Expression of mRNA for Adenosine A_1, A_{2a}, A_{2b}, and A_3 Receptors in HL-60 Cells: Dependence on Cell Cycle Phases

M. HOFER[1], L. DUŠEK[2], Z. HOFEROVÁ[1], L. STIXOVÁ[3], M. POSPÍŠIL[1]

[1]Laboratory of Experimental Hematology, Department of Molecular Cytology and Cytometry, Institute of Biophysics, Academy of Sciences of the Czech Republic, Brno, Czech Republic, [2]Institute of Biostatistics and Analyses, Masaryk University, Brno, Czech Republic, [3]Laboratory of Structure and Function of the Cell Nucleus, Department of Molecular Cytology and Cytometry, Institute of Biophysics, Academy of Sciences of the Czech Republic, Brno, Czech Republic

Received June 14, 2011
Accepted August 19, 2011
On-line October 12, 2011

Summary

The present studies investigated changes in expression of mRNA for adenosine A_1, A_{2a}, A_{2b}, and A_3 receptors in samples of HL-60 promyelocytic cells differing in the actual presence of cells in various phases of the cell cycle induced by the double thymidine block method. Real-time PCR technique was used for obtaining data on mRNA expression. Statistical analysis of the data revealed that the mRNA expression of adenosine A_1, A_{2a}, and A_3 receptors is dependent on the cell cycle phase. G_0/G_1 and G_2/M phases were characterized by a higher mRNA expression of adenosine A_1 receptors and a lower one of adenosine A_{2a} and A_3 receptors whereas the opposite was true for the S phase. Interestingly, expression of mRNA of the adenosine A_{2b} receptors was independent on the cell cycle phase. The results indicate the plasticity of mRNA expression of adenosine receptors in the investigated promyelocytic cells and its interaction with physiological mechanisms of the cell cycle.

Key words

Adenosine receptors • Messenger RNA expression • Cell cycle • HL-60 promyelocytic cells

Corresponding author

M. Hofer, Institute of Biophysics, v.v.i., Academy of Sciences of the Czech Republic, Královopolská 135, CZ-61265 Brno, Czech Republic. Fax: +420 541211293. E-mail: hofer@ibp.cz

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 many cellular functions including proliferation and differentiation (Abbracchio and Burnstock 1998, Poulsen and Quinn 1998, Schulte and Fredholm 2003). The regulatory function of extracellular adenosine is based on the activation of cell surface receptors, namely A_1, A_{2a}, A_{2b}, and A_3. Receptor activation can be achieved either non-selectively, by adenosine, or selectively, using various adenosine analogs (Jacobson 2002).

We have demonstrated that pharmacologically induced elevation of extracelullar adenosine, i.e. non-selective activation of adenosine receptors, stimulates hematopoiesis in mice (for review, see Hofer and Pospíšil 2006, Hofer *et al*. 2011). Furthermore, we have aimed our effort at direct determination of mRNA expression for adenosine A_1, A_{2a}, A_{2b}, and A_3 receptors in four mouse hematopoietic precursor cell lines, namely granulopoietic/monocytopoietic, erythropoietic, B-lymphopoietic, and T-lymphopoietic ones. Similar studies were carried out with mouse RAW 264.7 macrophages taken as a model object representing the hematopoietic microenvironent. We have demonstrated that mRNA for all the four adenosine receptors is expressed in all cell types tested with the exception of the A_1 receptor in the RAW 264.7 macrophages (Štreitová

et al. 2010a,b). Thus, the ability of important cells of the hematopoietic system to respond to activation by selective adenosine receptor agonists has been proven. Furthermore, we have used the model of hematopoietic suppression evoked by the cytotoxic drug 5-fluorouracil, killing predominantly cycling cells, and we have found that particularly administration of two tested selective adenosine receptor agonists, i.e. N^6-cyclopentyladenosine (CPA) and N^6-(3-iodobenzyl)adenosine-5'-N-methyluronamide (IB-MECA), selective for A_1 and A_3 receptors, respectively, influences cycling of hematopoietic progenitor cells: activation of adenosine A_1 receptors has been found to inhibit whereas activation of adenosine A_3 receptors to stimulate proliferation in the compartments of both bone marrow progenitor and precursor cells (Pospíšil *et al*. 2004, 2005, Hofer *et al*. 2006, 2007, 2008).

Starting from the knowledge platform summarized above, we have addressed the problem of adenosine receptor expression and we have studied whether or not is the mRNA expression of individual adenosine receptors related to the varying functional state of the hematopoietic tissue induced by the variable presence of cells occurring in different phases of the cell cycle. HL-60 promyelocytic cells have been used as a model of the hematopoietic cell renewal system. The mRNA expression for individual adenosine receptors has been determined by the quantitative real-time PCR technique.

Materials and Methods

Cell system and culture conditions

Human promyelocytic leukemic cell line HL-60 was obtained from the American Tissue Culture Collection (ATCC). The cells were cultivated in RPMI-1640 medium (Sebak, Aidenbach, Germany) supplemented with 10 % inactivated fetal bovine serum (Pan Biotech GmbH, Aidenbach, Germany), 1 mM sodium pyruvate (ICN Biomedicals, Costa Mesa, CA, USA), 100 μg/ml streptomycin (Gibco, Paisley, UK), 100 IU/ml penicillin (Gibco), 0.1 mg/ml gentamicin (PAN Systems, Nürnberg, Germany), 8 mM L-glutamine (Gibco), 1 % nonessential amino acids (ICN Biomedicals), 5 mM HEPES (Serva, New York, NY, USA), and 50 μM 2-mercaptoethanol (Fluka, Seelz, Germany). The cells were kept at 37 °C in a humidified atmosphere containing 95 % air and 5 % CO_2, transferred into a fresh medium twice a week and used for experiments during the exponential growth phase.

Double thymidine block of HL-60 cells

To obtain populations with varying percentages of cells in individual cell cycle phases, HL-60 cells were exposed to the double thymidine block. Stock solution of 200 mM thymidine (Sigma, St.Louis, MO, USA) in PBS was prepared and used at a 1/50 dilution to produce a 4 mM thymidine solution. The cells in the exponential growth phase (analysed as sample 1) (5×10^5) were blocked for 18 h with 4 mM thymidine, released by washing out the thymidine once in phosphate-bufferred saline (PBS, pH 7.4), and cultivated in fresh RPMI medium for 9 h. Then the cells were blocked again with 4 mM thymidine for 17 h to arrest a significant percentage of the cells at the beginning of S-phase. The arrest was then released by washing out thymidine and the cells were cultivated in fresh medium for 72 h. Further analyses were carried out at hours 1 (sample 2), 2 (sample 3), 3 (sample 4), 6 (sample 5), 24 (sample 6), 48 (sample 7) and 72 (sample 8). Relative representation of the cells in G_0/G_1, S, and G_2/M cell cycle phases in the cell samples is shown in Table 1.

Table 1. Per cent representation of G_0/G_1, S, and G_2/M cell cycle phases in individual cell populations (samples described in the text).

Sample	G_0/G_1 phase	S phase	G_2/M phase
1	67.0	32.5	0.5
2	35.8	60.3	3.9
3	36.0	61.7	2.3
4	37.9	60.2	1.9
5	39.6	40.6	19.8
6	41.7	40.3	18.0
7	54.0	35.9	10.1
8	69.2	25.0	5.8

Cell cycle studies

The sampled cells were collected by centrifugation, washed once in PBS and suspended for 12 h in 70 % ethanol for cell fixation at the temperature of 4 °C. The fixed cells were again collected by centrifugation, ethanol was aspirated off and the cells were resuspended in a staining buffer containing 5 mg/ml propidium iodide, 1M TRIS (Roth, Karlsruhe, Germany) (pH 8), 10 mg/ml RNase (Sigma) and 10 % Triton X-100 (AppliChem, Darmstadt, Germany). After 30 min of incubation at 37 °C, samples were analyzed with a

FACScan flow-cytometer (Becton-Dickinson, San Jose, CA, USA) equipped with an argon laser (excitation at 488 nm). To estimate the percentage of cells in each phase of cell cycle (G_o/G_1, S and G_2/M), histogram analyses of relative DNA contents were caried out with Mod Fit LT 2,0 software (VERITY, Software House, Inc., Topsham, ME, USA).

Isolation of total RNA

In all samples (1 to 8), isolation of total RNA of the cells was carried out using the High Pure Isolation Kit (Roche, Mannheim, Germany). RNA concentration and purity were measured on Nano-Drop ND1000 Spectrophotometer (Thermo Scientific, USA). The 260/280 ratio was not less than 1.8 for each RNA sample. Total RNA was stored at –80 °C prior to cDNA synthesis.

Reverse trancription of total RNA to cDNA

The subsequent cDNA synthesis for a two-step real-time polymerase chain reaction (RT-PCR) was carried out with the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany). One μg of RNA template was used per each RNA-to-cDNA reaction. The templates were mixed with anchored-oligo(dT18) primers (2.5 μM) and random hexamer primers (60 μM). The samples were placed in DNA Engine Peltier thermal cycler (BioRad, USA) and incubated for 10 min at 25 °C, followed by 30 min at 55 °C, and at the end heated to 85 °C for 5 min. Synthesized cDNA was stored at –20 °C.

Quantitative real-time PCR (RT-PCR)

Using FastStart SYBR Green Master (Roche) in combination with appropriate PCR primers, RT-PCR detection and quantification of DNA sequences for A_1, A_{2a}, A_{2b}, and A_3 subtypes of adenosine receptors were performed. Sequences of primers (see Štreitová *et al.* 2010a) were taken over from Ashton *et al.* (2003) and Overbergh *et al.* (1999) and the primers were synthesized by Generi Biotech (Hradec Králové, Czech Republic). RT-PCR was done in duplicates for each adenosine receptor primer on Rotor-Gene 6000 (Corbett Research, Sydney, Australia). The final reaction volume (20 μl) included 10 μl of FastStart SYBRGreen Master Mix, 2.5 μl of cDNA, and 300 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, 30 s at 60 °C and 30 s at 72 °C. To calculate relative gene expression, the delta-delta Ct method based on the difference of treshold cycles (Ct) of the target gene and the β-actin sequence was used. A twofold increase in PCR products per cycle was assumed. Any receptor was taken for relative quantification if the treshold cycle number was less than 36. If the threshold cycle number was greater than 36, the receptor mRNA 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.

Statistical analysis

Primary data were obtained on the basis of two repeated experiments. In each experiment, two determinations of the mRNA expression from each sample were performed. Arithmetic means were calculated from the two repeated determinations in each sample and then calculations of arithmetic means from both experiments were carried out. Thus, a single value representing the expression of a particular receptor in each sample was obtained. This aggregation of primary data was carefully checked and it was proved not to reduce overall experimental variance.

Mutual association of examined parameters was evaluated on the basis of rank Spearman's non-parametric correlation and its statistical significance. Variance associated with the estimated correlations was assessed and the statistical significance of mutual differences between correlation coefficients was evaluated by a standard test based on normally distributed test statistics Z (Cornbleet and Shea 1978, Borkowf 2000).

The value $p \leq 0.05$ was considered to be a boundary of statistical significance in all performed analyses. The analyses were carried out after removal of outliers (4.2 %) identified by Dixon's test (Dean and Dixon 1951, Rorabacher 1991). All analyses were performed using the SPSS 18.0.3. software (IBM Corporation, 2010).

Results

The experimental protocol employing the promyelocytic HL-60 cell line was based on investigations of relationships between mRNA expression of individual adenosine receptors and the presence of cells occurring in individual phases of the cell cycle, i.e. G_0/G_1, S, and G_2/M. These analyses were performed separately for each receptor type and each cell cycle phase. Because of the four analysed adenosine

receptors and the three investigated cell cycle phases, twelve correlations in total have been computed. Spearman's correlation coefficients r_s were used as a robust measure of association between given adenosine receptor mRNA expression and given cell cycle phase. Thus, a positive value of r_s indicates that the given phase of the cell cycle is linked with a higher production of the given receptor mRNA. On the contrary, a negative value of r_s indicates that the given cell cycle phase is linked with a lower level of the given receptor mRNA production. Results of the experiments are presented in Figures 1-3 which characterize the tested functional dependences by regression lines and Spearman's correlation coefficients (r_s) with corresponding statistical significances (p).

The first distinct phenomenon of the results is the evidence that the mRNA expression of the adenosine A_{2b} receptors is inert to the structure of cell cycle phases in the individual samples: in all samples it reaches nearly the same level. The experimental variability associated with the mRNA expression of the adenosine A_{2b} receptors is very low, at the level of random error of fluctuations and no significant correlation occurred. This is in contrast to the experimental variability observed when analysing the expression of mRNA of all other adenosine receptor subtypes (see Figs 1-3).

Concerning the cells in the G_0/G_1 phase of the cell cycle (Fig. 1), the following observations are worth mentioning: there is a positive and significant correlation for the expression of adenosine A_1 receptor mRNA, a negative and significant correlation for the expression of adenosine A_{2a} receptor mRNA, as well as a negative correlation (p=0.052) for the adenosine A_3 receptor mRNA. These findings suggest a higher level of mRNA expression of the A_1 receptors and a lower one of the A_{2a} and A_3 receptors in cells occurring in the G_0/G_1 phase of the cell cycle.

Differently pronounced results have been found by calculations of association between the mRNA expression of individual adenosine receptors and the presence of the cells in the S phase of the cell cycle (Fig. 2). Here, the negative r_s for adenosine A_1 receptor mRNA and positive r_s for mRNA of adenosine A_{2a} and A_3 receptors occur, even if not attaining statistical significance. These findings seem to indicate that the manifestations of the mRNA expression of the adenosine A_1, A_{2a}, and A_3 receptors in the S phase of the cell cycle are opposite to those obtained in the G_0/G_1 phase in terms of negativity or positivity of r_s. For confirmation of this phenomenon, correlation coefficients between the mRNA expression of the three adenosine receptors and the presence of the cells in the cell cycle phases G_0/G_1 and S have been compared and the results are presented in Table 2. It is apparent that the mRNA expression of adenosine A_1, A_{2a}, and A_3 receptors differs significantly between the G_0/G_1 and the S phases of the cell cycle. For the G_0/G_1 phase an increased mRNA expression of the adenosine A_1 receptor is typical whereas in the S phase an expression of the adenosine A_{2a} and A_3 receptors predominates.

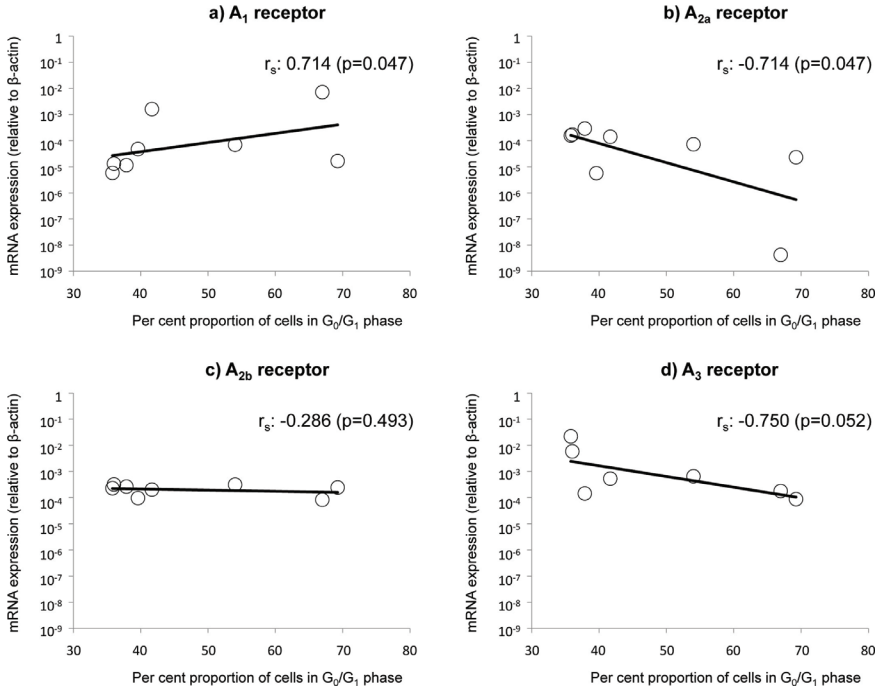

Fig. 1. Dependence of expression of mRNA for adenosine A_1, A_{2a}, A_{2b}, and A_3 receptors on the per cent proportion of HL-60 cells in the G_0/G_1 cell cycle phase, as presented in Table 1. r_s – Spearman's correlation coefficient; p – statistical significance of r_s.

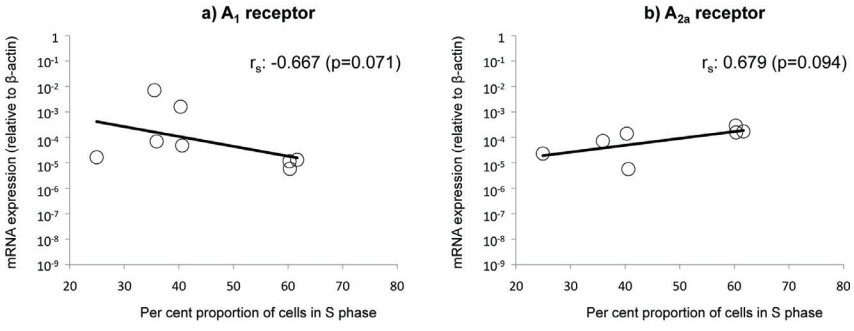

Fig. 2. Dependence of expression of mRNA for adenosine A_1, A_{2a}, A_{2b}, and A_3 receptors on the per cent proportion of HL-60 cells in the S cell cycle phase, as presented in Table 1. r_s – Spearman's correlation coefficient; p – statistical significance of r_s.

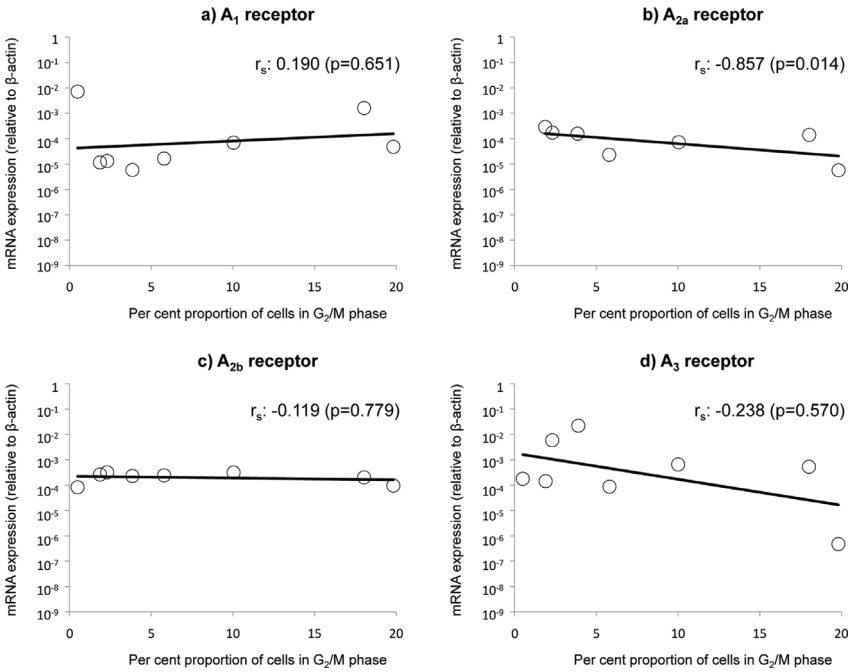

Fig. 3. Dependence of expression of mRNA for adenosine A_1, A_{2a}, A_{2b}, and A_3 receptors on the per cent proportion of HL-60 cells in the G_2/M cell cycle phase, as presented in Table 1. r_s – Spearman's correlation coefficient; p – statistical significance of r_s.

Table 2. Comparison of correlation coefficients r_s between cell cycle phases G_0/G_1 and S, and between cell cycle phases S and G_2/M, for mRNA expression of adenosine A_1, A_{2a}, and A_3 receptors.

Receptor type	G_0/G_1 phase (r_s)	S phase (r_s)	G_2/M phase (r_s)	Comparison of r_s (G_0/G_1 vs. S)	Comparison of r_s (S vs. G_2/M)
A_1	0.714	-0.667	0.190	p=0.021	p=0.141
A_{2a}	-0.714	0.679	-0.857	p=0.027	p=0.014
A_3	-0.750	0.714	-0.238	p=0.025	p=0.118

p – value of statistical significance of comparison of correlation coefficients r_s between the compared cell cycle phases.

Table 3. Relations of mRNA expression indices between individual pairs of adenosine A_1, A_{2a}, and A_3 receptors.

Receptor type	A_1		A_{2a}		A_3	
	r_s	p	r_s	p	r_s	p
A_1	-	-	-0.143	0.760	-0.393	0.383
A_{2a}	-0.143	0.760	-	-	0.786	0.036
A_3	-0.393	0.383	0.786	0.036	-	-

r_s – correlation coefficients, p – values of statistical significance of the pertinent r_s.

Data given in Figure 3 demonstrate the dependence of the mRNA expression of adenosine receptors on the presence of the cells in the G_2/M phase of the cell cycle. Here, a negative statistically significant r_s for the mRNA expression of the A_{2a} receptor dominates. This situation is reflected also by the significant difference of correlation coefficients for the expression of mRNA for the adenosine A_{2a} receptor between the S and G_2/M phases, as shown in Table 2.

Additional statistical processing of the data consisted in computing of correlation coefficients representing the mutual relations in mRNA expression in pairs of adenosine receptors (A_1 – A_{2a}, A_1 – A_3, A_{2a} – A_3) when using data from all cell cycle phases taken as a whole. The results are given in Table 3 and show a statistically significant positive correlation between the mRNA expression of adenosine A_{2a} and A_3 receptors. There is also a negative correlation in receptor mRNA expression between A_1 and A_{2a} receptors, as well as between that of A_1 and A_3 receptors, but without statistical significance.

Discussion

To our knowledge, the obtained data present the first study investigating the expression of mRNAs of adenosine receptors in different phases of the cell cycle. Even if the rate of mRNA expression needs not be fully reflected at the corresponding protein levels, the presented data suggest at least tendencies for the synthesis of functional adenosine receptors and allows to formulate certain general conclusions.

The experiments performed on HL-60 promyelocytic cells suggest a diversity of mRNA expression of individual adenosine receptors in different phases of the cell cycle. The preparatory G_0/G_1 phase, as well as the G_2/M phase, are positively linked to the mRNA expression of the A_1 receptors and negatively to that of the A_{2a} and A_3 adenosine receptors. On the contrary, the synthetic S phase is characterized by the decrease of the mRNA expression of the A_1 adenosine receptors and the increase of that of the A_{2a} and A_3 adenosine receptors. These findings might be analogical to our earlier considerations ensuing from *in vivo* experiments. We have observed positive effects of an adenosine A_3 receptor agonist on cycling of hematopoietic cells (Pospíšil *et al*. 2004, Hofer *et al*. 2006, 2007, 2008), as well as an inhibitory action of an agonist of adenosine A_1 receptors on the proliferation of cells in the murine hematopoietic system *in vivo* (Pospíšil *et al*. 2004, 2005, Hofer *et al*. 2008). Of particular interest are our findings in mice whose hematopoiesis was suppressed by 5-fluorouracil (Hofer *et al*. 2008). In these mice, the proliferation-stimulating effects of the adenosine A_3 receptor agonist were most pronounced in the phase of maximum cell depletion when maximum cell proliferation was to be expected. On the other hand, the proliferation-inhibiting action of the adenosine A_1 receptor agonist was most pronounced in the phase of maximum cell regeneration when a feedback suppressing the overshooting cell proliferation was needed (Hofer *et al*. 2008). Taking into account these findings and considerations, the data presented here on expression of mRNA of adenosine A_1 and A_3 receptors, which varies between the G_0/G_1, S, and G_2/M phases of the cell cycle, might be a further evidence of the regulatory and homeostatic role of these two adenosine receptors.

Although an analogical behavior of premyelocytic HL-60 cells with hematopoietic progenitor and precursor cells *in vivo*, as discussed in the previous paragraph, can be expected, it cannot be completely excluded that the very process of malignization of the HL-60 cells could influence the mRNA expression of adenosine receptors. Therefore, certain caution should accompany the interpretation of the results obtained.

The opposite role of the adenosine A_1 and A_3

receptors in the regulation of cell cycling might be surprising because both these receptors are known to inhibit adenylyl cyclase. However, they differ in their coupling to different G proteins (Fredholm *et al*. 2000) and to phospholipases C and D (Parsons *et al*. 2000). Another example of opposite effects of the two adenosine receptors was shown in experiments of Lee and Emala (2000) demonstrating that adenosine A_1 receptor activation reduces, while adenosine A_3 receptor activation worsens renal ischemia-reperfusion injury.

An interesting phenomenon of the presented experiments is the behavior of the expression of mRNA for adenosine A_{2a} receptors which significantly positively correlates with that for adenosine A_3 receptors. Activation of adenosine A_{2a} receptors inhibits inflammatory processes in neutrophils, platelets, macrophages, and T cells (Linden 2001). Our earlier experiments investigating effects of the agonist of these receptors exhibited no significant effects on the cycling of haematopoietic cells (Pospíšil *et al*. 2004). Thus, the role of the adenosine A_{2a} receptors in hematopoiesis and the relationships between the adenosine A_{2a} and A_3 receptor mRNA expression deserve further attention.

Worth noting is also the evidence of the independence of the expression of mRNA of adenosine A_{2b} receptors on the different phases of the cell cycle. The reason might be the low affinity of these receptors towards the endogenous agonist adenosine (Fredholm 2007) and thus operation of adenosine A_{2b} receptors may be important only under pathophysiological conditions.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

Suported by grants from Ministry of Defense of the Czech Republic (Project Receptor, No. 1001 8 5090), Grant Agency of the Czech Republic (No.s 305/08/0158 and P303/11/0128), and Academy of Sciences of the Czech Republic (No.s AV0Z50050507 and AV0Z50040702).

References

ABBRACCHIO MP, BURNSTOCK G: Purinergic signalling: Pathophysiological roles. *Jap J Pharmacol* **78**: 113-145, 1998.

ASHTON KJ, NILSSON U, WILLEMS L, HOLMGREN K, HEADRICK JP: Effects of aging and ischemia on adenosine receptor transcription in mouse myocardium. *Biochem Biophys Res Commun* **312**: 367-372, 2003.

BORKOWF CB: A new nonparametric method for variance estimation and confidence interval construction for Spearman's rank correlation. *Comput Stat Data Anal* **34**: 219-241, 2000.

CORNBLEET PJ, SHEA MC: Comparison of product moment and rank correlation coefficients in the assessment of laboratory method-comparison data. *Clin Chem* **24**: 857-861, 1978.

DEAN RB, DIXON WJ: Simplified statistics for small numbers of observations. *Anal Chem* **23**: 636-638, 1951.

FREDHOLM BB: Adenosine, an endogenous distress signal, modulates tissue damage and repair. *Cell Death Differ* **14**: 1315-1323, 2007.

FREDHOLM BB, ARSLAN G, HALLDNER L, KULL B, SCHULTE G, WASSERMAN W: Structure and function of adenosine receptors and their ligands. *Naunyn-Schmiederberg's Arch Pharmacol* **362**: 364-374, 2000.

HOFER M, POSPÍŠIL M: Role of adenosine signaling in hematopoiesis – a short review. *Med Hypotheses Res* **3**: 629-635, 2006.

HOFER M, POSPÍŠIL M, VACEK A, HOLÁ J, ZNOJIL V, WEITEROVÁ L, ŠTREITOVÁ D: Effects of adenosine A_3 receptor agonist on bone marrow granulocytic system in 5-fluorouracil-treated mice. *Eur J Pharmacol* **538**: 163-167, 2006.

HOFER M, POSPISIL M, WEITEROVA L, HOFEROVA Z: The role of adenosine receptor agonists in regulation of hematopoiesis. *Molecules* **16**: 675-685, 2011.

HOFER M, POSPÍŠIL M, ZNOJIL V, HOLÁ J, ŠTREITOVÁ D, VACEK A: Homeostatic action of adenosine A_3 and A_1 receptor agonists on proliferation of hematopoietic precursor cells. *Exp Biol Med* **233**: 897-900, 2008.

HOFER M, POSPÍŠIL M, ZNOJIL V, HOLÁ J, VACEK A, ŠTREITOVÁ D: Adenosine A_3 receptor acts as a homeostatic regulator of bone marrow hematopoiesis. *Biomed Pharmacother* **61**: 356-359, 2007.

JACOBSON MA: Adenosine receptor agonists. *Expert Opin Ther Patents* **12**: 489-501, 2002.

LEE HT, EMALA CW: Protective effects of renal ischemic preconditioning and adenosine pretreatment: role of A_1 and A_3 receptors. *Am J Physiol Ren Physiol* **278**: F380-F387, 2000.

LINDEN J: Molecular approach to adenosine receptors: Receptor-mediated mechanisms of tissue protection. *Annu Rev Pharmacol Toxicol* **41**: 775-787, 2001.

OVERBERGH L, VALCKX D, WAER M, MATHIEU C: Quantification of murine cytokine mRNAs using real time quantitative reverse transcriptase PCR. *Cytokine* **11**: 305-312, 1999.

PARSONS M, YOUNG L, LEE JE, JACOBSON KA, LIANG BT: Distinct cardioprotective effects of adenosine mediated by differential coupling of receptor subtypes to phospholipases C and D. *FASEB J* **14**: 1423-1431, 2000.

POSPÍŠIL M, HOFER M, VACEK A, ZNOJIL V, PIPALOVÁ I: Effects of stable adenosine receptor agonists on bone marrow hematopoietic cells as inferred from the cytotoxic action of 5-fluorouracil. *Physiol Res* **53**: 549-556, 2004.

POSPÍŠIL M, HOFER M, VACEK A, HOLÁ J, PIPALOVÁ I, ZNOJIL V: N^6-cyclopentyladenosine inhibits proliferation of murine haematopoietic progenitor cells *in vivo*. *Eur J Pharmacol* **507**: 1-6, 2005.

POULSEN S-A, QUINN RJ: Adenosine receptors. New opportunities for future drugs. *Bioorg Med Chem* **6**: 619-641, 1998.

RORABACHER DB: Statistical treatment for rejection of deviant values: Critical values of Dixon Q parameter and related subrange ratios at the 95 percent confidence level. *Anal Chem* **63**: 139-146, 1991.

SCHULTE G, FREDHOLM BB: Signalling from adenosine receptors to mitogen-activated protein kinases. *Cell Signal* **15**: 813-827, 2003.

ŠTREITOVÁ D, ŠEFC L, SAVVULIDI F, POSPÍŠIL M, HOLÁ J, HOFER M: Adenosine A_1, A_{2a}, A_{2b}, and A_3 receptors in hematopoiesis. 1. Expression of receptor mRNA in four mouse hematopoietic precursor cells. *Physiol Res* **59**: 133-137, 2010a.

ŠTREITOVÁ D, HOFER M, HOLÁ J, VACEK A, POSPÍŠIL M: 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. *Physiol Res* **59**: 139-144, 2010b.