Adenosine A_1, A_{2a}, A_{2b}, and A_3 receptors in hematopoiesis. 2. Expression of receptor mRNA in resting and lipopolysaccharide-activated mouse RAW 264.7 macrophages

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Short title: Adenosine receptors and macrophages

Summary

Expression of mRNA for adenosine receptor subtypes A_1, A_{2a}, A_{2b}, and A_3 in normal and lipopolysaccharide (LPS)-activated murine RAW 264.7 macrophages has been investigated using the method of quantitative real-time polymerase chain reaction. The results have shown a very low, unquantifiable expression of adenosine A_1 receptor mRNA both in normal and LPS-activated macrophages. The other three adenosine receptor mRNAs have been found to be expressed at various but always quantifiable levels. Activation of the macrophages with LPS induced upregulation of the expression of adenosine receptor A_{2a} and A_{2b} mRNA whereas the expression of adenosine receptor A_3 mRNA was downregulated. Unstimulated macrophages exhibited a high expression of the A_{2b} adenosine receptor mRNA. The findings are discussed from the point of view of the antiinflammatory and hematopoiesis-stimulating roles of the adenosine receptor signaling.

Key words: Adenosine receptors; Macrophage; mRNA expression

Introduction

Adenosine, an ubiquitous purine nucleoside released into the extracellular environment from metabolically active or stressed cells, has been recognized to act as a paracrine regulator of a number of cellular functions (Poulsen and Quinn 1998, Schulte and Fredholm 2003). The regulatory role of extracellular adenosine is based on the activation of specific receptors located on the cell surface. Functional and molecular studies made it possible to classify adenosine recptors as A_1, A_{2a}, A_{2b}, and A_3 subtypes. These G-protein-coupled receptors differ in their abilities to modulate various functions and their roles in various areas of medicine have been widely investigated (Olah and Stiles 1995).

An important regulatory system, which is believed to involve both macrophages and adenosine receptor signaling, is that of modulation of hematopoietic processes. Hematopoiesis-modulating effects of adenosine receptor agonists have been reported (reviewed in Hofer and Pospíšil 2006). Activated macrophages have been found to produce hematopoiesis-stimulating factors, e.g., interleukin-6 (IL-6) or granulocyte colony-stimulating factor (G-CSF) (e.g., Hofer *et al.* 2007, Kamio *et al.* 2008). Administration of drugs elevating extracellular adenosine and strengthening, thus, its receptor-mediated action, has been found to increase the ability of serum of the treated mice to support the proliferation of granulocyte/macrophage progenitor cells (Weiterová *et al.* 2007). It may be hypothesized from these observations that the actions of adenosine receptor agonists on hematopoietic cells

are indirect, mediated by other cells and that macrophages might play a role in this phenomenon.

A considerable attention is also paid to the participation of adenosine receptor signaling in immunological and inflammatory processes. Here, the interest is focused on the functions of macrophages and to their modulation by the adenosine receptor system, i.e. on the expresion of individual adenosine receptors by these cells, as well as on their role in mechanisms of inflammation. The accumulated evidence indicates that it is especially the activation of adenosine A_{2a} receptor subtype which downregulates the inflammation and mediates protection from tissue damage (Ohta and Sitkovsky 2001, Haskó and Pacher 2008). An increased expression of mRNA for adenosine A_{2a} receptor in macrophages under inflammatory states has also been reported (Khoa *et al.* 2001, Bshesh *et al.* 2002) and confirmed recently in experiments of Murphree et al. (2005) on mouse peritoneal macrophages, human macrophages, and Wehi-3 cells.

Experiments, the results of which are presented here, are focused on investigations of levels of adenosine receptor mRNAs of all four adenosine receptor subtypes in resting and LPS-activated mouse RAW 264.7 macrophages. They comprise determinations of mRNA expression in three time intervals up to 24 hours following the initiation of macrophage activation by LPS as well as statistical processing of the data obtained.

Methods

Murine macrophage RAW 264.7 cell line

Murine macrophage cell line RAW 264.7, purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) was maintained in D-MEM medium supplemented with 10% fetal heat-inactivated bovine serum. For experiments $1x10^6$ cell/well (1.0 ml) were seeded onto 24-well plates. The cells were allowed to adhere for 1 hour at 37^oC , non-adherent cells were removed by aspiration and wells were filled with 1.0 ml of D-MEM medium with 10% fetal bovine serum. The cells were cultivated in a thermostat (Forma Scientific, U.S.A.) at 37 ^oC in a fully humidified atmosphere containing 5% CO_2 in air. The density of the cells in the cultures during the experiments was approximately 1 x 10^6 cells per ml.

Stimulation of RAW 264.7 cells with LPS

One μg LPS (Sigma, St. Louis, MO, USA) in a volume of 100 μL was added to the cell culture to obtain the final concentration of 0.1 μg/ml. Corresponding volume of medium (saline) was added to control cell cultures.

RNA isolation

RNA was isolated from the RAW 264.7 murine macrophages by a kit with Dnase treatment (RNeasy Mini Kit, QIAGEN, Hilden, Germany). RNA concentration and its purity were quantified by UV spectrophotometry. The 260/280 ratio was not less than 1.8 for each RNA sample.

Quantitative real-time RT-PCR

cDNA was prepared using iScript cDNA Synthesis Kit (Bio-Rad, CA, USA). 1 μg of RNA template was used per each reverse transcription. The mRNA levels of selected genes in RNA from mouse RAW 264.7 macrophages were measured by real-time reverse-transcription-polymerase chain reaction (RT-PCR) in triplicates on RotorGene 6000 (Corbett Research, Sydney, Australia) using SYBR Green Master Mix reagent (Roche Diagnostics, Mannheim, Germany). The final reaction volume (20 μl) included 10 μl SYBR Green Master Mix reagent, 5μl of diluted cDNA, and 1000 nM concentration of each primer. The first reaction cycle was carried out at 95°C for 10 min and was followed by 40 cycles, each of which consisted in 15 s at 95°C, 20 s at 60°C and 20 s at 72°C. Sequences of primers were taken over from publications of Ashton et al. [9] and Overbergh et al. [10] (the sequences are shown in Štreitová *et al*. (*Physiol Res*, submitted concommitantly). To calculate relative gene expression, we used delta-delta Ct method based on the difference of threshold cycles (Ct) of the target gene and the β-actin sequence. We assumed a 2-fold increase in PCR products per cycle. A receptor was taken for relative quantification if the threshold cycle number was less than 36. If the threshold cycle number was greater than 36, the receptor was considered to be present in minimal quantities and relative quantification was not performed. A gene was considered not to be expressed if no amplification was detected by cycle 40.

Statistics

All experiments were performed three times. The results are expressed as means ± SD. For statistical evaluation of the differences between control and LPS-activated

macrophages, ANOVA and Tukey-HSD test were used. The significance level was set at $P < 0.05$.

Results

In initial experiments, relative mRNA expression for A_1, A_{2a}, A_{2b}, and A_3 receptors was evaluated in control untreated RAW 264.7 macrophages. The threshold cycle number for A_1 receptor mRNA occurred in the interval between 36 and 40. Therefore, the A_1 receptor mRNA was considered to be present in minimal quantities not suitable for quantification (see Materials and Methods). The values for the relative mRNA expression for A_{2a}, A_{2b}, and A_3 receptors are shown in Fig. 1. The relative mRNA level for A_{2a} receptors was rather low, though quantifiable ($1.06 \pm 0.47 \times 10^{-6}$), those for A_{2b} and A_3 receptors were markedly higher ($5.80 \pm 1.41 \times 10^{-4}$ and $1.73 \pm 0.28 \times 10^{-4}$, respectively).

The relative changes of the above-given mRNA levels induced by LPS-activation represented the subject of consequential studies. The level of mRNA expression for A_1 receptor did not attain values suitable for quantification of the experiments. Results obtained on A_{2a}, A_{2b}, and A_3 mRNA expression are shown in Fig. 2. It follows from the results that an up-regulation was induced in the expression of mRNA of adenosine A_{2a} and A_{2b} receptor, whereas a down-regulation occurred in the expression of mRNA of the adenosine A_3 receptor. A particularly expressive increase was found in the A_{2a} mRNA expression, namely to more than 120 times of that found in the controls at 3 and 6 hours after the beginning of the stimulation with LPS. It was followed by a decrease to values not significantly different in comparison with control values. The elevation of the level of the A_{2b} receptor mRNA expression attained values approximately 8 times higher than those of the controls at 3 and 6 hours and returned to nearly control levels after 24 hours. The A_3 adenosine receptor mRNA levels significantly decreased to about one half of those in the controls at hour 3 after the beginning of the stimulation with a subsequent gradual return to control levels after 24 hours.

Discussion

Our results on the induction of changes in the expression of mRNA for adenosine A_{2a}, A_{2b}, and A_3 receptors following stimulation of RAW 264.7 murine macrophages by LPS confirm that the action of these receptors can be included into the spectrum of the macrophage activities initiated by their LPS-induced activation. Such a definite statement cannot be expressed for adenosine A_1 receptors since their mRNA has been found to be present only in minimal levels not enabling quantification. Adenosine receptor agonists have been shown to

modulate production of many cytokines and other regulatory factors by macrophages, e.g., of interleukin-10 (Haskó *et al.* 1996, Le Moine *et al.* 1996), tumor necrosis factor-α (TNF-α) (Haskó *et al.* 1996, 2000), interleukin-12 (Haskó *et al.* 2000), and prostaglandin E_2 (Ezeamuzie and Khan 2007). As mentioned, earlier studies have shown that activation of macrophages by LPS induces production of proinflammatory (TNF-α) (Franco-Molina *et al.* 2005) and hematopoiesis-stimulating (IL-6, G-CSF) (Hofer *et al.* 2007, Kamio *et al.* 2008) cytokines by these cells. Thus, LPS-mediated macrophage activation has been shown to change the profile of active substances released from macrophages towards an up-regulation of immune reactions and production of blood cells.

The data from our experiments have shown that LPS induces an up-regulation of the expression of adenosine A_{2a} and A_{2b} receptor mRNA and a down-regulation of the expression of adenosine A_3 receptor mRNA in RAW 264.7 macrophages. These effects are similar to those observed by Murphree *et al.* (2005) who used other cell lines of macrophages. As stated by these authors, the up-regulation of the expression of adenosine A_{2a} and A_{2b} receptor mRNA can be understood as a feedback mechanism aimed at macrophage deactivation. This is logical in terms of the evidence of antiinflammatory action of both these receptors deduced from studies using mice deficient in A_{2a} (Ohta and Sitkovsky 2001) and A_{2b} receptors (Yang *et al.* (2006). The reason for the down-regulation of expression of adenosine A_3 receptor mRNA after LPS-induced activation of macrophages observed in the experiments of Murphree *et al.* (2005), as well as in ours, is unknown and remains to be elucidated.

In context of the considered role of adenosine receptor signaling in regulation of hematopoietic processes by macrophages it should be mentioned that a positive regulatory role in stimulation of proliferation of hematopoietic progenitor cells has been reported for an agonist of the adenosine A_3 receptors whereas suppression of their proliferation for an agonist of the adenosine A_{2a} receptors (Pospíšil *et al.* 2004). Since activation of the RAW 264.7 macrophages has been described in the present communication to down-regulate the expression of the A_3 receptors and to up-regulate that of the A_{2a} receptors, it seems that activation of the macrophages does not increase the ability of these cells to respond to adenosine receptor agonists in a hematopoiesis-stimulating manner. Further studies, including those on primary mammalian macrophages, are needed to supply more data on this topic and to elucidate the biological meaning of the observed phenomena.

As shown, the changes in the expression of mRNA of adenosine receptors induced by LPS are temporary. Maximum of the changes can be observed at hours 3 and 6 after activation of the macrophage by LPS, whereas the level of mRNA expression returns back to

control values at hour 24 (see Fig. 2). However maximum manifestations of functional changes in macrophages after their activation with LPS, like an increase of nitric oxide, prostaglandin E_2 or production of granulocyte colony-stimulating factor, are recorded at time interval within 18 to 24 hours (Hulkower *et al.* 1996, Hofer *et al.* 2007). Our data document well that the changes in mRNA expression are primary and precede the modulation of the functional state of the macrophages.

Besides changes in the mRNA expression of the adenosine receptors induced by LPS, also differences in mRNA expression of individual subtypes of adenosine receptors in resting conditions of the cell system can provide an interesting information. Assuming that the adenosine receptor signaling presents the retaliatory mechanism (Newby 1984), the higher expression of some of the adenosine receptor mRNAs under resting conditions can indicate a higher readiness of the cells to activate such regulatory mechanism under stress conditions. As shown by our experiments, such an emergency role in the activated macrophage system can be played by the adenosine A_{2b} receptor whose mRNA has been found to exhibit a relatively high level of expression. It is interesting that Németh *et al.* (2005) detected A_{2b} receptors in membrane protein fractions from RAW 264.7 cells.

The level of mRNA needs not automatically correlate with levels of respective proteins. In our experiments protein levels could not been determined since antibodies against mouse adenosine receptor proteins, with the exception of those against A_{2a}, are not available. However, the convincing data on the mRNA expression for the adenosine A_{2a}, A_{2b}, and A_3 receptors in control, as well as LPS-activated RAW 264.7 mouse macrophages enable us to express an opinion that adenosine receptor signaling plays a role in macrophage-mediated regulatory processes. Based on the results shown here taken together with those obtained in studies on mouse primary hematopoietic precursor cells (Štreitová *et al.*, *Physiol Res*, concommitantly submitted), it is possible to conclude that cells of the hematopoietic system express, though in various levels, mRNA for all four subtypes of adenosine receptors and are, thus, able to interact with both non-selective and selective adenosine receptor agonists. Nevertheless, more data are needed for specifying the regulatory pathways through which the effects of adenosine receptor agonists on the hematopoietic system are executed.

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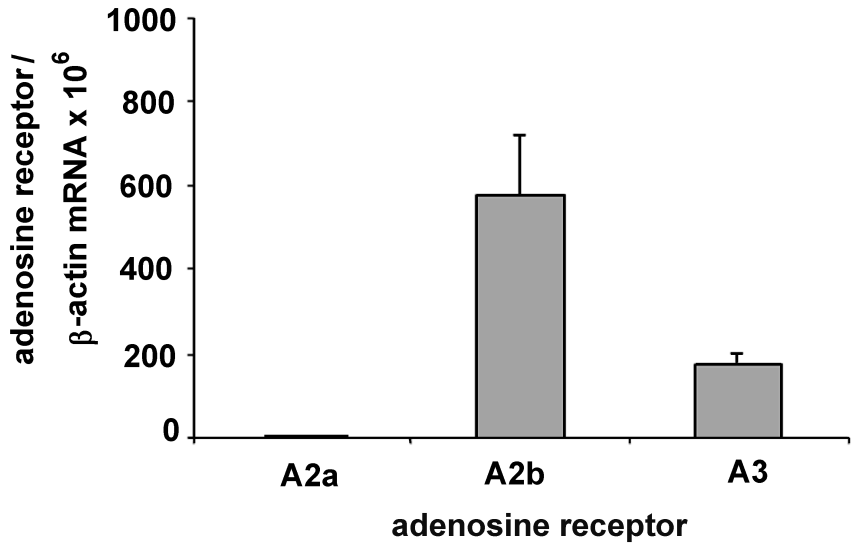
Legends to Figures

Fig. 1. Adenosine A_{2a}, A_{2b}, and A_3 receptor mRNA expression in control untreated RAW 264.7 macrophages. Relative values of mRNA expression are given as means ± SD. Numeric values: A_{2a} receptors: 1.06 ± 0.47 x 10^{-6}; A_{2b} receptors: 5.80 x 1.41 x 10^{-4}; A_3 receptors: 1.73 x 0.28 x 10^{-4}.

Fig. 2. Effects of LPS-induced activation on the expression of adenosine A_{2a}, A_{2b}, and A_3 receptor mRNA in RAW 264.7 macrophages. Data are expressed as relative changes compared to controls (control untreated cells, value = 1) and given as means ± SD. Time interval (hours) – time interval between the beginning of induction of activation and material sampling. *, ** - $P<0.05$, $P<0.01$, respectively, in comparison with control; + - $P<0.05$ in comparison with 24 hours.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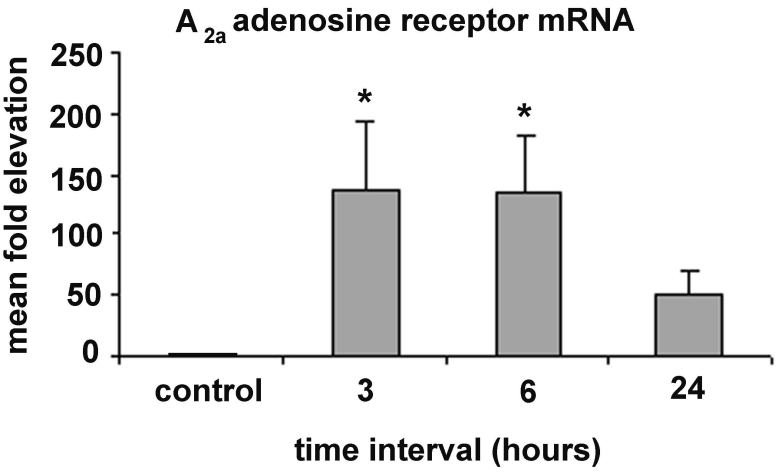
A2a
A2b
A3
adenosine receptor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200
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3
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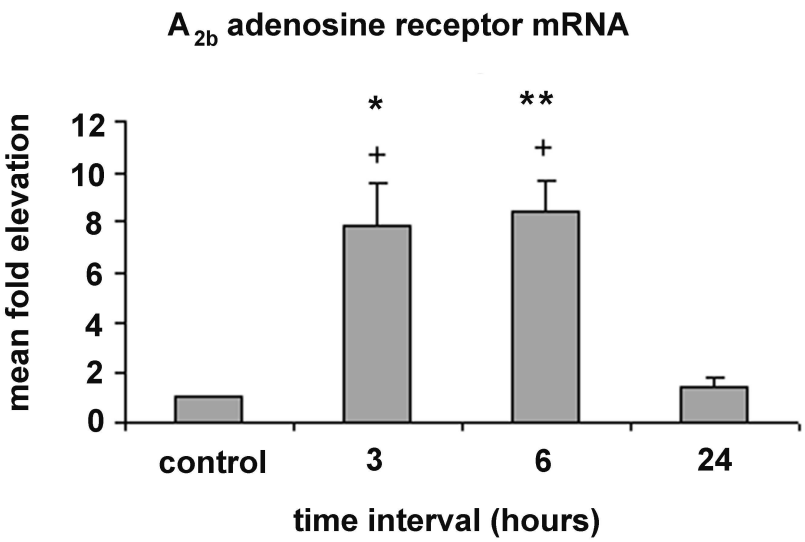
time interval (hours)


A 2b adenosine receptor mRNA
mean fold elevation
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10
8
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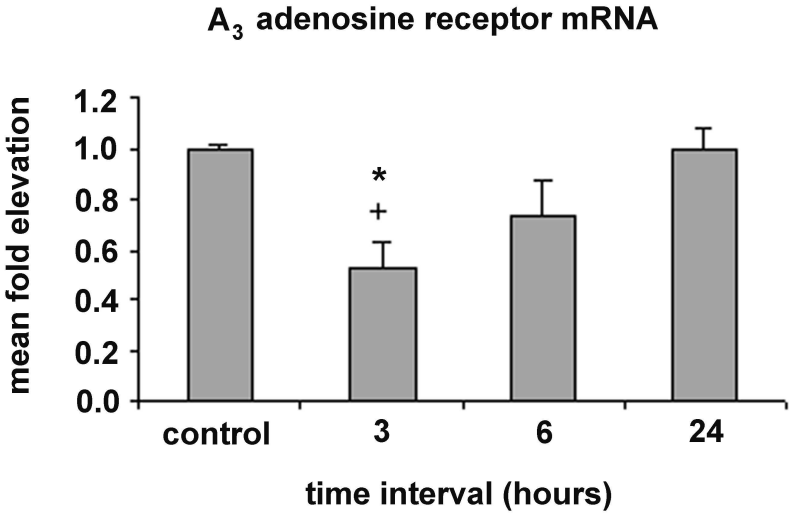
time interval (hours)


A 3 adenosine receptor mRNA
mean fold elevation
1.2
1.0
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time interval (hours)