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Activation of adenosine A_3 receptors on macrophages inhibits tumor necrosis factor-α

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Abstract

Murine macrophage-derived tumor necrosis factor alpha (TNF-α) gene expression has been shown to be dramatically induced by bacterial lipopolysaccharide, and to be dependent upon nuclear factor-κB (NF-κB) binding sites in its promoter for the lipopolysaccharide induction. Murine J774.1 macrophage cells were found to predominately express the adenosine A_3 receptor RNA relative to adenosine A_1 receptor or adenosine A_2 receptor RNA. Adenosine receptor agonists, in a dose-dependent manner characteristic of the adenosine A_3 receptor, blocked the endotoxin induction of the TNF-α gene and TNF-α protein expression in the J774.1 macrophage cell line. The adenosine A_3 receptor antagonist BW-1433 dose-dependently reversed this adenosine inhibitory effect on TNF-α gene expression. Thus, the binding of adenosine receptor agonists to the adenosine A_3 receptor interrupts the endotoxin CD14 receptor signal transduction pathway and blocks induction of cytokine TNF-α, revealing a novel cross-talk between the murine adenosine A_3 receptor and the endotoxin CD14 receptor in J774.1 macrophages.

Keywords: TNF-α (tumor necrosis factor α) inhibition; Adenosine A_3 receptor; Macrophage; Receptor; Cross-talk

1. Introduction

Adenosine has been shown to regulate a variety of physiological processes through activation of different classes of adenosine cell surface receptors (Van Calker et al., 1979; Londos et al., 1980; Ali et al., 1990; Daval et al., 1991; Stiles, 1992; Zhou et al., 1992; Collis and Hourani, 1993; Ramkumar et al., 1993; Linden, 1994; Van Galen et al., 1994). Adenosine and related analogs have been shown to inhibit lipopolysaccharide induction of tumor necrosis factor alpha (TNF-α) in monocytes and macrophages (Han et al., 1990; Schrier et al., 1990; Cerri et al., 1993; LeVraux et al., 1993; Reinstein et al., 1994), and inhibit the activation of neutrophils, mast cells, lymphocytes and monocytes (Lappin and Whaley, 1984; Riches et al., 1985; De LaHarpe and Nathan, 1989; Salmon and Cronstein, 1990; Sullivan et al., 1990; Peachell et al., 1991; Richter, 1992; Bouma et al., 1994; Hoskin et al., 1994; MacKenzie et al., 1994). Monocytes and murine macrophages have been shown to express adenosine A_1 receptor and adenosine A_2 receptor (Eppell et al., 1989; Najar et al., 1990; Salmon et al., 1993), while adenosine A_3 receptor in rat mast cells has been shown to be linked to adenosine suppression of these cells (Peachell et al., 1991). Murine

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macrophage-derived TNF-α gene expression has been shown to be dramatically induced by lipopolysaccharide or endotoxin through the CD14 receptor (Wright et al., 1990; Heumann et al., 1992), and is dependent upon nuclear factor κB (NF-κB) binding sites in the murine TNF-α promoter for the lipopolysaccharide induction (Collart et al., 1990; Drouet et al., 1991; Delude et al., 1994). The lipopolysaccharide signal transduction pathway (Geng et al., 1993; Liu et al., 1994; Naumann and Scheidereit, 1994) activates NF-κB transcription factor and promotes its translocation into the nucleus, where it binds to the TNF-α promoter and activates gene transcription (Collart et al., 1990; Drouet et al., 1991; Delude et al., 1994). Induction of murine macrophage TNF-α mRNA has recently been shown to be blocked by adenosine and adenosine-like analogs (Parmely et al., 1993; Edwards et al., 1994).

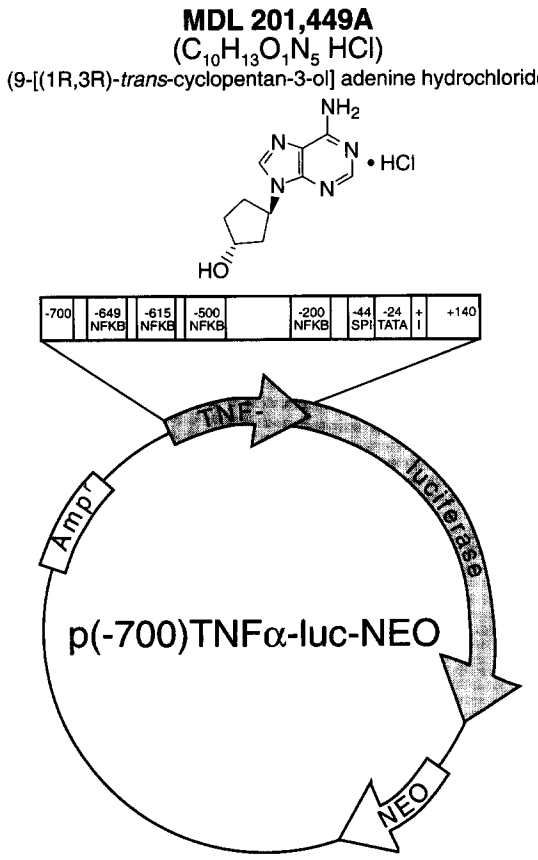

Fig. 1. The murine tumor necrosis factor alpha (TNF-α) promoter region and adenosine analog (MDL 201,449A). (A) The chemical structure of adenosine analog, MDL 201,449A, (9-(1*R*,3*R*)-*trans*-cyclopentan-3-ol]adenine hydrochloride). (B) The murine 5′ flanking sequences of the TNF-α promoter. The murine TNF-α promoter fragment (−700 to +140) contains four NF-κB transcription (−649, −615, −500 and −200) factor binding sites required for lipopolysaccharide induction, a TATAA box (−24), the initiation site (+1), and 140 bases of downstream sequence. This TNF-α promoter region was cloned into the pGL-2 basic luciferase expression vector (Promega) to generate the p-(700)-TNF-α-luciferase-neomycin reporter construct with the neomycin gene cloned downstream of the polyadenylation signal for neomycin (Geneticin, G418) selection of stable transfectants.

Regulation of macrophage activation by adenosine-like compounds has led us to investigate the effects of selective adenosine receptor agonists and antagonists on both TNF-α gene expression and TNF-α protein secretion in vitro. Furthermore, we investigated whether the novel adenosine analog, MDL 201,449A (Fig. 1A), (9-[(1*R*,3*R*)-*trans*-cyclopentan-3-ol]adenine hydrochloride), which we have recently shown to inhibit lipopolysaccharide-induced TNF-α mRNA and secreted protein in bone marrow-derived murine macrophage cultures, may do so through adenosine receptors (Parmely et al., 1993; Edwards et al., 1994).

2. Materials and methods

2.1. Northern protocol

Total RNA samples were prepared from murine J774.1 macrophage cells stimulated with lipopolysaccharide (1 μg/ml, Sigma) for 90 min. Total RNA samples (30 μg/lane; Chomczynski and Sacchi, 1987; Sambrook et al., 1989) were electrophoresed on a 1% agarose gel containing formaldehyde, transferred to nylon membranes (Nytran, S & S) and cross-linked. Membranes were prehybridized and then hybridized with random primer-labeled cDNA probes (adenosine A_1 receptor, adenosine A_2 receptor or adenosine A_3 receptor) at 42°C for 16–20 h (panel A; Ali et al., 1990; Reppert et al., 1991; Stehle et al., 1992). Adenosine A_1 receptor, adenosine A_2 receptor and adenosine A_3 receptor rat cDNA probes (> 300 bp) were generated by polymerase chain reaction (PCR) methods using oligonucleotide primers from rat adenosine receptor sequences. The probe for the adenosine A_2 receptor hybridizes to adenosine A_{2a} receptor and adenosine A_{2b} receptor subtypes. The Northern blot was stripped and reprobed with the housekeeping gene, human gylceraldehyde-3-phosphate dehydrogenase gene probe. The 1161-bp cDNA probe for glyceraldehyde-3-phosphate dehydrogenase (Gene Bank HSG3PDC) was generated by PCR using primers annealing at +26 and +1187. Probes were labeled using Boehringer Random Primer Labeling Kit and [α-^{32}P]dATP from Dupont NEN. Nytran membranes were purchased from Schleicher & Schuell. Buffers (20× sodium chloride, sodium monophosphate, disodium ethylenediaminetetraacetate buffer (SSPE) and 10% sodium dodecyl sulfate (SDS)) were purchased from Gibco (BRL).

2.2. Adenosine agonists and antagonists

Adenosine agonists were purchased from Research Biochemicals International (RBI): N^6-cyclopentyladenosine (CPA); 2-*p*-(2-carboxyethyl)phenethylamino-5′-*N*-ethylcarboxamidoadenosine hydrochloride (CGS21680); *R*(−)-N^6-(2-phenylisopropyl)adenosine (RPIA); 5′-*N*-ethylcarboxamidoadenosine (NECA); CCPA (2-chloro-N^6-cyclopentyladenosine); APNEA, N^6-2-(4-amino-3-

phenyl)ethyladenosine. BW-1433 (1,3-dipropyl-8-(4-acrylate)phenylxanthine) was obtained from Burroughs Welcome (Salvatore et al., 1993).

2.3. TNF-luciferase construct

Plasmid p(−700)-TNF-α-luciferase-neomycin reporter construct contains the murine TNF-α promoter fragment (−700 to +140) with NF-κB binding sites necessary for lipopolysaccharide induction (Collart et al., 1990; Shakhov et al., 1990; Drouet et al., 1991; Delude et al., 1994). The TNF-α promoter was cloned into Promega pGL2-Basic Vector to drive expression of the luciferase gene. The TK promoter driving expression of the neomycin gene was cloned downstream of the polyadenylation signal. The final p(−700)-TNF-α-luciferase-neomycin construct was electroporated (Gibco Gene Pulser, 800 μFad, Opti-MEM media (Gibco (BRL)) into J774.1 macrophages and Geneticin (G418, 800 μg/ml, Gibco (BRL)) selection was applied. J774.1 cells are a murine macrophage-like cell line which was derived from a tumor of a female BALB/c mouse and has been shown to possess characteristics typical of macrophages (Muroi and Suzuki, 1993). J774.1 macrophage cells were grown on tissue culture plates (Corning) at 37°C in 5% CO_2 in Dulbecco's modified Eagle's minimal medium with 5% fetal calf serum, penicillin (100 units/ml), streptomycin (100 μg/ml), all from Gibco (BRL). Stably transfected J774.1 macrophage cells were treated with *E. coli*-derived lipopolysaccharide (055:B5 Sigma; 1 ng/ml − 5 μg/ml) for 16 h for luciferase assays or 90 min for Northern analysis. Geneticin (G418) was purchased from Gibco (BRL) Life Technologies.

2.4. Luciferase assay

The effect of adenosine receptor agonists or MDL 201,449A on endotoxin-stimulated murine TNF-α-luciferase gene expression (p(−700)-TNF-α-luciferase-neomycin construct) was measured in a stably transfected J774.1 murine macrophage cell line (Muroi and Suzuki, 1993). Adenosine receptor agonists (APNEA, R-PIA, NECA and CGS21680) or MDL 201,449A were added to stably transfected J774.1 macrophage cells 15 min prior to the addition of lipopolysaccharide (1 μg/ml), incubated for 16 h and cell lysates were assayed for luciferase gene expression using a Berthold luminometer and Promega lysis buffer and substrate. In all luciferase experiments, background luciferase was subtracted from data, and induction with lipopolysaccharide (1 μg/ml) was normalized to 100%. The data presented are representative of 3–5 independent experiments and the results are expressed as means ± S.E.M. of determinations.

2.5. TNF-α protein secretion

Lipopolysaccharide induction of murine TNF-α protein secretion from a stably transfected J774.1 macrophage cell line was measured (Genzyme murine TNF-α ELISA kit) at 16 h. The data presented are representative of 3–5 independent experiments. Values are obtained from triplicate plates and shown as means ± S.E.M. of determinations.

2.6. Adenosine A_3 receptor radioligand binding experiments

APNEA, N^6-2-(4-amino-3-phenyl)ethyladenosine, was synthesized at Marion Merrell Dow Research Institute. Iodinated [^{125}I]APNEA (N^6-2-(4-amino-3-phenyl)ethyladenosine) was labeled with ^{125}I by Dupont NEN. RBL-2H3 cells, a rat mast cell line expressing only the adenosine A_3 receptor (Ramkumar et al., 1993), were cultured in Earle's modified Eagle's medium supplemented with 15% fetal bovine serum. RBL-2H3 membranes were prepared using the protocol of Ramkumar et al. (1993) by differential centrifugation and used for radioligand binding experiments. Saturation curves were performed by incubating approximately 50 μg of membranes with increasing concentrations of [^{125}I]APNEA in the absence (total binding) or presence (nonspecific binding) of theophylline (10 μM). Competition curves were performed using 3–5 nM [^{125}I]APNEA and increasing concentrations of the different adenosine analogs. Incubations were performed at 37°C for 1 h. Values were reported as means ± S.E.M., and IC_{50} values were derived from competition binding data analyzed by a computer curve-fitting program equipped with a statistical package (Baron and Siegel, 1990). The data presented are representative of three independent experiments.

3. Results

3.1. TNF-α-luciferase gene expression is inhibited by adenosine agonists

TNF-α-luciferase gene expression (Fig. 1B) was detected in stably transfected J774.1 macrophage cells (Muroi and Suzuki, 1993) and was induced in a dose-dependent manner upon the addition of *E. coli*-derived lipopolysaccharide to the transfected macrophage (Fig. 2). Treatment of J774.1 cells with 1 μg/ml lipopolysaccharide gave a 5–10-fold induction of TNF-α-luciferase gene expression (Fig. 2A). Murine TNF-α ELISA assays detected increasing amounts of secreted TNF-α protein from stably transfected J774.1 macrophage cells with the addition of increasing amounts of lipopolysaccharide (Fig. 2B). Treatment of J774.1 cells with 1 μg/ml lipopolysaccharide

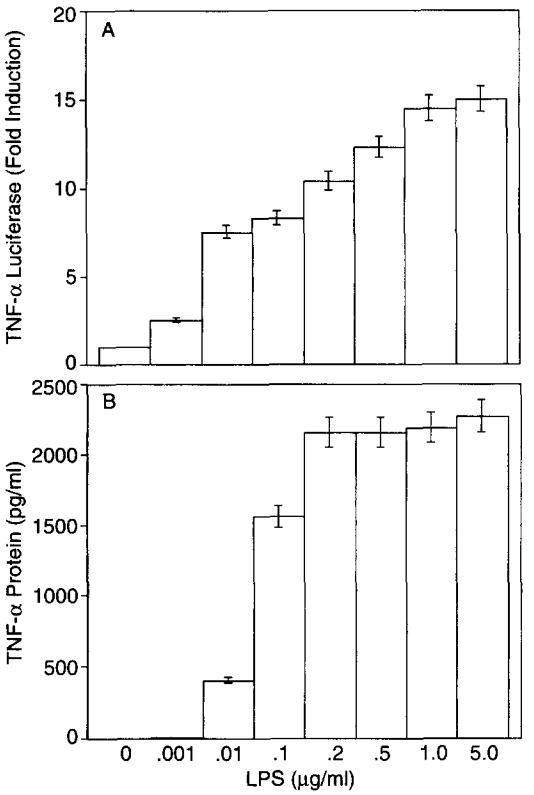

Fig. 2. Lipopolysaccharide induction of TNF-α luciferase gene expression and endogenous TNF-α protein secretion in murine J774.1 macrophage cell line. Stably transfected J774.1 macrophage cells were treated with increasing amounts of *E. coli*-derived lipopolysaccharide (055:B5) for 16 h (0.001–5.0 μg/ml lipopolysaccharide, lanes 2–8). (A) Cell lysates were assayed for TNF-α-luciferase gene expression using Promega reagents (lysis buffer and substrate) in a Berthold luminometer. (B) TNF-α secreted protein detected from J774.1 macrophage cells upon lipopolysaccharide stimulation. Cell supernatants from the above experiment were assayed for secreted TNF-α protein (pg/ml) using a Genezyme murine TNF-α ELISA kit as the result of treatment with increasing amounts of *E. coli*-derived lipopolysaccharide (panel B, lanes 2–8). No secreted TNF-α protein was detected without lipopolysaccharide treatment (lane 1). Each lipopolysaccharide dose was assayed in triplicate and the results are expressed as the means ± S.E.M. of determinations.

resulted in secretion of 2200 pg/ml of TNF-α protein (Fig. 2B).

3.2. J774.1 macrophage cells express the adenosine A_3 receptor

Selective treatment of J774.1 macrophages with adenosine A_1 receptor agonist CPA (N^6-cyclopentyladenosine) or adenosine A_2 receptor agonist CGS21680 (2-*p*-(2-carboxyethyl)phenethylamino-5′-*N*-ethylcarboxamidoadenosine hydrochloride (Kikugawa and Ichino, 1973; Londos et al., 1978; Williams, 1984; Lohse et al., 1988; Jarvis et al., 1989) at 25 nM inhibited lipopolysaccharide-induced TNF-α-luciferase gene expression and TNF-α protein secretion from J774.1 macrophages by 40%. Since the selective adenosine A_1 receptor or adenosine A_2 receptor agonists were equally effective at inhibiting TNF-α-luciferase gene expression and TNF-α protein secretion, a possible interaction with the newly described adenosine A_3 receptor subtype (Ali et al., 1990; Zhou et al., 1992; Ramkumar et al., 1993; Linden, 1994) detected on mast cells and lymphocytes was suggested. Northern analysis with rat adenosine A_1 receptor, adenosine A_2 receptor and adenosine A_3 receptor cDNA probes (Reppert et al., 1991; Stehle et al., 1992; Zhou et al., 1992) revealed that J774.1 macrophage cells do express the adenosine A_3 receptor at levels 6- and 10-fold higher than the adenosine A_1 receptor or adenosine A_2 receptor, respectively (Fig. 3). These data provide the first report that the newly identified adenosine A_3 receptor is present on murine macrophages. To determine which adenosine receptor subtype modulates TNF-α gene regulation in this cell line, a series of adenosine receptor agonists was analyzed for their ability to inhibit TNF-α-luciferase gene expression (Fig. 4), and matched to the reported rank order affinity of agonists to the adenosine A_3 receptor (Ali et al., 1990; Zhou et al., 1992; Ramkumar et al., 1993; Linden, 1994).

3.3. Adenosine A_3 receptor agonists inhibit TNF-α-luciferase expression

Treatment of J774.1 macrophage with the adenosine receptor agonists (Fig. 4) inhibited TNF-α-luciferase gene

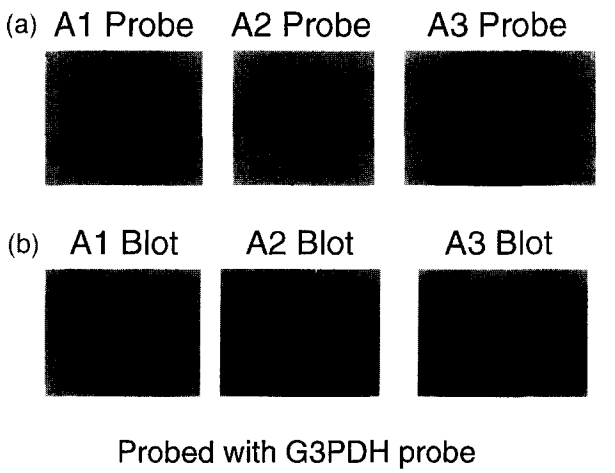

Fig. 3. Identification of J774.1 macrophage cell adenosine A_3 receptor RNA by Northern blotting. Total RNA (30 μg per lane) was extracted from murine J774.1 macrophage cells (lanes 1–3) stimulated with lipopolysaccharide (1 μg/ml, Sigma) for 90 min, and electrophoresed in a 1.0% agarose, formaldehyde gel and transferred to nylon membrane (Nytran). The Northern blot was subdivided into three subsections with 30 μg of total RNA per lane in each subsection. Each Northern blot subsection was probed with [α-^{32}P]dATP-labeled, random-primed DNA probe complementary to the murine cDNA adenosine receptor subtype sequences (adenosine A_1 receptor probe, lane 1; adenosine A_2 receptor probe, lane 2; or adenosine A_3 receptor probe, lane 3; panel A). Each Northern blot subsection was then was stripped and reprobed with the glyceraldehyde-3-phosphate dehydrogenase gene probe (panel B; lane 1, adenosine A_1 blot; lane 2, adenosine A_2 blot; lane 3, adenosine A_3 blot). Northerns were quantitated using an SF PhosphoImager (Molecular Dynamics) utilizing the ImageQuant Program.

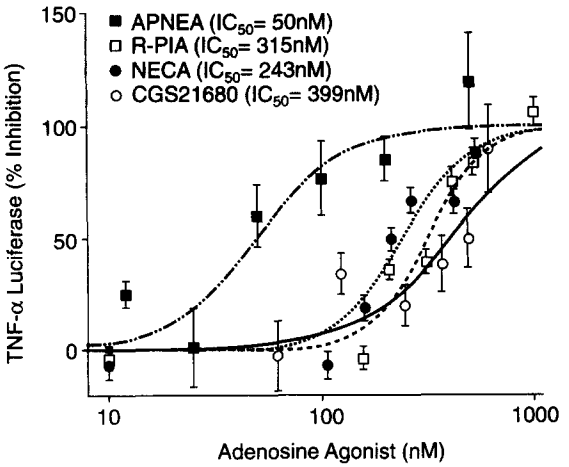

Fig. 4. Effects of adenosine receptor agonists on lipopolysaccharide-stimulated TNF-α-luciferase gene expression. Adenosine receptor agonists [APNEA (closed square), R-PIA (open square), NECA (closed circle), CGS21680 (open circle)] were added to stably transfected J774.1 macrophage cells 15 min prior to the addition of lipopolysaccharide (1 μg/ml), incubated for 16 h, and cell lysates isolated. Cell lysates were used for determination of TNF-α-luciferase gene expression using a Berthold luminometer with Promega reagents (lysis buffer and substrate). The inhibitory adenosine receptor agonist dose–response curves are representative of single experiments in which each point is determined in triplicate (means ± S.E.M. of determinations). The data presented are representative of 3–5 independent experiments. Curves and IC_{50} values were generated by a computer curve-fitting program which was equipped with a statistical package. IC_{50} values were established and they produced a rank order of adenosine receptor agonists inhibiting TNF-α-luciferase gene expression as follows: APNEA (50 nM), NECA (243 nM), R-PIA (315 nM), and CGS21680 (399 nM).

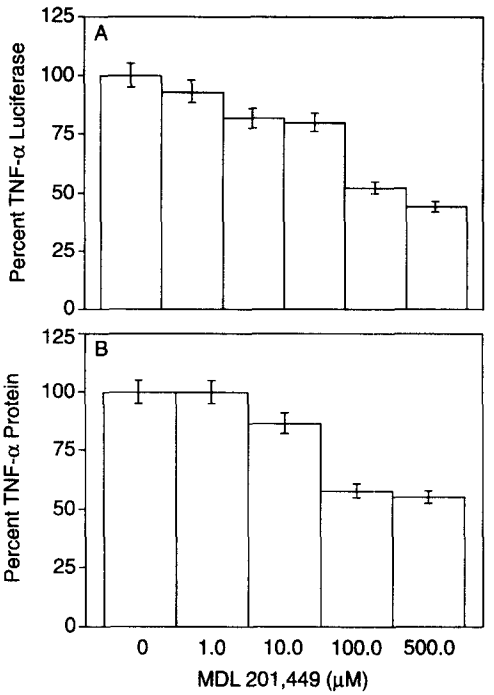

Fig. 5. (A) The effect of an adenosine analog (MDL 201,449A) on lipopolysaccharide-induced TNF-α-luciferase gene expression. Stably transfected murine J774.1 macrophage cells were pretreated 15 min with an adenosine analog (0.1–500 μM MDL 201,449A, lanes 2–5), induced with lipopolysaccharide (1 μg/ml) for 16 h (lanes 1–5), and cell lysates isolated. Cell lysates were used for determination of TNF-α-luciferase gene expression using a Berthold luminometer with Promega reagents (lysis buffer and substrate). An inhibitory dose–response of MDL 201,449A was generated from a single experiment in which each point was determined in triplicate (means ± S.E.M. of determinations). The data presented are representative of 3–5 independent experiments. IC_{50} values were generated by a computer curve-fitting program which was equipped with a statistical package. (B) The effect of MDL 201,449A on lipopolysaccharide-induced endogenous TNF-α protein secretion from stably transfected J774.1 macrophage cells. TNF-α protein secretion into the media was measured using a murine TNF-α ELISA kit (Genezyme) at 16 h in response to increasing concentrations of MDL 201,449A (lanes 2–5) and lipopolysaccharide (1 μg/ml, lanes 1–5). The data presented are representative of 3–5 independent experiments. Values are obtained from triplicate plates and shown as means ± S.E.M. of determinations.

expression in a dose-dependent manner, and produced a rank order (Ali et al., 1990; Zhou et al., 1992; Ramkumar et al., 1993; Linden, 1994) of inhibition (Fig. 4, Table 1), expressed as IC_{50} values, as follows: APNEA (50 nM), N^6-2-(4-amino-3-phenyl)ethyladenosine > NECA (243 nM), 5′-*N*-ethylcarboxamidoadenosine > R-PIA (315 nM), *R*(−)-N^6-(2-phenylisopropyl)adenosine > CGS-21680 (399 nM). This rank order is inconsistent with either

Table 1
Adenosine receptor agonists that inhibit TNF-α-luciferase also bind adenosine A_3 receptor

Compound	IC_{50} (nM)	K_i (nM)	
	Inhibition of TNF-α-luciferase	Binding for RBL-2H3 cells (adenosine A_3R)	Literature values for adenosine A_3 receptor affinity
APNEA	50 ± 13.4	46 ± 18.0	30
NECA	243 ± 25.6	60 ± 14.2	113
R-PIA	315 ± 26.4	57 ± 5.3	158
CGS21680	399 ± 79.9	255 ± 96.0	584
MDL 201,449A	30000 ± 3050	26080 ± 7637	–

Adenosine receptor agonists (APNEA, R-PIA, NECA and CGS21680) were added to stably transfected J774.1 macrophage cells 15 min prior to the addition of lipopolysaccharide (1 μg/ml, Sigma), incubated for 16 h and TNF-α-luciferase gene expression was determined using a Berthold luminometer and Promega reagents (lysis buffer and substrate). The IC_{50} values were calculated from the adenosine dose–response curves (Fig. 4) and are representative of single experiments in which each point is determined in triplicate (means ± S.E.M. of determinations). The data presented are representative of 3–5 independent experiments.

an adenosine A_1 receptor or adenosine A_2 receptor mediated event, but is consistent with the reported affinity of these agonists for the adenosine A_3 receptor (Zhou et al., 1992; Ramkumar et al., 1993). Treatment of J774.1 macrophage with the adenosine A_3 receptor antagonist BW-1433 (Salvatore et al., 1993; Table 2) produced a dose-dependent reversal of the effect of APNEA on TNF-α gene expression (Table 2). Reversal of APNEA-induced inhibition of TNF-α gene expression by a selective adenosine A_3 receptor antagonist (BW-1433) demonstrates that TNF-α-luciferase gene expression is regulated through adenosine A_3 receptors on J774.1 macrophage cells.

3.4. Adenosine analog, MDL 201,449A, binds the adenosine A_3 receptor

Receptor binding studies (Table 1) were conducted to characterize binding of the adenosine analog MDL 201,449A (Fig. 1) to the adenosine A_3 receptor on RBL-2H3 mast cells (Ali et al., 1990; Zhou et al., 1992; Ramkumar et al., 1993; Linden, 1994). Binding to the adenosine A_3 receptor on RBL-2H3 mast cells with the adenosine A_3 receptor-specific ligand, [^{125}I]APNEA, produced a K_i of 26 μM for the adenosine analog, MDL 201,449A (Fig. 1A; Table 1). Adenosine agonists (Table 1) competitively bound to the adenosine A_3 receptor of RBL-2H3 cells with a rank order and affinity characteristic of the adenosine A_3 receptor (Ali et al., 1990; Zhou et al., 1992; Ramkumar et al., 1993; Linden, 1994). MDL 201,449A dose-dependently (IC_{50} = 30 μM) inhibited endotoxin-induced TNF-α-luciferase gene expression and TNF-α protein secretion in the same stably transfected J774.1 macrophage cell line (Fig. 5). Inhibition of TNF-α gene expression with 50 μM MDL 201,449A was reversed by the selective adenosine A_3 receptor antagonist, BW-1433 (Table 2), demonstrating that adenosine analog MDL 201,449A inhibits TNF-α induction through adenosine A_3 receptors on these macrophage cells. The binding of MDL 201,449A to the adenosine A_3 receptor at a value close to its IC_{50} value (30 μM) for inhibiting TNF-α-luciferase gene expression along with reversal of this affect by an adenosine A_3 receptor antagonist, demonstrates that the mechanism of action of MDL 201,449A is through activation of the predominantly expressed adenosine A_3 receptor subtype on these J774.1 macrophages.

4. Discussion

TNF-α-luciferase gene expression and TNF-α protein secretion (Fig. 2) were induced in a dose-dependent manner upon addition of *E. coli*-derived lipopolysaccharide in stably transfected J774.1 macrophage cells. Adenosine A_1 receptor or adenosine A_2 receptor agonists equally inhibited lipopolysaccharide-induced TNF-α-luciferase gene expression and TNF-α protein secretion from J774.1 macrophages. Since the selective adenosine A_1 receptor or adenosine A_2 receptor agonists were equally effective at inhibiting TNF-α-luciferase gene expression and TNF-α protein secretion, interaction with the newly described adenosine A_3 receptor subtype (Ali et al., 1990; Zhou et al., 1992; Ramkumar et al., 1993) detected on mast cells and lymphocytes was suggested. Northern analysis with rat

Table 2
Effect of adenosine A_3 receptor antagonist on adenosine agonist inhibition of TNF-α luciferase gene expression

Adenosine A_3 receptor agonists (μM)		Adenosine A_3 receptor antagonist BW-1433 (μM)	TNF-α-luciferase [a] relative activity (%)
APNEA	0.0	0.0	100
APNEA	0.0	20.0	101.8 ± 8.0
APNEA	0.10	0.0	25.0 ± 2.43
APNEA	0.10	4.2	35.0 ± 3.04
APNEA	0.10	6.3	60.0 ± 2.35
APNEA	0.10	8.4	70.0 ± 2.82
APNEA	0.10	20.0	110.0 ± 1.14
MDL 201,449A	0.0	0.0	100
MDL 201,449A	0.0	20.0	100.9 ± 8.0
MDL 201,449A	50.0	0.0	11.2 ± 5.13
MDL 201,449A	50.0	1.0	24.5 ± 4.34
MDL 201,449A	50.0	5.0	33.1 ± 0.30
MDL 201,449A	50.0	10.0	45.5 ± 3.56
MDL 201,449A	50.0	20.0	58.9 ± 7.60

Selective adenosine A_3 receptor agonist APNEA, or MDL 201,449A (Fig. 1A), plus or minus adenosine A_3 receptor antagonist BW-1433, were added to stably transfected J774.1 macrophage cells for 15 min prior to lipopolysaccharide (1 μg/ml, Sigma) induction of TNF-α-luciferase and incubated for 16 h. Cell lysates were used for determination of TNF-α-luciferase gene expression using a Berthold luminometer. Luciferase values were expressed as % of lipopolysaccharide-induced TNF-α gene expression.

[a] Each value represents the means ± S.E.M. of determinations and is representative of 3–5 different experiments. BW-1433 with APNEA treatment gave an IC_{50} of 6.2 μM for reversal of TNF-α inhibition. BW-1433 with MDL 201,449A treatment gave an IC_{50} of 13 μM for reversal of TNF-α inhibition.

adenosine A_1 receptor, adenosine A_2 receptor and adenosine A_3 receptor cDNA probes (Reppert et al., 1991; Stehle et al., 1992; Zhou et al., 1992) revealed that J774.1 macrophage cells express 6- to 10-fold more adenosine A_3 receptor than either adenosine A_1 receptor or adenosine A_2 receptor (Fig. 3). A series of adenosine receptor agonists inhibited TNF-α-luciferase gene expression (Fig. 4) in a rank order similar to that reported for activation of the adenosine A_3 receptor (Ali et al., 1990; Zhou et al., 1992; Ramkumar et al., 1993). Reversal of adenosine-induced inhibition of TNF-α gene expression by a selective adenosine A_3 receptor antagonist (BW-1433; Salvatore et al., 1993) demonstrates that TNF-α-luciferase gene expression is regulated through adenosine A_3 receptors on J774.1 macrophage cells.

Receptor binding studies (Table 1) revealed that adenosine analog (MDL 201,449A) bound to the adenosine A_3 receptor on RBL-2H3 mast cells (Ali et al., 1990; Zhou et al., 1992; Collis and Hourani, 1993; Ramkumar et al., 1993; Van Galen et al., 1994) with a K_i of 26 μM (Fig. 1A; Table 1), and dose-dependently (IC_{50} = 30 μM) inhibited lipopolysaccharide-induced TNF-α-luciferase gene expression and TNF-α protein secretion in stably transfected J774.1 macrophage cells (Fig. 5). This inhibition of TNF-α gene expression with MDL 201,449A was reversed by the selective adenosine A_3 receptor antagonist BW-1433 (Table 2), demonstrating that this adenosine analog inhibits TNF-α induction through the adenosine A_3 receptor subtype on these J774.1 macrophage cells.

In summary, these results provide direct evidence that murine TNF-α gene expression and TNF-α protein secretion are negatively regulated in vitro by adenosine A_3 receptor agonists in the J774.1 macrophage-like cell line. In vitro, adenosine receptor agonists and an adenosine analog (MDL 201,449A) were able to dose-dependently inhibit TNF-α-luciferase gene expression and TNF-α protein secretion in stably transfected J774.1 macrophage cells in a rank order that was consistent with activation of the adenosine A_3 receptor. Inhibition of TNF-α-luciferase gene and TNF-α protein expression by adenosine A_3 receptor agonists and analogs was reversed by the adenosine A_3 receptor antagonist BW-1433. Adenosine analog (MDL 201,449A) bound to the adenosine A_3 receptor on RBL-2H3 mast cell membranes near its IC_{50} for inhibiting TNF-α-luciferase gene and TNF-α protein expression in J774.1 macrophage-like cells. Thus, the binding of adenosine analogs to adenosine A_3 receptor interrupts the endotoxin CD14 receptor signal transduction pathway, and blocks induction of TNF-α transcription and TNF-α protein secretion in macrophages. These data provide the first report that the newly identified adenosine A_3 receptor is present on murine macrophages, and provide the first evidence of an adenosine A_3 receptor function in murine macrophages. These data further reveal the novel cross-talk between the murine adenosine A_3 receptor and the endotoxin CD14 receptor in J774.1 macrophages.

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