Potentiation of neutrophil cyclooxygenase-2 by adenosine: an early anti-inflammatory signal

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Summary

Neutrophils, which are often the first to migrate at inflamed sites, can generate leukotriene B_4 from the 5-lipoxygenase pathway and prostaglandin E_2 through the inducible cyclooxygenase-2 pathway. Adenosine, an endogenous autacoid with several anti-inflammatory properties, blocks the synthesis of leukotriene B_4 while it potentiates the cyclooxygenase-2 pathway in fMLP-treated neutrophils, following activation of the A_{2A} receptor. Using the murine air pouch model of inflammation, we observed that inflammatory leukocytes from mice lacking the A_{2A} receptor have less cyclooxygenase-2 induction than wild-type animals. In human leukocytes, A_{2A} receptor activation specifically elicited potentiation of cyclooxygenase-2 in neutrophils, but not in monocytes. Signal transduction studies indicated that the cAMP, ERK1/2, PI-3K and p38K intracellular pathways are implicated both in the direct upregulation of cyclooxygenase-2 and in its potentiation. Together, these results indicate that neutrophils are particularly important mediators of adenosine's effects. Given the uncontrolled inflammatory phenotype observed in knockout mice and in view of the potent inhibitory actions of prostaglandin E_2 on inflammatory cells, an increased cyclooxygenase-2 expression resulting from A_{2A} receptor activation, observed particularly in neutrophils, may take part in an early modulatory mechanism promoting anti-inflammatory activities of adenosine.

Key words: Polymorphonuclear leukocytes, Experimental animal models, Adenosine A_{2A} receptor, Cyclic AMP, Resolution of inflammation

Introduction

Polymorphonuclear neutrophils (PMNs) are often the first blood cells to migrate toward inflammatory lesions where they accumulate and contribute to initial phases of the inflammatory response. Their host defense functions include the phagocytosis of invading microorganisms and cell debris, the release of proteolytic enzymes and the generation of oxygen-derived reactive agents (Edwards, 1994). PMNs are potent inflammatory cells that can, when not under appropriate control, worsen disease states (Sawyer et al., 1989). Reactive oxygen metabolic products may promote the development of acute inflammatory responses and contribute to the development of tissue injury in a variety of diseases including emphysema, myocardial infarction, adult respiratory distress syndrome and rheumatoid arthritis (Fantone et al., 1983a; Fantone and Ward, 1985). Therefore, endogenous mechanisms of control must be in place to regulate and limit the activation of PMNs. Such immunoregulatory signals, however, remain poorly documented.

In response to specific stimuli, PMNs can synthesize and release an array of cytokines and chemokines, including: interleukin (IL)-1β, IL-1 receptor antagonist (IL-1RA), IL-8, macrophage inflammatory peptides (MIP)-1α and β, MIP-3α and interferon-α (Cassatella, 1999; Scapini et al., 2000; Scapini et al., 2001), each of which differentially contributing to the orchestration of the inflammatory response. Also, PMNs are well recognized as a major source of the lipid mediator leukotriene (LT)B_4, a potent chemoattractant and agonist for leukocytes and implicated in asthma, allergy and inflammation (Busse, 1998; Nicosia et al., 2001). Moreover, PMNs can generate thromboxane (TX)A_2 and prostaglandin (PG)E_2, predominantly through the inducible cyclooxygenase (COX-2) pathway (Fasano et al., 1998; Maloney et al., 1998; Niiro et al., 1997; Pouliot et al., 1998). While TXA_2 activates platelet aggregation, PGE_2 is a potent inhibitor of leukocyte functions (Fantone and Kinnes, 1983; Fantone et al., 1981; Fantone et al., 1983b; Ham et al., 1983; Lehmeyer and Johnston, 1978; Marone et al., 1980; O'Flaherty et al., 1979; Pouliot, M. et al., 2002; Rivkin et al., 1975; Zurier et al., 1974) by raising intracellular cAMP ($cAMP_i$) above basal levels (Ham et al., 1983; Lehmeyer and Johnston, 1978; Marone et al., 1980; Rivkin et al., 1975; Zurier et al., 1974), in addition to participating in blood flow regulation and vascular permeability, bronchial airway contraction, nociceptor activation and hyperresponsiveness. Thus, an upregulation of COX-2 in inflammatory PMNs and the resulting generation of PGE_2 may well contribute to attenuate their functions and those of surrounding inflammatory cells.

Adenosine is a ubiquitous autacoid with immunomodulatory properties, including the promotion of wound healing (Montesinos et al., 1997) and a protective and downregulatory role in several infectious diseases (Thiel et al., 2003). Recently,

A_{2A} adenosine receptors ($A_{2A}R$) were identified as a crucial part of a nonredundant physiological negative feedback mechanism for limitation and termination of both tissue-specific and systemic inflammatory responses (Ohta and Sitkovsky, 2001). However, the cellular targets responsible for exerting anti-inflammatory effects of adenosine are not well defined. High concentrations of extracellular adenosine can be found in vivo in traumatized tissues; therefore, the autacoid may impact on leukocyte inflammatory functions (Cronstein et al., 1993). Accordingly, in vitro, adenosine has showed to attenuate major leukocyte functions including superoxide anion production, phagocytosis and cell adhesion, through activation of the $A_{2A}R$ (Gessi et al., 2000). Several studies have shown that endogenous adenosine and $A_{2A}R$ agonists are potent inhibitors of the generation of leukotrienes (LTs) and platelet-activating factor (Krump and Borgeat, 1999; Krump et al., 1996; Krump et al., 1997). Our laboratory recently reported that activation of the $A_{2A}R$ potentiates the expression of COX-2 in fMLP-treated PMNs, both at the mRNA and protein levels, in turn increasing their capacity to generate PGE_2 from exogenous arachidonic acid, while not altering that of TXA_2 (Pouliot et al., 2002). This shift in the profile of lipid mediator generation triggered by adenosine may contribute to attenuate subsequent PMN-elicited inflammatory events.

In stimulated PMNs, anti-inflammatory activities of adenosine have been shown to be mediated by the $A_{2A}R$; its activation results in an elevation of intracellular cAMP which is thought to cause an inhibition of functions such as degranulation, locomotion and H_2O_2 synthesis (Ignarro and George, 1974). It is not clear, however, if all of the inhibitory activities of adenosine on PMN functions depend on an elevation of intracellular cAMP levels (Spisani et al., 1996), and some studies have shown that the inhibition of superoxide anion synthesis was not blocked by a protein kinase A inhibitor (Cronstein et al., 1992), underlining the probable involvement of other kinases or signaling pathways. Moreover, intracellular signaling events that mediate the actions of adenosine are often cell-specific, and are certainly not thoroughly characterized in leukocytes. In the present study, we sought to delineate the impact of $A_{2A}R$ on COX-2 expression, in vivo and in vitro. Our results indicate that the effect of adenosine on COX-2 expression, through $A_{2A}R$ activation, is largely specific to PMNs. Signal transduction pathways mediating this effect are assessed.

Materials and Methods

Materials

Adenosine deaminase (ADA) was purchased from Roche Applied Science (Indianapolis, IN). COX-2 mouse monoclonal antibody was from Cayman Chemicals (Ann Arbor, MI) and CGS 21680 was from Research Biochemicals International (Natik, MA). Rabbit polyclonal antibody specific to cAMP responsive element binding protein (CREB) phosphorylated on Ser133 was obtained from Novus Biologicals (Littleton, CO). Cytochalasin B, fMLP, forskolin, RO-20-1724 and wortmannin (from *Penicillium fumiculosum*) were obtained from Sigma-Aldrich (St Louis, MO). DFP (diisopropylfluorophosphate) was from Serva Electrophoresis (Carl-Benz-Str7, Heidelberg). HRP-linked sheep anti-mouse, and donkey anti-rabbit antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA). KT5720, H89 and SB 203580 were from Biomol Research Laboratories (Plymouth Meeting, PA). Leupeptin and aprotinin were obtained from ICN Biomedicals (Irvin, CA). Lipopolysaccharide (LPS; from *Escherichia coli* 0111;B4), PD 98059 (2′-amino-3′-methoxyflavone), Sp-cAMPs-am (Adenosine 3′,5′-cyclic monophosphothioate acetoxymethyl ester) and U0126 (1,4-diamino-2,3-dicyano-1,4-bis(2-aminophenylthio)butadiene) were purchased from Calbiochem-Novalbiochem (San Diego, CA).

Mouse $A_{2A}R$ knockout

Couples of $A_{2A}R$ heterozygotes ($A_{2A}R^{+/-}$) CD1 mice were bred; offsprings were genotyped in order to select $A_{2A}R^{-/-}$ and $A_{2A}R^{+/+}$ animals. Aggressiveness, hypoalgesia and high blood pressure were reported in $A_{2A}R^{-/-}$ mice; they otherwise appeared normal and bred normally (Ledent et al., 1997). Genotyping was performed as follows. Tail ends (0.5-1 cm) were cut from anesthetized animals and immersed in 500 µl tail buffer (40 mM TRIS, 200 mM NaCl, 20 mM EDTA, 0.5% SDS) supplemented with 20 µl proteinase K and 2.5 µl β-mercaptoethanol, and incubated overnight at 55° C. Saturated NaCl (250 µl) was added to samples and incubated on ice for 15 minutes, then centrifuged for 60 minutes at 12,000 ***g***. Supernatants were transferred in fresh tubes and 750 µl ethanol were added. After mixing, tubes were centrifuged for 4 minutes at 6000 ***g***, supernatants were discarded and pellets were washed with 70% ethanol then centrifuged again (5 minutes, 12,000 ***g***). Air dried pellets were dissolved in 100 µl TE. PCR were performed using the following conditions: 45 seconds at 94°C; 45 seconds at 94°C; 45 seconds at 72°C, 35 repetitions, followed by 5 minutes at 72°C. Primers used to detect endogenous $A_{2A}R$ were: 5′-TGG GTA ACG TGC TTG TGT GCT-3′ (forward) and 5′-AAC CAG GCT ACT CTT TTC CAT-3′ (reverse). To detect knocked out $A_{2A}R$ gene, primers for internal Neo cassette were used: AGA GGC TAT TCG GCT ATG ACT G-3′ (forward) and 5′-TTC GTC CAG ATC ATC CTG ATC-3′ (reverse). The presence of the native $A_{2A}R$ generated a PCR product of 466 bp, whereas that elicited from the Neo cassette was 400 bp.

Dorsal air pouches

All experimental designs involving animals have been approved by the institution's Committee for animal protection. Air pouches were raised on the dorsum by s.c. injection of 5 ml of sterile air on day 0, and 3 ml on day 3. Experiments were conducted on day 6. Individual air pouches (one per mouse) were injected either with vehicle alone (endotoxin-free PBS) or with 500 ng LPS suspended in 1 ml PBS. Four hours later, mice were killed and individual air pouches were lavaged two times with ice-cold PBS (total of 2 ml). Leukocyte suspensions were enumerated with an automated cell counter (Model ZM, Coulter Electronics, Luton, England). In each mouse, a 1 cm^2 section of lining tissue was excised from the pouch and processed for RNA isolation or for immunoblotting procedure.

RNA isolation and cDNA reverse-transcription

Leukocyte total RNA was isolated using Trizol (Gibco, Burlington, VT) according to the manufacturer's protocol, with modifications. Briefly, 5×10^5 leukocytes (or a 1 cm^2 of lining tissue) were homogenized in 1 ml Trizol and 200 µl of chloroform were added. After mixing, samples were centrifuged at 10,000 ***g*** for 15 minutes (4°C). The upper aqueous phase was transferred in a tube containing an equal volume of isopropanol. Mixtures were thoroughly vortexed and centrifuged at 12,000 ***g*** for 10 minutes (4°C). Supernatants were discarded and the precipitated RNA pellets were washed using 1 ml of 75% ethanol. RNA pellets were centrifuged at 12,000 ***g*** for 5 minutes (RT). After discarding supernatants, pellets were allowed to air-dry for 2-3 minutes, then resuspended in DEPC-treated water. RNA was quantitated by UV absorbance at 260 nm. First strand cDNA synthesis was performed using 1 µg of total RNA with Superscript II

(Invitrogen Lifetechnology, Carlsbad, CA) in recommended conditions, using 200 ng of random hexamers.

Real-time PCR

Amplification of mouse cDNA was carried out in a Rotor-Gene 3000 operated with Rotor Gene software version 5.0.53 (Corbett Research, Mortlake, 2137 NSW, Australia). Each sample consisted of: 1 μg cDNA, 1.5 mM $MgCl_2$, 0.2 mM dNTP, 100 pmoles of primers, 1 unit of Taq polymerase (Amersham Biosciences, Piscataway, NY) and Sybr Green dye (Molecular Probes, Eugene, OR; 1:30,000 dilution) in a reaction volume of 20 μl. Amplification conditions were as follows: 95°C (20 seconds), 58°C (20 seconds), 72°C (20 seconds); 40 repetitions. Specificity of each reaction was ascertained by performing the Melt® procedure (50-99°C; 1°C/5 seconds) after completion of the amplification protocol, following the manufacturer's instructions.

Mouse primers were: GAPDH: 5′-AAC TTT GGC ATT GTG GAA GG-3′ (forward), 5′-ACA CAT TGG GGG TAG GAA CA-3′ (reverse), COX-1: 5′-CCT CCG ACC TAC AAC TCA GC-3′ (forward), 5′-GCC TAA GGC CTT GGT AAA GC-3′ (reverse), COX-2: 5′-GGC CAT GGA GTG GAC TTA AA-3′ (forward), 5′-AAG TGG TAA CCG CTC AGG TG-3′ (reverse).

Human leukocyte isolation

PMNs were isolated as originally described (Böyum, 1968) with modifications (Pouliot et al., 2002). Briefly, venous blood collected on isocitrate anticoagulant solution from healthy volunteers was centrifuged (250 ***g***, 10 minutes), and the resulting platelet-rich plasma was discarded. Leucocytes were obtained following erythrocytes sedimentation in 3% Dextran T-500. PMNs were then separated from other leukocytes by centrifugation on a 10 ml Ficoll-Paque cushion. Contaminating erythrocytes were removed by a 15 second hypotonic lysis, and purified granulocytes (>95% PMNs, <5% eosinophils) contained fewer than 0.2% monocytes, as determined by esterase staining. Viability was greater than 98%, as determined by trypan blue dye exclusion. The whole cell isolation procedure was carried out sterilely at room temperature (RT). Human monocytes were purified by positive selection from mononuclear cell preparations, with the AutoMACS apparatus (Myltenyi Biotech, Auburn, CA), according to the manufacturer's instructions (www.miltenyibiotec.com). Briefly, PBMC were obtained from anti-coagulated whole blood by gradient centrifugation on ficoll cushion, then passed through a 30 μm nylon filter. Cells were resuspended in running buffer (PBS with 2 mM EDTA+0.5% BSA; 1×10^7 total cells/80 μl) and incubated with MACS CD14 Microbeads for 15 minutes at 6°-12°C. Cells were washed by adding 20 times the labeling volume of buffer and by centrifuging at 300 ***g***, 10 minutes. Supernatant was removed and pellet resuspended in running buffer (500 μl/10^8 total cells). Magnetic separation was performed using appropriate column and program. Monocyte suspensions prepared with this technique were typically >93% pure, as assessed by flow cytometry.

Cell incubations

PMNs were resuspended at a concentration of 5×10^6 cells/ml in Hank's balanced salt solution (HBSS; 37°C) containing 10 mM HEPES pH 7.4, 1.6 mM Ca^{2+} and no Mg^{2+}. Monocytes were plated in 12-well Costar® cell culture flasks (Corning, NY; 1×10^6 cells/well) in RPMI 1640 supplemented with 10% FBS for 60 minutes. Experiments were conducted in RPMI+1% FBS (37°C, 5% CO_2, 95% humidity). Where mentioned, adenosine deaminase (ADA; 0.1 U/ml) was added to cell suspensions 20 minutes before stimulation. Pharmacological agents dissolved in DMSO and cytochalasin B were added to cell suspensions 10 minutes before stimulation with indicated agonists. The final organic solvent concentration never exceeded 0.1% (v/v).

Immunoblots

Following appropriate treatments, samples (PMNs, monocytes or mouse leukocytes) were pelleted and resuspended in 100 μl of HBSS (containing the following antiprotease cocktail: 0.2 μg/ml diisopropylfluorophosphate (DFP), 10 μg/ml leupeptin, 10 μg/ml aprotinin), then in 100 μl of 2× sample buffer (125 mM Tris-Hcl pH 6.8, 8% SDS, 10% 2-ME, 17% glycerol, supplemented with the antiprotease cocktail). Tissue section excised from mouse air pouch lining were dissolved directly in 500 μl 1× sample buffer. Samples were boiled for 5 minutes; aliquots (50 μl) were subjected to SDS-PAGE and transferred to Immobilon membranes (Millipore, Bedford, MA). Equal protein loading and transfer efficiency were visualized by Ponceau Red staining. Membranes were soaked for 30 minutes at RT in TBS (25 mM Tris-HCl pH 7.6, 0.2 M NaCl, 0.15% Tween 20) containing 5% (w/v) dried milk, washed three times in TBS. For human cells, membranes were exposed for 45 minutes to an anti-COX-2 mouse monoclonal antibody (Cayman Chemicals; 1/1000 dilution), or to an anti-CREB (specific for the Ser133-phosphorylated form) polyclonal antibody (dilution 1/500). Membranes were then washed three times in TBS and incubated for 45 minutes with an HRP-linked sheep anti-mouse antibody, or an HRP-linked donkey anti-rabbit antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) (1/10,000 dilution). For mouse leukocytes and resident cells from lining tissue, membranes were either exposed to an anti-COX-1 or an anti-COX-2 rabbit polyclonal antibody (Cayman Chemicals; 1/1000 dilution), then to an HRP-linked donkey anti-rabbit antibody. Enzyme expression was revealed on X-Omat Blue XB-1 Kodak films (Kodak, Rochester, NY) with ECL-Plus (PerkinElmer Life Sciences, Boston, MA).

Determination of extracellular adenosine

Adenosine concentrations were determined as described previously (Krump et al., 1997) with modifications. Briefly, PMN incubations (1 ml) were stopped by adding 100 μl of 22% TCA. NECA was added (10 ng/sample) as an internal standard and the denatured cell suspensions were placed at –20°C for at least 30 minutes. The samples were then centrifuged at 2000 ***g*** for 10 minutes and the supernatants were extracted on C_{18} SPE cartridges (Oasis HLB, Waters) as follows. The samples were loaded on the cartridges which were washed with water; adenosine was then eluted with 3.5 ml of methanol/water (50:50, containing 0.1% acetic acid). Eluates were evaporated to dryness using a Speed Vac evaporator. The residues were dissolved in 200 μl of methanol/water (25:75, containing 0.05% acetic acid). Samples were analyzed by liquid chromatography–mass spectrometry using nebulizer-assisted electrospray (turbo ionspray) ionization (P.E. Sciex, Thornhill, Canada) in the positive mode and by monitoring the transitions m/z 309 and m/z 268 (protonated parent ions) to m/z 136 (protonated adenine), corresponding to the loss of the carbohydrate moieties from NECA and adenosine. The samples (5 μl) were injected onto a C-18 column Brownlee column (Spheri-5, 2.1×30 mm cartridge, 5 μm particles) (Applied Biosystems, San Jose, CA) and eluted at a flow rate of 150 μl/minute using acetonitrile/water (70:30, containing 2 mM ammonium) as the mobile phase. Adenosine was quantitated by extrapolating the measured adenosine/NECA ratio on a calibration curve generated from standard solutions containing 1 ng NECA and 0-4 ng adenosine in 5 μl. The limit of detection for adenosine was ~25 pg injected (signal to noise ratio $\geqslant$5).

Densitometry

Immunoblot autoradiograms were digitalized using a snapscan 1236 scanner (Agfa, Woburn, MA). Densitometric analyses

of autoradiograms were performed with NIH Image (http://rsb.info.nih.gov).

Statistical analysis

Where applicable, statistical analysis was performed by Student's nonpaired test (two-tailed), and significance (*) was considered to be attained when $P<0.05$.

Results

The impact of $A_{2A}R$ activation on COX-2 expression was addressed in vivo using wild-type ($A_{2A}R^{+/+}$) and knockout ($A_{2A}R^{-/-}$) CD1 mice along with the well-characterized murine air pouch model of inflammation (Garcia-Ramallo et al., 2002). Dorsal air pouches were injected with LPS for 4 hours, which elicited the recruitment of approximately twice as much leukocytes, predominantly PMNs (>75% as determined by giemsa staining), as PBS did (Insert, Fig. 1A). No significant difference was observed between males or females, or between $A_{2A}R^{+/+}$ and $A_{2A}R^{-/-}$ mice, for the number of recruited leukocytes. The mRNA expression of GAPDH, COX-1 and COX-2 were determined in migrated leukocytes as well as in resident cells of the pouch lining. LPS caused the upregulation of the expression of COX-2 mRNA in leukocytes by eightfold when compared with PBS-injected animals, while that of COX-1 and GAPDH remained stable, as determined by real-

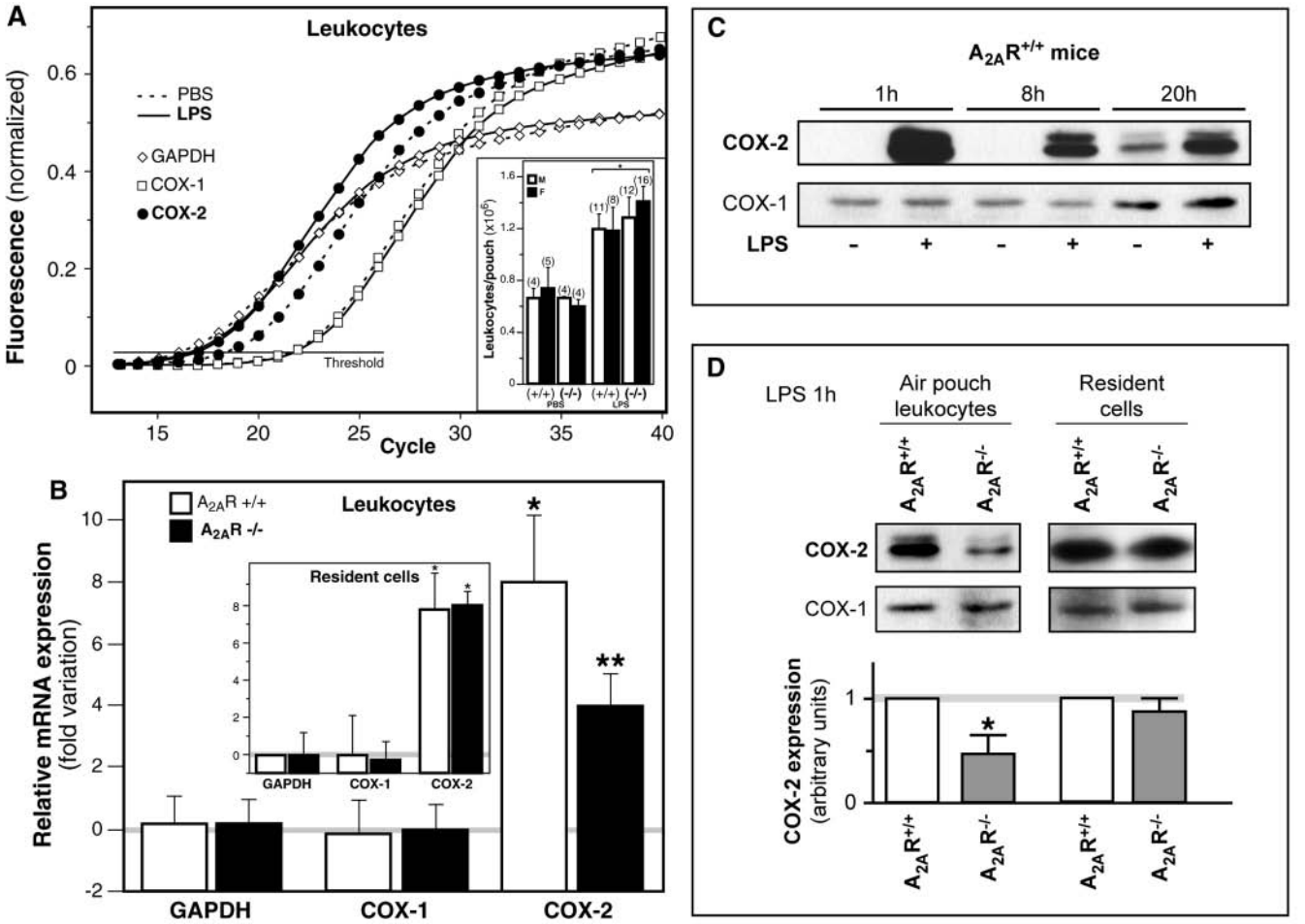


Fig. 1. Leukocytes from wild-type ($A_{2A}R^{+/+}$) mice express more COX-2 in response to LPS than do knockout ($A_{2A}R^{-/-}$) mice. (A) Dorsal air pouches from wild-type ($A_{2A}R^{+/+}$) CD1 mice were injected with PBS alone or containing 500 ng LPS for 4 hours; migrated leukocytes were collected and processed for the determination of indicated mRNA expression by real-time PCR, as described in Materials and Methods. Typical real-time PCR results from one mouse are shown. (Inset) Enumeration of leukocytes which migrated into the air pouch of wild-type (+/+) or homozygote knockout mice (–/–). Results are expressed as the mean±s.e.m., with values between brackets being the number (n) of animals in each condition. *Statistically different from PBS-injected mice. (B) Comparative GAPDH, COX-1 and COX-2 mRNA expression in migrated leukocytes between LPS-injected $A_{2A}R^{+/+}$ and $A_{2A}R^{-/-}$ mice. Results show the mean±s.e.m. from at least four animals. *Statistically different from GAPDH and COX-1. **Statistically different from $A_{2A}R^{+/+}$. (Inset) Comparative mRNA expression in resident cells (mean±s.e.m., n=4). (C) Air pouches were injected with LPS for the indicated times. Migrated leukocytes were collected, enumerated, and samples, equalized by cell numbers, were processed for the determination of COX-2 by western immunoblotting, as described in Materials and Methods. (D) Air pouches were injected with LPS for 60 minutes. Migrated leukocytes were collected, enumerated, and samples were processed for the determination of COX-2 by western immunoblotting. *Statistically different from $A_{2A}R^{+/+}$. In each panel, immunoblots from one experiment typical of at least three separate experiments are shown. Bar graph depicts the expression of COX-2 protein levels in leukocytes from LPS-injected mice, relative to that observed in $A_{2A}R^{+/+}$ mice, as determined by densitometric analysis of the bands in three independent experiments (mean±s.e.m.; n=3).

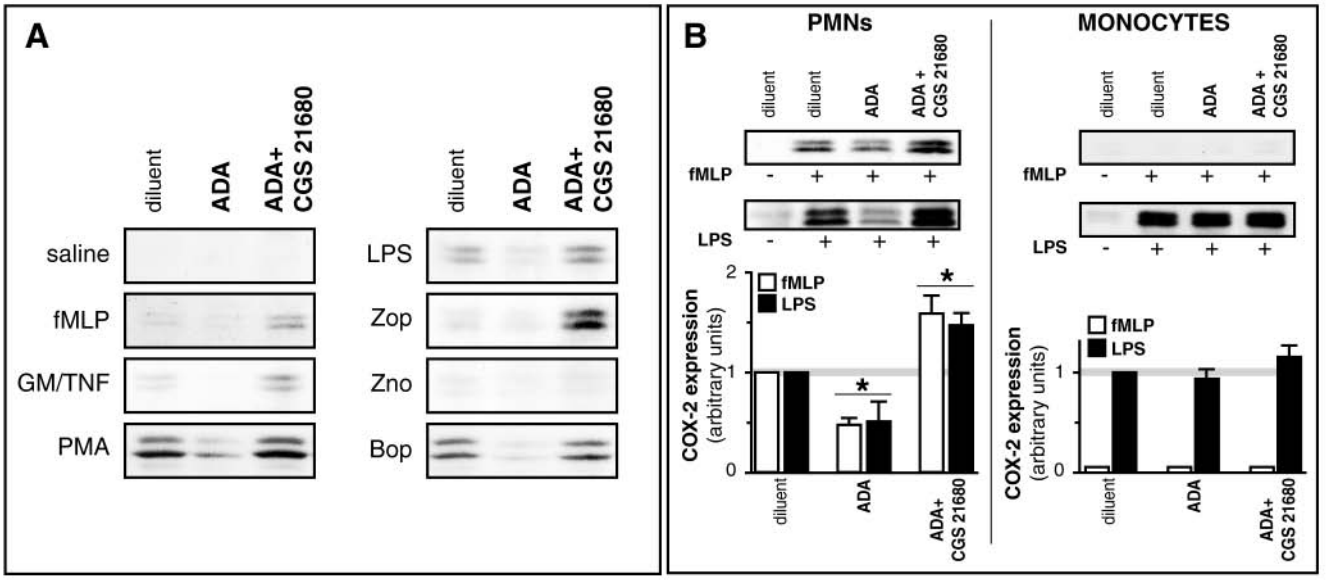


Fig. 2. Activation of the A_{2A} receptor specifically potentiates the expression of COX-2 from human neutrophils. (A) Human PMNs were stimulated for 60 minutes with indicated agonists, alone or in the presence of adenosine deaminase (ADA; 0.1 U/ml), or with ADA and the $A_{2A}R$ agonist CGS 21680 (1 μM). Reactions were stopped and samples were processed for the determination of COX-2 by western immunoblotting. Shown are immunoblots from one experiment, typical of at least *n*=3 experiments performed in identical conditions with separate donors. fMLP, formyl-methionyl-leucyl-phenylalanine (100 nM); GM/TNF, mixture of granulocyte-macrophage colony-stimulating factor and tumor necrosis factor-α (1 nM and 100 nM, respectively); PMA (10 nM), phorbol 12-myristate 13-acetate; LPS, lipopolysaccharide (100 ng/ml); Zop, opsonized zymosan (300 μg/ml); Zno, nonopsonized zymosan (300 μg/ml); Bop, opsonized bacteria (10^8 bacteria/ml). (B) Human leukocytes were stimulated with fMLP or LPS, for 1 hour (PMNs) or 4 hours (Monocytes) alone or in the presence of ADA or with ADA and CGS 21680; samples were processed for the determination of COX-2 by western immunoblotting. In each condition, one typical immunoblot is shown and is representative of at least *n*=3 experiments performed in identical conditions with different donors. Bar graphs depict the expression of COX-2 protein levels, relative to those observed in leukocytes stimulated in the absence of ADA and CGS 21680, as determined by densitometric analysis of the bands in three independent experiments (mean±s.e.m.; *n*=3). *Statistically different from diluent-treated cells.

time PCR (Fig. 1B). Comparable results were obtained with resident cells of the pouch lining tissues where COX-2 mRNA expression was also specifically increased by LPS (Insert, Fig. 1B). The amplitude of the LPS-elicited COX-2 mRNA increase in leukocytes from $A_{2A}R^{+/+}$ mice was almost twice as strong as that observed in $A_{2A}R^{-/-}$ mice. Resident cells from $A_{2A}R^{+/+}$ or from $A_{2A}R^{-/-}$ mice, however, displayed comparable COX-2 mRNA levels.

Evidence for the potentiating impact of $A_{2A}R$ activation on leukocyte COX-2 was obtained at the protein level also. LPS injection caused a robust and rapid upregulation of COX-2 protein in migrated leukocytes, reaching maximal levels within 1 hour of injection, and sustained for at least 20 hours (Fig. 1C). COX-2 upregulation was approximately twice as much in LPS-stimulated leukocytes elicited from $A_{2A}R^{+/+}$, when compared with $A_{2A}R^{-/-}$ mice (Fig. 1D, left panel). In lining tissues, the impact of $A_{2A}R$ activation on COX-2 levels was not significant.

In addition to obtaining in vivo evidence for the implication of the $A_{2A}R$ receptor in increasing COX-2 expression, this first set of results indicated that, among inflammatory tissues, the impact of $A_{2A}R$ on COX-2 may be of particular importance in leukocytes. Thus, a series of experiments were undertaken to characterize the impact of adenosine on COX-2 in human leukocytes.

To this end, we sought to determine whether the $A_{2A}R$-driven potentiation of COX-2 observed in fMLP-stimulated PMNs (Pouliot et al., 2002) could be extended to other stimuli. When stimulations were performed in the presence of adenosine deaminase (ADA), which prevents the accumulation of endogenous adenosine in cell suspensions, the upregulation of COX-2 expression was markedly decreased with each of the tested agonists. By contrast, addition of the specific $A_{2A}R$ agonist CGS 21680 entirely re-established upregulation of COX-2 in cases of stimulation with PMA or opsonized bacteria, and potentiated COX-2 levels in cases of fMLP-stimulated or opsonized zymosan-stimulated PMNs (Fig. 2A). These results indicate a central role for $A_{2A}R$ in regulating COX-2 expression in PMNs.

Circulating leukocytes which may express COX-2 include PMNs and monocytes. $A_{2A}R$ activation nearly tripled leukocyte COX-2 expression, both in fMLP- and LPS-stimulated PMNs (Fig. 2B). In monocytes, fMLP did not induce COX-2 and $A_{2A}R$ activation had no significant impact on LPS-stimulated COX-2 expression, underlining cell-specific differences in mechanisms controlling COX-2 expression, even in closely related cell types. This result is in line with previous observations made by Flamand et al., who reported that PMNs are 100 times more sensitive than monocytes to the action of CGS 21680 for the inhibition of LTB_4 (Flamand et al., 2000). Along with the in vivo data, these results indicate that among inflammatory cells, PMNs can be particularly affected by $A_{2A}R$ activation. The action of the $A_{2A}R$ on COX-2, specifically observed in PMNs, indeed suggests that these cells may constitute an important

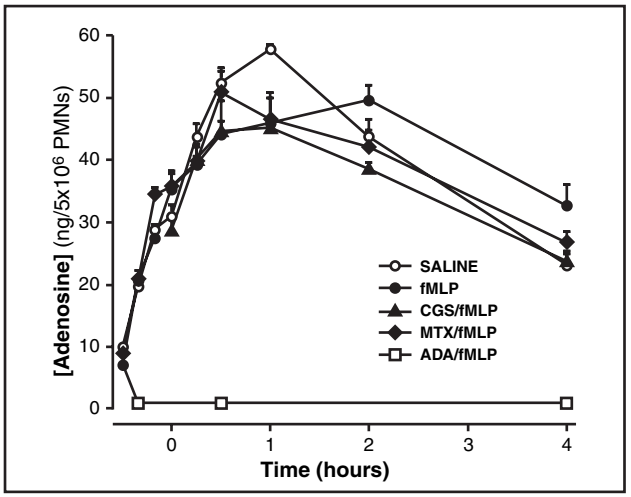


Fig. 3. The anti-inflammatory drug methotrexate does not influence the release of adenosine from fMLP-stimulated neutrophils. Human PMNs were stimulated with fMLP, alone or in the presence of CGS 21680, or with the anti-inflammatory compound methotrexate (MTX; 1 μM), or with adenosine deaminase (ADA), as described in Materials and Methods. At indicated time points, samples were processed for the measurement of extracellular adenosine by mass-spectrometry.

component of the anti-inflammatory responses triggered by adenosine.

In view of the impact of adenosine on many PMNs inflammatory functions, we verified the possibility that endogenous adenosine levels produced by PMNs might be influenced by cell stimulation and/or by incubation with methotrexate. Methotrexate is a widely prescribed anti-inflammatory drug with an efficacy that is nearly unsurpassed in the treatment of arthritis and whose mode of action probably includes an increased release of adenosine at inflammatory sites (Chan and Cronstein, 2002; Cronstein et al., 1993). As can be observed in Fig. 3, adenosine rapidly accumulated in the extracellular milieu of PMN suspensions and plateaued within 60 minutes of stimulation, after which it gradually decreased. The accumulation of adenosine was not significantly affected by stimulation of the cells by fMLP, nor by the addition of CGS 21680. These results confirm previous observations made in platelet-activating factor-stimulated PMNs (Krump et al., 1997). Similarly, methotrexate did not markedly affect the accumulation profile of adenosine, thus suggesting that this anti-inflammatory drug may be acting on other cell types, rather than directly on PMNs, for the increased accumulation of adenosine. Addition of ADA, however, efficiently eliminated extracellular adenosine at all the times tested.

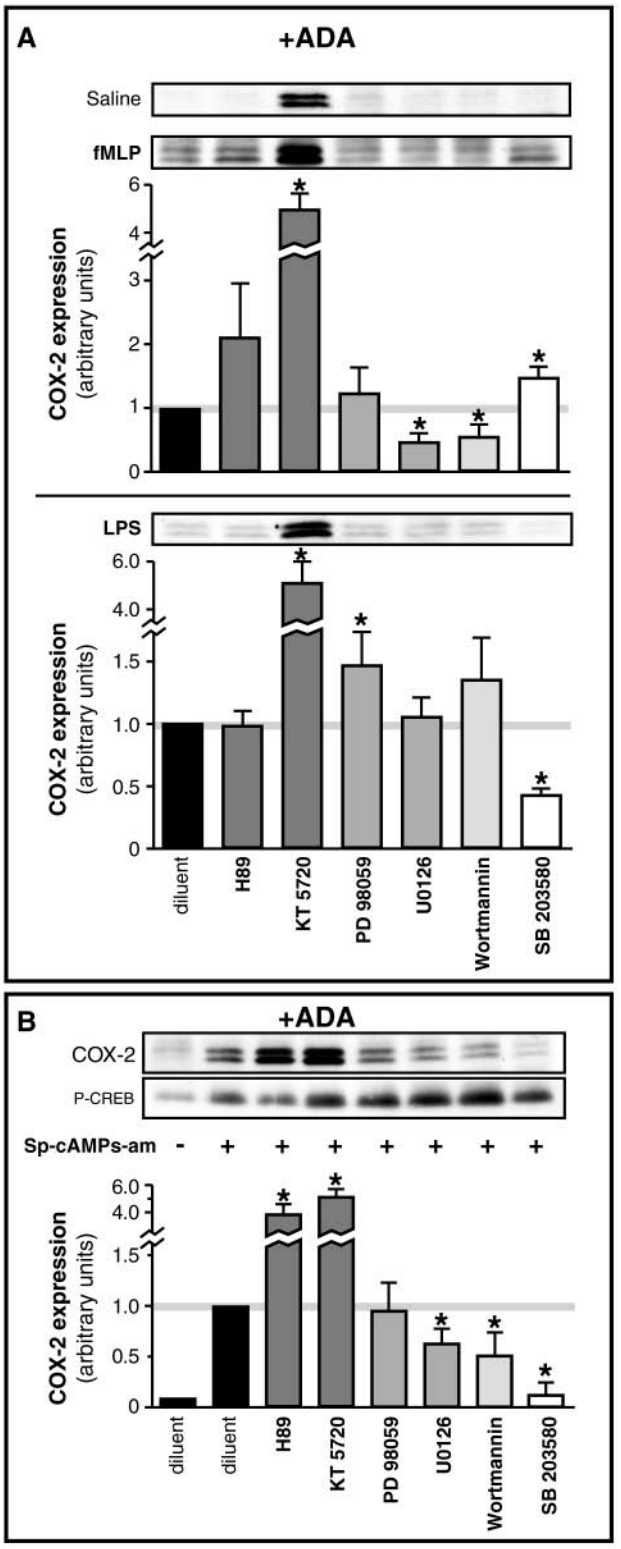
Given the specific and apparently central effect of $A_{2A}R$ activation on COX-2 in inflammatory PMNs, we undertook to identify signal transduction pathways which can lead to its induction or which mediate the potentiating impact of $A_{2A}R$ on this important enzyme in human PMNs. From the abovementioned series of agonists, two were selected – fMLP and LPS – because these agonists utilize distinct classes of receptors and also because their effects on PMNs are well-documented. An initial set of incubations was performed in the presence of ADA to minimize possible intervention of adenosine receptors. The implications of the major PMN metabolic pathways: protein kinase A (PKA), ERK1/2, PI-3K, p38 MAPK and cAMP were addressed. In fMLP-stimulated PMNs, ERK1/2- and PI-3K-specific inhibitors prevented COX-2 upregulation by approximately 50% (Fig. 4A, upper panel). By contrast, p38 MAPK appeared to be solely implicated in LPS-stimulated PMNs (approx. 50% inhibition by SB 203580), as none of the other inhibitors interfered with COX-2 induction (Fig. 4A, lower panel). Surprisingly, the PKA inhibitor KT 5720 superinduced the expression of COX-2 in inflammatory PMNs. Caution must be taken in this case, however, because KT 5720 by itself (in nonstimulated PMNs) caused induction of COX-2 (Fig. 4A, upper panel). The other structurally distinct PKA inhibitor used, H89, doubled fMLP-induced COX-2 expression but had no effect in LPS-stimulated PMNs. These data identify the ERK1/2 and PI-3K pathways as being directly implicated in fMLP-triggered induction of COX-2, and the p38 MAPK pathway in LPS-stimulated PMNs.

Elevated concentrations of intracellular cAMP ($cAMP_i$) have been reported to upregulate COX-2 in human PMNs (Pouliot et al., 2002) as well as in other cell types, in a PKA-dependent fashion. In view of the somewhat surprising results obtained herein with PKA inhibitors, we looked into PMN signal transduction pathways that could be regulated by cAMP. To this end, intracellular cAMP in PMNs was elevated pharmacologically using a cell permeable and stable cAMP analog, Sp-cAMPs-am, alone or in combination with metabolic inhibitors. This receptor-independent rise in $cAMP_i$ caused induction of COX-2, supporting the implication of the classic cAMP/PKA/CREB pathway (Fig. 4B). Inhibition of the p38 pathway efficiently blocked COX-2 induction. The ERK1/2 and PI-3K pathways appeared to be involved also, albeit to a lesser extent. Again, inhibition of PKA by two structurally distinct specific inhibitors superinduced (by approximately fivefold) COX-2 expression. In turn, we assessed CREB activation by performing immunoblots specifically for the Ser133 phospho-CREB isoform. As expected, elevated $cAMP_i$ was sufficient to activate CREB (Fig. 4B). However, in the presence of metabolic inhibitors, CREB activation did not correlate with levels of COX-2 induction indicating that in addition to CREB, other transcription factors are likely to be important in regulating COX-2 induction. PKA inhibitors had differential effects on CREB activation levels, perhaps reflecting incomplete inhibition of PKA at the concentrations used. Delineation of signal transduction pathways leading to the induction of COX-2 in PMNs is depicted in Fig. 5.

Signal transduction pathways responsible for the potentiation of COX-2 in response to $A_{2A}R$ activation were next addressed. PMNs were stimulated in the presence of ADA and a specific and stable agonist of the $A_{2A}R$, CGS 21680 (Jarvis et al., 1989), alone or in combination with metabolic inhibitors. In fMLP-stimulated cells, inhibition of PI-3K by wortmannin efficiently blocked COX-2 induction (approx. 80% inhibition; Fig. 6A, upper panel) but it was only moderately effective in LPS-stimulated PMNs (approx. 30% inhibition; Fig. 6A, lower panel). ERK1/2 inhibition was more also effective in fMLP-stimulated PMNs, inhibitions ranging from 40% to 70%. The inhibition of p38 markedly blocked COX-2 induction in LPS-stimulated PMNs (approx. 80% inhibition), whereas it only had a marginal effect in fMLP-stimulated cells. Blocking PKA had no inhibitory effect on the

potentiation of COX-2 by CGS 21680, either in fMLP- or in LPS-stimulated PMNs. These results indicate that in inflammatory PMNs, downstream events following $A_{2A}R$ engagement which lead to the potentiation of COX-2 involve similar pathways to those involved in its direct induction.

Because elevated $cAMP_i$ has been suggested to mediate several of the documented anti-inflammatory events following



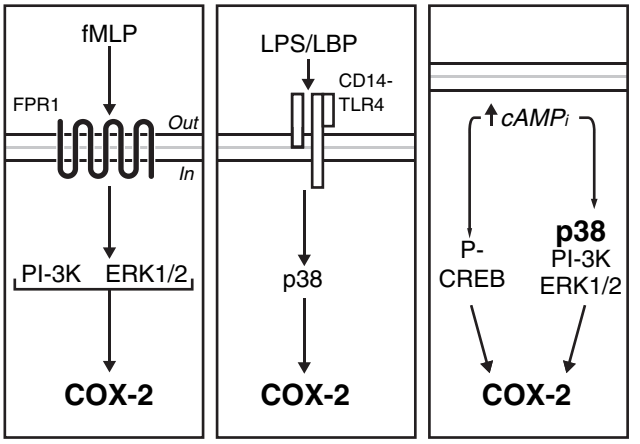


Fig. 5. Cellular pathways leading to upregulation of COX-2 in neutrophils. Left*:* In adenosine-free conditions, the PI-3K and ERK pathways are primarily involved in the induction of COX-2, following engagement of formylated peptide receptor-1 (FPR1). Center*:* In response to LPS, the p38 pathway is almost exclusively solicited. Right*:* An elevation of intracellular cAMP concentrations can cause COX-2 induction, through a classic PKA-dependent activation of CREB, or through activation of the p38, PI-3K and ERK1/2 pathways. LBP, LPS-binding protein; TLR4, Toll-like receptor-4.

$A_{2A}R$ activation (Flamand et al., 2000; Flamand et al., 2002; Visser et al., 2000), we mimicked the activation of $A_{2A}R$ by a pharmacological elevation in $cAMP_i$ in fMLP- or LPS-stimulated PMNs to compare its impact on COX-2 potentiation. This elevation was achieved by two distinct approaches: either by using Sp-cAMPs-am, or a mixture of forskolin and RO-20-1724 (adenylyl cyclase activator and phosphodiesterase IV inhibitor, respectively). Although with some degree of variation and respective of the used agonist, the profile of COX-2 expression obtained in Fig. 6B,C roughly resembled that obtained in Fig. 6A, supporting the paradigm

Fig. 4. Distinct metabolic pathways can lead to the upregulation of neutrophil COX-2: implication of PI-3K, ERK1/2, p38 MAPK and cAMP pathways. (A) PMNs were stimulated in adenosine-free conditions (i.e. in the presence of ADA) with fMLP (upper) or LPS (lower), alone or in combination with the indicated metabolic inhibitors. Samples were processed for the determination of COX-2 by western immunoblotting. (B) PMNs were incubated in adenosine-free conditions (i.e. in the presence of ADA) with a cell-permeable stable analog of cAMP, Sp-cAMPs-am (50 µM), alone or in combination with indicated metabolic inhibitors. Samples were processed for the determination of COX-2 and phosphorylated (P-)CREB by western immunoblotting. In each panel, one typical immunoblot is shown and is representative of at least n=3 independent experiments performed in identical conditions with different donors. Bar graphs depict the expression of COX-2 protein levels, relative to those observed in A: PMNs stimulated with fMLP or LPS only (solid black bars) and in B: PMNs incubated with Sp-cAMPs-am only (solid black bar) as determined by densitometric analysis of the bands in three independent experiments (mean±s.e.m.; n=3). *Statistically different from diluent-treated cells. H89 and KT 5720 (two structurally distinct inhibitors of PKA; 10 µM and 3 µM, respectively), PD 98059 (MEK-ERK1/2 inhibitor; 10 µM), U0126 (ERK1/2 inhibitor; 10 µM), wortmannin (PI-3K inhibitor; 200 nM), SB 203580 (p38 inhibitor; 10 µM).

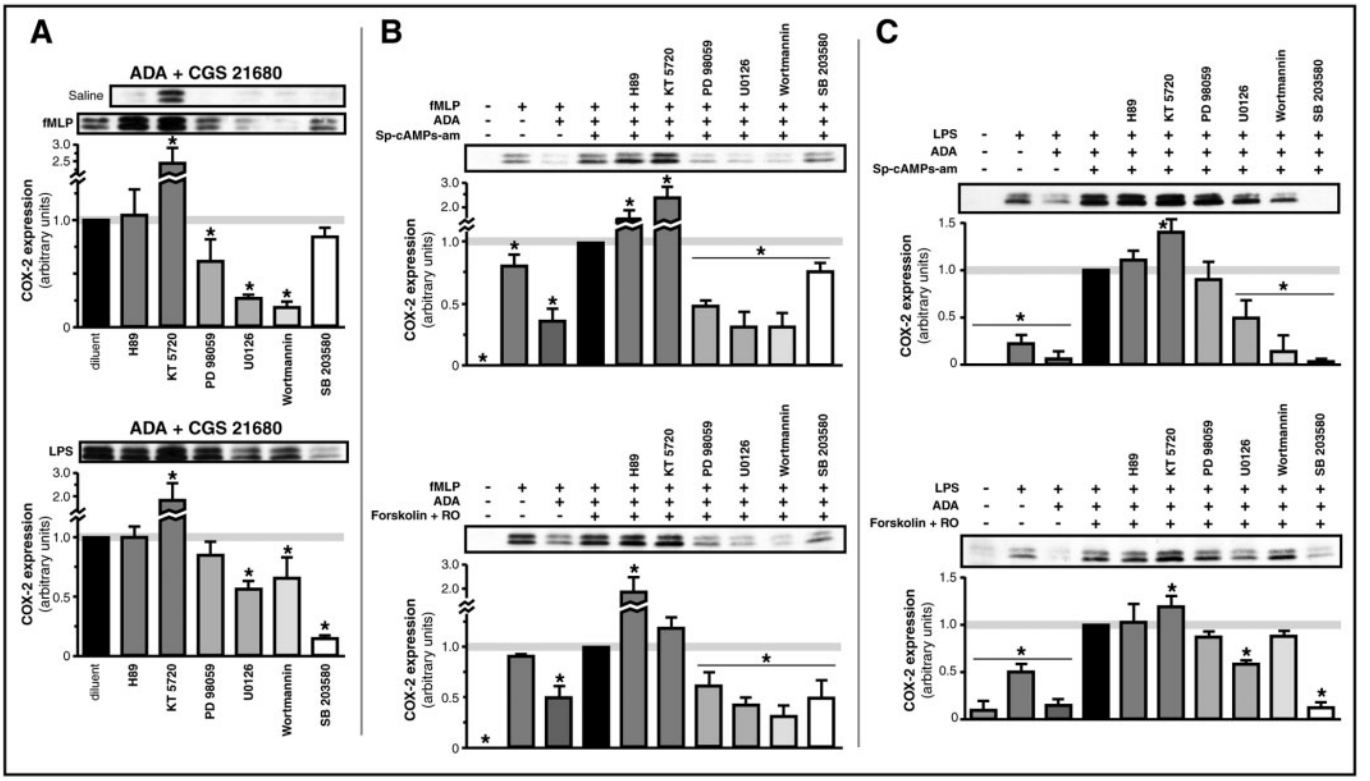


Fig. 6. Metabolic pathways implicated in potentiating COX-2 expression in neutrophils following A_{2A} receptor activation. (A) PMNs were stimulated with fMLP (upper) or LPS (lower) in the presence of ADA and of CGS 21680 (A_{2A}R agonist), alone or in combination with indicated metabolic inhibitors. Samples were processed for the determination of COX-2 by western immunoblotting. Bar graphs depict the expression of COX-2 protein levels, relative to those observed in PMNs stimulated in absence of metabolic inhibitors (solid black bars), as determined by densitometric analysis of the bands in three independent experiments (mean±s.e.m.; *n*=3). (B, C) PMNs were stimulated with fMLP (B) of LPS (C), in combination either with a membrane-permeable cAMP analog, Sp-cAMPs-am (upper), or with a mixture of an activator of adenylyl cyclase and an inhibitor of phosphodiesterase IV, RO-20-1724 and forskolin; 50 μM and 10 μM, respectively (lower), alone or in combination with the indicated metabolic inhibitors. Bar graphs depict the expression of COX-2 protein levels, relative to that observed in PMNs stimulated in absence of metabolic inhibitors, as determined by densitometric analysis of the bands in three independent experiments (mean±s.e.m.; *n*=3). In each panel, one typical immunoblot is shown and is representative of at least *n*=3 independent experiments performed in identical conditions with different donors. *Statistically different from cells treated with stimulus+ADA+cAMP-elevating agents.

that A_{2A}R activation and elevation in $cAMP_i$ levels do indeed share several downstream events. Thus, similar to A_{2A}R activation, elevated $cAMP_i$ may cause activation of the ERK1/2 and PI-3K pathways and also clearly implicates the p38 MAPK pathway in LPS-stimulated cells. Once more, inhibition of PKA did not block COX-2 expression. A summary of observations relating to A_{2A}R activation is depicted in Fig. 7.

Discussion

Adenosine receptors play an increasingly recognized modulatory role in inflammatory responses. In particular, A_{2A}R have been identified as being part of a a nonredundant signaling system in attenuating inflammation and tissue damage; no other mechanism of downregulation of inflammation in vivo appears able to fully compensate for the lack of A_{2A}R; this was clearly shown with mice deficient in the A_{2A}R, which developed extensive tissue damage in response to subthreshold concentrations of inflammatory stimuli (Ohta and Sitkovsky, 2001). Accordingly, there is clinical interest for the use of adenosine and its analogues in the treatment of inflammatory diseases such as arthritis and asthma, as well as for more general processes such as wound healing, and this interest stresses the need for a better understanding of the downstream events triggered by anti-inflammatory A_{2A}R agonists (for a review, see Kaiser and Quinn, 1999).

We determined in the present study that A_{2A}R positively impacts on the expression of COX-2. In the murine air pouch model of inflammation, using wild-type and A_{2A}R knockout mice, the potentiation was observed in migrated leukocytes, which are predominantly neutrophils (Nakamura et al., 2001). Resident cells of the pouch lining tissue are also actively implicated in the inflammatory response and serve in this model as a major mechanism for leukocyte recruitment during local inflammation (Garcia-Ramallo et al., 2002). Lining tissue harbors fibroblasts, smooth muscle cells and epithelial cells. In these tissues, COX-2 expression was similar in wild-type and A_{2A}R knockout animals, indicating that this particular effect of adenosine is directed mainly at leukocytes. In human circulating leukocytes, the impact of A_{2A}R activation on COX-2 was observed specifically in neutrophils, but not in monocytes. These results contribute to identify the neutrophil as an important effector for A_{2A}R-derived anti-inflammatory signaling. In support of this, activation of the A_{2A}R has been

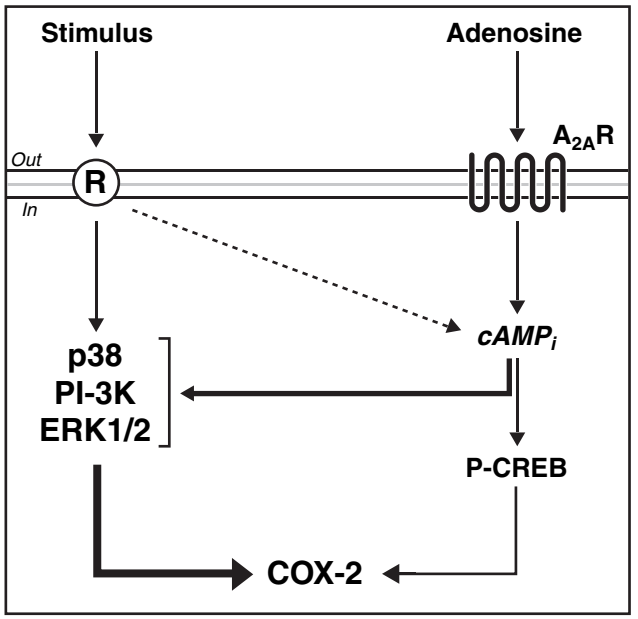


Fig. 7. The cellular pathways implicated in potentiating the expression of COX-2 in neutrophils, following activation of the A_{2A} receptor. Different agonists cause the induction of COX-2 in human neutrophils by activating distinct classes of receptors, which in turn differentially activate the p38, PI-3K and ERK1/2 pathways. Some of these agonists can also transiently elevate $cAMP_i$. Activation of $A_{2A}R$ elevates $cAMP_i$, which has a positive impact on COX-2 by further activating the p38, PI-3K and ERK1/2 pathways and also through the classic PKA/CREB pathway.

shown to centrally mediate the downregulatory impact of adenosine on many of the neutrophil's cytotoxic functions, including the expression of β2-integrins, adhesion, oxygen radical production, degranulation and production of TNF-α (reviewed by Kaiser and Quinn, 1999; Thiel et al., 2003).

Several classes of agonists activate distinct signaling pathways, and they can then converge further downstream to regulate neutrophil COX-2 expression (Pouliot et al., 1998). Agents activating seven transmembrane domain receptors linked to heterotrimeric GTP-binding proteins (e.g. fMLP); receptors comprising tyrosine kinase activity (e.g. GM-CSF), $F_{C\gamma}$ receptors (opsonized particles, LPS), as well as ceramide-linked receptors (TNF-α), can each upregulate COX-2 in neutrophils. In cell incubations made in the presence of ADA, experimental conditions which minimize the implication of adenosine receptors, the PI-3K and ERK1/2, and the p38 MAPK pathways appear to be directly involved in COX-2 induction, following PMN stimulation with fMLP or LPS. An elevation in $cAMP_i$, an event sufficient to induce COX-2 in neutrophils (Pouliot et al., 2002), also caused stimulation of these intracellular pathways; cAMP had a predominant effect on p38 MAPK and also activated PI-3K and ERK1/2. This finding indicates that these typically inflammatory pathways can also lead to anti-inflammatory events in neutrophils. In numerous cell types, the *COX-2* gene can be activated through the relatively direct PKA-dependent activation of CREB, through phosphorylation of its Ser133. Results obtained within the present study further support this paradigm. Indeed, pharmacogically achieved elevations of $cAMP_i$ were seen to be sufficient to elicit upregulation of COX-2 in PMNs, and also caused increased Ser133 phosphorylation of CREB. But PKA inhibitors did not prevent COX-2 induction; in fact, both PKA inhibitors further elevated COX-2 levels. This somewhat surprising result may find an interpretation in considering the regulatory mechanisms of CREB activation. Cyclic AMP stimulates transcription with burst-attenuation kinetics that mirror the rapid phosphorylation and subsequent protein phosphatase 1 (PP1)-mediated dephosphorylation of the CREB at Ser133 (Canettieri et al., 2003; Hagiwara et al., 1992), both events being PKA dependent (Beullens et al., 1993; Van Eynde et al., 1994). Part of PP1 is present in the nucleus in a latent form, composed of PP1 complexed to an inhibitory (NIPP-1) polypeptide (Wera and Hemmings, 1995). NIPP-1 loses its inhibitory potency after phosphorylation by PKA, resulting in an increase in PP1 activation and, in turn, CREB deactivation. At the concentrations of inhibitors used, residual PKA activity may have remained, favoring classic CREB phosphorylation events at the expense of NIPP1/PP1-mediated CREB deactivation, resulting in higher COX-2 expression. Other PP1 (or NIPP-1)-related events, including effects on mRNA stability and translation, cannot be ruled out at this time; further experiments are necessary to fully elucidate this particular point.

Activation of $A_{2A}R$, while having no effect by itself on the expression of COX-2, did activate the p38, PI-3K and ERK1/2 pathways in neutrophils stimulated with inflammatory agonists. The amplitude of its impact on each of these pathways differed, depending on the inflammatory stimulus. For example, while LPS-induced COX-2 expression appears only to involve p38, $A_{2A}R$ activation has a stimulatory effect on each of the studied pathways, accounting for its more-than-additive effect. Nonetheless, the same pathways directly involved in COX-2 induction also seem solicited upon $A_{2A}R$ activation.

In addition to its association with inflammation, COX-2 on the one hand plays a role in regulating cellular proliferation, differentiation and tumorogenesis (Bornfeldt et al., 1997; DuBois et al., 1996), and is emerging as a therapeutic target in the treatment and prevention of many human cancers, including colon, prostate, esophageal and breast cancers (Bertagnolli, 1999; Higashi et al., 2000; Howe et al., 2001; Zimmerman et al., 1986). On the other hand, COX-2 is associated with many physiological functions and possesses anti-inflammatory properties. COX-2 inhibition was found to exacerbate inflammation in an animal model of carrageenin-induced pleurisy (Gilroy and Colville-Nash, 2000). Also, clinical trials and case reports support biochemical evidence that COX-2 inhibitors may promote or worsen a tendency to thrombosis (Cleland et al., 2001) and indicate that the use of anti-COX-2 drugs should be monitored with caution. Furthermore, analyses from COX-2-deficient mice suggest that COX-2 is important for postnatal development and multiple female reproductive processes, including ovulation and fertilization (Dinchuk et al., 1995; Jun et al., 1999; Morham et al., 1995). In neutrophils, PGE_2 is predominantly generated from the COX-2 pathway (Pouliot et al., 1998); PGE_2 inhibits chemotaxis, aggregation, superoxide production, lysosomal release and LTB_4 generation by raising intracellular cAMP above basal levels. In view of the potent anti-inflammatory actions of PGE_2 in inflammatory cells, an augmentation in

COX-2 expression in neutrophils may reflect a potentially important role for these cells in limiting inflammation.

In summary, we determined in the present study that activation of the $A_{2A}R$ positively impacts on the expression of COX-2, with particular amplitude in neutrophils. The ERK1/2, PI-3K and p38 MAPK signaling pathways are directly involved in the induction of COX-2 in these cells and are also solicited in the potentiation of its expression following $A_{2A}R$ activation, a process probably mediated by an elevation in intracellular cAMP. In view of the potent anti-inflammatory activities of PGE_2 and other cAMP-elevating agents in leukocytes, our results identify the neutrophil as a potentially important actor in the limitation of an inflammatory response. The recent clinical interest in the development of cAMP-elevating agents as anti-inflammatory drugs is a promising concept that our results further support.

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References

Bertagnolli, M. M. (1999). Cyclooxygenase-2 as a target for prevention of colorectal cancer. *Curr. Oncol. Rep.* **1**, 173-178.

Beullens, M., Van Eynde, A., Bollen, M. and Stalmans, W. (1993). Inactivation of nuclear inhibitory polypeptides of protein phosphatase-1 (NIPP-1) by protein kinase A. *J. Biol. Chem.* **268**, 13172-13177.

Bornfeldt, K. E., Campbell, J. S., Koyama, H., Argast, G. M., Leslie, C. C., Raines, E. W., Krebs, E. G. and Ross, R. (1997). The mitogen-activated protein kinase pathway can mediate growth inhibition and proliferation in smooth muscle cells. Dependence on the availability of downstream targets. *J. Clin. Invest.* **100**, 875-885.

Böyum, A. (1968). Isolation of mononuclear cells and granulocytes from human blood: isolation of mononuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1 g. *Scand J. Clin. Invest. (Suppl.)* **97**, 77-89.

Busse, W. W. (1998). Leukotrienes and inflammation. *Am. J. Respir. Crit. Care Med.* **157**, S210-S213; discussion S247-S248.

Canettieri, G., Morantte, I., Guzman, E., Asahara, H., Herzig, S., Anderson, S. D., Yates, J. R., III and Montminy, M. (2003). Attenuation of a phosphorylation-dependent activator by an HDAC-PP1 complex. *Nat. Struct. Biol.* **10**, 175-181.

Cassatella, M. A. (1999). Neutrophil-derived proteins: selling cytokines by the pound. *Adv. Immunol.* **73**, 369-509.

Chan, E. S. and Cronstein, B. N. (2002). Molecular action of methotrexate in inflammatory diseases. *Arthritis Res.* **4**, 266-273.

Cleland, L. G., James, M. J., Stamp, L. K. and Penglis, P. S. (2001). COX-2 inhibition and thrombotic tendency: a need for surveillance. *Med. J. Aust.* **175**, 214-217.

Cronstein, B. N., Haines, K. A., Kolasinski, S. and Reibman, J. (1992). Occupancy of GalphaS-linked receptors uncouples chemoattractant receptors from their stimulus-transduction mechanisms in the neutrophil. *Blood* **80**, 1052-1057.

Cronstein, B. N., Naime, D. and Ostad, E. (1993). The antiinflammatory mechanism of methotrexate. Increased adenosine release at inflamed sites diminishes leukocyte accumulation in an in vivo model of inflammation. *J. Clin. Invest.* **92**, 2675-2682.

Dinchuk, J. E., Car, B. D., Focht, R. J., Johnston, J. J., Jaffee, B. D., Covington, M. B., Contel, N. R., Eng, V. M., Collins, R. J., Czerniak, P. M. et al. (1995). Renal abnormalities and an altered inflammatory response in mice lacking cyclooxygenase II. *Nature* **378**, 406-409.

DuBois, R. N., Shao, J., Tsujii, M., Sheng, H. and Beauchamp, R. D. (1996). G1 delay in cells overexpressing prostaglandin endoperoxide synthase-2. *Cancer Res.* **56**, 733-737.

Edwards, S. W. (1994). *Biochemistry and Physiology of the Neutrophil*. Cambridge, UK: Cambridge University Press.

Fantone, J. C., Johnson, K. J., Till, G. O. and Ward, P. A. (1983a). Acute and progressive lung injury secondary to toxic oxygen products from leukocytes. *Chest* **83**, 46S-48S.

Fantone, J. C. and Kinnes, D. A. (1983a). Prostaglandin E1 and prostaglandin I2 modulation of superoxide production by human neutrophils. *Biochem. Biophys. Res. Commun.* **113**, 506-512.

Fantone, J. C., Kunkel, S. L. and Ward, P. A. (1981). Suppression of human polymorphonuclear function after intravenous infusion of prostaglandin E1. *Prostaglandins Med.* **7**, 195-198.

Fantone, J. C., Marasco, W. A., Elgas, L. J. and Ward, P. A. (1983b). Anti-inflammatory effects of prostaglandin E1: imodulation of the formyl peptide chemotactic receptor on the rat neutrophil. *J. Immunol.* **130**, 1495-1497.

Fantone, J. C. and Ward, P. A. (1985). Polymorphonuclear leukocyte-mediated cell and tissue injury: oxygen metabolites and their relations to human disease. *Hum. Pathol.* **16**, 973-978.

Fasano, M. B., Wells, J. D. and Mccall, C. E. (1998). Human neutrophils express the prostaglandin G/H synthase 2 gene when stimulated with bacterial lipopolysaccharide. *Clin. Immunol. Immunopathol.* **87**, 304-308.

Flamand, N., Boudreault, S., Picard, S., Austin, M., Surette, M. E., Plante, H., Krump, E., Vallee, M. J., Gilbert, C., Naccache, P. et al., (2000). Adenosine, a potent natural suppressor of arachidonic acid release and leukotriene biosynthesis in human neutrophils. *Am. J. Respir. Crit. Care Med.* **161**, S88-S94.

Flamand, N., Surette, M. E., Picard, S., Bourgoin, S. and Borgeat, P. (2002). Cyclic AMP-mediated inhibition of 5-lipoxygenase translocation and leukotriene biosynthesis in human neutrophils. *Mol. Pharmacol.* **62**, 250-256.

Garcia-Ramallo, E., Marques, T., Prats, N., Beleta, J., Kunkel, S. L. and Godessart, N. (2002). Resident cell chemokine expression serves as the major mechanism for leukocyte recruitment during local inflammation. *J. Immunol.* **169**, 6467-6473.

Gessi, S., Varani, K., Merighi, S., Ongini, E. and Borea, P. A. (2000). A(2A) adenosine receptors in human peripheral blood cells. *Br. J. Pharmacol.* **129**, 2-11.

Gilroy, D. W. and Colville-Nash, P. R. (2000). New insights into the role of COX 2 in inflammation. *J. Mol. Med.* **78**, 121-129.

Hagiwara, M., Alberts, A., Brindle, P., Meinkoth, J., Feramisco, J., Deng, T., Karin, M., Shenolikar, S. and Montminy, M. (1992). Transcriptional attenuation following cAMP induction requires PP-1-mediated dephosphorylation of CREB. *Cell* **70**, 105-113.

Ham, E. A., Soderman, D. D., Zanetti, M. E., Dougherty, H. W., McCauley, E. and Kuehl, F. A., Jr (1983). Inhibition by prostaglandins of leukotriene B4 release from activated neutrophils. *Proc. Natl. Acad. Sci. USA* **80**, 4349-4353.

Higashi, Y., Kanekura, T. and Kanzaki, T. (2000). Enhanced expression of cyclooxygenase (COX)-2 in human skin epidermal cancer cells: evidence for growth suppression by inhibiting COX-2 expression. *Int. J. Cancer* **86**, 667-671.

Howe, L. R., Subbaramaiah, K., Brown, A. M. and Dannenberg, A. J. (2001). Cyclooxygenase-2: a target for the prevention and treatment of breast cancer. *Endocr. Relat. Cancer* **8**, 97-114.

Ignarro, L. J. and George, W. J. (1974). Hormonal control of lysosomal enzyme release from human neutrophils: elevation of cyclic nucleotide levels by autonomic neurohormones. *Proc. Natl. Acad. Sci. USA* **71**, 2027-2031.

Jarvis, M. F., Schulz, R., Hutchison, A. J., Do, U. H., Sills, M. A. and Williams, M. (1989). [3H]CGS 21680, a selective A2 adenosine receptor agonist directly labels A2 receptors in rat brain. *J. Pharmacol. Exp. Ther.* **251**, 888-893.

Jun, H. S., Santamaria, P., Lim, H. W., Zhang, M. L. and Yoon, J. W. (1999). Absolute requirement of macrophages for the development and activation of beta-cell cytotoxic CD8+ T-cells in T-cell receptor transgenic NOD mice. *Diabetes* **48**, 34-42.

Kaiser, S. M. and Quinn, R. J. (1999). Adenosine receptors as potential therapeutic targets. *Drug Discovery Today* **4**, 542-551.

Krump, E. and Borgeat, P. (1999). Adenosine. An endogenous inhibitor of arachidonic acid release and leukotriene biosynthesis in human neutrophils. *Adv. Exp. Med. Biol.* **447**, 107-115.

Krump, E., Lemay, G. and Borgeat, P. (1996). Adenosine A2 receptor-induced inhibition of leukotriene B4 synthesis in whole blood ex vivo. *Br. J. Pharmacol.* **117**, 1639-1644.

Krump, E., Picard, S., Mancini, J. and Borgeat, P. (1997). Suppression of leukotriene B4 biosynthesis by endogenous adenosine in ligand-activated human neutrophils. *J. Exp. Med.* **186**, 1401-1406.

Ledent, C., Vaugeois, J. M., Schiffmann, S. N., Pedrazzini, T., El Yacoubi, M., Vanderhaeghen, J. J., Costentin, J., Heath, J. K., Vassart, G. and Parmentier, M. (1997). Aggressiveness, hypoalgesia and high blood pressure in mice lacking the adenosine A2a receptor. *Nature* **388**, 674-678.

Lehmeyer, J. E. and Johnston, R. B., Jr (1978). Effect of anti-inflammatory drugs and agents that elevate intracellular cyclic AMP on the release of toxic oxygen metabolites by phagocytes: studies in a model of tissue-bound IgG. *Clin. Immunol. Immunopathol.* **9**, 482-490.

Maloney, C. G., Kutchera, W. A., Albertine, K. H., Mcintyre, T. M., Prescott, S. M. and Zimmerman, G. A. (1998). Inflammatory agonists induce cyclooxygenase type 2 expression by human neutrophils. *J. Immunol*. **160**, 1402-1410.

Marone, G., Thomas, L. L. and Lichtenstein, L. M. (1980). The role of agonists that activate adenylate cyclase in the control of cAMP metabolism and enzyme release by human polymorphonuclear leukocytes. *J. Immunol.* **125**, 2277-2283.

Montesinos, M. C., Gadangi, P., Longaker, M., Sung, J., Levine, J., Nilsen, D., Reibman, J., Li, M., Jiang, C. K., Hirschhorn, R. et al. (1997). Wound healing is accelerated by agonists of adenosine A2 (G alpha s-linked) receptors. *J. Exp. Med.* **186**, 1615-1620.

Morham, S. G., Langenbach, R., Loftin, C. D., Tiano, H. F., Vouloumanos, N., Jennette, J. C., Mahler, J. F., Kluckman, K. D., Ledford, A., Lee, C. A. et al. (1995). Prostaglandin synthase 2 gene disruption causes severe renal pathology in the mouse. *Cell* **83**, 473-482.

Nakamura, H., Herzenberg, L. A., Bai, J., Araya, S., Kondo, N., Nishinaka, Y. and Yodoi, J. (2001). Circulating thioredoxin suppresses lipopolysaccharide-induced neutrophil chemotaxis. *Proc. Natl. Acad. Sci. USA* **98**, 15143-15148.

Nicosia, S., Capra, V. and Rovati, G. E. (2001). Leukotrienes as mediators of asthma. *Pulm. Pharmacol. Ther.* **14**, 3-19.

Niiro, H., Otsuka, T., Izuhara, K., Yamaoka, K., Ohshima, K., Tanabe, T., Hara, S., Nemoto, Y., Tanaka, Y., Nakashima, H. et al., (1997). Regulation by interleukin-10 and interleukin-4 of cyclooxygenase-2 expression in human neutrophils. *Blood* **89**, 1621-1628.

O'Flaherty, J. T., Kreutzer, D. L. and Ward, P. A. (1979). Effect of prostaglandins E1, E2 and F2alpha on neutrophil aggregation. *Prostaglandins* **17**, 201-210.

Ohta, A. and Sitkovsky, M. (2001). Role of G-protein-coupled adenosine receptors in downregulation of inflammation and protection from tissue damage. *Nature* **414**, 916-920.

Pouliot, M., Fiset, M. E., Masse, M., Naccache, P. H. and Borgeat, P. (2002). Adenosine up-regulates cyclooxygenase-2 in human granulocytes: impact on the balance of eicosanoid generation. *J. Immunol.* **169**, 5279-5286.

Pouliot, M., Gilbert, C., Borgeat, P., Poubelle, P. E., Bourgoin, S., Creminon, C., Maclouf, J., Mccoll, S. R. and Naccache, P. H. (1998). Expression and activity of prostaglandin endoperoxide synthase-2 in agonist-activated human neutrophils. *FASEB J.* **12**, 1109-1123.

Rivkin, I., Rosenblatt, J. and Becker, E. L. (1975). The role of cyclic AMP in the chemotactic responsiveness and spontaneous motility of rabbit peritoneal neutrophils. The inhibition of neutrophil movement and the elevation of cyclic AMP levels by catecholamines, prostaglandins, theophylline and cholera toxin. *J. Immunol.* **115**, 1126-1134.

Sawyer, D. W., Donowitz, G. R. and Mandell, G. L. (1989). Polymorphonuclear neutrophils: an effective antimicrobial force. *Rev. Infect. Dis.* **11 (Suppl 7)**, S1532-S1544.

Scapini, P., Lapinet-Vera, J. A., Gasperini, S., Calzetti, F., Bazzoni, F. and Cassatella, M. A. (2000). The neutrophil as a cellular source of chemokines. *Immunol. Rev.* **177**, 195-203.

Scapini, P., Laudanna, C., Pinardi, C., Allavena, P., Mantovani, A., Sozzani, S. and Cassatella, M. A. (2001). Neutrophils produce biologically active macrophage inflammatory protein-3alpha (MIP-3alpha)/CCL20 and MIP-3beta/CCL19. *Eur. J. Immunol.* **31**, 1981-1988.

Spisani, S., Pareschi, M. C., Buzzi, M., Colamussi, M. L., Biondi, C., Traniello, S., Pagani Zecchini, G., Paglialunga Paradisi, M., Torrini, I. and Ferretti, M. E. (1996). Effect of cyclic AMP level reduction on human neutrophil responses to formylated peptides. *Cell Signal.* **8**, 269-277.

Thiel, M., Caldwell, C. C. and Sitkovsky, M. V. (2003). The critical role of adenosine A2A receptors in downregulation of inflammation and immunity in the pathogenesis of infectious diseases. *Microbes Infect.* **5**, 515-526.

Van Eynde, A., Beullens, M., Stalmans, W. and Bollen, M. (1994). Full activation of a nuclear species of protein phosphatase-1 by phosphorylation with protein kinase A and casein kinase-2. *Biochem. J.* **297**, 447-449.

Visser, S. S., Theron, A. J., Ramafi, G., Ker, J. A. and Anderson, R. (2000). Apparent involvement of the A(2A) subtype adenosine receptor in the anti-inflammatory interactions of CGS 21680, cyclopentyladenosine, and IB-MECA with human neutrophils. *Biochem. Pharmacol.* **60**, 993-999.

Wera, S. and Hemmings, B. A. (1995). Serine/threonine protein phosphatases. *Biochem. J.* **311**, 17-29.

Zimmerman, K. A., Yancopoulos, G. D., Collum, R. G., Smith, R. K., Kohl, N. E., Denis, K. A., Nau, M. M., Witte, O. N., Toran-Allerand, D., Gee, C. E. et al. (1986). Differential expression of myc family genes during murine development. *Nature* **319**, 780-783.

Zurier, R. B., Weissmann, G., Hoffstein, S., Kammerman, S. and Tai, H. H. (1974). Mechanisms of lysosomal enzyme release from human leukocytes. II. Effects of cAMP and cGMP, autonomic agonists, and agents which affect microtubule function. *J. Clin. Invest.* **53**, 297-309.