 6. 4. To investigate whether adenosine can modify the NF-κB transcription factor system in LPS-treated RAW 264.7 macrophages.

3. 6. 5. To examine the effect of adenosine receptor stimulation on the activation of the CREB transcription factor pathway in RAW 264.7 macrophages.

3. 6. 6. To study whether gene expression can be up- or down-regulated by adenosine treatment in LPS-stimulated RAW 264.7 macrophages

6. MATERIALS AND METHODS

Cell culture

The mouse macrophage cell line RAW 264.7 was grown in Dulbeccos's modified Eagle's Medium (Invitrogen Corp., Carlsbad, CA) supplemented with 10% fetal bovine serum, 50 U/ml penicillin (Invitrogen Corp.), 50 μg/ml streptomycin (Invitrogen Corp.), and 1.5 mg/ml sodium bicarbonate (Invitrogen Corp.) in a humidified atmosphere of 95% air and 5% CO_2.

Drugs and reagents

The non-selective adenosine receptor agonist 5'-N-ethylcarboxamidoadenosine (NECA), the selective A_1 receptor agonists N^6-cyclopentyladenosine (CPA), 2-chloro-N^6-cyclopentyl-adenosine (CCPA), N^6-cyclohexyladenosine (CHA), R(-)N^6-phenylisopropyladenosine (R-PIA), the A_{2a} receptor agonist 2-p-(2-carboxyethyl)phenethylamino-5'-*N*-ethyl-carboxamidoadenosine (CGS-21680), and the A_3 receptor agonist N^6-(3-iodobenzyl)-adenosine-5'-*N*-methyluronamide (IB-MECA) were obtained from Research Biochemicals Inc. (Natick, MA). Adenosine, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), and LPS (*Escherichia coli* 055:B5) were purchased from Sigma (St. Louis, MO). The p38 pathway inhibitor SB203580 and p42/44 pathway inhibitor PD98059 were purchased from Calbiochem (San Diego, CA). Stock solutions of SB203580 and PD98059 were prepared using dimethylsuphoxide (DMSO).

TNF-α determination from cell supernatants and cell extracts

Cells in 96 well plates were treated with adenosine or various adenosine receptor agonists 30 min before the addition of 10 μg/ml LPS. Supernatants for TNF-α determination were obtained 4 hours after stimulation with LPS. For the determination of intracellular TNF-α, macrophages in 24 well plates were pretreated with adenosine followed by LPS (10 μg/ml) stimulation 30 min later. After an additional 6-hour incubation, the supernatants were removed and the cells were lysed as described

previously (Haskó et al., 2002a). TNF-α levels in cell supernatants or cell lysates were determined by ELISA (R&D Systems Inc. Minneapolis, MN), as we have previously described (Haskó et al., 2002a; Németh et al., 2002a; Németh et al., 2003b).

Measurement of IL-10 levels in the supernatant

Cells in 24 well plates were treated with adenosine (100 μM) followed by the addition of 10 μg/ml LPS. Supernatants for IL-10 determination were obtained 5 hours after stimulation with LPS (10 μg/ml) as we have previously described (Haskó et al., 2002a). IL-10 levels in cell supernatants were determined by enzyme-linked immunosorbent assay (ELISA) as described previously (Németh et al., 1998).

NF-κB and CREB electromobility shift assay (EMSA)

RAW 264.7 cells were stimulated with LPS (10 μg/ml) for 45 min and nuclear protein extracts were prepared as described previously (Németh et al., 2002a). To determine the effect of adenosine receptor agonists, cells were pretreated with these agents or their vehicle 30 min before stimulation. All nuclear extraction procedures were performed on ice with ice-cold reagents. Cells were washed with PBS and harvested by scraping into 1.5 ml of PBS and pelleted at 1,500 x *g* for 10 min.

The pellet was resuspended in 60 μl of cytosolic lysis buffer (20% v/v glycerol, 10 mM HEPES pH 8.0, 10 mM KCl, 0.5 mM EDTA pH 8.0, 1.5 mM $MgCl_2$, 0.5 % v/v NP-40, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin A) and incubated for 15 min on ice with occasional vortexing.

After centrifugation at 4,500 x *g* for 10 min, supernatants (cytosolic extracts) were discarded. Two cell pellet volume of nuclear extraction buffer (20% v/v glycerol, 20 mM HEPES pH 8.0, 420 mM NaCl, 0.5 mM EDTA pH 8.0, 1.5 mM $MgCl_2$, 50 mM glycerol-phosphate, 0.5 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin A, all from Sigma) was added to the nuclear pellet and incubated on ice for 30 min with occasional vortexing.

Nuclear proteins were isolated by centrifugation at 14,000 x *g* for 15 min. Protein concentrations were determined using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA). Nuclear extracts were aliquoted and stored at -80 °C until used for electromobility shift assay (EMSA).

The nuclear factor κB (NF-κB) or cAMP responsive element binding protein (CREB) consensus oligonucleotide probe used for the EMSA was purchased from Promega (Promega Corp., Madison, WI). Oligonucleotide probes were labeled with [γ-^{32}P] ATP using T4 polynucleotide kinase (Invitrogen Corp.) and purified in MicroSpin G-50 columns (Amersham Pharmacia, Piscataway, NJ).

For the EMSA analysis, 8-12 μg of nuclear proteins were pre-incubated with EMSA binding buffer (8 % glycerol v/v, 10 mM Tris-HCl pH 8.0, 2 mM $MgCl_2$, 0.5 mM EDTA pH 8.0, and 0.5 mM dithiothreito, all from Sigma) as well as 15 ng/μl poly(dI)-poly(dC) Reagent (Amersham Pharmacia Biotech), 0.4 ng/μl of ssDNA Reagent (Amersham Pharmacia Biotech), and 0.2 mg/ml of bovine serum albumin (Sigma) at room temperature for 10 min before addition of the radiolabeled oligonucleotide for an additional 25 min. Protein-nucleic acid complexes were resolved using a non-denaturing polyacrylamide gel consisting of 4 % acrylamide (29:1 ratio of acrylamide: bisacrylamide) and run in 0.5X TBE buffer (44.5 mM Tris-Base, 44.5 mM boric aid, 1 mM EDTA, all from Sigma) for approximately 2.5 h at constant current (35 mA). Gels were transferred to Whatman 3M paper, dried under vacuum at 80 °C for 40 min, and exposed to photographic film at -80 ºC with an intensifying screen.

For NF-κB supershift studies, before addition of the radiolabeled probe, samples were incubated for 30 min with 4 μg of isotype control (rabbit polyclonal IgG Mad 1 antibody, sc-222X), p65 (sc-109X), or p50 (sc-114X) Ab (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). And for CREB supershift studies, before addition of the radiolabeled probe, samples were incubated for 30 min with 4 μg of isotype control (rabbit polyclonal IgG Mad 1 antibody, (sc-222X) (Santa Cruz Biotechnology, Inc.), ATF-1 (sc-243X) (Santa Cruz Biotechnology, Inc.) or CREB-1 (sc-186X) antibody (Santa Cruz Biotechnology, Inc.).

Transient transfection and luciferase activity

For transient transfections, $3x10^5$ RAW 264.7 cells were seeded per well of a 24-well tissue culture dish (Corning Incorporated, Corning, NY) 1 day prior to transient transfection. Cells were transfected with 30 μl/ml of FuGENE 6 Transfection Reagent (Roche Applied Science Applied Science, Indianapolis, IN) in 160 μl of medium per well. The medium contained 5 μg/ml of DNA containing a NF-κB promoter construct (Clontech, San Diego, CA). In the case of CREB luciferase the transfection medium contained 5 μg/ml CREB luciferase (pCRE-luc) promoter construct (Clontech) and 0.3 μg/ml of control Renilla luciferase (pRL-CMV) plasmid (Clontech).

After an overnight transfection with NF-κB luciferase vector, the cells were pretreated with adenosine (100 μM) or its vehicle (medium) for 30 min, which was followed by stimulation with LPS (10 μg/ml) for 6 hours. In the case of the measurement of CREB activation, after an overnight transfection, the cells were exposed to adenosine or its vehicle (medium) for 8 hours. NF-κB luciferase activity was measured by the Luciferase Reporter Assay System (Promega, Madison, WI) and normalized relative to μg of protein, as we have described previously (Németh et al., 2002b; Németh et al., 2003b) for the NF-κB luciferase assay. CREB luciferase activity was measured by the Dual Luciferase Reporter 1000 Assay System (Promega).

Western blot analysis of inhibitory κB (IκB) and p65

Levels of protein p65 were analyzed using the nuclear extracts prepared for the EMSA assays. For IκB Western blotting, RAW cells in 6-well plates were pretreated with adenosine (100 μM) or vehicle and 30 min later the cells were stimulated with LPS (10 μg/ml) for 30 min. After washing with PBS, the cells were lysed by the addition of modified radioimmunoprecipitation (RIPA) buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.25% Na-deoxycholate, 1% NP-40, 1 μg/ml pepstatin, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 1 mM Na_3VO_4).

The lysates were transferred to Eppendorf tubes, centrifuged at 15,000 x *g* and the supernatant was recovered. Protein concentrations were determined using the Bio-Rad Protein Assay. 10-20 μg of sample was separated on a 8-16% Tris-Glycine gel

(Invitrogen Corp.) and transferred to a nitrocellulose membrane. The membranes were probed with anti-p65 (Santa Cruz Biotechnology) or anti-IκBα (Cell Signaling, Beverly, MA) and subsequently incubated with a secondary horseradish peroxidase-conjugated donkey anti-rabbit antibody (Boehringer Mannheim Corp., Indianapolis, IN). Bands were detected using ECL Western Blotting Detection Reagent (Amersham Pharmacia Biotech).

Western blot analysis of phospho-CREB, phospho-p38, and phospho-p42/44

Phospho-CREB levels were analyzed using nuclear extracts prepared as described for the EMSA assays. Western blotting to detect p38 and p42/44 phosphorylation was performed using cytosolic extracts prepared as described above. Protein concentrations were determined using the Bio-Rad Protein Assay. 10-20 μg of sample was separated on a 8-16% Tris-Glycine gel (Invitrogen) and transferred to a nitrocellulose membrane. The membranes were probed with Anti-ACTIVE p38 pAb (V1211) or anti-ACTIVE MAPK (p42/44) pAb (V8031) (Promega Corp.) and subsequently incubated with a secondary horseradish peroxidase-conjugated goat anti-rabbit antibody (Santa Cruz Biotechnology). Bands were detected using ECL Western Blotting Detection Reagent (Amersham Pharmacia Biotech).

Affymetrix gene chip analysis

RAW 264.7 cells were plated in 6-well plates 1 day prior to the experiment. The cells were activated with LPS (10 μg/ml) for 3 h or exposed to the vehicle for LPS (medium). Adenosine (100 μM) or its vehicle was added to the cells 30 min before the LPS challenge.

Thus, the following 4 experimental groups were designed: vehicle for adenosine + vehicle for LPS, vehicle for adenosine + LPS, adenosine + LPS, and adenosine + vehicle for LPS. With the exception of the adenosine + vehicle group, where *n* was 3, all groups contained an *n* of 4. These numbers were the result of two independent experiments performed on two different experimental days (two samples from both experiments for all the groups with the exception of the adenosine + vehicle group, where two samples were obtained from the first experiment and one from the other).

Total RNA was prepared using TRIzol (Invitrogen Corp.) and further purified using Rneasy Mini Kit (Qiagen, Valencia, CA). Thereafter, total RNA was reverse transcribed in 20 μl using superscript II (Invitrogen). Double stranded DNA was created using a replacement reaction involving Rnase H, ligase, and DNA polymerase I. The in vitro transcription was done using the Enzo High Yield Transcription Kit which incorporates the biotinylated ribonucleotides UTP and CTP.

Equal amounts of fragmented cRNA were then hybridized to MG-U74Av2 gene chips according to Affymetrix protocols (Affymetrix, Inc., Santa Clara, CA) at the Biopolymers Facility, Harvard Medical School (Boston, MA). Chips were scanned and analyzed using GeneChip® Analysis Suite software. Data sets of intensities of 12,488 probe sets per array were compared using Microsoft Excel (Microsoft, Redmond, WA) software. To identify differentially expressed genes, we excluded all genes from the analysis that were scored absent in any of the samples. Furthermore, the ESTs were excluded from the analysis.

RT-PCR

These experiments were performed using RNA isolated for the microarray experiment. 5 μg of RNA was transcribed in a 20 μl reaction containing 10.7 μl of RNA (5 μg), 2 μl of 10x PCR buffer, 2 μl of 10 mM dNTP mix, 2 μl of 25 mM $MgCl_2$, 2 μl of 100 mM dithiothreitol, 0.5 μl of RNase inhibitor (PerkinElmer, Inc., 20 U/μl), 0.5 μl 50 mM oligo d(T) and 0.3 μl of reverse transcriptase (Roche Applied Science, Indianapolis, IN). The reaction mix was incubated at 42˚C for 15 minutes for reverse transcription. Thereafter, the reverse transcriptase was inactivated at 99˚C for 5 min. RT generated DNA was amplified using Expand™ High Fidelity PCR System (Roche Applied Science). The reaction buffer (25 μl) contained 2 μl of cDNA, water, 2.5 μl of PCR buffer, 1.5 μl of 25 mM $MgCl_2$, 1 μl of 10 mM dNTP mix, 0.5 μl of 10 μM oligonucleotide primer (each), and 0.2 μl of Taq polymerase (Roche Applied Science Applied Science).

cDNA was amplified using the primer sets described in Table 2. PCRs were carried out using the following conditions for TNF-α, A_{2a} receptor, A_{2b} receptor an initial denaturation at 94˚C X 5 min, 22, 30, and 30 cycles of 94˚C x 30 s for TNF-α, A_{2b}

receptor, and A_{2a} receptor, respectively, 58˚C x 45 s, 72˚C x 45 s; a final dwell at 72˚C x 7 min.

PCR products were resolved on a 1.5% agarose gel and stained with ethidium bromide (Németh et al., 2002a).

Table 2.

Primer sets used for PCR

Gene	Direction	Sequence	Reference
TNF-α	sense	5'-GGCAGGTCTACTTTGGAGTCATTGC -3'	Murray et al., 1990
TNF-α	antisense	5'-ACATTCGAGGCTCCAGTGAATTCGG -3'	Murray et al., 1990
A_{2a} rec.	sense	5'-CACGCAGAGTTCCATCTTCA-3'	Hoskin et al., 2002
A_{2a} rec.	antisense	5'-AGCAGTTGATGATGTGCAGG-3'	Hoskin et al., 2002
A_{2b} rec	sense	5'- TGGCGCTGGAGCTGGTTA -3'	Zhao et al., 2002
A_{2b} rec	antisense	5'- GCAAAGGGGATGGCGAAG -3'	Zhao et al., 2002

Measurement of mitochondrial respiration

Mitochondrial respiration, an indicator of cell viability, was assessed by the mitochondria-dependent reduction of MTT to formazan (Németh et al., 2002b). After the various treatments for cytokine measurements (see above), supernatants were removed and cells incubated with MTT (0.5 mg/ml) for 60 min at 37 ^{o}C. Culture medium was removed by aspiration and the cells were solubilized in Me_2SO (100 µl/well). The extent of reduction of MTT to formazan within cells was quantitated by measurement of optical density at 550 nm using a Spectramax 250 microplate reader (Global Medical Instrumentation, Inc., Albertville, MN).

Statistical evaluation

Values in the figures, tables and text are expressed as mean ± SEM of *n* observations. Statistical analysis of the data was performed by Student *t* test or one-way analysis of variance followed by Dunnett's test, as appropriate.

5. RESULTS

5. 1. Adenosine receptor agonists decrease TNF-α production and intracellular TNF-α levels in LPS-stimulated RAW 264.7 macrophages

First, we examined whether adenosine receptor stimulation decreased the production of the NF-κB-regulated cytokine TNF-α by macrophages. Stimulation of cells with LPS for 4 hours induced the release of TNF-α into the medium. Adenosine (10-100 μM) pretreatment of cells 30 min before the LPS challenge reduced the release of TNF-α, which occurred in a concentration-dependent fashion (Figure 2). The adenosine receptor agonists CPA, CCPA, CGS-21680, NECA, and IB-MECA, all mimicked the effect of adenosine in suppressing the production of TNF-α by LPS-stimulated RAW 264.7 cells (Figure 3). None of these purinergic agents had any effect on cell viability, as determined using the MTT assay (data not shown). These data obtained using LPS-stimulated RAW 264.7 cells confirm the previous observations of studies using other macrophage systems (Bouma et al., 1994; Haskó et al., 1996; McWhinney et al., 1996; Sajjadi et al., 1996; Mayne et al., 1999; Mayne et al., 2001; Leibovich et al., 2002) that adenosine receptor stimulation attenuates the production of TNF-α.

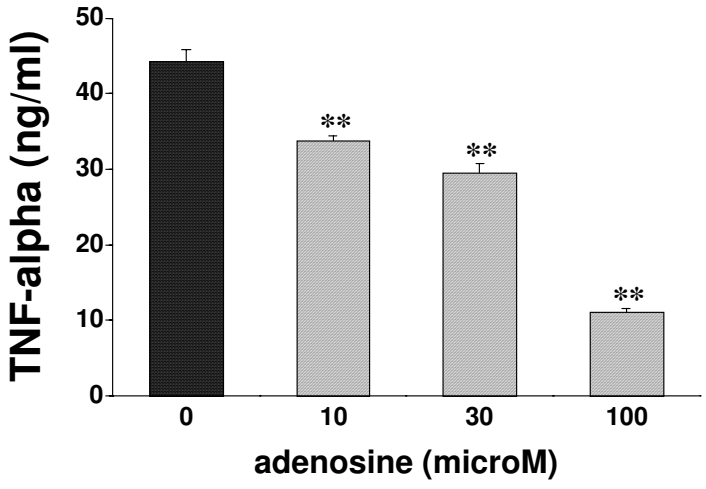
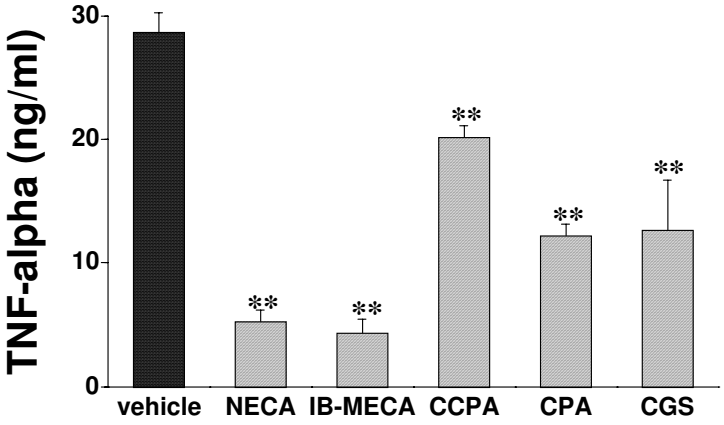


Figure 2.
Adenosine inhibits TNF-α production by RAW 264.7 macrophages stimulated with LPS (10 μg/ml) for 4 hours. TNF-α was measured from the supernatant using ELISA. Data are expressed as the mean ± SEM of 6 wells. **, indicates $p < 0.01$.

Figure 3.

Effect of various adenosine receptor agonists (10 μM) on LPS-induced TNF-a production by RAW cells. Adenosine or the adenosine agonists were added to the cells 30 minutes prior to LPS administration. TNF-α was measured from the supernatants using ELISA. Data are expressed as the mean ± SEM of 6 wells. **, indicates $p < 0.01$.



Next, we asked the question, whether adenosine acted by decreasing the accumulation of intracellular TNF-α or if it affected the release of this cytokine. The results of this experiment showed that treatment of the cells with LPS induced the appearance of intracellular TNF-α, which was suppressed by adenosine pretreatment (Figure 4). These results indicate that adenosine does not interfere with the release process of TNF-α.

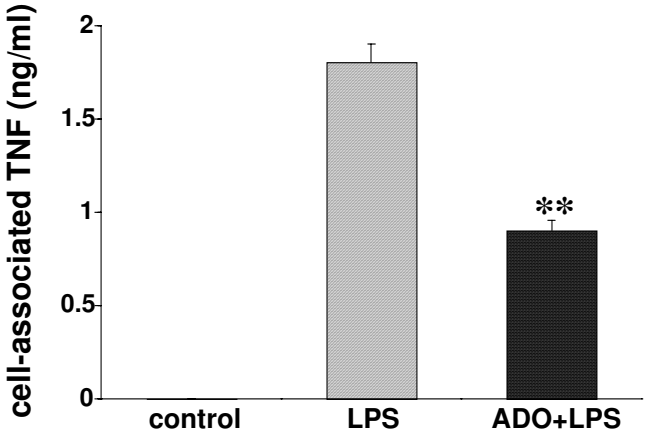


Figure 4.

Adenosine decreases intracellular TNF-α levels in RAW 264.7 macrophages. Cells were pretreated with adenosine (100 μM) and 30 min later the cells were exposed to LPS (10 μg/ml) for another 6 hours. At the end of the incubation period, the adherent cells were lysed for the determination of intracellular TNF-α. TNF-α levels were determined by ELISA. Data are expressed as the mean ± SEM of 8 wells. **, Indicates $p < 0.01$.

5. 2. Lack of effect of adenosine on NF-κB activation in RAW 264. 7 macrophages

Because NF-κB is an important regulator of TNF-α production by macrophages (Baeuerle and Henkel, 1994), we next tested the possibility that adenosine decreased activation of the NF-κB transcription factor system. As shown in Figures 5A and 5B, using nuclear extracts from LPS-treated RAW 264.7 cells, we observed an increase in NF-κB binding, when compared to LPS-untreated cells. Supershift studies confirmed the observation by previous reports (Baeuerle and Henkel, 1994) that the DNA binding complex induced by LPS contained both p65 and p50 (Figure 4A, right panel). However, neither adenosine (Figure 5) nor adenosine receptor agonists (Figures 6, 7, and 8) affected this induction of NF-κB DNA binding.

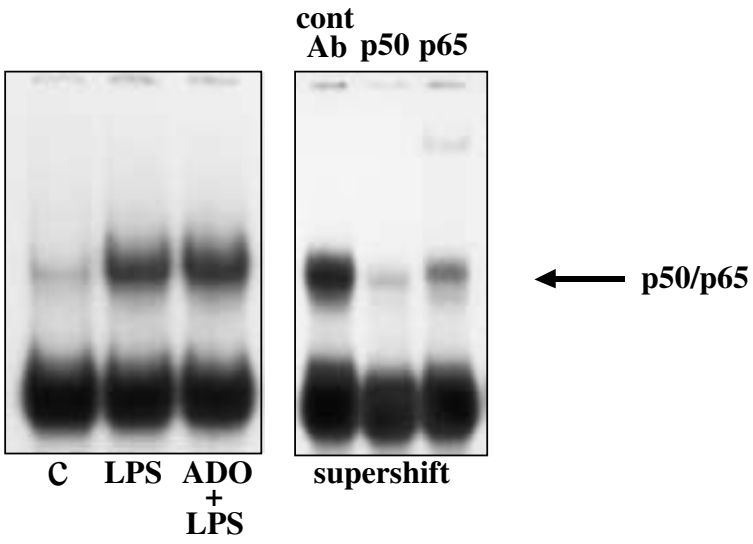


Figure 5.
Adenosine pretreatment (100 μM) fails to decrease LPS (10 μg/ml)-induced NF-κB DNA binding in RAW 264.7 cells. Adenosine or the adenosine agonists were added to the cells 30 minutes prior to LPS administration. NF-κB DNA binding was assessed from nuclear extracts obtained 45 min after LPS (10 μg/ml) stimulation using EMSA. These figures are representatives of 3 separate experiments.

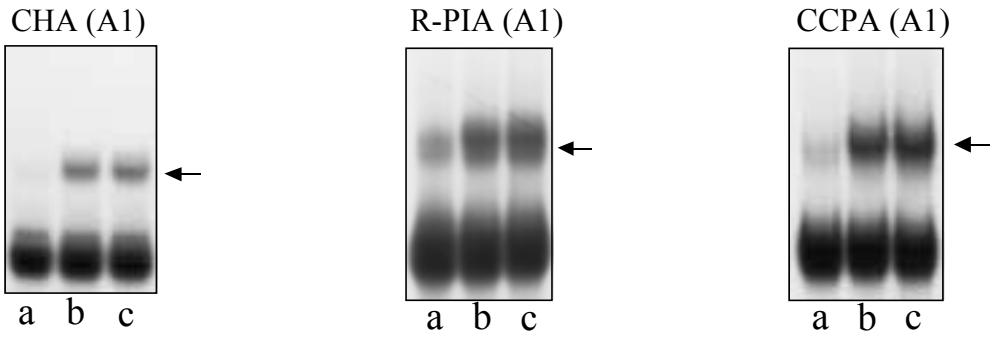


Figure 6.

Pretreatment with 10 μM of adenosine A_1 receptor agonists (CHA, R-PIA, CCPA) does not affect LPS (10 μg/ml)-induced NF-κB DNA binding in these cells.

a: no LPS; b: LPS; c: agonist+LPS.

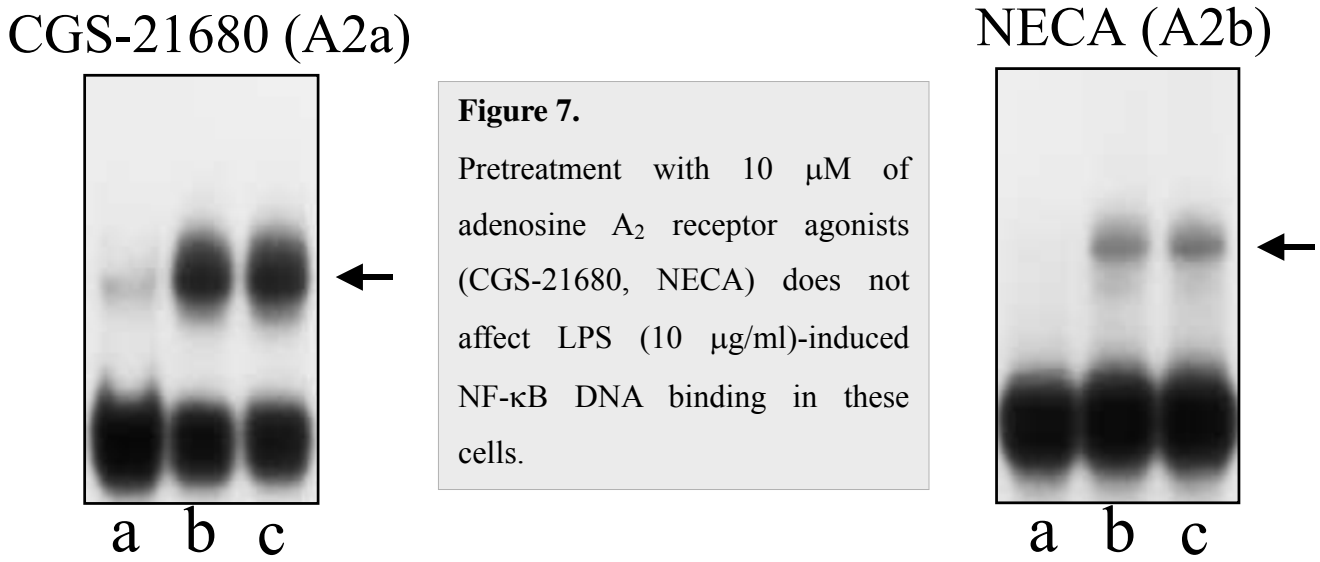


Figure 7.

Pretreatment with 10 μM of adenosine A_2 receptor agonists (CGS-21680, NECA) does not affect LPS (10 μg/ml)-induced NF-κB DNA binding in these cells.

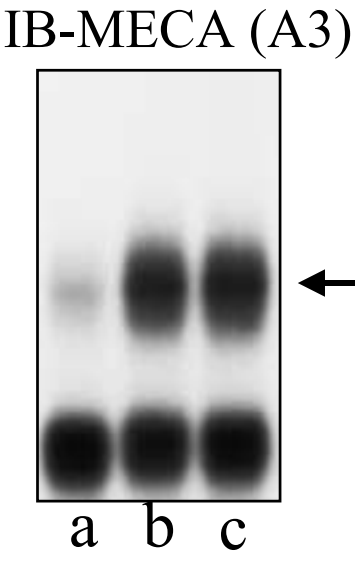


Figure 8.

Pretreatment with 10 μM of adenosine A_3 receptor agonists (IB-MECA) does not affect LPS (10 μg/ml)-induced NF-κB DNA binding in these cells.

a: no LPS; b: LPS; c: agonist+LPS.

Further, adenosine did not prevent either the LPS-induced accumulation of p65 in the nucleus or LPS-elicited IκB degradation (Figure 9).

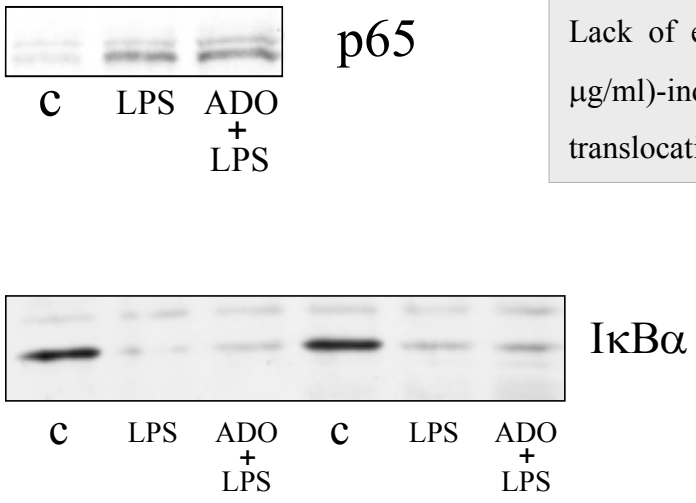


Figure 9.

Lack of effect of adenosine (100 μM) on LPS (10 μg/ml)-induced degradation of IκB and the nuclear translocation of p65.

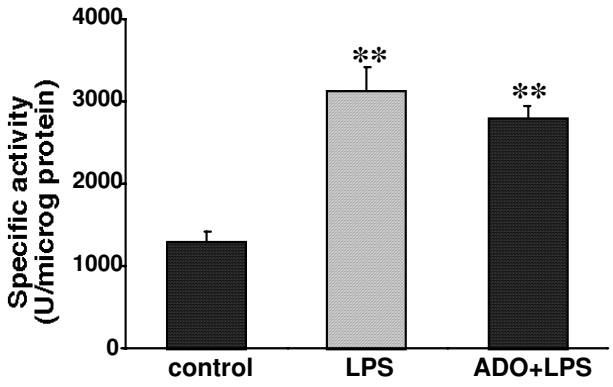
RAW 264.7 cells were pretreated with vehicle (c) or adenosine (ADO) for 30 min followed by an LPS challenge for 30 min. The degradation of IκB and p65 nuclear translocation activation were determined using Western blotting. This figure is representative of 3 separate experiments.

5. 3. Adenosine does not affect LPS-induced NF-κB-dependent transcriptional activity

The possibility still existed that adenosine could prevent NF-κB transcriptional activity without interfering with NF-κB DNA binding. To test this hypothesis, we transiently transfected cells with a NF-κB-luciferase reporter construct. Then, the transfectants were pretreated with adenosine or its vehicle for 30 min, which was followed by stimulation with LPS for 6 hours. The effect of adenosine on NF-κB-dependent gene transcription was assessed using the luciferase assay. Similar to results of the DNA binding experiments, adenosine failed to suppress LPS-stimulated NF-κB-dependent gene transcription (Figure 10). Finally, adenosine alone failed to affect NF-κB-dependent gene transcription (data not shown).

Figure 10.

Adenosine pretreatment (100 μM) has no effect on LPS-induced NF-κB-dependent transcriptional activity in RAW cells transiently transfected with a NF-κB-luciferase promoter construct. Adenosine was added to the cells 30 minutes prior to LPS administration. Luciferase activity was measured from cells lysed 6 hours after LPS treatment and normalized to protein content. Data are mean ± SEM of n=14-16 wells from two separate experiments. **, Indicates $p < 0.01$ vs. control



5. 4. Adenosine upregulates IL-10 production by LPS-stimulated RAW 264.7 macrophages

IL-10 was initially described as a T helper (Th)2 product that inhibited the secretion of cytokines by Th1 T cell clones (Mosmann, 1994). Recently, it has been demonstrated that monocytes and macrophages also produce IL-10 (Fiorentino at al., 1991; de Waal Malefyt at al., 1991) and that macrophages appear to be a major source of circulating IL-10 response to LPS (Barsig et al., 1995). IL-10 inhibits the synthesis of various cytokines (TNF-α, IL-1, IFN-γ, IL-6 and granulocyte-macrophage CSF) secreted by monocytes/macrophages in response to activation by LPS (Mosmann, 1994).

Since our previous results (Haskó et al., 1996) show a clear anti-inflamatory effect of adenosine receptor stimulation, we examined whether treatment with adenosine increases the production of the anti-inflammatory cytokine IL-10 by LPS-stimulated macrophages. Stimulation of RAW 264.7 macrophages with LPS (10 μg/ml) for 5 hours resulted in an increase in IL-10 production. Exposure of the cells to 100 μM adenosine for 5 hours upregulated this LPS-induced IL-10 response (Figure 11).

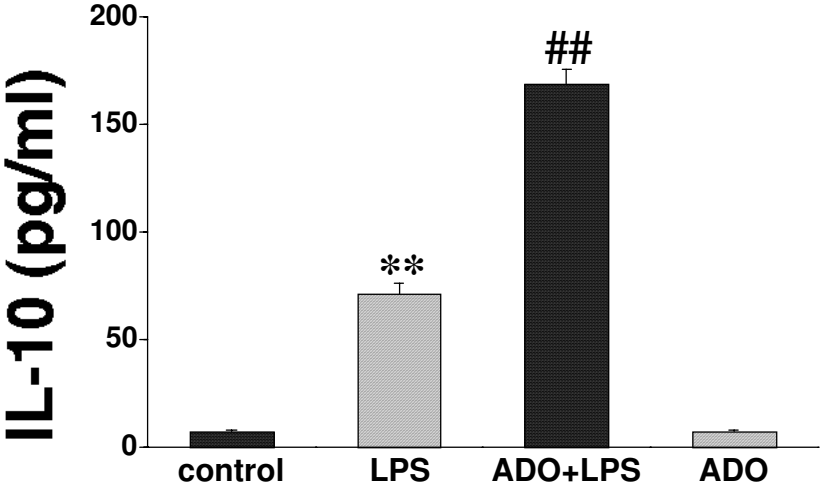


Figure 11.
Adenosine augments IL-10 production by RAW 264.7 macrophages. Data are expressed as the mean ± SEM of 8 wells.
**, indicates $p < 0.01$; and
##, indicates $p < 0.01$.

5. 5. Adenosine augments CREB-dependent transcriptional activity

Since IL-10 production can be increased following CREB activation (Hasko and Szabo, 1998), we next studied whether extracellular adenosine affected transcriptional activity of a CREB-firefly-luciferase construct transfected into RAW 264.7 macrophages. To control transfection efficacy, cells were cotransfected with pCMV-*Renilla*-luciferase vector. Exposure of the cells to adenosine (10-100 μM) increased the firefly/*Renilla* ratio as compared to vehicle-treated cells, which indicates that adenosine augments CREB-dependent transcriptional activity (Figure 12).

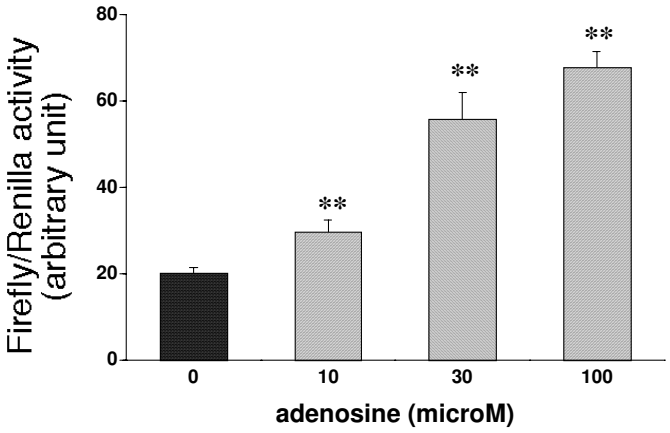


Figure 12.
Adenosine increases CREB-dependent transcriptional activity in macrophages. RAW 264.7 cells were transiently transfected with a firefly CREB-luciferase promoter construct and control *Renilla*-luciferase vector. Exposure of the cells to adenosine for 8 hours induced a significant increase in the Firefly/*Renilla* ratio. Data are mean ± SEM of n=6-8, **$p < 0.01$. This figure is representative of 3 separate experiments.

In the next set of experiments, we determined whether this increase in CREB- driven transcriptional activity following adenosine treatment was secondary to increased CREB DNA binding.

To assess CREB DNA binding, cells were treated with adenosine (100 μM) for 0, 30, and 90 minutes and nuclear extracts were prepared and subjected to EMSA using a CREB consensus oligonucleotide. The intensity of the only major CREB DNA binding complex (CREB-1) that was observed at the 0 minute time point did not change 30 or 90 min after adenosine administration (Figure 13). Furthermore, the composition of this complex was not altered by adenosine treatment, because it contained CREB-1 but not ATF-1 at all of the time points. That is because a CREB-1 antibody but not an ATF-1 antibody caused a complete supershift of this complex at all time points examined.

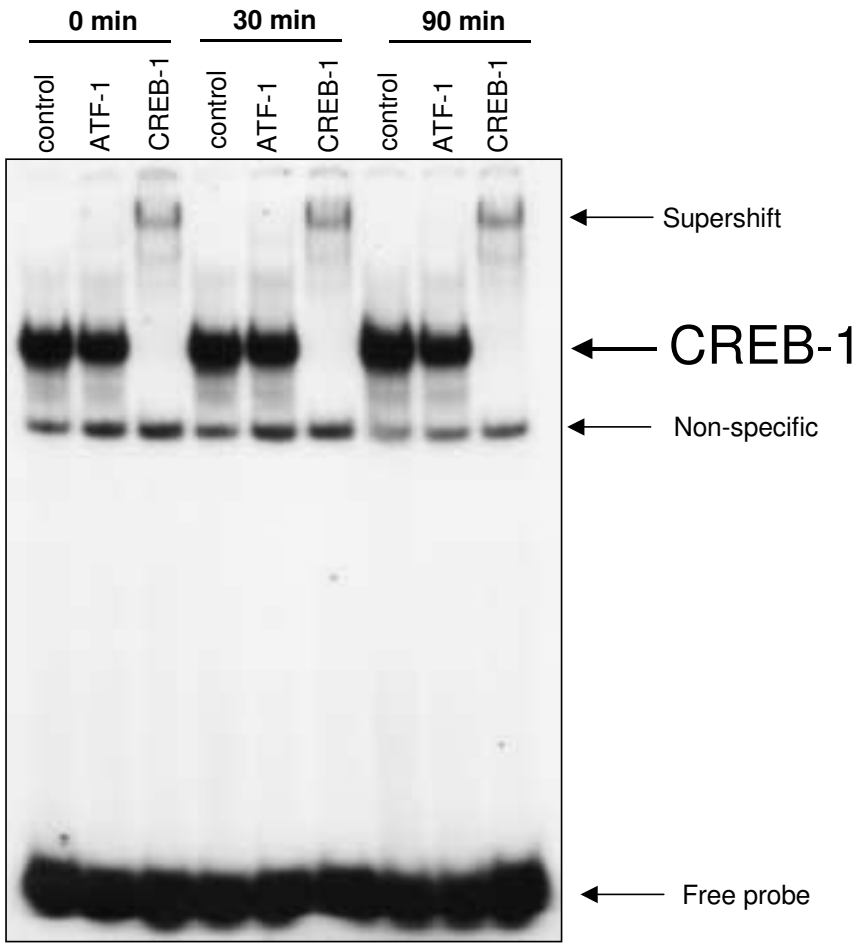


Figure 13.

Adenosine fails to affect CREB DNA binding in RAW 264.7 macrophages.

Cells were incubated with adenosine (100 μM) for 0, 30, or 90 min and after the incubation period nuclear extracts were prepared. DNA binding was determined from the nuclear extracts using EMSA. To determine the composition of the DNA binding complex, nuclear extracts were preincubated for 30 min with antibodies to CREB family members or an isotype control antibody. This figure is representative of 3 separate experiments.

5. 6. Adenosine induces CREB phosphorylation

Since adenosine did not alter CREB DNA binding, the next possibility was that theadenosine-induced increment in CREB transcriptional activity was a consequence of increased CREB phosphorylation on Ser133.

The reason for this proposition is that Ser133 phosphorylation of CREB is one of the predominant mechanisms by which CREB-driven transcriptional activity is stimulated (Shaywitz and Greenberg, 1999). Adenosine (100 μM) administration to RAW cells for 30 minutes elicited an increase in CREB phosphorylation (Figure 14), indicating that adenosine enhances CREB-driven transcriptional activity via CREB phosphorylation.

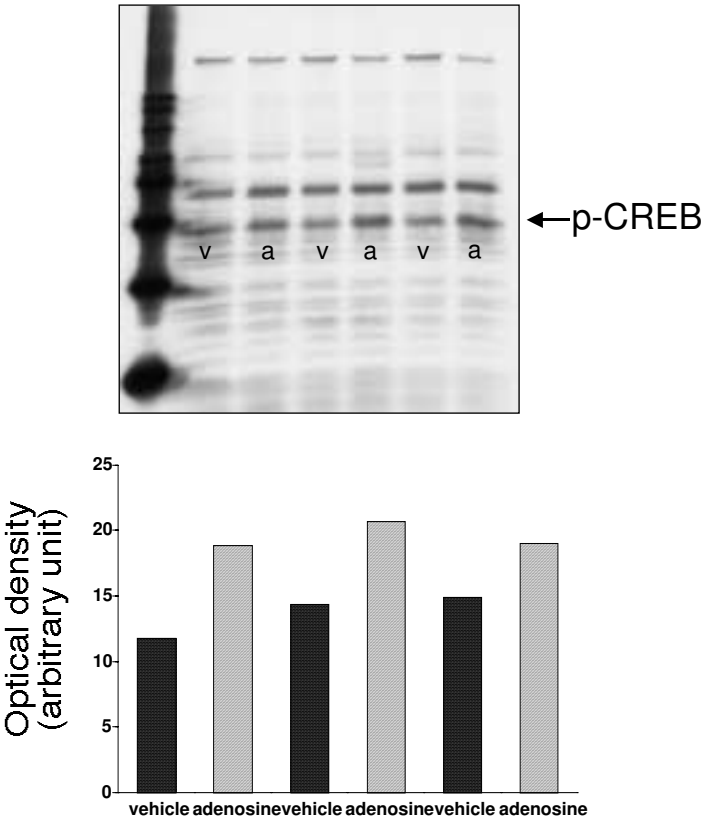


Figure 14.
Adenosine triggers phosphorylation of nuclear CREB on Ser133 in RAW 264.7 macrophages. Cells were treated with adenosine for 30 minutes, following which nuclear extracts were prepared.
CREB phosphorylation was assessed using Western blotting with an antibody raised against Ser133 phospho-CREB. This figure is representative of 3 separate experiments.
v-vehicle; a-adenosine

Extracellular adenosine has been reported to activate both p38 and p42/44 (Sexl et al., 1997; Dickenson et al., 1998; Grant et al., 2001; Feoktistov et al., 2001). In addition, both of these mitogen-activated protein (MAP) kinases have been shown to be intermediaries of CREB activation following extracellular stimuli (Tan 1996, Cammarota

2001). Therefore, we tested the possibility that either p38 or p42/44 was involved in mediating the stimulatory effect of adenosine on CREB transcriptional activity.

First, we examined whether adenosine triggered activation of p38 and p42/44 in RAW macrophages using Western blotting utilizing antibodies against the active, double-phosphorylated form of p38 and p42/44. We found that adenosine (100 μM) increased the activation of p38 (Figure 15).

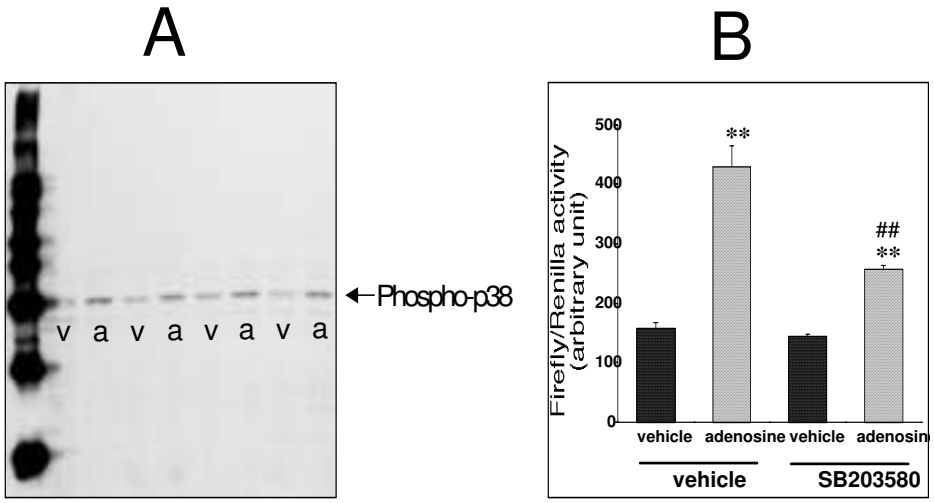


Figure 15.

Adenosine (100 μM) induces p38 activation in RAW 264.7 cells (A). Cells were incubated with adenosine for 30 minutes. p38 activation was determined from cytosolic extracts using Western blotting employing antibodies raised against the active, double-phosphorylated form of p38. This figure is representative of three separate experiments. Treatment of RAW 264.7 cells with the selective p38 pathway inhibitor SB203580 suppresses the adenosine-induced CREB transcriptional activity (B). To measure CREB-driven transcriptional activity, cells were transiently transfected with a firefly CREB-luciferase promoter construct and control *Renilla*-luciferase vector. Cells were treated with adenosine in the presence or absence of SB203580 for 8 hours. Firefly luciferase reporter activities were normalized against *Renilla* luciferase activities and CREB-driven transcriptional activity was expressed as the Firefly/*Renilla* ratio. Data are mean ± SEM of n=6-8 wells wells. Three experiments with similar results were performed. **p < 0.01 indicates a significant increase in CREB-transcriptional activity following adenosine administration. ## p < 0.01 indicates a significant suppression of adenosine-induced transcriptional activity by SB203580 treatment. v-vehicle; a-adenosine

Furthermore, adenosine enhanced activation of p42/44 with a more pronounced effect on p44 (Figure 15). To examine, whether this activation of p38 and p42/44 caused by adenosine contributed to the stimulatory effect of adenosine on CREB transcriptional activity, we next investigated whether MAP kinase inhibition decreased adenosine-stimulated CREB transcriptional activity. Treatment of RAW cells with the selective p38 pathway inhibitor SB203580 produced a blunting of the CREB transcriptional response to adenosine (Figure 15), because adenosine increased CREB transcriptional activity by 172% in vehicle-treated cells, whereas the adenosine-induced increase in CREB-driven transcriptional activity amounted to only 79% in SB203580-treated cells. On the other hand, the selective p42/44 pathway inhibitor PD98059 failed to affect CREB transcriptional activity, because adenosine increased CREB transcriptional activity to the same extent in both vehicle-treated and PD98059-treated cells (Figure 16).

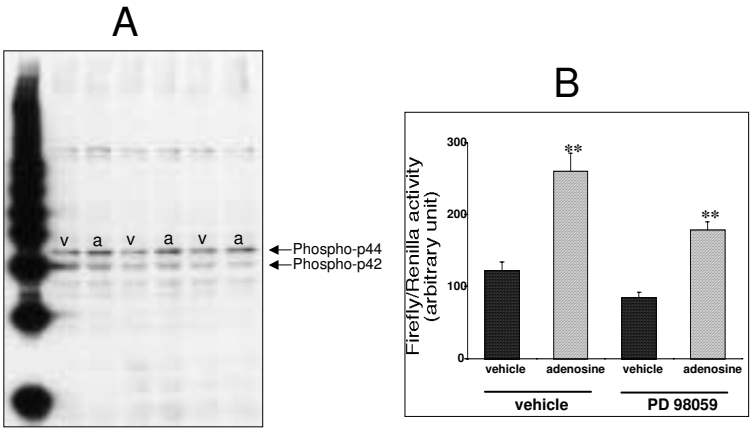


Figure 16.

Adenosine (100 μM) induces p42/44 activation in RAW cells (A). Cells were incubated with adenosine for 30 minutes. p42/44 activation was determined using Western blotting employing antibodies raised against the active, double-phosphorylated form of p42/44. This figure is representative of three separate experiments. Treatment of RAW cells with the selective p42/44 pathway inhibitor PD98059 fails to suppress adenosine-induced CREB transcriptional activity (B). Cells were transiently transfected with a firefly CREB-luciferase promoter construct and control *Renilla*-luciferase vector. RAW cells were treated with adenosine in the presence or absence of SB203580 for 8 hours. Firefly luciferase reporter activities were normalized against *Renilla* luciferase activities and CREB-driven transcriptional activity was expressed as the Firefly/*Renilla* ratio. Data are mean ± SEM of n=6-8 wells wells. Three experiments with similar results were performed. **p < 0.01 indicates a significant increase in CREB-transcriptional activity following adenosine administration. v-vehicle; a-adenosine

5. 7. Microarray analysis of gene expression in RAW 264.7 cells treated with adenosine and/or LPS

Stimulation with LPS induced a ≥ 2-fold induction of 98 genes after 3 hours (Table 3) whereas 32 genes were repressed ≥ 2-fold by LPS at this time point (Table 4).

Table 3.
Genes induced after 3 hours in LPS-stimulated RAW 264.7 macrophages

Accession number	Descriptions	LPS/control	Adenosine+LPS /LPS
M88242	glucocortoid-regulated inflammatory prostaglandin G/H synthase	48.10	0.73
M31418	Interferon activated gene 202	21.15	0.80
X87128	p75 TNF receptor	16.62	1.04
J04491	small inducible cytokine A3	15.64	0.94
D84196	tumor necrosis factor alpha	13.94	0.92
M31419	interferon activated gene 204	13.86	0.82
X67644	gly96	9.88	0.93
AF099973	schlafen2	9.44	0.90
U53219	GTPase IGTP	8.54	0.87
M12330	Ornithine decarboxylase	8.16	0.98
U23781	Hematopoietic-specific early-response A1-d protein	8.15	0.96
L32838	interleukin 1 receptor antagonist	7.97	1.01
AB011665	BAZF	7.92	0.82
L09737	GTP cyclohydrolase 1	7.80	1.00
X61800	CCAAT/enhancer binding protein (C/EBP), delta	7.47	0.77
U57524	I kappa B alpha	7.39	0.91
AF099974	schlafen3	7.27	0.77
U19118	transcription factor LRG-21	6.25	0.86
AJ007972	GTPI protein	6.24	0.86
L35528	manganese superoxide dismutase (MnSOD)	5.72	0.81
U23778	hematopoietic-specific early-response A1-b protein	5.49	0.97
M90551	intercellular adhesion molecule	4.89	0.81
U06924	signal transducer and activator of transcription 1	4.81	0.85
J03023	Hemopoietic cell kinase	4.57	0.88
Y14041	CASH alpha	4.53	0.92
U09507	cyclin-dependent kinase inhibitor 1A (P21)	4.51	1.08
AB013137	glutaredoxin	4.47	1.06
AF099977	schlafen4	4.42	0.85
U20159	76 kDa tyrosine phosphoprotein SLP-76	4.28	0.82
U05265	glycoprotein 49 B	4.28	0.89

M22998	solute carrier family 2 (facilitated glucose transporter)	4.26	0.81
L15435	tumor necrosis factor (ligand) superfamily, member 9	4.21	1.06
U60020	transporter 1, ABC (ATP binding cassette)	4.13	0.88
U94828	retinally abundant regulator of G-protein signaling mRGS-r (RGS-r)	4.11	1.17
Y13089	caspase-11	4.04	0.85
U40930	oxidative stress-induced protein	3.73	0.90
AJ001616	myeloid associated differentiation protein	3.73	0.85
AJ249706	myosin X (myo 10 gene)	3.69	0.94
M57999	nuclear factor of kappa light chain gene enhancer in B-cells 1	3.65	0.91
U16985	lymphotoxin-beta	3.56	0.71
J04103	E26 avian leukemia oncogene 2, 3 domain	3.55	0.88
X66084	CD44	3.53	0.89
L35049	Bcl2-like	3.53	0.68
X80638	rhoC	3.41	1.03
U09928	protein kinase, interferon inducible double stranded RNA dependent	3.37	1.00
U48403	glycerol kinase	3.37	0.88
AF052506	double-stranded RNA-specific adenosine deaminase	3.34	0.98
L10244	spermidine/spermine N1-acetyl transferase	3.26	0.89
K02236	metallothionein 2	3.12	0.92
M73696	Glvr-1	3.10	0.81
AF002719	secretory leukoprotease inhibitor gene	3.03	0.96
AJ242778	ABINl (A20-binding inhibitor of NF-kappa B activation	3.02	0.91
U35374	purine nucleoside phosphorylase (Np-b)	2.99	1.00
X61399	F52	2.96	0.89
M31312	beta Fc receptor type II (FCRII)	2.91	0.72
AF020772	importin alpha Q2	2.83	0.82
Z50159	Sui1	2.82	0.96
S46665	C5a anaphylatoxin receptor=peptidergic G-protein-coupled receptor	2.82	0.94
AF075136	Sin3-associated protein (sap30)	2.81	0.78
M65027	glycoprotein 49 A	2.80	0.80
Y08460	degenerative spermatocyte homolog	2.71	0.81
D63902	zinc finger protein 147	2.67	0.83
U49513	small inducible cytokine A9	2.57	0.96
U33626	promyelocytic leukemia	2.54	0.95
D86176	phosphatidylinositol 4-phosphate 5-kinase type I-alpha	2.49	0.92
Y17860	ganglioside-induced differentiation associated protein 10	2.48	0.86
U95498	AF1q	2.47	0.72
X60304	protein kinase C, delta	2.46	0.85
J04509	jun proto-oncogene related gene d1	2.38	0.91

X70956	TOP gene for topoisomerase I	2.38	0.83
U77461	complement component 3a receptor 1	2.34	0.85
U18869	mitogen-responsive 96 kDa phosphoprotein p96	2.33	0.86
M59821	growth factor-inducible protein (pip92)	2.31	0.84
L13732	natural resistance-associated macrophage protein 1	2.29	0.91
D13003	reticulocalbin	2.29	0.74
AB024427	Sid1669p	2.25	0.81
U68064	ceroid lipofuscinosis,	2.25	0.99
AF038008	tyrosylprotein sulfotransferase-1	2.23	0.91
AB033887	mACS4 variant2 mRNA for Acyl-CoA synthetase 4 variant 2	2.23	0.78
D13695	lymphocyte antigen 84	2.20	0.77
AJ009862	transforming growth factor-beta 1	2.19	0.92
AF020313	proline-rich protein 48	2.17	0.80
M34603	proteoglycan core protein	2.14	1.00
M59446	scavenger receptor	2.12	0.79
X76850	MAP kinase-activated protein kinase 2	2.09	0.94
V00756	interferon beta	2.09	0.81
AF061272	C-type lectin (Mcl) /	2.07	0.93
X07888	3-hydroxy-3-methylglutaryl coenzyme A reductase	2.07	0.88
Y15163	mrg1 protein	2.06	0.82
X54056	proprotein convertase subtilisin/kexin type 3	2.06	0.95
AF033186	WSB-1 mRNA	2.04	0.76
L02526	protein kinase, mitogen activated, kinase 1, p45	2.02	0.84
D89728	LOK	2.02	0.83
V00835	metallothionein 1	2.02	0.93
D87691	eRF1	2.01	0.86
M35247	histocompatibility 2, T region locus 17	2.01	0.93
X84797	similar to human hematopoietic specific protein 1	2.00	1.02
AB027565	TXNRD1 mRNA for thioredoxin reductase 1	2.00	0.92

Data are the average of 4 independent experiments and were analyzed as described in Materials and Methods. Genes shown were induced ≥ 2-fold.

However none of the LPS-induced induced genes, including the NF-κB-regulated ones, such as TNF-α, IκBα, and IL-1 receptor antagonist were altered by at least 1.5 fold by adenosine. In addition, none of the LPS-repressed genes were changed (at least 1.5 fold) by adenosine treatment. Adenosine (no LPS) treatment did not affect gene expression as compared to treatment with vehicle (no LPS). Interestingly, while the A_{2a} receptor mRNA was not expressed in either LPS-untreated or LPS-treated cells, the mRNA for A_{2b} receptor was not present in LPS non-stimulated cells, but became detectable in LPS-stimulated cells (data not shown).

Table 4.

Genes down-regulated after 3 hours in LPS-stimulated RAW 264.7 macrophages.

Accession number	Descriptions	Control/LPS	Control/ Adenosine+LPS
X57687	LYL gene (clone L6)	7.69	9.09
M26270	stearoyl-coenzyme A desaturase 2	3.57	5
AJ007360	ORC5-related protein	2.85	3.33
U07159	acetyl coenzyme A dehydrogenase, medium chain	2.85	2.63
U70674	m-Numb (m-nb)	2.77	2.7
M33988	mouse histone H2A.1 gene	2.63	2.63
U23921	osmotic stress protein 94 (Osp94)	2.63	2.5
U80932	serine/threonine kinase 6	2.63	2.7
L26320	flap structure specific endonuclease 1	2.63	2.63
U67187	G protein signaling regulator RGS2 (rgs2)	2.5	2.43
M29260	mouse histone 1-0 gene	2.5	2.63
AF053959	putative ras effector Nore1	2.5	3.7
Z47766	cyclin F	2.38	2.56
X86000	N-glycan alpha 2,8-sialyltransferase	2.38	2.94
M58566	TIS11 primary response gene	2.32	3.33
AF074600	LIM domain transcription factor LMO4	2.32	2.32
D90374	APEX nuclease	2.32	2.63
L07508	Golli-mpb	2.27	2.5
V00727	FBJ osteosarcoma oncogene	2.27	2.85
U22262	apolipoprotein B editing complex 1	2.27	2.56
AF016583	checkpoint kinase Chk1 (Chk1)	2.22	2.43
L38822	Max interacting protein 1	2.17	2.38
U75680	histone stem-loop binding protein (SLBP) mRNA	2.08	2.85
X53176	integrin alpha 4 (Cd49d)	2.08	2.43
X82786	antigen identified by monoclonal antibody Ki 67	2.08	2.85
AF100956	major histocompatibility locus class II region; Fas-binding protein Daxx (DAXX) gene	2.04	1.96
U25691	helicase, lymphoid specific	2.04	2.7
AF012923	p53-inducible zinc finger protein (Wig-1)	2.04	2.7
X75316	seb4	2.04	1.92
M97632	gamma-aminobutyric acid transporter protein (GABA transporter)	2.04	2.17
Z48745	ATP-binding cassette 8	2	2.38
AF017085	BAP-135 homolog	2	2.5

Data are the average of 4 independent experiments and were analyzed as described in Materials and Methods. Genes shown were decreased ≥ 2-fold.

5. 8. RT-PCR analysis of TNF-α and A2b receptor gene expression

As shown in Figure 17, RT-PCR analysis confirmed that TNF-α mRNA was induced by LPS but was not affected by adenosine pretreatment. Furthermore, the A_{2b} receptor was upregulated in response to LPS, but was unchanged in adenosine-pretreated cells. Finally, it was confirmed using RT-PCR that the A2a receptor was not expressed in RAW cells (data not shown).

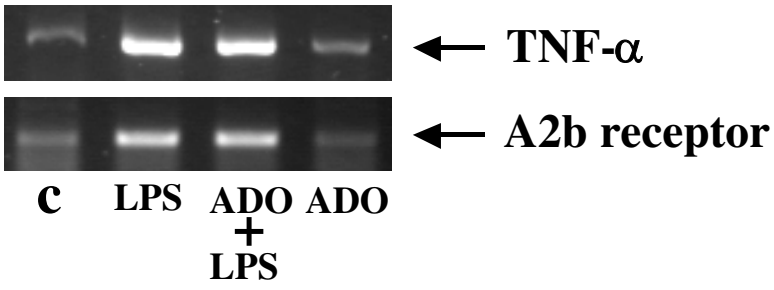


Figure 17.

LPS treatment for three hours induces mRNA accumulation of both TNF-α and the A_{2b} adenosine receptor in RAW 264.7 cells.

Adenosine pretreatment 30 min before LPS fails to affect mRNA levels of either TNF-α or the A_{2b} adenosine receptor. Furthermore, adenosine alone has no effect on mRNA of either TNF-α or the A_{2b} adenosine receptor compared to LPS-unstimulated (c) cells.

mRNA levels of both TNF-α and the A_{2b} adenosine receptor were quantitated using RT-PCR.

6. DISCUSSION

We examined the possibility that the modulatory effects of extracellular adenosine and adenosine receptor agonists on cytokine production observed in macrophages were mediated by interference with activation of the NF-κB and/or CREB transcription factor systems. One of the major finding of our study is that despite the fact that adenosine receptor stimulation decreased both extracellular and intracellular concentrations of TNF-α, a prototype NF-κB-regulated proinflammatory cytokine, adenosine did not interfere with NF-κB activation. There are three lines of evidence to support this proposition. First, adenosine as well as a series of adenosine receptor agonists failed to decrease DNA binding of NF-κB. Secondly, adenosine was unable to decrease NF-κB-driven promoter activity of a luciferase construct. Finally, global analysis of gene expression using cDNA microarray demonstrated that while LPS induced expression of a number of NF-κB regulated genes, adenosine failed to alter this response.

While these results argue against a role of NF-κB and even a transcriptional effect of adenosine in macrophages, there are several caveats that need to be discussed. First, gene expression was assessed only at the 3-hour time point, whereas it is possible that adenosine may affect gene expression at other time points. Secondly, although adenosine itself had no effect on the expression of cytokine mRNAs in the current study using RAW 264.7 macrophages, we found that the selective A_3 adenosine receptor agonist IB-MECA decreased MIP-1α mRNA levels in the same cell type in an earlier study (Szabó et al., 1998). Since adenosine itself is a relatively week agonist at A_3 receptors (Linden 2001) it is possible that selective A_3 receptor stimulation can decrease the levels of cytokine mRNAs.

The mechanism of action for the macrophage deactivating effect of adenosine is incompletely understood. A recent study by Sajjadi et al. (1996) demonstrated that adenosine decreased TNF-α mRNA steady state levels in an LPS-stimulated human monocytic cell line, which results are contradictory to our findings in LPS-stimulated mouse macrophages showing a failure of adenosine to inhibit TNF-α mRNA accumulation. Nevertheless, this reduction in TNF-α mRNA steady state levels following

adenosine receptor stimulation in human macrophages was not associated with a decrease in NF-κB activation. On the other hand, it appears that under certain conditions, adenosine can decrease NF-κB activation. For example, adenosine suppressed NF-κB activation in both myeloid and lymphocytic, as well as epithelial cells, when TNF-α but not when LPS was used to stimulate the cells (Majumdar and Aggarwal, 2003). Clearly, further studies will be necessary to dissect the signaling pathways whereby adenosine exerts its anti-inflammatory effects.

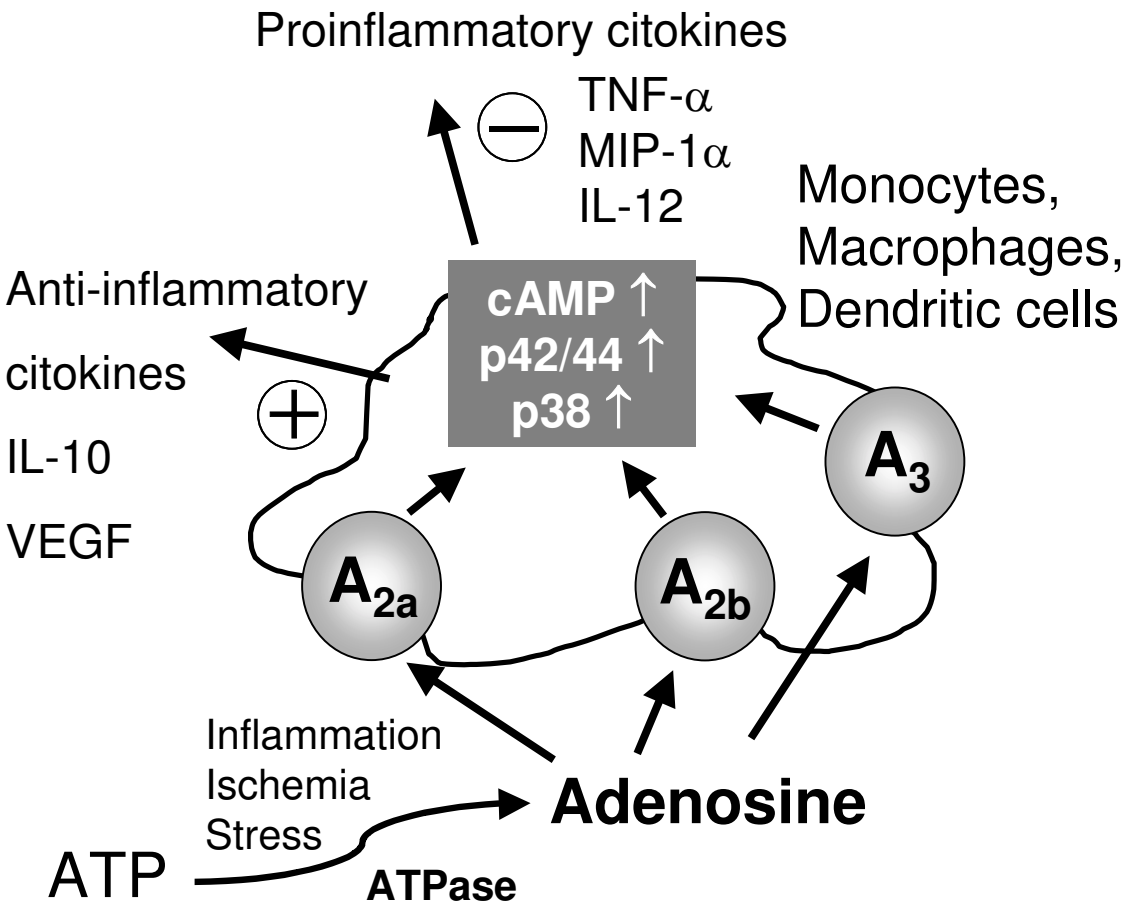


Figure 18.
Schematic representation of various intracellualar pathways whereby adenosine receptor agonists modulate immune cell functions
Adenosine receptor occupancy signals through alteration of intracellular cyclic AMP and Ca2+ concentrations and induction of the mitogen-activated protein kinases p38 and p42/44. Adenosine receptor occupancy on monocytes and macrophages diminishes production of the proinflammatory mediators TNF-α, IL-12, MIP-1α and nitric oxide while augmenting secretion of the anti-inflammatory cytokine IL-10 and VEGF.

It is also important to point out that at this point it is unclear, which receptors mediated the suppressive effect of adenosine on TNF-α production in the current study. While the general view is that the A_{2a} receptor may be the most important one in regulating cytokine production and macrophage activation (Cronstein, 1998), it is clear that this was not the case here. That is because the microarray analysis found no A_{2a} receptor mRNA expression in the RAW cells. In addition, in our previous study (Szabó et al., 1998), the selective A_{2a} receptor CGS-21680 was much less potent (EC 50 in the low micromolar range) in suppressing MIP-1α production by RAW cells than would have been expected. On the other hand, in a study utilizing primary peritoneal macrophages (Haskó et al., 2000), we found that the potency of CGS-21680 in decreasing cytokine production was much more consistent with an effect on A_{2a} receptors (EC 50 in the nanomolar range). A further support for the role of A_{2a} receptors in peritoneal cells came from the observation that CGS-21680 lost its efficacy in cells taken from A_{2a} receptor knockout mice (Haskó et al., 2000). Nevertheless, adenosine itself, although to a lesser extent, was still capable of decreasing cytokine production by peritoneal cells from A_{2a} knockout mice, suggesting that both A_{2a} and other receptors are involved in the anti-inflammatory effects of adenosine. Since RAW 264.7 cell do not appear to express A_{2a} receptors, this cell line may be a powerful tool to study the A_{2a} receptor-independent effects of adenosine on macrophage function.

Interestingly, the results presented in the current study found evidence, for the first time, for a profound upregulation of A_{2b} receptors following LPS stimulation. Thus, the A_{2b} receptor may have been a possible mediator of the anti-inflammatory effects of adenosine in RAW cells. Of note, it has been reported that IFN-γ up-regulates A_{2b} receptor expression in murine bone marrow-derived macrophages, and through this receptor, adenosine suppresses the induction of inducible nitric oxide synthase expression (Xaus et al., 1999).

In addition to the NF-κB transcription system we also investigated the possible involvement and function of CREB transcriptional pathway. Our study provides evidence for the first time that adenosine activates the CREB transcription factor system in macrophages. Thus, macrophages can be added to the growing list of cell types, including intestinal epithelial cells (Sitaraman et al., 2001) endothelial cells (Grant et al., 2001),

smooth muscle cells (Yaar et al., 2002), neurons (Cheng et al. 2002), and skeletal muscle cells (Lynge et al., 2003) that exhibit an increment in CREB activation following adenosine receptor occupancy. This adenosine-induced activation of CREB transcriptional activity in macrophages follows the conventional route of CREB activation (Shaywitz and Greenberg, 1999; Mayr and Montminy, 2001) in that it is regulated primarily by Ser133 phosphorylation of CREB without major changes in CREB DNA binding. Another major novel finding of the current study is that adenosine stimulates the phosphorylation of both p38 and p42/44 in macrophages. In addition, p38 activation but not p42/44 activation contributes to the adenosine-elicited increase in CREB transcriptional activity, because blockade of the p38 pathway decreases adenosine-induced CREB activation.

These results raise the interesting question of which genes are the targets of adenosine-induced CREB activation in macrophages. In a recent study using cDNA microarray analysis of approximately 12,000 genes, we found that adenosine exposure of RAW macrophages failed to induce expression of any gene when measured 3.5 hours after adenosine treatment (Németh et al., 2003b). Furthermore, while the prototypical macrophage activating agent lipopolysaccharide stimulated expression of a few hundred genes, adenosine co-treatment of the cells did not alter the expression of these LPS-induced genes (Németh et al., 2003b). One obvious explanation for our failure to detect any effect of adenosine on gene expression despite a substantial stimulation of CREB activation is that mRNA levels of CREB-induced genes may have subsided by 3.5 hours after adenosine treatment. This possibility is underlined by the general observation that the CREB transcriptional response to extracellular stimuli is very rapid and mRNA levels of CREB-regulated genes fade by 3-4 hours post-stimulation (Shaywitz and Greenberg, 1999; Mayr and Montminy, 2001). A further possibility is that CREB activation itself is not sufficient to activate gene transcription in these cells. Rather, CREB activation may play a regulatory role in gene expression stimulated by some other pathway. It has to be emphasized at this point that it is possible that the expression IL-10 was upregulated following adenosine administration, however, IL-10 did not show up on the gene chip. This lack of expression of IL-10 mRNA on the gene chip is not completely unexpected, because IL-10 mRNA is notoriously difficult to detect due to the low copy number of

mRNA for this cytokine. Another possibility, supported by previous data is that IL-10 mRNA has a delayed kinetics following LPS stimulation of macrophages and the 3 hour time point may have been too early for the generation of sufficient IL-10 mRNA levels. Further studies will be required to exactly pinpoint the mechanisms whereby adenosine enhances IL-10 production by LPS-stimulated macrophages.

7. SUMMARY

While it is likely that adenosine receptor ligation targets a common major intracellular pathway to exert a general anti-inflammatory effect in macrophages, the nature of this intracellular target remains unclear. The possibility that NF-κB, a central transcription factor mediating most of the proinflammatory effects of LPS, could be such a target was dismissed, since not only adenosine failed to decrease LPS-mediated NF-κB activation but it also had no effect on cytokine transcript levels as assessed by cDNA array analysis. Thus, our data seem to support the proposition that many of the regulatory actions of adenosine on cytokine production are post-transcriptional.

On the other hand, our results demonstrating that adenosine induces CREB activation, uncovers a major intracellular regulatory mechanism, which may explain some of the changes in macrophage phenotype following adenosine exposure. Furthermore, since both p38 and p42/44 are central factors in conveying signals from the extracellular space to regulate cell function, activation of these two MAPKs by adenosine may represent an important mechanism contributing to macrophage deactivation under conditions of ischemia, inflammation, and sepsis. Future studies will be required to examine how these various pathways initiated by extracellular adenosine regulate macrophage function.

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- Department of Medical Chemistry, Medical and Health Science Center, University of Debrecen, Debrecen, Hungary.

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10. APPENDIX

10. 1. Relevant publications (in chronological order)

Peer Reviewed Articles:

1. Haskó G, Szabó C, **Németh ZH**, Kvetan V, Pastores SM, and Vizi ES. (1996) Adenosine receptor agonists differentially regulate IL-10, TNF-α, and nitric oxide production in RAW 264.7 macrophages and in endotoxemic mice. *J Immunol* 157:4634-4640.
   Impact Factor: 7.014

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