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cDNA microarray analysis reveals a nuclear factor-κB-independent regulation of macrophage function by adenosine

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Running title: Adenosine and NF-κB

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Abbreviations used: CGS-21680, 2-p-(2-carboxyethyl) phenethylamino-5'-*N*-ethyl-carboxamidoadenosine; CPA, N^6-cyclopentyladenosine; CCPA, 2-chloro-N^6-cyclopentyladenosine; CHA, N^6-cyclohexyladenosine; EMSA, electromobility shift assay; IB-MECA, N^6-(3-iodobenzyl)-adenosine-5'-N-methyluronamide; LPS, IκB, inhibitory κB; bacterial lipopolysaccharide; MIP, macrophage inflammatory protein; NECA, 5'-N-ethylcarboxamidoadenosine; NF, nuclear factor; R-PIA, N^6-phenylisopropyladenosine; RIPA, radioimmunoprecipitation; TNF, tumor necrosis factor

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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 by these cells. However, the mechanisms of this anti-inflammatory effect have not been well characterized. 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 bacterial lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophages, we found that adenosine as well as adenosine receptor agonists decreased the production of tumor necrosis factor (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 A2b 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 degradation. Taken together, our results suggest that the anti-inflammatory effects of adenosine are independent of NF-κB.

Adenosine is an endogenous nucleoside that regulates a variety of physiological processes including function of the central nervous, circulatory and gastrointestinal systems (Fredholm et al., 2001). Adenosine has also been implicated as a regulator of a number of pathophysiological conditions, including ischemic processes and inflammatory states (Dubyak and el-Moatassim, 1993; Meldrum et al., 1993; Cohen et al., 2000; Narravula et al., 2000; Chunn et al., 2001; Linden, 2001; Sitaraman et al., 2001; Banerjee et al., 2002; Okusa, 2002; Sitkovsky, 2003). The adenosine modulation of these pathophysiological processes is mediated, in a large part, by effects on the innate immune system (Cronstein, 1994; Haskó et al., 2002b).

Monocytes/macrophages have recently emerged as prime targets of the immunomodulatory effects of adenosine (Haskó and Szabó, 1998; Haskó et al., 2002b). Adenosine exerts its biological effects by engaging cell surface receptors. Adenosine receptors have been subdivided according to molecular, biochemical, and pharmacological evidence into four subtypes, which are the A1, A2a, A2b and A3 receptors (Ralevic and Burnstock, 1998). Cells of the monocyte/macrophage lineage have been documented to express all four adenosine receptors (Haskó and Szabó, 1998; Haskó et al., 2002b). In most in vitro and in vivo studies utilizing macrophages, stimulation of adenosine receptors has been shown to result in an anti-inflammatory, deactivated macrophage phenotype (Haskó and Szabó, 1998; Haskó et al., 2002b). In bacterial lipopolysaccharide (LPS)-stimulated monocytes/macrophages, adenosine receptor stimulation reduces the production of a variety of proinflammatory cytokines, including tumor necrosis factor (TNF)-α (Bouma et al., 1994; Haskó et al., 1996; McWhinney et al., 1996;

Sajjadi et al., 1996; Mayne et al., 1999; Elenkov et al., 2000; Mayne et al., 2001; Leibovich et al., 2002), interleukin-12 (Hasko et al., 2000, Link et al., 2000; Khoa et al., 2001, Bshesh et al., 2002), and macrophage inflammatory protein (MIP)-1α (Szabó et al., 1998). Furthermore, adenosine receptor stimulation prevents the induction of inducible nitric oxide synthase and the formation of nitric oxide (Haskó et al., 1996; Xaus et al., 1999). In addition to the suppressive effect of adenosine on the production of these various soluble macrophage products, adenosine decreases expression of the membrane protein major histocompatibility complex II (Edwards et al., 1994; Xaus et al., 1999).

Based on the above evidence that adenosine suppresses the expression of proinflammatory chemokines, inducible nitric oxide synthase, and major histocompatibility complex II, which are all dependent on the transcription factor nuclear factor κB (NF-κB) (Baeuerle and Henkel 1994; Karin and Ben-Neriah, 2000; Haddad, 2002), we hypothesized that adenosine exerts its anti-inflammatory effects by diminishing the activation of this transcription factor system.

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Materials and Methods

Cell culture

The mouse macrophage cell line RAW 264.7 was grown in Dulbeccos's modified Eagle's Medium supplemented with 10% fetal bovine serum, 50 U/ml penicillin, 50 μg/ml streptomycin, and 1.5 mg/ml sodium bicarbonate 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-cyclopentyladenosine (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, MTT, and LPS (*Escherichia coli* 055:B5) were purchased from Sigma (St. Louis, MO).

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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, as we have previously described (Haskó et al., 2002a).

NF-κB 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., 2002). 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) 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 EMSA. The NF-κB consensus oligonucleotide probe used for the EMSA was purchased from Promega. Oligonucleotide probes were labeled with [γ-^{32}P] ATP using T4 polynucleotide kinase (Invitrogen) 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 dithiothreitol) as well as 15 ng/μl poly(dI)-poly(dC), 0.4 ng/μl of ssDNA, and 0.2 mg/ml of bovine serum albumin 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) 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°C with an intensifying screen. For 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, Santa Cruz, CA).

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 1 day prior to transient transfection. Cells were transfected with 15 μl/ml of FuGENE 6 Transfection Reagent (Roche, Indianapolis, IN) in 160 μl of medium per well. The medium contained 5 μg/ml of DNA containing a NF-κB luciferase promoter construct (Clontech, San Diego, CA). After an overnight transfection, 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. 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., 2002).

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Western blot analysis of inhibitory κB (IκB) and p65

p65 levels were analyzed using the nuclear extracts prepared for the EMSAs. 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, Carlsbad, CA) 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, Indianapolis, IN). Bands were detected using ECL Western Blotting Detection Reagent (Amersham Pharmacia Biotech, Piscataway, NJ).

Affymetrix gene chip analysis

RAW cells were plated in 6-well plates 1 day prior to the experiment. The cells were activated with LPS 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) 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 (Perkin, 20 U/µl), 0.5 µl 50 mM oligo d(T) and 0.3 µl of reverse transcriptase (Roche, 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). 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. cDNA was amplified using the following primers and conditions: TNF-α (Murray et al., 1990) - 5'-GGCAGGTCTACTTTGGAGTCATTGC -3' (sense) and 5'-ACATTCGAGGCTCCAGTGAATTCGG -3' (antisense); A2b receptor (Zhao et al., 2002)- 5'-TGGCGCTGGAGCTGGTTA -3' (sense) and 5'- GCAAAGGGGATGGCGAAG -3' (anti-sense), A2a receptor (Hoskin et al., 2002) 5'-CACGCAGAGTTCCATCTTCA-3' (sense) and 5'-AGCAGTTGATGATGTGCAGG-3' (antisense), an initial denaturation at 94˚C X 5 min, 22, 30, and 30 cycles of 94˚C x 30 s for TNF-α, A2b receptor, and A2a 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., 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., 2002). 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.

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.

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Results

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 (Fig. 1A). 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 (Fig. 1B). 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.

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

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showed that treatment of the cells with LPS induced the appearance of intracellular TNF-α, which was suppressed by adenosine pretreatment (Fig. 2). These results indicate that adenosine does not interfere with the release process of TNF-α.

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 Fig. 3A and B, 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 (Fig. 3A, right panel). However, neither adenosine (Fig. 3A) nor adenosine receptor agonists (Fig. 3B) affected this induction of NF-κB DNA binding. Further, adenosine did not prevent either the LPS-induced accumulation of p65 in the nucleus or LPS-elicited IκB degradation (Fig 4).

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

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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 (Fig. 5). Finally, adenosine alone failed to affect NF-κB-dependent gene transcription (data not shown).

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 1), whereas 32 genes were repressed ≥2-fold by LPS at this timepoint (Table 2). 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 A2a receptor mRNA was not expressed in either LPS-untreated or LPS-treated cells, the mRNA for A2b receptor was not present in LPS non-stimulated cells, but became detectable in LPS-stimulated cells (data not shown).

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

As shown in Fig. 6, RT-PCR analysis confirmed that TNF-α mRNA was induced by LPS but was not affected by adenosine pretreatment. Furthermore, the A2b 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).

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Discussion

In this paper, we examined the possibility that some of the anti-inflammatory effects of extracellular adenosine and adenosine receptor agonists observed in macrophages are mediated by a suppressive effect on the NF-κB transcription factor system. 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 A3 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 A3 receptors (Linden 2001), it is possible that selective A3 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.

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 A2a 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 A2a receptor mRNA expression in the RAW cells. In addition, in our previous study (Szabó et al., 1998), the selective A2a 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 A2a receptors (EC 50 in the nanomolar range). A further support for the role of A2a receptors in peritoneal cells came from the observation that CGS-21680 lost its efficacy in cells taken from A2a 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 A2a knockout mice, suggesting that both A2a and other receptors are involved in the anti-inflammatory effects of adenosine. Since RAW 264.7 cell do not appear to express A2a receptors, this cell line may be a powerful tool to study the A2a 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 A2b receptors following LPS stimulation. Thus, the A2b 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 A2b 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).

Taken together, it appears that the anti-inflammatory effects of adenosine may be mediated by different receptors depending on the experimental conditions. Consequently, it can be proposed that the intracellular pathways mediating the anti-inflammatory effects of adenosine in macrophages may vary with the adenosine receptors expressed and/or coupling mechanisms.

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Footnotes

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Legends to the Figures

Figure 1. (A) Adenosine inhibits TNF-α production by RAW 264.7 macrophages stimulated with LPS (10 μg/ml) for 4 hours. (B) 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 $p < 0.05$; **, indicates $p < 0.01$.

Figure 2. 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 $p < 0.01$.

Figure 3. Adenosine (ADO) pretreatment (100 μM) fails to decrease LPS (10 μg/ml)-induced NF-κB DNA binding in RAW 264.7 cells (A). Pretreatment with a variety of adenosine receptor agonists (10 μM) does not affect LPS (10 μg/ml)-induced NF-κB DNA binding in these cells (B). a: no LPS; b: LPS; c: agonist+LPS. 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. This figure is representative of 3 separate experiments.

Figure 4. 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 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.

Figure 5. Adenosine pretreatment (100 μM) has no effect on LPS-induced NF-κB-dependent transcriptional activity in RAW 264.7 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

Figure 6. LPS treatment for 3 hours induces mRNA accumulation of both TNF-α and the A2b adenosine receptor in RAW 264.7 cells. Adenosine pretreatment 30 min before LPS fails to

affect mRNA levels of either TNF-α or the A2b adenosine receptor. Furthermore, adenosine alone has no effect on mRNA levels of either TNF-α or the A2b adenosine receptor as compared to LPS-unstimulated (c) cells. mRNA levels of both TNF-α and the A2b adenosine receptor were quantitated using RT-PCR.

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Table 1. 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

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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.

Table 2. 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.

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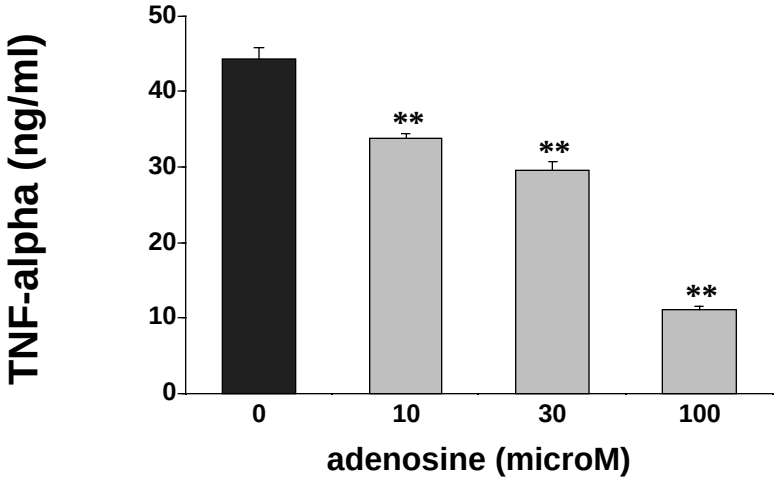

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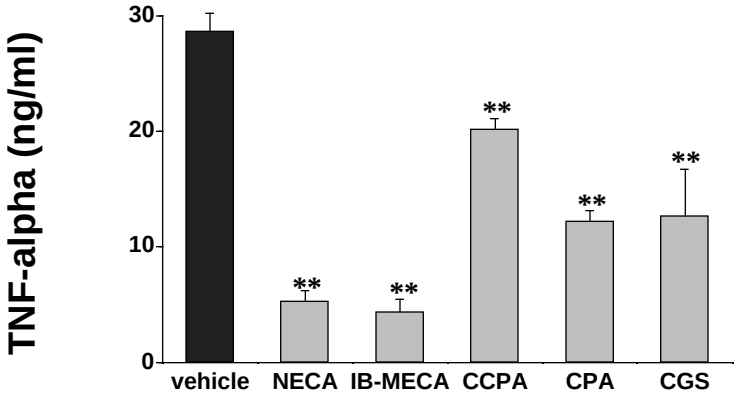

Fig. 1

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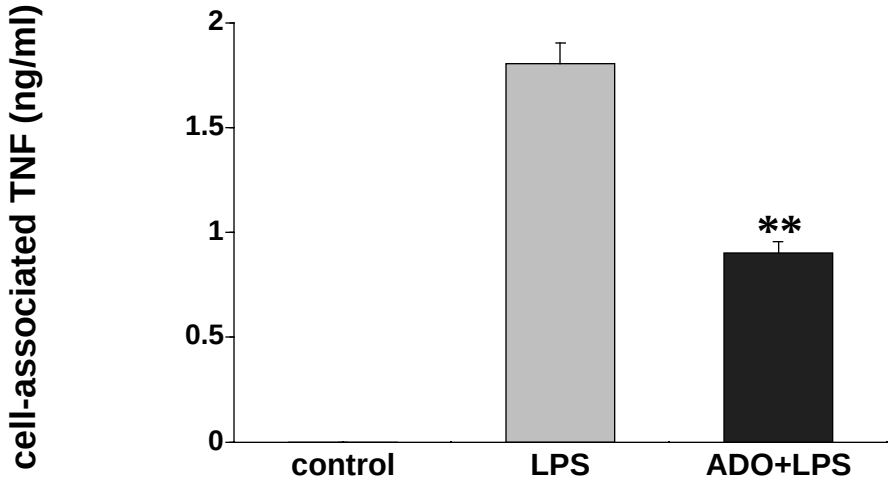

Fig. 2

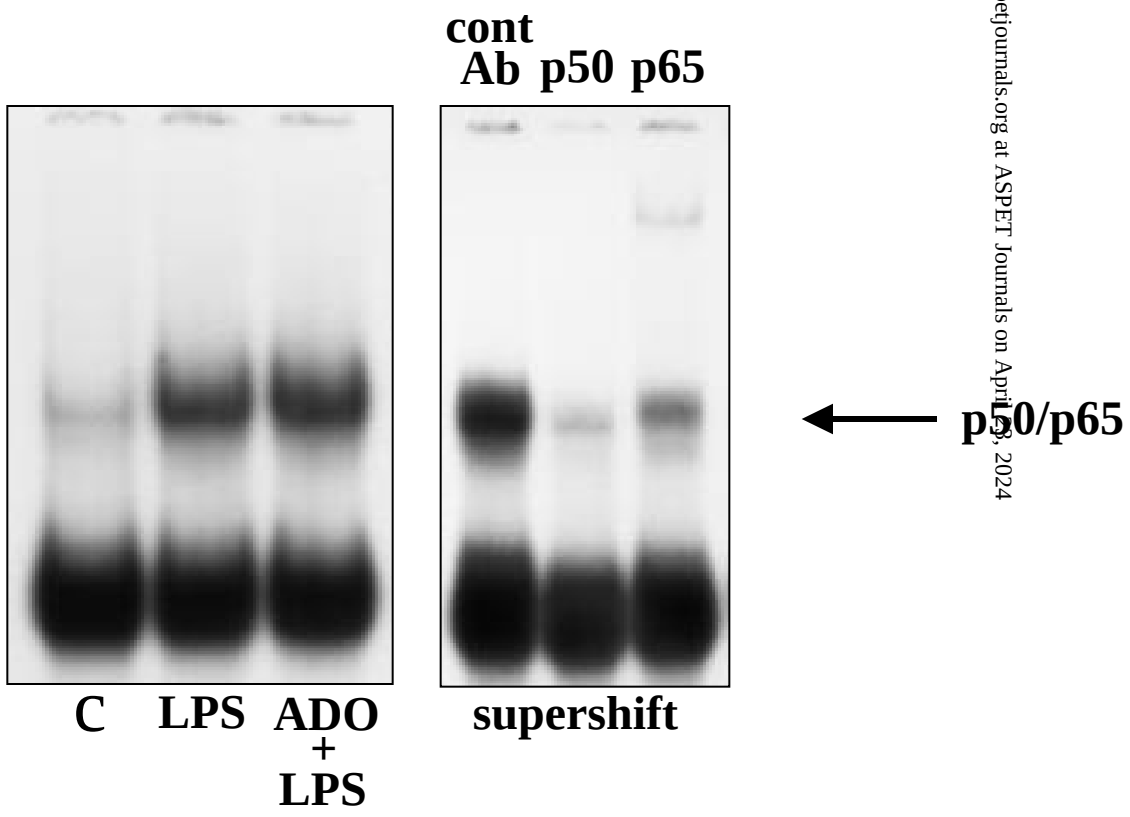

Fig. 3A

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Fig. 3B

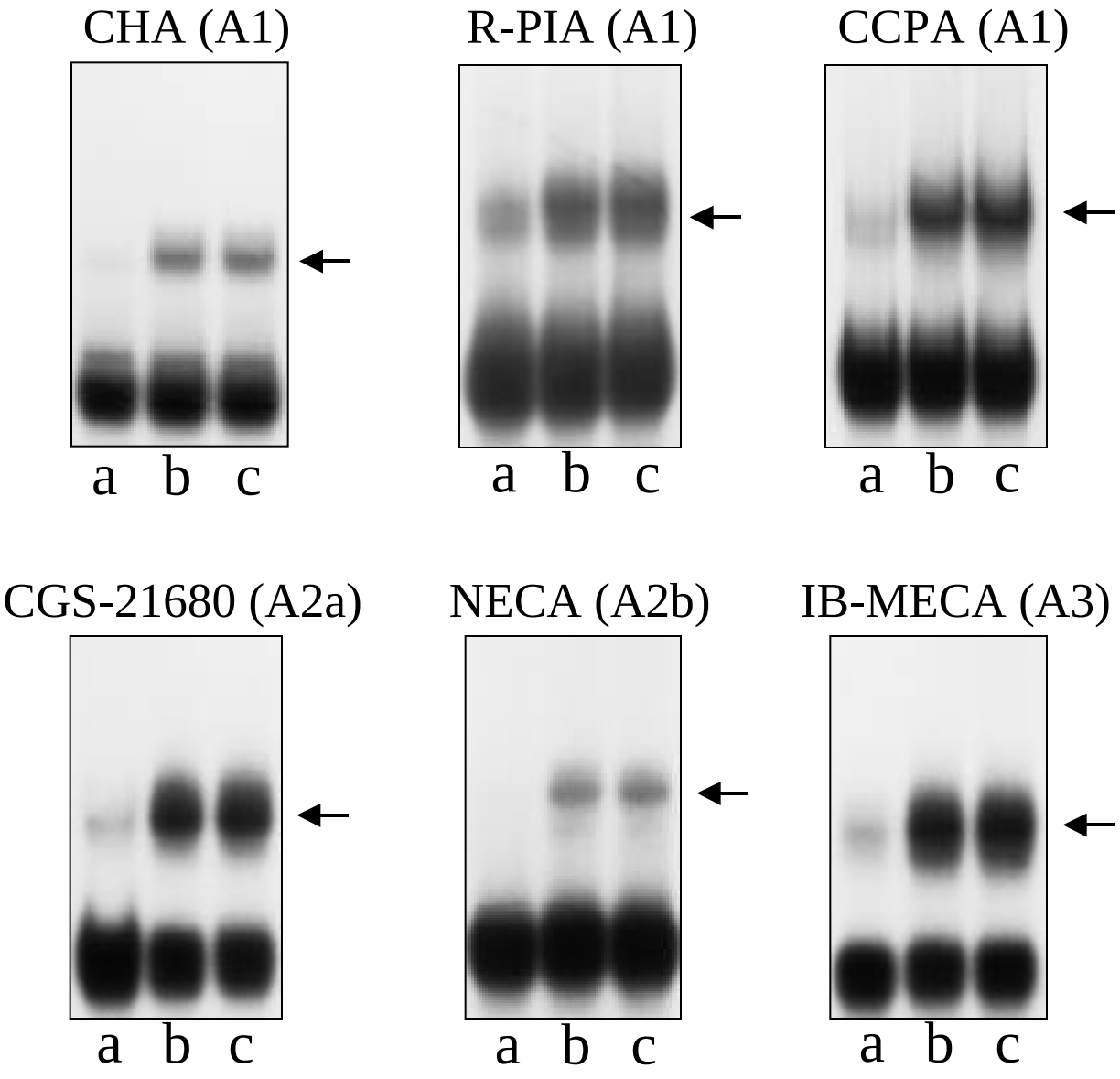

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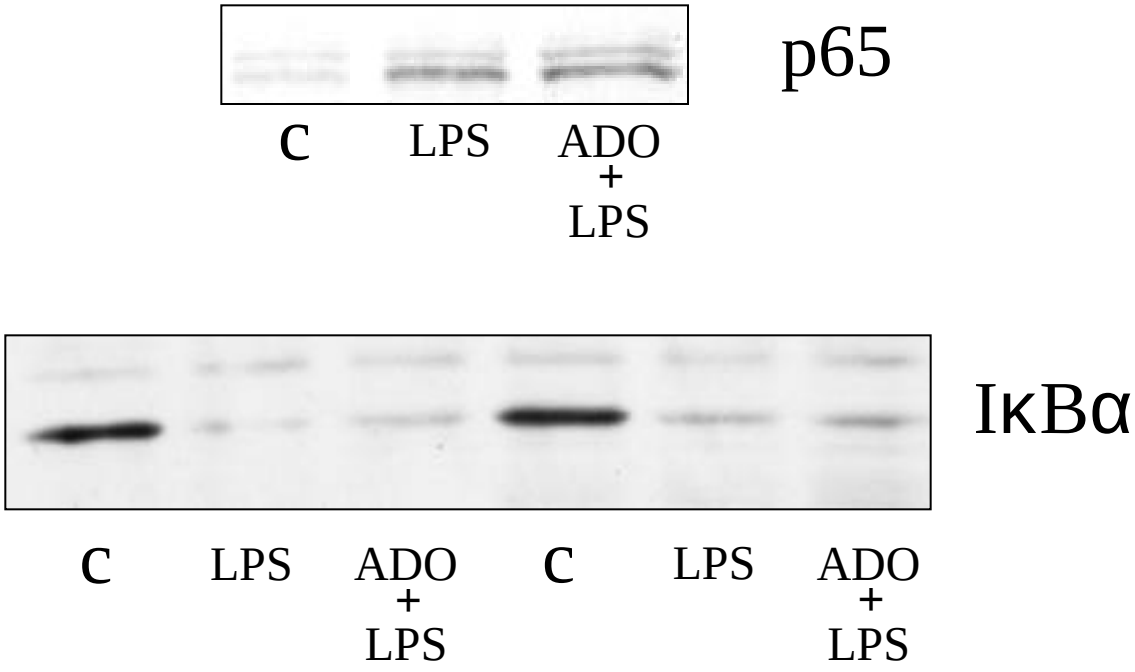
Fig. 4



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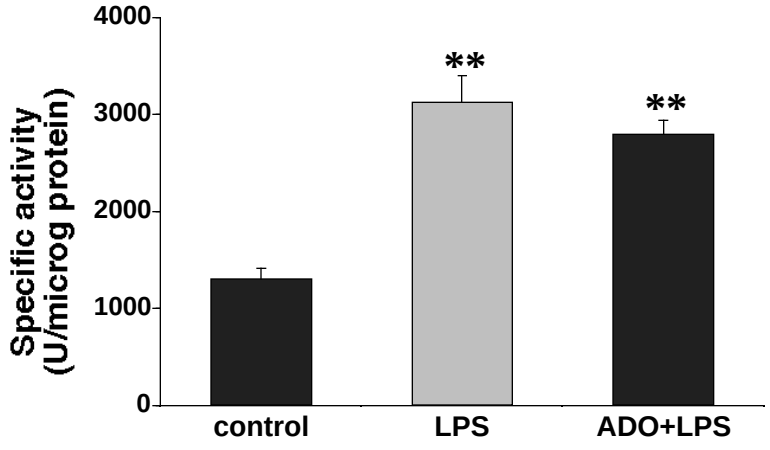

Fig.5

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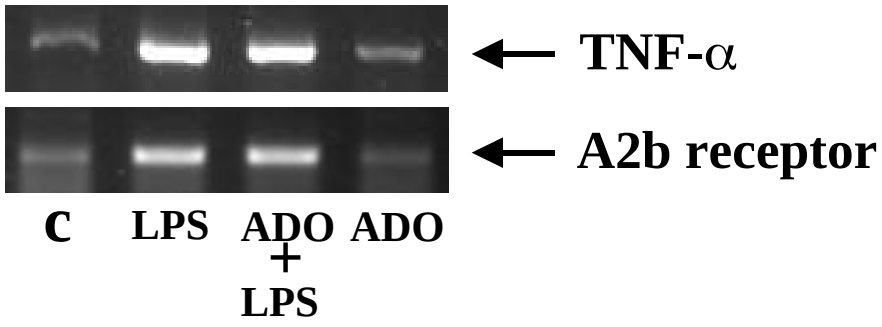

Fig. 6