SHORT COMMUNICATION

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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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 in both 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 by 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

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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 receptors 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 have been widely investigated in various areas of medicine (Olah and Stiles 1995).

An important regulatory system, which is believed to involve both macrophages and adenosine receptor signaling, is the modulation of hematopoietic processes. Hematopoiesis-modulating effects of adenosine receptor agonists have been reported (for review see 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) (Hofer et al. 2007, Kamio et al. 2008). Administration of drugs increasing extracellular adenosine and strengthening its receptor-mediated action has been found to enhance 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 has also been paid to the participation of adenosine receptor signaling in immunological and inflammatory processes. Here, the interest was focused on the functions of macrophages and to their modulation by the adenosine receptor system, i.e.
on the expression 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 A2a 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 A2a 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, were focused on the 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 h following the initiation of macrophage activation by LPS as well as statistical processing of the data obtained.

**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 one hour at 37 °C, 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 °C in a fully humidified atmosphere containing 5 % CO2 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** from the RAW 264.7 murine macrophages was done using a kit with Dnase treatment (RNase 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, 5 μl 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 Ashton et al. (2003) and Overbergh et al. (1999) (the sequences are shown in Štreitová et al. 2010). 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 twofold 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.

In initial experiments, relative mRNA expression for A1, A2a, A2b, and A3 receptors was evaluated in control
untreated RAW 264.7 macrophages. The threshold cycle number for A1 receptor mRNA occurred in the interval between 36 and 40. Therefore, the A1 receptor mRNA was considered to be present in minimal quantities not suitable for quantification (see above). The values for the relative mRNA expression for A2a, A2b, and A3 receptors are shown in Figure 1. The relative mRNA level for A2a receptors was rather low, though quantifiable (1.06±0.47 x 10^-6), those for A2b and A3 receptors were markedly higher (5.80±1.41 x 10^-4 and 1.73±0.28 x 10^-4, respectively).

The relative changes of the above mRNA levels induced by LPS-activation represented the subject of consequential studies. The level of mRNA expression for A1 receptor did not attain values suitable for quantification of the experiments. Results on A2a, A2b, and A3 mRNA expression are shown in Figure 2. It follows from the results that an up-regulation was induced in the expression of mRNA of adenosine A2a and A2b receptor, whereas a down-regulation occurred in the expression of mRNA of the adenosine A3 receptor. A particularly expressive increase was found in the A2a mRNA expression, namely to more than 120 times of that found in the controls at 3 and 6 h 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 A2b receptor mRNA expression attained values approximately 8 times higher than those of the controls at 3 and 6 h and returned to nearly control levels after 24 h. The A3 adenosine receptor mRNA levels significantly decreased to about one half of those in the controls at 3 h after the beginning of the stimulation with a subsequent gradual return to control levels after 24 h.

Our results on the induction of changes in the expression of mRNA for adenosine A2a, A2b, and A3 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 A1 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. 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), or prostaglandin E2 (Ezeamuzie and Khan 2007). As mentioned, earlier studies have shown that the activation of macrophages by LPS induces a 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 A3 receptors and A2b receptor mRNA and a down-regulation of the expression of adenosine A2 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 A3 receptors and A2b receptor mRNA can be understood as a feedback mechanism aimed at macrophage deactivation. This is logical in terms of the evidence on antiinflammatory action of both these receptors deduced from studies using mice deficient in A2a (Ohta and Sitkovsky 2001) and A2b receptors (Yang et al. 2006). The reason for down-regulated expression of adenosine A3 receptor mRNA after LPS-induced activation of macrophages observed by Murphree et al. (2005), as well as in our study, 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 their activation with LPS, like an increase in production of nitric oxide, prostaglandin E2 or granulocyte colony-stimulating factor, are recorded at time interval between 18 and 6 h after activation of the macrophage by LPS, temporary. Maximum of the changes can be observed at 3 and 6 h after activation of the macrophage by LPS, whereas the level of mRNA expression returns back to control values at 24 h (Fig. 2). However, maximal manifestations of functional changes in macrophages after their activation with LPS, like an increase in production of nitric oxide, prostaglandin E2 or granulocyte colony-stimulating factor, are recorded at time interval between 18 to 24 h (Hulkower et al. 1996, Hofer et al. 2007). Our data document well that the changes in mRNA expression are primary and precede modulation of the functional state of the macrophages.

Besides the changes in mRNA expression of the adenosine receptors induced by LPS, the differences in mRNA expression of individual subtypes of adenosine receptors in resting conditions of the cell system can also 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 A2b receptor whose mRNA has been found to exhibit a relatively high level of expression. It is interesting that Németh et al. (2005) detected A2b receptors in membrane protein fractions from RAW 264.7 cells.

The level of mRNA does not always correlate with levels of respective proteins. In our experiments protein levels could not be determined since antibodies against mouse adenosine receptor proteins, with the exception of those against A2a, are not available. However, the convincing data on the mRNA expression for the adenosine A2a, A2b, and A3 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. 2010), 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 exerted.

Conflict of Interest
There is no conflict of interest.

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