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Chronic mild hyperhomocysteinemia alters ectonucleotidase activities and gene expression of ecto-5′-nucleotidase/CD73 in rat lymphocytes

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Abstract Since mild hyperhomocysteinemia is a risk factor for cardiovascular and cerebral diseases and extracellular nucleotides/nucleosides, which are controlled by the enzymatic action of ectonucleotidases, can induce an immune response, in the present study, we investigated the effect of chronic mild hyperhomocysteinemia on ectonucleotidase activities and expression in lymphocytes from mesenteric lymph nodes and serum of adult rats. For the chronic chemically induced mild hyperhomocysteinemia, Hcy (0.03 μmol/g of body weight) or saline (control) were administered subcutaneously from the 30th to the 60th day of life. Results showed that homocysteine significantly decreased ATP, ADP, and AMP hydrolysis in lymphocytes of adult rats. E-NTPDases transcriptions were not affected, while the ecto-5′-nucleotidase transcription was significantly decreased in mesenteric lymph nodes of hyperhomocysteinemic rats. ATP, ADP, and AMP hydrolysis were not affected by homocysteine in rat serum. Our findings suggest that Hcy in levels similar to considered risk factor to development of vascular diseases modulates the ectonucleotidases, which could lead to a pro-inflammatory status.

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Introduction

Homocysteine (Hcy) is a thiol amino acid synthesized during the metabolic conversion of methionine to cysteine [1]. Hyperhomocysteinemia occurs when the plasma concentration of Hcy exceeds 15 μmol/l, being classified into three ranges; mild (15–30 μmol/l), moderate (31–100 μmol/l), and severe (>100 μmol/l) [2]. Mild hyperhomocysteinemia is prevalent in the general population and can be caused mainly by nutritional deficiencies of folate or vitamin B_{12}, renal disease, and certain medications [3–5]. Some studies support an association between mild hyperhomocysteinemia and vascular diseases [6–8]. The mechanisms mediating Hcy-induced vascular changes are not completely defined, but it is established that hyperhomocysteinemia leads to endothelial dysfunction by promoting oxidative stress and inflammation [9].

Since the strategy of using animal models is useful to better understand the pathophysiology of diseases, we have developed an experimental model of chronic mild hyperhomocysteinemia in adult rats [10], whose plasma levels of Hcy were similar to those considered as risk factor for cardiovascular and cerebral diseases [11, 12]. Animals subjected to this experimental model present oxidative damage in blood and cerebral cortex [10].

Extracellular tri- and diphosphate nucleotides, such as adenosine 5′-triphosphate (ATP) and adenosine 5′-diphosphate (ADP), together with adenosine (Ado), modulate a multiplicity of tissue functions, including development, blood flow, inflammation, and immune reactions [13]. These molecules exert their effects by binding to two types

of purinoceptors: P1 and P2. There are four subtypes of P1 receptors: A_1, A_{2A}, A_{2B}, and A_3, which respond to the nucleoside Ado. P2 receptors are activated by ATP and ADP and classified into two families: ionotropic P2X, subdivided in seven subtypes ($P2X_{1-7}$), and G-protein-coupled P2Y, characterized by eight subtypes ($P2Y_{1,2,4,6,11-14}$) [14]. It has been shown that ATP exerts a pro-inflammatory role and induces cytokine release by acting at $P2X_7$ receptors, while the Ado has anti-inflammatory actions via binding to A_{2A} receptors, found in several cells, such as neutrophils, monocytes/macrophages, lymphocytes, platelets, and neurons [15, 16].

The levels of nucleotides in the extracellular space are controlled by the enzymatic action of ectonucleotidases. Ecto-nucleoside triphosphate diphosphohydrolases (NTPDases) are a family of ecto-enzymes that hydrolyze extracellular ATP and ADP to AMP, and this nucleotide monophosphate is converted to Ado by ecto-5′-nucleotidase, also identified as CD73. Four members of the NTPDase family (NTPDase1, 2, 3, and 8) are typical cell surface-located and have an active site facing to the extracellular milieu [13, 14, 17].

Considering that (a) hyperhomocysteinemia induces lymphocytes proliferation and differentiation [18, 19], (b) ATP, ADP, and Ado are signaling molecules that might play a role in the regulation of lymphocytes function [14], (c) Hcy added to the medium assay (in vitro studies) inhibits nucleotide hydrolysis in rat serum [20], and (d) to our knowledge there are no studies showing the in vivo effect of mild hyperhomocysteinemia on nucleotide hydrolysis, in the present study, we investigated the effect of chronic mild hyperhomocysteinemia on ectonucleotidase activities in lymphocytes and serum of adult rats. We also evaluated the E-NTPDases and ecto-5′-nucleotidase expression in mesenteric lymph nodes, to better understand the involvement of extracellular nucleotide hydrolysis in mild hyperhomocysteinemia.

Materials and methods

Animals and reagents

Wistar rats (30 days old) were obtained from the Central Animal House of Biochemistry Department, Institute of Basic Health Sciences, Federal University of Rio Grande do Sul, Porto Alegre, Brazil. They were maintained on a 12:12 h light/dark cycle (lights on 07:00–19:00 h) in air-conditioned constant temperature (22 ± 1°C) colony room, with free access to water and 20% (w/w) protein commercial chow. Animal care followed the ''Principles of Laboratory Animal Care'' (NIH publication 85-23, revised 1985) and was approved by the Ethical Committee of the Universidade Federal do Rio Grande do Sul, Brazil (#19634).

Chronic mild hyperhomocysteinemia

Hcy (0.03 μmol/g of body weight) was administered subcutaneously from the 30th to the 60th day of life. Plasma Hcy concentration in rats subjected to this treatment achieved levels similar to those described in the plasma of patients with mild hyperhomocysteinemia (30 μM) [10]. Control rats received saline solution in the same volumes (0.5 ml/100 g of body weight). The animals were killed by decapitation 12 h after the last injection of Hcy, and the blood and mesenteric lymph nodes were removed.

Isolation of lymphocytes

Mesenteric lymph nodes were removed and passed through a mesh grid in saline 0.9% [21]. Cells were washed three times with saline, and centrifuged at 200 g for 10 min. Afterward, cells were centrifuged 2 times at $200\times g$ for 10 min with the same buffer used in the enzyme assays, without divalent cations [22]. The cells were counted with Trypan Blue, and only the groups with more than 95% of viability were used for the experiments.

Isolation of blood serum fraction

Blood samples were drawn after decapitation of rats and were soon centrifuged in plastic tubes at $5000\times g$ for 5 min at 20°C. The serum samples obtained were then stored on ice and immediately used in the experiments [23].

Assays of ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase) and ecto-5′-nucleotidase activities in lymphocytes

The reaction medium contained 2 mM $CaCl_2$ (for ATP and ADP) or $MgCl_2$ (for AMP), 120 mM NaCl, 5 mM KCl, 60 mM glucose, 1 mM sodium azide, 0.1% mM albumin, and 20 mM Hepes buffer, pH 7.6, in a final volume of 200 μl. About 10^6 cells of lymphocytes were added to the reaction medium, and the enzyme reaction was started by the addition of ATP, ADP, or AMP to a final concentration of 2 mM and incubated for 30 min at 37°C. The reaction was stopped by the addition of 200 μl of 10% trichloroacetic acid (TCA). The samples were chilled on ice, and the amount of inorganic phosphate (Pi) released was measured as described by Chan et al. [24]. In order to correct nonenzymatic hydrolysis, we performed controls by adding the cells after reaction was stopped with TCA. All samples were assayed in triplicate. Enzyme activities were

generally expressed as nmol Pi released per min per 10^6 cells [22].

Assays of ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase) and ecto-5′-nucleotidase activities in serum

ATP, ADP, and AMP hydrolysis were determined using a method described by Yegutkin [25] with modification prescribed by Oses et al. [23]. The reaction mixture containing 3 mM ATP, ADP, or AMP as substrate, and 112.5 mM Tris–HCl, pH 8.0, was incubated with approximately 1.0 mg of serum protein at 37°C for 40 min in a final volume of 200 µl. The reaction was stopped by the addition of 200 µl of 10% TCA. The samples were chilled on ice, and the amount of Pi released was measured as described by Chan et al. [24]. In order to correct non-enzymatic hydrolysis, we performed controls by adding the serum after the reaction was stopped with TCA. All samples were centrifuged at $5000 \times g$ for 5 min to eliminate precipitated protein and the supernatant was used for the colorimetric assay. All the samples were assayed in triplicate. Specific activities were generally expressed as nmol Pi released per min per milligram of protein.

Analysis of gene expression by semi-quantitative RT-PCR

Analysis of NTPDase1 (Entpd1), 2 (Entpd2), 3 (Entpd3), and ecto-5′-nucleotidase (Nt5e) gene expression was carried out by a semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) assay. 12 h after the last injection of Hcy, mesenteric lymph nodes of rats were isolated for total RNA extraction with the Trizol® Reagent (Invitrogen) in accordance with the manufacturer's instructions. RNA purity was quantified spectrophotometrically and assessed by electrophoresis in a 1.0% agarose gel using GelRed™. The cDNA species were synthesized with SuperScript™ III First-Strand Synthesis SuperMix (Invitrogen) from 3 µg of total RNA following suppliers. RT reactions were performed for 50 min at 42°C. cDNA (1 µl) was used as a template for PCR with specific primers for Entpd1, 2, 3, and Nt5e. β-actin (Actb) was used for normalization as a constitutive gene. PCR reactions have a volume of 25 µl using a concentration of 0.4 µM of each primer indicated below and 200 µM and 1 U Taq polymerase (Invitrogen) in the supplied reaction buffer. Conditions for all PCR were as follows: Initial 1 min denaturation step at 94°C, 1 min at 94°C, 1 min annealing step (Entpd1, 3, and Nt5e: 65°C; Entpd2: 66°C; Actb: 58.5°C), 1 min extension step at 72°C for 35 cycles and a 10 min final extension at 72°C. The amplification products were Entpd1—543 bp; Entpd 2—331 bp; Entpd 3—267 bp; Nt5e—403 bp; Actb—210 bp. For each set of PCR reactions, negative control was included. Ten microliters of the PCR reaction mixture were analyzed on a 1% agarose gel using GelRed™ and photographed under UV light. The Low DNA Mass Ladder was used as a molecular marker and normalized employing Actb (β-actin) as a constitutive gene. The images of stained PCR products were analyzed by optical densitometry and semi-quantified (enzyme/Actb mRNA ratios) using the computer software Image J. Table 1 shows the set of primers used.

Protein determination

Protein was measured by the Comassie Blue method according to Bradford [26], using bovine serum albumin as standard.

Statistical analysis

Data were analyzed by Student's t test. All analyses were performed using the statistical package for the social sciences (SPSS) software in a PC-compatible computer. Differences were considered statistically significant if $P < 0.05$.

Table 1 PCR primers sequences

Enzyme		Sequence (5′–3′)
Entpd1	Sense	GAT CAT CAC TGG GCA GGA GGA AGG
Entpd1	Antisense	AAG ACA CCG TTG AAG GCA CAC TGG
Entpd2	Sense	GCT GGG TGG GCC GGT GGA TAC G
Entpd2	Antisense	ATT GAA GGC CCG GGG ACG CTG AC
Entpd3	Sense	CGG GAT CCT TGC TGT GCG TGG CAT TTC TT
Entpd3	Antisense	TCT AGA GGT GCT CTG GCA GGA ATC AGT
Nt5e	Sense	CCC GGG GGC CAC TAG CAC CTC A
Nt5e	Antisense	GCC TGG ACC ACG GGA ACC TT
Actb	Sense	TAT GCC AAC ACA GTG CTG TCT GG
Actb	Antisense	TAC TCC TGC TTC CTG ATC CAC AT

Results

Effect of chronic mild hyperhomocysteinemia on ectonucleotidase activities in lymphocytes of adult rats

We measured extracellular ATP, ADP, and AMP hydrolysis in lymphocytes obtained of mesenteric lymph nodes of rats subjected to chronic mild hyperhomocysteinemia. Figure 1 shows that Hcy significantly decreased the hydrolysis of ATP [$t(8) = 6.144$; $P < 0.001$], ADP [$t(9) = 4.743$; $P < 0.01$], and AMP [$t(9) = 4.128$; $P < 0.01$] as compared to control.

Effect of chronic mild hyperhomocysteinemia on E-NTPDases and ecto-5′-nucleotidase expression in mesenteric lymph nodes of adult rats

As reported in Fig. 2, Entpd1, 2, and 3 transcript levels were not affected by chronic mild hyperhomocysteinemia [$t(6) = -1.974$; $P > 0.05$; $t(6) = 1.851$; $P > 0.05$; $t(7) = -1.246$; $P > 0.05$, respectively]. However, Nt5e mRNA transcript [$t(6) = 4.057$; $P < 0.01$] was decreased in mesenteric lymph nodes of rats.

Effect of chronic mild hyperhomocysteinemia on ectonucleotidase activities in serum of adult rats

We investigated the effect of chronic mild hyperhomocysteinemia on nucleotide hydrolysis in serum of adult rats. As observed in Fig. 3, ATP [$t(10) = -1.851$; $P > 0.05$], ADP [$t(12) = 0.406$; $P > 0.05$] and AMP [$t(15) = 0.092$; $P > 0.05$] hydrolysis were not affected by Hcy administration.

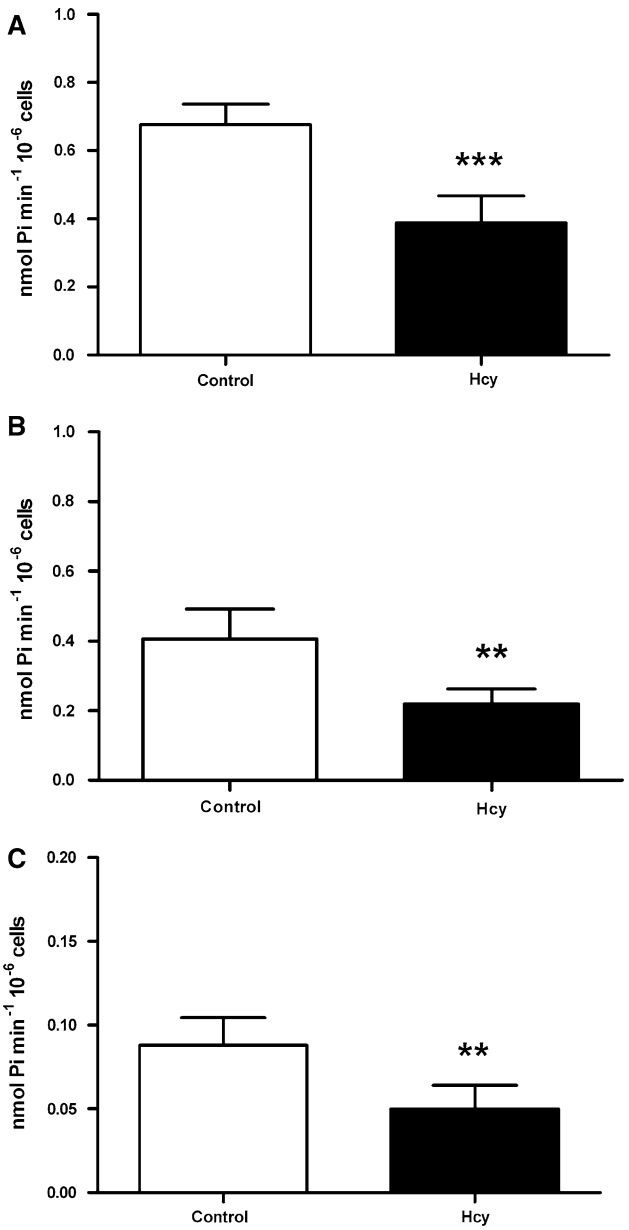

Fig. 1 Effect of chronic homocysteine administration on ATP (**a**), ADP (**b**), and AMP (**c**) hydrolysis in lymphocytes of adult rats. Results are expressed as mean ± SD for 4–6 animals in each group. Different from control, $^{**}P < 0.01$; $^{***}P < 0.001$ (Student's *t* test). *Hcy* homocysteine

Discussion

Mild hyperhomocysteinemia is a risk factor for cerebral and cardiovascular diseases [12, 27, 28]. Although the mechanisms by which Hcy leads to these alterations are not well fully elucidated, there are studies showing that this amino acid may induce oxidative stress and inflammation, which may contribute to the development and progression of atherosclerotic plaques [9, 29–32].

ATP is present in high levels in the extracellular space during inflammation [33, 34]. This nucleotide may modulate inflammatory responses through several ways, including activating cells (endothelium, leukocytes), cytokine and chemokine release and increase adhesion molecules expression via activation of $P2X_7$ receptor [35–37]. ADP is the most important promoter of platelet aggregation, which may be secondary to vascular injury as a consequence of inflammatory processes [38]. On the other hand, nucleoside Ado exhibits potent anti-inflammatory and immunosuppressive action in tissues subjected to various forms of injurious stimuli such as ischemia and inflammation [39].

Since ectonucleotidases are important enzymes in the regulation of extracellular nucleotides and nucleosides levels, acting as signaling molecules in inflammatory processes, in the present study we evaluate the effect of chronic mild hyperhomocysteinemia on nucleotide hydrolysis in lymphocytes from mesenteric lymph nodes of adult

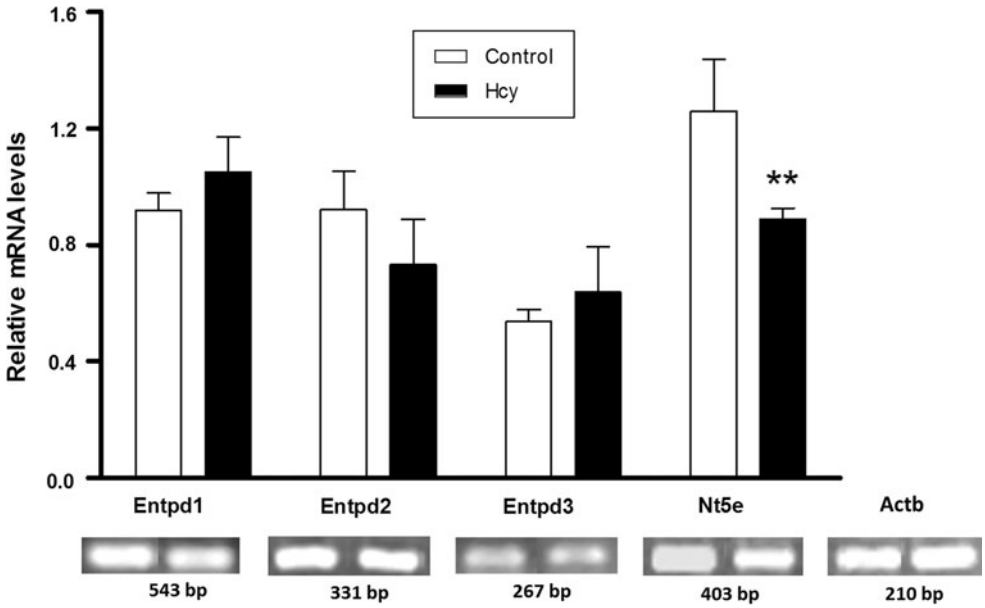

Fig. 2 Gene expression patterns after chronic Hcy treatment for NTPDase1 (Entpd1), NTPDase2 (Entpd2), NTPDase3 (Entpd3), ecto-5′-nucleotidase (Nt5e), and β-actin in mesenteric lymph nodes of rats. Results are expressed as mean ± SD for 4–5 animals in each group. Different from control, $^{**}P < 0.01$ (Student's *t* test). *Hcy* homocysteine

rats. Present reports show that extracellular ATP, ADP, and AMP hydrolysis were significantly decreased in lymphocytes from hyperhomocysteinemic rats.

In order to evaluate whether the mild hyperhomocysteinemia causes transcriptional modifications in the ecto-nucleotidases in lymphocytes, we also investigated the expression patterns of these enzymes after Hcy treatment in mesenteric lymph nodes of adult rats. A semi-quantitative RT-PCR analysis showed that E-NTPDase1, 2, and 3 transcriptions were not affected by chronic mild hyperhomocysteinemia. These findings demonstrated that the decrease in ATP and ADP hydrolysis caused by Hcy was not followed by alterations in E-NTPDases mRNA transcript levels. In this context, it well known that the gene expression is regulated by various factors involving cell machinery and signal transduction pathways, being that the enzymatic activity cannot be directly correlated with the gene expression pattern or with protein levels because of the existence of several post-translational events [40].

On the other hand, ecto-5′-nucleotidase/CD73 mRNA transcriptional level was significantly decreased in mesenteric lymph nodes of hyperhomocysteinemic rats. Considering that ATP and ADP can exert inhibitory effects on ecto-5′-nucleotidase/CD73 [41], it is possible that the inhibition in nucleotide hydrolysis caused by Hcy may lead to increase of ATP levels that consequently, inhibit the ecto-5′-nucleotidase/CD73. Taken together, these data could result in activation of inflammatory responses, since ATP could accumulate in the extracellular milieu, and AMP hydrolysis is decreased, leading to decreased Ado production.

Riksen et al. [42] showed that in patients with severe hyperhomocysteinemia, the cellular adenosine uptake is enhanced due to S-adenosyl-homocysteine (AdoHcy) formation, limiting Ado-induced vasodilation. However, to our knowledge, there are no studies showing the involvement of adenine nucleotides and nucleosides in mild hyperhomocysteinemia. In the present study, we suggest that the Hcy can reduce the Ado concentration through of inhibition of nucleotide hydrolysis in lymphocytes of rats. These findings corroborate with studies demonstrating that a decreased in Ado levels could be associated with the adverse effects of hyperhomocysteinemia, since several cardio- and vaso-protective actions are attributed to this nucleoside, such vasodilation [43], inhibition of platelet aggregation [44], and activation of cellular antioxidant enzyme systems [45].

Moreover, Ado regulates inflammatory actions on lymphocytes [36, 46]. The activation of the A_{2A} receptors on CD_4^+ T lymphocytes prevents myocardial ischemia–reperfusion injury by inhibiting the accumulation and activation of CD_4^+ T cells in the reperfused heart [47] and limits the production of inflammatory mediators such as IL-12, TNF-α, and INFγ by lymphocytes [48, 49]. In addition, Vuaden et al. [50] showed that a selective A_{2A} receptor agonist prevents the endotoxin-induced effects on nucleotide catabolism in mouse lymphocytes.

ATP and other nucleotides of adenine can be degraded by nucleotidases both in the membrane-bound form, and in the soluble form, located in the interstitial medium or within body fluids [51]. It is currently accepted that the exogenous ATP levels may be increased in several inflammatory and shock conditions, mainly as a consequence of nucleotide release from platelets, endothelial, and blood vessel cells [52–54]. This rise in exogenous ATP concentration is usually accompanied by concurrent secretion and/or cleavage of various enzymes to the extracellular milieu [55]. Therefore, serum enzymes might reduce the excess of the extracellular nucleotides levels and

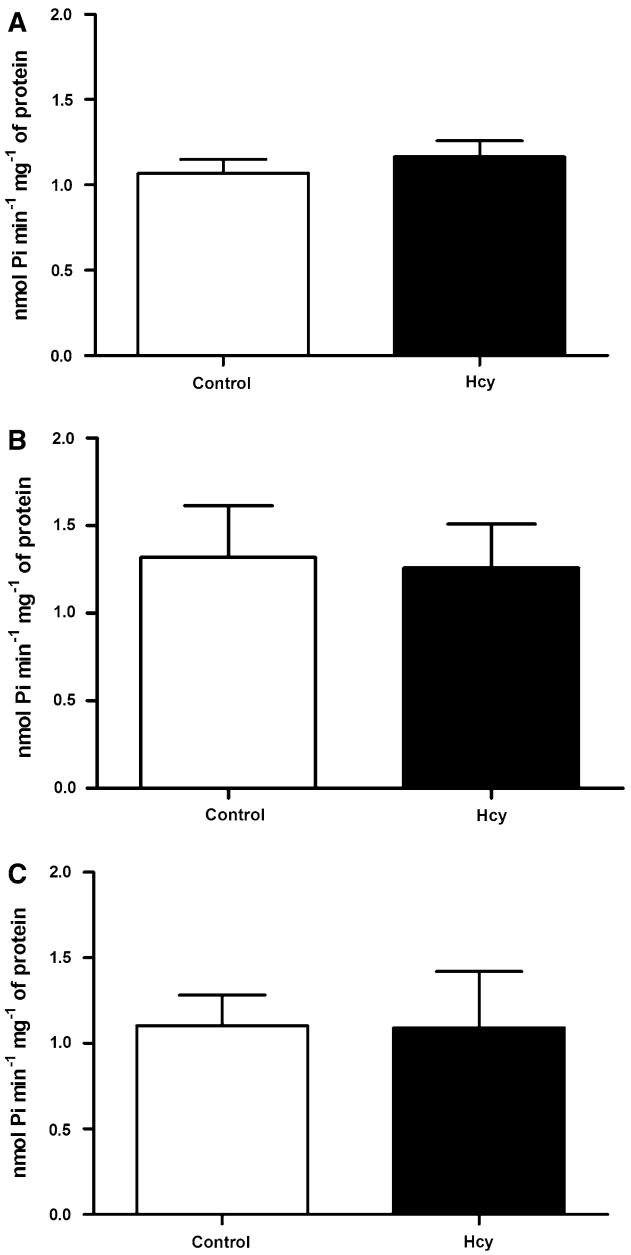

Fig. 3 Effect of chronic homocysteine administration on ATP (**a**), ADP (**b**), and AMP (**c**) hydrolysis in serum of adult rats. Results are expressed as mean ± SD for 6–9 animals in each group. *Hcy* homocysteine

to have an important role in maintaining normal physiology [23].

Our results also demonstrated that extracellular ATP, ADP, and AMP hydrolysis were not altered in serum of rats subjected to experimental mild hyperhomocysteinemia. The lack of effect of mild hyperhomocysteinemia on these parameters in serum is puzzling; however, Böhmer et al. [20] showed that the Hcy in vitro inhibits the nucleotide hydrolysis in the millimolar range, and that this inhibition is concentration dependent. It is important to consider that in vivo other factors could affect the enzymatic cascade and that our results represent concentrations of Hcy in the micromolar range, similar to those considered as a risk factor to cardio- and cerebrovascular diseases [11, 12].

In summary, in the present study, we demonstrated that experimental mild hyperhomocysteinemia significantly decreases the extracellular ATP, ADP, and AMP hydrolysis and ecto-5′-nucleotidase/CD73 gene expression pattern in lymphocytes of adult rats. Our findings suggest that the Hcy modulates the ectonucleotidases, probably decreasing the Ado levels, which could lead to a pro-inflammatory status.

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References

1. Williams KT, Schalinske KL (2010) Homocysteine metabolism and its relation to health and disease. Biofactors 36:19–24
2. Hansrani M, Gillespie JI, Stansby G (2002) Homocysteine in myointimal hyperplasia. Eur J Vasc Endovasc Surg 23:3–10
3. Selhub J, Jacques PF, Wilson PW, Rush D, Rosenberg IH (1993) Vitamin status and intake as primary determinants of homocysteinemia in an elderly population. JAMA 270:2693–2698
4. Jacques PF, Rosenberg IH, Rogers G, Selhub J, Bowman BA, Gunter EW, Wright JD, Johnson CL (1999) Serum total homocysteine concentrations in adolescent and adult Americans: results from the third National Health and Nutrition Examination Survey. Am J Clin Nutr 69:482–489
5. Lentz SR, Haynes WG (2004) Homocysteine: is it a clinically important cardiovascular risk factor? Cleve Clin J Med 71:729–734
6. Balogh E, Bereczky Z, Katona E, Kőszegi Z, Edes I, Muszbek L, Czuriga I (2011) Interaction between homocysteine and lipoprotein(a) increases the prevalence of coronary artery disease/myocardial infarction in women: a case-control study. Thromb Res (in press)
7. Boushey CJ, Beresford SA, Omenn GS, Motulsky AG (1995) A quantitative assessment of plasma homocysteine as a risk factor for vascular disease. Probable benefits of increasing folic acid intakes. JAMA 274:1049–1057
8. Maron BA, Loscalzo J (2009) The treatment of hyperhomocysteinemia. Annu Rev Med 60:39–54
9. Papatheodorou L, Weiss N (2007) Vascular oxidant stress and inflammation in hyperhomocysteinemia. Antioxid Redox Signal 9:1941–1958
10. Scherer EB, da Cunha AA, Kolling J, da Cunha MJ, Schmitz F, Sitta A, Lima DD, Delwing D, Vargas CR, Wyse AT (2011) Development of an animal model for chronic mild hyperhomocysteinemia and its response to oxidative damage. Int J Dev Neurosci 29:693–699
11. Weiss N, Keller C, Hoffmann U, Loscalzo J (2002) Endothelial dysfunction and atherothrombosis in mild hyperhomocysteinemia. Vasc Med 7:227–239
12. Herrmann W, Obeid R (2011) Homocysteine: a biomarker in neurodegenerative diseases. Clin Chem Lab Med 49:435–441

13. Robson SC, Sevigny J, Zimmermann H (2006) The E-NTPDase family of ectonucleotidases: structure function relationships and pathophysiological significance. Purinergic Signal 2:409–430
14. Yegutkin GG (2008) Nucleotide-and nucleoside-converting ectoenzymes: Important modulators of purinergic signalling cascade. Biochim Biophys Acta 1783:673–694
15. Solle M, Labasi J, Perregaux DG, Stam E, Petrushova N, Koller BH, Griffiths RJ, Gabel CA (2001) Altered cytokine production in mice lacking P2X(7) receptors. J Biol Chem 276:125–132
16. Sullivan GW (2003) Adenosine A2A receptor agonists as anti-inflammatory agents. Curr Opin Investig Drugs 4:1313–1319
17. Bigonnesse F, Lévesque SA, Kukulski F, Lecka J, Robson SC, Fernandes MJ, Sévigny J (2004) Cloning and characterization of mouse nucleoside triphosphate diphosphohydrolase-8. Biochemistry 43:5511–5519
18. Zhang Q, Zeng X, Guo J, Wang X (2001) Effects of homocysteine on murine splenic B lymphocyte proliferation and its signal trasduction mechanism. Cardiovasc Res 52:328–336
19. Dawson H, Collins G, Pyle R, Deep-Dixit V, Taub DD (2004) The immunoregulatory effects of homocysteine and its intermediates on T-lymphocyte function. Mech Ageing Dev 125:107–110
20. Böhmer AE, Pochmann D, Sarkis JJ (2006) In vitro effect of homocysteine on nucleotide hydrolysis by blood serum from adult rats. Chem Biol Interact 160:159–164
21. Wu GY, Field CJ, Marliss EB (1991) Glutamine and glucose metabolism in rat splenocytes and mesenteric lymph node lymphocytes. Am J Physiol 260:E141–E147
22. Vuaden FC, de Paula Cognato G, Bonorino C, Bogo MR, Sarkis JJ, Bonan CD (2007) Lipopolysaccharide alters nucleotidase activities from lymphocytes and serum of rats. Life Sci 80:1784–1791
23. Oses JP, Cardoso CM, Germano RA, Kirst IB, Rücker B, Fürstenau CR, Wink MR, Bonan CD, Battastini AM, Sarkis JJ (2004) Soluble NTPDase: an additional system of nucleotide hydrolysis in rat blood serum. Life Sci 74:3275–3284
24. Chan KM, Delfert D, Junger KD (1986) A direct colorimetric assay for Ca^{2+}-stimulated ATPase activity. Anal Biochem 157:375–380
25. Yegutkin GG (1997) Kinectic analysis of enzymatic hydrolysis of ATP in human and rat blood serum. Biochemistry (Mosc) 62:619–622
26. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
27. De Bree A, Verschuren WM, Kromhout D, Kluijtmans LA, Blom HJ (2002) Homocysteine determinants and the evidence to what extent homocysteine determines the risk of coronary heart disease. Pharmacol Rev 54:599–618
28. Benninger DH, Herrmann FR, Georgiadis D, Kretschmer R, Sarikaya H, Schiller A, Baumgartner RW (2009) Increased prevalence of hyperhomocysteinemia in cervical artery dissection causing stroke: a case-control study. Cerebrovasc Dis 27:241–246
29. Upchurch GR Jr, Welch GN, Fabian AJ, Freedman JE, Johnson JL, Keaney JF Jr, Loscalzo J (1997) Homocyst(e)ine decreases bioavailable nitric oxide by a mechanism involving glutathione peroxidase. J Biol Chem 272:17012–17017
30. Heydrick SJ, Weiss N, Thomas SR, Cap AP, Pimentel DR, Loscalzo J, Keaney JF Jr (2004) L-Homocysteine and L-homocystine stereospecifically induce endothelial nitric oxide synthase-dependent lipid peroxidation in endothelial cells. Free Radic Biol Med 36:632–640
31. Ravaglia G, Forti P, Maioli F, Servadei L, Martelli M, Arnone G, Talerico T, Zoli M, Mariani E (2004) Plasma homocysteine and inflammation in elderly patients with cardiovascular disease and dementia. Exp Gerontol 39:443–450
32. Gori AM, Corsi AM, Fedi S, Gazzini A, Sofi F, Bartali B, Bandinelli S, Gensini GF, Abbate R, Ferrucci L (2005) A pro-inflammatory state is associated with hyperhomocysteinemia in the elderly. Am J Clin Nutr 82:335–341
33. Pellegatti P, Raffaghello L, Bianchi G, Piccardi F, Pistoia V, Di Virgilio F (2008) Increased level of extracellular ATP at tumor sites: in vivo imaging with plasma membrane luciferase. PLoS One 3:e2599
34. Di Virgilio F, Ceruti S, Bramanti P, Abbracchio MP (2009) Purinergic signalling in inflammation of the central nervous system. Trends Neurosci 32:79–87
35. Di Virgilio F, Borea PA, Illes P (2001) P2 receptors meet the immune system. Trends Pharmacol Sci 22:5–7
36. Bours MJ, Swennen EL, Di Virgilio F, Cronstein BN, Dagnelie PC (2006) Adenosine 5-triphosphate and adenosine as endogenous signaling molecules in immunity and inflammation. Pharmacol Ther 112:358–404
37. Yu HB, Finlay BB (2008) The caspase-1 inflammasome: a pilot of innate immune responses. Cell Host Microbe 4:198–208
38. Piro M, Giubilato G, Pinnelli M, Giordano Sciacca P, Biasucci LM (2005) Endothelium and inflammation. Panminerva Med 47:75–80
39. Sitkovsky M, Lukashev D (2005) Regulation of immune cells by local-tissue oxygen tension: HIF1 alpha and adenosine receptors. Nat Rev Immunol 5:712–721
40. Nedeljkovic N, Banjac A, Horvat A, Stojiljkovic M, Nikezic G (2005) Developmental profile of NTPDase activity in synaptic plasma membranes isolated from rat cerebral cortex. Int J Dev Neurosci 23:45–51
41. Cunha RA (2001) Regulation of the ecto-nucleotidase pathway in rat hippocampal nerve terminals. Neurochem Res 26:979–991
42. Riksen NP, Rongen GA, Boers GH, Blom HJ, van den Broek PH, Smits P (2005) Enhanced cellular adenosine uptake limits adenosine receptor stimulation in patients with hyperhomocysteinemia. Arterioscler Thromb Vasc Biol 25:109–114
43. Tabrizchi R, Bedi S (2001) Pharmacology of adenosine receptors in the vasculature. Pharmacol Ther 91:133–147
44. Deguchi H, Takeya H, Urano H, Gabazza EC, Zhou H, Suzuki K (1998) Adenosine regulates tissue factor expression on endothelial cells. Thromb Res 91:57–64
45. Maggirwar SB, Dhanraj DN, Somani SM, Ramkumar V (1994) Adenosine acts as an endogenous activator of the cellular antioxidant defense system. Biochem Biophys Res Commun 201:508–515
46. Marone G, Petracca R, Vigorita S, Genovese A, Casolaro V (1992) Adenosine receptors on human leukocytes. IV. Characterization of an A1/Ri receptor. Int J Clin Lab Res 22:235–242
47. Yang Z, Day YJ, Toufektsian MC, Xu Y, Ramos SI, Marshall MA, French BA, Linden J (2006) Myocardial infarct-sparing effect of adenosine A2A receptor activation is due to its action on $CD4^+$ T lymphocytes. Circulation 114:2056–2064
48. Pinhal-Enfield G, Ramanathan M, Hasko G, Vogel SN, Salzman AL, Boons GJ, Leibovich SJ (2003) An angiogenic switch in macrophages involving synergy between Toll-like receptors 2, 4, 7, and 9 and adenosine A(2A) receptors. Am J Pathol 163:711–721
49. Lappas CM, Rieger JM, Linden J (2005) A2A adenosine receptor induction inhibits IFN-gamma production in murine $CD4^+$ T cells. J Immunol 174:1073–1080
50. Vuaden FC, Savio LE, Bastos CM, Bogo MR, Bonan CD (2011) Adenosine A(2A) receptor agonist (CGS-21680) prevents endotoxin-induced effects on nucleotidase activities in mouse lymphocytes. Eur J Pharmacol 651:212–217
51. Zimmermann H (2000) Extracellular metabolism of ATP and other nucleotides. Naunyn-Schmiedeberg's Arch Pharmacol 362:299–309

52. Hantgan RR (1984) A study of the kinetics of ADP-triggered platelet shape change. Blood 64:896–906
53. Bodin P, Burnstock G (1996) ATP-stimulated release of ATP by human endothelial cells. J Cardiovas Pharmacol 27:872–875
54. Dubyak GR (2000) Purinergic signaling at immunological synapses. J Auton Nerv Syst 81:64–68
55. Yegutkin G, Bodin P, Burnstock G (2000) Effect of shear stress on the release of soluble ecto-enzymes ATPase and 5 V-nucleotidase along with endogenous ATP from vascular endothelial cells. Br J Pharmacol 129:921–926