Regulation of Adenosine Receptor Subtypes during Cultivation of Human Monocytes: Role of Receptors in Preventing Lipopolysaccharide-Triggered Respiratory Burst

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Adenosine is a potent anti-inflammatory agent that modulates the function of cells involved in the inflammatory response. Here we show that it inhibits lipopolysaccharide (LPS)-induced formation of reactive oxygen intermediates (ROI) in both freshly isolated and cultured human monocytes. Blocking of adenosine uptake and inactivation of the adenosine-degrading enzyme adenosine deaminase enhanced the inhibitory action of adenosine, indicating that both pathways regulate the extracellular adenosine concentration. Adenosine-mediated inhibition could be reversed by XAC (xanthine amine congener), an antagonist of the adenosine receptor A2A, and MRS 1220 (N-9-chloro-2-(2-furanyl)[1,2,4]triazolo[1,5-c]quinazolin-5-benzeneacetamide), an A3 receptor antagonist, in both cell populations, whereas DPCPX (1,3-dipropyl-8-cyclopentylxanthine), an A1 receptor antagonist, had no effect. Similar to what was seen with adenosine, CGS 21680, an A2A receptor agonist, and IB-MECA, a nonselective A1 and A3 receptor agonist, dose dependently prevented ROI formation, indicating the involvement of A3 and probably also A2A in the suppressive effect of adenosine. Pretreatment of monocytes with adenosine did not lead to changes in the LPS-induced increase in intracellular calcium levels ([Ca2+]i). Thus, participation of [Ca2+]i in the action of adenosine seems unlikely. The adenosine-mediated suppression of ROI production was found to be more pronounced when monocytes were cultured for 18 h, a time point at which changes in the mRNA expression of adenosine receptors were observed. Most prominent was the increase in the A2A receptor mRNA. These data demonstrate that cultivation of monocytes is accompanied by changes in the inhibitory action of adenosine mediated by A3 and probably also the A2A receptor and that regulation of adenosine receptors is an integral part of the monocyte differentiation program.

Adenosine is a ubiquitous purine nucleoside that regulates a variety of physiologic processes through ligation of the four known adenosine receptors, designated A1, A2A, A2B, and A3 (13, 36, 38). The receptors have been cloned and the deduced sequences revealed that all four are members of the large family of seven transmembrane-spanning, G-protein-coupled receptors (35). A1 and A3 receptors inhibit adenyl cyclase, whereas A2A and A2B receptors activate the enzyme (41, 45). Furthermore, it was found that adenosine receptors can also couple to other second messenger systems, such as calcium or potassium channels (A1), and phospholipase C (A1, A2B, and A3) (38).

Recently, it was shown that adenosine, acting at one or more of its receptors, mediates the anti-inflammatory effects of drugs such as methotrexate, commonly used in the treatment of inflammation and chronic arthritis (5, 8). During conditions associated with metabolic stress, such as ischemia, tissue injury, and inflammation, adenosine can be formed and released into the extracellular space as a result of a rapid degradation of intracellular ATP (10, 24, 30, 44). At these concentrations, adenosine modulates functional responses of inflammatory cells, including monocytes (31). By the occupancy of A2 receptors, adenosine inhibits the production of tumor necrosis factor alpha and interleukin 12 (IL-12) (3, 17, 29, 37), whereas secretion of IL-10, a protective cytokine that suppresses release of IL-12 and tumor necrosis factor alpha, was found to be enhanced (16, 25). Besides diminishing cytokine production, adenosine exerts other anti-inflammatory effects, including inhibition of phagocytosis and C2 production (11, 23). Testing the ability of adenosine to inhibit phagocytosis by monocytes and macrophages, Eppell et al. (11) found that while adenosine had no effect on phagocytosis of freshly isolated monocytes, it acts as a powerful inhibitor in macrophages. According to their data, differentiation of monocytes to macrophages is accompanied by a dramatic increase of A2 receptors, implying that inhibition of the phagocytic activity is regulated by A2 receptors.

Another important biological response associated with inflammation is the release of reactive oxygen intermediates (ROI) by phagocytes. ROI, including hydrogen peroxide (H2O2), hydroxyl radical (OH·), and hypochlorous acid (HOCI), are derived from O2−, which is produced in the so-
called respiratory burst via an NADPH oxidase. By generating these toxic oxidants, phagocytes kill and degrade invading microorganisms. However, an uncontrolled production of ROI in various inflammatory conditions may result in pathological tissue injury (2, 19).

Whereas much attention has been paid to the inhibitory action of adenosine on the oxidative burst in neutrophils (6, 7, 9, 14, 42), little is known about its action in monocytes. Therefore, we tested the ability of adenosine to modulate lipopolysaccharide (LPS)-induced ROI production in human monocytes and studied the role of adenosine receptors involved in this process. Furthermore, we investigated whether differentiation of monocytes to macrophages is accompanied by changes in the inhibitory action of adenosine on ROI production and whether differentiation has any impact on the mRNA expression of the four adenosine receptor subtypes.

MATERIALS AND METHODS

Materials. LPS (Escherichia coli 055:BS), adenosine, N9-carboxy-2-(2-furanyl)1, 2, 4-triazolo[1, 5-c]quinazolin-5-benzeneacetic acid (MRS 1220), 2p-(2-carboxyethyl)phenylmethylamine-5-N-ethylcarboxamidoadenosine (CGS 21680), N6-(3-isodobenzyl)adenosine-5-N-methyluron-amide (IB-MECA), 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), adenosine deaminase, dipyridamole, and ethyl-6-aminobenzofuran-5-oxy[2-(2-N-ethylcarboxamidoadenosine (CGS 21680), N6-(3-isodobenzyl)adenosine, andyethyl)-6-aminobenzofuran-5-oxy-2-(2-N-ethylcarboxamidoadenosine (CGS 21680), N6-(3-isodobenzyl)adenosine-5-N-methyluron-amide (IB-MECA), 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), adenosine deaminase, dipyridamole, and were obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Xanthine anion generator (XAC) was obtained from ICN Biomedicals GmbH (Eschwege, Germany), and luminol was obtained from Roche (Basel, Switzerland). The oligonucleotides were synthesized by MWG Biotech AG (Ebersberg, Germany). The A3A primers were a kind gift of B. N. Cronstein (New York University School of Medicine, New York, N.Y.).

Cell preparation. Monocytes were prepared from human blood or buffy coats by using Ficoll-Isoaque density gradient centrifugation (Pharmacia, Freiburg, Germany). After being washed in phosphate-buffered saline containing 0.3 mM EDTA, the monocytes were isolated by counterflow centrifugation using the J6-MC elutriator system (Beckman Instruments, Palo Alto, Calif.) as described previously (15). Fractions of 80 to 90% human monocytes were used. Monocytes (5 × 10^6 cells/ml) were either used immediately after elutriation or incubated for 18 h or 7 days in RPMI 1640 containing 2% human AB serum, 1 mg of NaHCO3/ml, 1,3-dipropyl-8-cyclopentylxanthine (DPCPX), adenosine deaminase, dipyridamole, and streptomycin (Seromed Biochrom KG)/ml, 1 mM sodium pyruvate, nonessential amino acids (1%; Seromed Biochrom KG), and vitamin solution (0.4%; Gibco BRL, Egstein, Germany) in 6-well plates (TPP, Trasadingen, Switzerland) at 37°C in an atmosphere of 5% CO2.

Measurement of intracellular calcium levels ([Ca2+]i) by Ca2+ imaging. A sample (300 μl) of cell suspension (2 × 10^6 cells) was seeded onto 30-mm-diameter sterile glass coverslips (Marienfeld Laboratory, Bad Mergentheim, Germany) incubated for 30 min at 37°C in an atmosphere of 5% CO2. Monocytes that adhered to coverslips were incubated with 10 μM 1-[2-(5-carboxoyoxazol-2-yl)-6-amino]benzofuran-5-0xy-2’-(2’-aminino-5’-methylphenoxy)ethane-N,N,N’,N’-tetraacetic acid pentaacetoxymethyl ester (FURA-2/AM) (TEF Labs, Austin, Tex.) and 0.1% Pluronic F-127 (TEF Labs) in 1 ml of standard Ca2+ solution at room temperature for 30 min in the dark.

The standard solution contained 125 mM NaCl, 5 mM KCl, 2 mMCaCl2, 10 mM HEPES, and 7.5 mM glucose (adjusted to pH 7.4 with NaOH). The zero calcium solution contained 125 mM NaCl, 5 mM KCl, 2 mM CaCl2, 6 H2O, 1 mM EGTA, 10 mM HEPES, and 7.5 mM glucose (adjusted to pH 7.4 with NaOH). The coverslips were placed in a recording chamber and continuously perfused at room temperature at a rate of 2 ml/min. Solutions were removed by a vacuum pump.

Experiments were performed on a microscope (Axiovert 135; Carl Zeiss Jena GmbH, Jena, Germany) equipped with UV transparent optics (Axiovert 135). Dye excitation illumination was provided by a dual-wavelength illuminator system (T.I.L.L. Photonics GmbH, Gräfelfing, Germany) consisting of a xenon arc lamp, a variable-speed reflective optic chopper, and two monochromators, both under computer control. The excitation and emission wavelengths were 340 and 380 nm, respectively. Emitted fluorescence filtered at 510 nm was collected by a photomultiplier tube and photon-counting photometer. Changes in [Ca2+]i, were expressed as the ratio of dye fluorescence at 340 nm to that at 380 nm. Calcium measurements were performed on fields containing 45 to 70 cell bodies.

Lumino-luminescence assay. Chemiluminescence assays were performed at 37°C using a MicroLumat 900 luminometer (Berthold, Bad Wildbad, Germany). Monocytes (1.0 × 10^6/ml) were suspended in RPMI 1640 (without phenol red) supplemented with 1% fetal calf serum and 5 mM HEPES (Seromed Bioproducts, Berlin, Germany). The cells were allowed to equilibrate at 37°C and 5% CO2 for 30 min in white polystyrene 96 microtiters plates (Wallac, Turku, Finland) in the presence of 140 μM luminol in a final volume of 250 μl. Then the plates were transferred to the luminometer and background readings were recorded. After 30 min, the agonist, the antagonist, or, with a latency of 5 min, both the antagonist and then the agonist were added to the cells and incubation continued for an additional 30 min before LPS (100 ng/ml) was added. The readings continued for a further 120 min. All data are based on the calculation of the area under the curve by using the WinGlow 1.02 program (Berthold). Percentage inhibition was calculated in relation to the control. Lumino-dependent chemiluminescence (LDCL) is given in terms of relative light units per second.

Semiquantitative reverse transcription-PCR. Total RNA was isolated from 6 × 10^6 cells by using an RNasey mini kit (Qiagen, Chatsworth, Calif.) according to the manufacturer’s instructions. cDNA was prepared by annealing RNA with oligo(dT)20 for 10 min at 70°C and reverse transcription using SuperScript II reverse transcriptase (Life Technologies). The primers used in the PCR were as follows: for the A1 receptor cDNA, sense primer 5’-CAG CTG CTT CAT CGT GTC and antisense primer 5’-AGC CAA ACA TAG GGG TCA GTC; for the A2A receptor cDNA, sense primer 5’-ACC TGC AGA AGC TCA CCA AC and antisense primer 5’-TCT GCT TCA GCT GTC GC; for the A3A receptor cDNA, sense primer 5’-GGA ATG CTT GAG ACA CAG and antisense primer 5’-CTG GAG GGT GCT CCT CGA GTC; for the A3B receptor cDNA, (4), sense primer 5’-GCT TAT CTT AAC CGC CTC C and antisense primer 5’-CCG TCT TGA ACT CCC GTC CAT A; and for the GAPDH cDNA, sense primer 5’-AAC AGC GAC ACC AAC TCC TCT CC and antisense primer 5’-GGG GAG ATT CAG TGT GGT. All primer pairs used were intron spanning.

Reactions were performed in a Crocodile III DNA thermal cycler (Oncor Appligene, Heidelberg, Germany) under the following conditions: an initial denaturation step for 5 min at 95°C followed by cycles of 60 s at 95°C (A1a), 62°C (A2a), 68°C (A3B, and A3A), or 60°C (GAPDH), and 90 s at 72°C, with a prolongation of 2 s per cycle. The final extension phase was 5 min at 72°C. The numbers of cycles were 35 for A1; 30 for A2A, A3B, and A3A; and 20 for GAPDH. The PCR products were separated by electrophoresis on 1.8% agarose gels (FMC Bioproducts, Rockland, Mass.) containing 1 μg of ethidium bromide/ml and visualized under UV light. The 100-bp ladder (Life Technologies) served as a molecular weight standard. The amounts of GAPDH PCR product were used as a reference.

Statistics. Each experiment was performed in duplicate. The WinGlow 1.02 program (Berthold) was used for data analysis. Data are presented as means ± standard errors of the means (SEM) of three independent experiments. Multiple comparisons with a control value were performed by one-way analysis of variance followed by Bonferroni’s t test. All other comparisons were made by paired Student’s t test. A probability level of 0.05 or less was considered to be statistically significant. The concentration-response curves were confirmed by a nonlinear regression. The 50% inhibitory concentrations (IC50) were calculated from the concentration-response curves.

RESULTS

Adenosine inhibits LPS-induced ROI production in freshly isolated and cultured human monocytes. Treatment of freshly isolated monocytes with LPS (100 ng/ml) led to a rapid production of ROI (Fig. 1). In the absence of LPS, no ROI were produced. When the monocytes were preincubated with adenosine (1, 10, 50, 100, and 200 μM), LPS-triggered ROI production was suppressed in a concentration-dependent manner (IC50 ≈ 33.6 μM) (Fig. 2). The course of the concentration-
response curve was similar when cultured monocytes were used (IC_{50} = 8.5 \mu M) (Fig. 2). A cultivation time of 18 h was chosen because at later time points monocytes cease to produce ROI in response to LPS (27, 33, 43, 46). At an adenosine concentration of 200 \mu M, ROI production in freshly isolated and cultured monocytes was inhibited by 41.1\% \pm 3.6\% (n = 3) and 44.3\% \pm 4\% (n = 3), respectively.

**Effect of adenosine deaminase, EHNA, and dipyridamole on LPS-induced and adenosine-mediated inhibition of ROI production.** As adenosine inhibited ROI production in monocytes less efficiently (IC_{50} in the micromolar range) than has been described for neutrophils (IC_{50} in the micromolar range [9, 42]), we measured ROI production in the presence of adenosine deaminase, which hydrolyzes adenosine to inosine, to exclude an effect of endogenous adenosine. As shown in Fig. 3A, the enzyme did not enhance ROI production induced by LPS, nor did it abolish the inhibitory action of adenosine. It even caused a slight decrease in ROI production in the absence or presence of adenosine.

We can exclude the possibility that the adenosine deaminase-mediated decrease was due to an accumulation of inosine, a degradation product of adenosine (Fig. 3B), which recently has been suggested to suppress IL-12 production (18). Despite the addition of adenosine deaminase to the cells, adenosine still exerted its effects. It is possible that adenosine deaminase is already present, as described previously (1, 12), so that a further addition of the enzyme has no effect or that the net concentration of adenosine remains constant due to the concerted action of adenosine-degrading and -producing enzymes in activated cells. To elucidate the specific action of adenosine deaminase, LPS-stimulated monocytes were incubated with the adenosine deaminase inhibitor EHNA in the presence or absence of adenosine (Fig. 3A). The presence of EHNA led to a decrease in the LPS-induced burst by 85\% and the adenosine-mediated inhibition was further potentiated, resulting in a nearly complete blocking of the burst. These data show that adenosine deaminase is highly active in LPS-stimulated monocytes and that adenosine, when spared from degradation by adenosine deaminase, very effectively inhibits the burst.

To test whether cellular uptake of adenosine by monocytes influences the inhibitory effect on ROI production, the cells were treated with dipyridamole (0.1, 1, and 10 \mu M) prior to the addition of adenosine (10, 100, and 200 \mu M) and LPS (100 ng/ml). As shown in Fig. 3C, dipyridamole by itself inhibited the ROI production in a concentration-dependent manner. Addition of adenosine potentiated this inhibitory effect.

**Analysis of adenosine receptor subtype mRNA expression in freshly isolated and cultured human monocytes and in macrophages.** The inhibitory action of adenosine on the LPS-induced oxidative burst and the potentiation of this effect when the adenosine uptake is blocked imply the involvement of adenosine receptor subtypes. Before identifying the receptor(s) mediating the action, we determined which of the four receptor mRNAs were present in monocytes and whether they were regulated during cultivation. The expression of the A_{2A} and A_{2B} receptor mRNA was far less pronounced in freshly isolated than in cells cultured for 18 h (Fig. 4). After 7 days in culture, A_{2B} receptor mRNA expression hardly changed whereas A_{2A} receptor mRNA was no longer detectable. The kinetics of the A_{1} and A_{3} receptor mRNA expression showed a slight increase after 7 days of culture.

**Effect of adenosine receptor antagonists on the adenosine-induced inhibition of LPS-induced ROI production.** Having shown that the mRNA of all receptors is present in freshly isolated monocytes and in monocytes cultured for 18 h, the role of the receptor(s) involved in the inhibitory action of adenosine on the LPS-induced oxidative burst was investigated. Cells were preincubated with receptor-selective antagonists for 5 min before adenosine was added. The antagonists, which were used at a concentration of 1 \mu M, by themselves did not induce ROI production either in the presence or in the absence of LPS. To block A_{1}, DPCPX was used (22). It did not.
A

LPS (100 ng/ml) + + + + + + +
Adenosine (100 μM) - - + + + + +
EHNA (5 μM) - - + - - + +
Adenosine deaminase (0.25 U/ml) - + - - + - +

B

LDCL [%]

120
100
80
60
40
20
0

Inosine (μM)

10 100 200 500

C

LDCL [%]

100
80
60
40
20
0

10 100 200

control 0.1 μM dipyridamole 1 μM dipyridamole 10 μM dipyridamole
reverse the effect of adenosine, with the inhibition being 38% in freshly isolated and 46% in cultured monocytes (Fig. 5). In the presence of XAC, which possesses high affinity at the A2A receptors (1 nM) (22) but also exhibits nanomolar affinity for the other three receptors (20, 22, 28), the adenosine-induced inhibition was prevented in freshly isolated monocytes. In cultured monocytes, XAC not only reversed the inhibitory action of adenosine but even stimulated ROI production. Similar to XAC, the A3 antagonist MRS 1220, a xanthine derivative, which is highly selective for human A3 receptor (21), but in the nanomolar range also blocks A2A (32), reversed the effect of adenosine in both cell populations.

**Effect of receptor agonists on LPS-induced ROI production.** As XAC and MRS 1220 reversed the inhibitory action of adenosine, we tested whether CGS 21680, a nonselective A2A and A3 receptor agonist (22), and IB-MECA, a nonselective A1 and A3 receptor agonist (22), have similar effects as adenosine. In freshly isolated and cultured monocytes, CGS 21680 suppressed the production of ROI in a dose-dependent manner (Fig. 6A). At a concentration of 50 μM, inhibition of ROI production in cultured monocytes was more pronounced (68%) than in freshly isolated monocytes (39%) (Fig. 6A).

To define the action of CGS 21680 more precisely, the cells were preincubated with the antagonists DPCPX, MRS 1220, and XAC before CGS 21680 and LPS were added. As shown in Fig. 6B, DPCPX had no effect, MRS 1220 slightly reduced the CGS 21680-induced inhibition, and XAC partly prevented the inhibition.

Compared to CGS 21680, IB-MECA suppressed the LPS-induced ROI production more efficiently (Fig. 7A). At a concentration of only 10 μM, inhibitions in freshly isolated and cultured monocytes were found to be 45 and 67%, respectively. The inhibitory effect of IB-MECA could be prevented by XAC, whereas MRS 1220 partly reversed the inhibition and DPCPX had no effect (Fig. 7B).

**Calcium signaling in response to adenosine.** Multiple intracellular signaling pathways can be induced by adenosine receptor stimulation. We tested how far modulation of Ca2+ mobilization could be involved in the adenosine-mediated inhibition of the LPS-induced oxidative burst. As shown in Fig. 8A, incubation of monocytes with LPS (100 ng/ml) led to a minor gradual increase in [Ca2+]i. The addition of adenosine, which by itself elicits a rapid rise in [Ca2+]i, prior to stimulation with LPS had no effect on the LPS-induced change in [Ca2+]i (Fig. 8B), indicating that the inhibitory action of adenosine is unlikely to be mediated by changes in [Ca2+]i.

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**DISCUSSION**

Here we show that differentiation of monocytes to macrophages is accompanied by a change in the mRNA expression of the four adenosine receptor subtypes. Already at 18 h after cultivation, an increase of both A2A and A2B receptor mRNA can be seen. Prolonging the cultivation time up to 7 days, during which monocytes differentiate to macrophages, results in the disappearance of A2A receptor mRNA expression. However, expression of A2B receptor mRNA is still higher than in freshly isolated monocytes. Consistent with this latter finding, Eppell et al. showed by functional analysis that differentiation of monocytes to macrophages is associated with an increase in A2 receptors (11), presumably the A2B receptor.

When studying the involvement of the adenosine receptors in mediating the adenosine-induced inhibition of ROI production, relatively high concentrations (micromolar) of adenosine were needed to exert an effect. This is in line with data from other reports in which the inhibitory action of adenosine on ROI production and other biological answers in monocytes have been described (3, 4, 17, 26, 40). According to our data, the IC50s may result from a rapid uptake and an extensive metabolism of adenosine.

We found that the blockade of adenosine uptake (by dipyr-
ridamole) and the inhibition of endogenous adenosine deaminase activity (by EHNA) both resulted in a decrease in LPS-induced ROI production even in the absence of exogenous adenosine, indicating that endogenous adenosine is produced in stimulated monocytes and that adenosine, if spared from uptake and degradation, exerts its inhibitory effects by acting at its receptors. To our surprise, the addition of adenosine deaminase to the cells in the absence or presence of adenosine did not result in an increase in ROI production. The fact that inhibition of adenosine deaminase by EHNA caused a drastic inhibition of the oxidative burst points to high adenosine deaminase activity so that further addition of adenosine deaminase may not affect the equilibrium between adenosine and adenosine deaminase in the cell culture.

A3 (monocytes) and A2A and probably A3 (neutrophils) have mainly been suggested to be the adenosine receptor subtypes that mediate the inhibitory action of adenosine on fMLP (formyl-Met-Leu-Phe)-induced respiratory burst in monocytes (4) and neutrophils (9, 14). In line with these observations, our data indicate that the action of adenosine on LPS-induced ROI production depends on A3 and probably also A2A activation. This conclusion is based on the observation that the A2A and A3 antagonists XAC and MRS 1220 blocked the inhibitory action of adenosine while the A1 antagonist DPCPX was without effect.

In humans, XAC has been described as A2A selective ($K_i$ ∼ 1 nM) (22), with the $K_i$ for A3 being 92 nM (22), and MRS 1220 has been described as A3 selective ($K_i$ ∼ 0.65 nM) (21), with the $K_i$ for A2A being 15 nM (32). It should be pointed out that at a concentration of 1 μM, as used in this study, both antagonists can block both receptor subtypes. Lowering the concentration of XAC and MRS 1220 to 10 nM, a concentration at which MRS 1220 might bind only to A3 and XAC only to A2A, did not prevent the inhibition induced by adenosine (100 μM), IB-MECA (10 μM), or CGS 21680 (50 μM) (data not shown). This might be due to the fact that the concentration of the antagonists is 10,000 to 1,000 times lower than that of the agonists.

Irrespective of these limitations, IB-MECA, a nonselective A1 and A3 receptor agonist (22), and CGS 21680, which shows some degree of selectivity for A2A receptors (22), were tested for their ability to block LPS-induced ROI production. Both agonists dose dependently inhibited ROI formation, with IB-MECA being more effective than CGS 21680. Similar to adenosine, IB-MECA and CGS 21680 exerted their effects only at concentrations in the micromolar range. The presence of adenosine deaminase had no influence on their inhibitory action (data not shown). As the enzyme did not prevent the inhibitory action of exogenous adenosine, it is possible that due to the endogenous enzyme activity the adenosine concentration is too low to be responsible for the low affinity of the agonist. However, despite its relatively low affinity to the adenosine receptor, IB-MECA induced a complete inhibition of LPS-induced ROI production while adenosine only partly prevented ROI production. It is most likely that adenosine, in contrast to IB-MECA, is metabolized by adenosine deaminase or that adenosine binds to several receptors whereas IB-MECA is more selective.

As IB-MECA is more efficient than CGS 21680 in inhibiting the LPS-induced respiratory burst, an involvement of A3 seems more likely than that of A2A. However, the fact that inhibition induced by CGS 21680 and IB-MECA were both prevented by XAC, only partly blocked by MRS 1220, but not prevented by DPCPX supports an involvement of A2A and A3. The ability of XAC to prevent the effects of both CGS 21680 and IB-MECA
suggests that this antagonist at a concentration of 1 μM blocked not only A2A but also A3.

It should be pointed out that the interpretation of the present data is based on $K_i$ and $K_d$ values of agonists and antagonists obtained from binding studies mainly carried out.
with transfected cell lines or isolated membrane preparations. In view of the tissue-specific differences in pharmacological properties, binding data for ligands may differ from functional data derived from experiments carried out with intact cells.

Comparing the inhibitory effects of A2A and A3 receptor agonists on freshly isolated monocytes with those on cultured monocytes clearly showed that the inhibitory action was stronger in the latter. Whether this is mirrored by an upregulation of the two receptors at the protein level remains to be clarified; an upregulation of the A2A receptor mRNA, however, could be observed.

The intracellular mechanisms involved in the inhibition of ROI production by adenosine have yet to be defined. A previous study by Broussas et al. (4) indicated that signaling pathways in monocytes do not include adenylate cyclase-dependent cyclic AMP (cAMP) elevation or changes in calcium mobilization.

According to our results and in line with those of Broussas et al. (4), a signaling role of calcium seems unlikely since the application of adenosine failed to inhibit the LPS-induced rise in [Ca2+]. However, in contrast to Broussas et al., we found an increase in [Ca2+] upon addition of adenosine to monocytes. The percentage of cells that responded to adenosine varied among donors but monocytes from nearly all donors (12 of 15) did react. Nevertheless, the rise in [Ca2+] induced by LPS was not affected by adenosine. Thus, if calcium signaling is a prerequisite for LPS to activate the NADPH oxidase, the adenosine-mediated rise in [Ca2+] obviously does not affect signaling pathways that interfere with LPS-induced calcium signaling.

While Sullivan et al. (42) suggested an involvement of cAMP in A2 receptor-mediated regulation of the oxidative burst in human neutrophils, other groups have questioned an enhancement of cAMP as an effector mechanism in these cells (6, 7). Alternative signal transduction mechanisms such as activation of a serine/threonine protein phosphatase (39) or participation of inositol 1,4,5-phosphate (IP3) in A2 receptor-mediated events (34) have been proposed.

In summary, our data show that differentiation of monocytes to macrophages is accompanied by the differential expression of adenosine receptor mRNA. This may provide potential means to regulate adenosine-mediated biological answers. Differences in the adenosine-mediated inhibition of the LPS-induced oxidative burst were seen between cultivated and freshly isolated monocytes. How far this effect, which we showed to be mediated by A3 and A2A receptors, relates to the expression of the corresponding adenosine receptor protein remains to be clarified.

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