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MILD HYPERHOMOCYSTEINEMIA ALTERS EXTRACELLULAR ADENINE METABOLISM IN RAT BRAIN

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Abstract—Since homocysteine (Hcy) is considered a risk factor to cerebral diseases and adenine nucleotides are important molecules to brain normal function, in the present study we investigated the effect of chronic mild hyperhomocysteinemia on ectonucleotidase activities and expression in rat cerebral cortex. The levels of ATP, ADP, AMP and adenosine (Ado) in cerebrospinal fluid (CSF) of adult rats also were evaluated by high-performance liquid chromatography. For the chronic chemically induced mild hyperhomocysteinemia, Hcy (0.03 μmol/g of body weight) was administered subcutaneously from the 30th to the 60th day of life. Control rats received saline solution in the same volumes. Results showed that Hcy significantly decreased nucleotide hydrolysis in the synaptosomal fraction and increased E-NTPDase1 and ecto-5′-nucleotidase transcripts in rat cerebral cortex. ATP levels were significantly increased, while Ado decreased in CSF of Hcy-treated rats. These findings suggest that the unbalance in ATP and Ado levels may be, at last in part, involved in the cerebral toxicity of mild hyperhomocysteinemia. **© 2012 IBRO. Published by Elsevier Ltd. All rights reserved.**

Key words: mild hyperhomocysteinemia, ectonucleotidases, ATP, adenosine, cerebral cortex, cerebrospinal fluid.

INTRODUCTION

Homocysteine (Hcy) is a sulfur-containing amino acid biosynthesized from methionine. In the first step of metabolic pathway, methionine is converted to S-adenosylmethionine (SAM), which serves as a methyl donor for methyl transferases. The major product of these transfer reactions is S-adenosylhomocysteine (SAH), which is rapidly hydrolyzed to Hcy and adenosine (Ado) in a reversible reaction catalyzed by SAH hydrolase (Williams and Schalinske, 2010).

Mild hyperhomocysteinemia, characterized by an elevated plasma Hcy concentration between 15 and 30 μmol/L (Raaf et al., 2011), is common in many populations whereas can be caused by thermolabile variant of methylenetetrahydrofolate reductase, vitamin deficiencies, drug treatment, aging, smoking, alcohol consumption, or can be secondary to systemic diseases such as insulin-dependent diabetes and hypothyroidism (Jacques et al., 2001; De Bree et al., 2002; Castro et al., 2006; Selhub, 2006; Troen et al., 2008). Furthermore, studies have shown that mild hyperhomocysteinemia is a risk factor to cardiovascular (De Bree et al., 2002; Huang et al., 2008) and neurodegenerative diseases (Obeid and Herrmann, 2006; Minagawa et al., 2010; Herrmann and Obeid, 2011).

ATP and Ado are signaling molecules that play crucial roles in the neurotransmission and neuromodulation acting by two types of purinoceptors: P1 receptors, activated by Ado, and P2 receptors that respond preferentially to ATP (Abbracchio et al., 2006). These molecules are metabolized by ectonucleotidases. Ecto-nucleoside triphosphate diphosphohydrolases (E-NTPDases) is a family of ecto-enzymes composed of eight members responsible for hydrolyzing ATP and ADP to AMP that, by the action of an ecto-5′-nucleotidase, is converted to Ado (Yegutkin, 2008). Four members of this family (NTPDase1, 2, 3, and 8) are tightly bound to the plasma membrane via two transmembrane domains. Ecto-5′-nucleotidase is a homodimer linked to the plasma membrane through a glycosyl phosphatidylinositol lipid anchor. These enzymes have their active site facing the extracellular milieu (Robson et al., 2006; Yegutkin, 2008).

We previously developed an experimental model of chronic mild hyperhomocysteinemia in adult rats (Scherer et al., 2011) and showed that this model was able to induce a decrease in extracellular nucleotide hydrolysis and ecto-5′-nucleotidase/CD73 gene expression pattern in rat lymphocytes (Scherer et al.,

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Abbreviations: Actb, β-actin; Ado, adenosine; CSF, cerebrospinal fluid; EDTA, ethylenediaminetetraacetic acid; E-NTPDases, Ecto-nucleoside triphosphate diphosphohydrolases; Hcy, homocysteine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-performance liquid chromatography; RT-PCR, reverse transcriptase-polymerase chain reaction; SAH, S-adenosylhomocysteine.

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2012). Therefore, in this study we sought to investigate the effect of chronic mild hyperhomocysteinemia on ectonucleotidase activities and gene expression in rat cerebral cortex. ATP, ADP, AMP and Ado concentrations in cerebrospinal fluid (CSF) of adult rats also were evaluated by high-performance liquid chromatography (HPLC).

EXPERIMENTAL PROCEDURES

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 1996) and was approved by the Ethics Committee of the Universidade Federal do Rio Grande do Sul, Brazil (#19634).

Chronic mild hyperhomocysteinemia

DL-Hcy (0.03 μmol/g of body weight) was administered subcutaneously twice a day from the 30th to the 60th day of life of rats; controls rats received saline solution in the same volumes (0.5 ml/100 g of body weight). 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) (Scherer et al., 2011). The animals were killed by decapitation 12 h after the last injection of Hcy and the cerebral cortex and CSF were removed.

Subcellular fraction

The cerebral cortex was removed and placed in ice-cold isolation medium (320 mM sucrose, 5 mM HEPES, pH 7.5, and 0.1 mM EDTA). The cerebral cortex was homogenized in five volumes of ice-cold isolation medium with a motor-driven Teflon-glass homogenizer. The synaptosomes were isolated as previously described (Nagy and Delgado-Escueta, 1984). Briefly, 0.5 ml of crude mitochondrial fraction was mixed with 4.0 ml of an 8.5% Percoll solution and layered onto an isoosmotic Percoll/sucrose discontinuous gradient (10/16%). The synaptosomes that banded at the 10/16% Percoll interface were collected with wide tip disposable plastic transfer pipettes. The synaptosomal fractions were washed twice at 15,000*g* for 20 min with the same ice-cold medium to remove the contaminating Percoll. The material was prepared fresh daily and maintained at 0–4 °C throughout preparation.

Enzyme assays

The reaction medium used to assay the ATP and ADP hydrolysis was essentially as described previously (Battastini et al., 1991) and contained 5.0 mM KCl, 1.5 mM $CaCl_2$, 0.1 mM EDTA, 10 mM glucose, 225 mM sucrose, and 45 mM Tris–HCl buffer, pH 8.0, in a final volume of 200 μl. The synaptosome preparation (10–20 μg protein) was added to the reaction mixture and preincubated for 10 min at 37 °C. The reaction was initiated by the addition of ATP or ADP to a final concentration of 1.0 mM and the reaction was stopped by the addition of 200 μl 10% trichloroacetic acid. The reaction medium used to assay the AMP hydrolysis contained 10 mM $MgCl_2$, 0.1 M Tris–HCl, pH 7.0, and 0.15 M sucrose in a final volume of 200 μl (Heymann et al., 1984). The synaptosome preparation (10–20 μg protein) was preincubated for 10 min at 37 °C. The reaction was initiated by the addition of AMP to a final concentration of 1.0 mM and was stopped by the addition of 200 μl 10% trichloroacetic acid. The released inorganic phosphate (Pi) was measured as previously described (Chan et al., 1986). In all enzyme assays, incubation times and protein concentration were chosen in order to ensure the linearity of the reactions (Heymann et al., 1984; Battastini et al., 1991). Controls with the addition of the enzyme preparation after addition of trichloroacetic acid were used to correct nonenzymatic hydrolysis of the substrates. All samples were run in triplicate.

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. Twelve hours after the last injection of Hcy, cerebral cortex of rats was isolated for total RNA extraction with the Trizol® Reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. The cDNA species were synthesized with Promega (Madison, Wisconsin, USA) 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 $MgCl_2$ 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; Entpd2 – 331 bp; Entpd3 – 267 bp; Nt5e – 403 bp; Actb – 210 bp. For each set of PCR reactions, negative control was included. Five microliters of the PCR reaction mixture was 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 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.

CSF purines measurement

Samples of CSF were centrifuged at 1 °C for 30 min at 16,000*g*, the supernatant taken and aliquots of 20 μl were applied to a reversed-phase HPLC system (Shimadzu, Kyoto, Japan) using a C_{18} column (Ultra C18, 25 cm × 4.6 mm × 5 μm, Restek, Bellefonte, PA, USA). Separation was carried out from 20 μl of CSF, with a reversed-phase column (Ultra C18, 25 cm × 4.6 mm × 5 μm, Restek) in a Shimadzu LC-10AD HPLC. The elution was carried out applying a linear gradient from 100% of solvent A (60 mM KH_2PO_4 and 5 mM of tetrabutylammonium phosphate, pH 6.0) to 100% of solvent B (solvent A plus 30% methanol) over a 30-min period (flow rate at 1.4 ml/min). The amounts of purines were measured by absorption at 260 nm. The retention time of standards was used as parameter for identification and quantification. Purines concentrations are expressed as nmol/ml.

Protein determination

Protein was measured by the Coomassie Blue method according to (Bradford, 1976), using bovine serum albumin as standard.

Table 1. PCR primers sequences

Enzyme		Sequence (5′–3′)	
Entpd1	Sense	GAT CAT CAC TGG GCA GGA GGA AGG	543 bp
	Antisense	AAG ACA CCG TTG AAG GCA CAC TGG	
Entpd2	Sense	GCT GGG TGG GCC GGT GGA TAC G	331 bp
	Antisense	ATT GAA GGC CCG GGG ACG CTG AC	
Entpd3	Sense	CGG GAT CCT TGC TGT GCG TGG CAT TTC TT	267 bp
	Antisense	TCT AGA GGT GCT CTG GCA GGA ATC AGT	
Nt5e	Sense	CCC GGG GGC CAC TAG CAC CTC A	403 bp
	Antisense	GCC TGG ACC ACG GGA ACC TT	
Actb	Sense	TAT GCC AAC ACA GTG CTG TCT GG	210 bp
	Antisense	TAC TCC TGC TTC CTG ATC CAC AT	

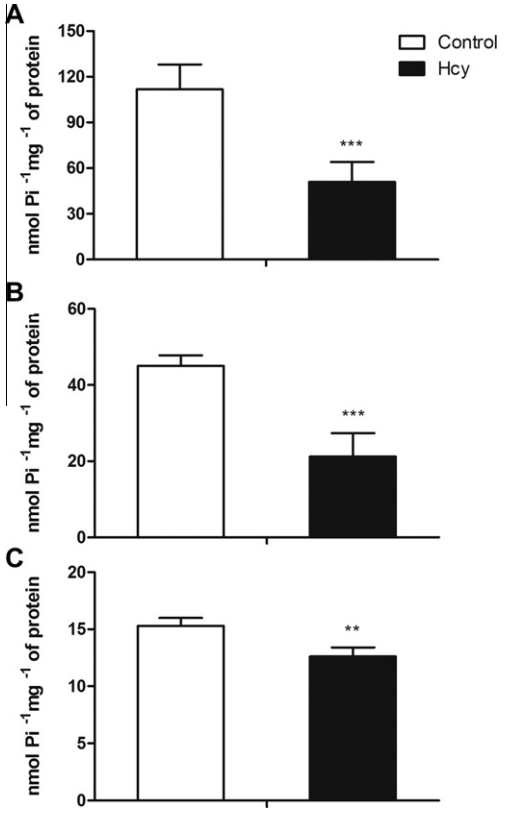

Fig. 1. Effect of chronic homocysteine administration on ATP (A), ADP (B) and AMP (C) hydrolysis in synaptosomal fraction of rat cerebral cortex. Results are expressed as mean ± S.D. for four to six animals in each group. Different from control, $^{**}p < 0.01$; $^{***}p < 0.001$ (Student's *t* test). Hcy – homocysteine.

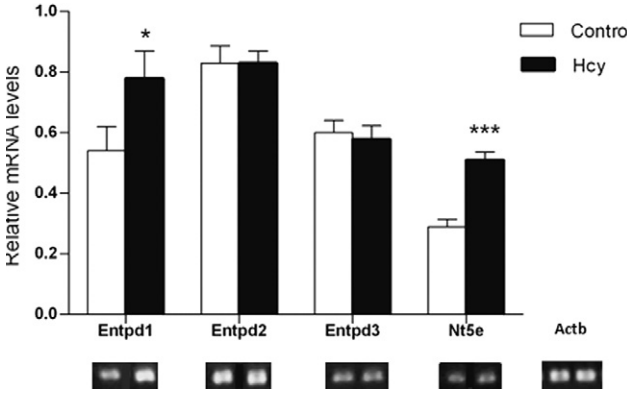

Fig. 2. Gene expression patterns after chronic Hcy treatment for NTPDase1 (Entpd1), NTPDase2 (Entpd2), NTPDase3 (Entpd3), ecto-5′-nucleotidase (Nt5e) and β-actin (Actb) in cerebral cortex of adult rats. Results are expressed as mean ± S.D. for four animals in each group. Different from control, $^{*}p < 0.05$; $^{***}p < 0.001$ (Student's *t* test). Hcy – homocysteine.

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 $p < 0.05$.

RESULTS

Effect of chronic mild hyperhomocysteinemia on ectonucleotidase activities in synaptosomal fraction from cerebral cortex of adult rats

Fig. 1 demonstrates the effect of chronic mild hyperhomocysteinemia on nucleotide hydrolysis in synaptosomal fraction of rat cerebral cortex. Our results showed that Hcy significantly decreased the hydrolysis of ATP [$t(9) = 6.733$; $p < 0.001$], ADP [$t(9) = 8.596$; $p < 0.001$] and AMP [$t(7) = 5.352$; $p < 0.01$] as compared to control.

Effect of chronic mild hyperhomocysteinemia on E-NTPDases and ecto-5′-nucleotidase gene expression in cerebral cortex of adult rats

As reported in Fig. 2, the chronic mild hyperhomocysteinemia increased Entpd1 gene expression [$t(5) = -3.522$; $p < 0.05$], but not affect Entpd2 and 3 transcript levels [$t(6) = 0.006$; $p > 0.05$; $t(6) = 0.749$; $p > 0.05$, respectively]. However, Nt5e mRNA transcript [$t(6) = -11.751$; $p < 0.001$] was increased in cerebral cortex of rats. We did not perform the E-NTPDase8 gene expression because it is not expressed in cerebral cortex (Bigonnesse et al., 2004).

Effect of chronic mild hyperhomocysteinemia on ATP, ADP, AMP and Ado concentration in CSF of adult rats

Considering that the chronic mild hyperhomocysteinemia alters the activity and expression of ectonucleotidases, we extend our study and investigated the effect of Hcy

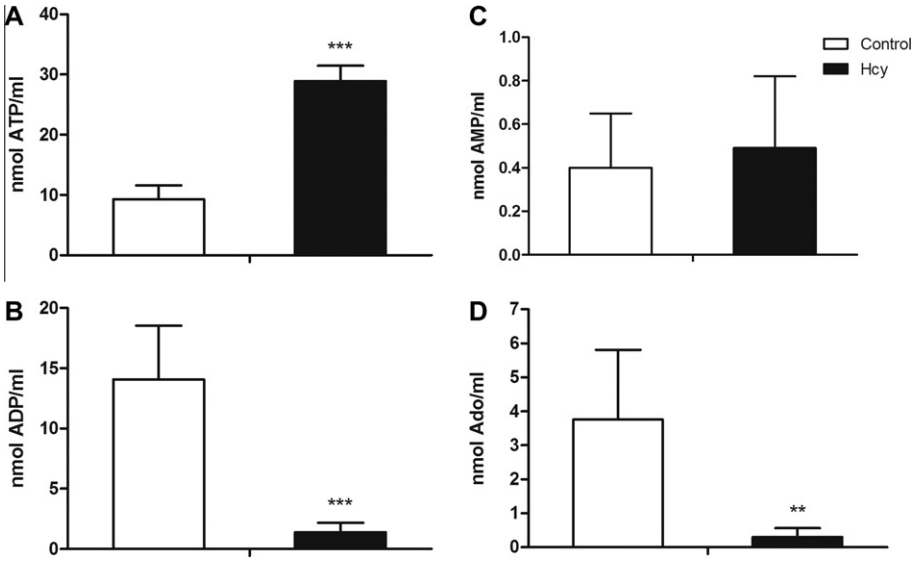

Fig. 3. Effect of chronic homocysteine administration on ATP (A), ADP (B), AMP (C) and Ado (D) concentration in cerebrospinal fluid of adult rats. Results are expressed as mean ± S.D. for four to five animals in each group. Different from control, $^{**}p < 0.01$; $^{***}p < 0.001$ (Student's *t* test). Hcy – homocysteine.

on adenine nucleotides and nucleoside concentration in CSF of adult rats. As can be observed in Fig. 3, ATP concentration was significantly increased in Hcy-treated group [$t(8) = -12.905$; $p < 0.001$] while ADP and Ado concentration were decreased [$t(7) = 6.321$; $p < 0.001$; $t(7) = 3.800$; $p < 0.01$, respectively]. AMP concentration was not affected by Hcy administration [$t(7) = -0.444$; $p > 0.05$].

DISCUSSION

It has been described that Hcy plasma concentration increases with age and that mild hyperhomocysteinemia may be associated with the physiopathology of neurodegenerative diseases (Herrmann et al., 1999; Obeid et al., 2004). Some proposed mechanisms for neurotoxic effects of Hcy involve oxidative stress (Zou and Banerjee, 2005), inflammation (van den Kommer et al., 2010) and glutamatergic excitotoxicity (Shi et al., 2003; Zieminska et al., 2003).

ATP and Ado exert important roles in the CNS, controlling excitatory glutamatergic synapses (Burnstock et al., 2011) and the releasing of neuroinflammatory mediators (Bours et al., 2006; Di Virgilio et al., 2009). In the extracellular space, the concentration of ATP and other nucleotides/nucleosides are tightly controlled by ectonucleotidases (Yegutkin, 2008). In this sense, we recently demonstrated that mild hyperhomocysteinemia decreases ATP, ADP and AMP hydrolysis in lymphocytes and ecto-5′-nucleotidase transcription in mesenteric lymph nodes of adult rats, suggesting that Hcy probably increases ATP and decreases Ado levels, leading to a pro-inflammatory status (Scherer et al., 2012).

In the present study we investigated the effect of chronic mild hyperhomocysteinemia on ectonucleotidase activities in cerebral cortex of adult rats. Results showed that rats subjected to this model presented a decrease in nucleotide hydrolysis promoted by E-NTPDases and ecto-5′-nucleotidase activities in the synaptosomal fraction of cerebral cortex, which could lead to an increase in ATP and a consequent reduction of Ado levels in the extracellular milieu. Ectonucleotidases are integral membrane proteins (Yegutkin, 2008), therefore, it is plausible to suggest that the oxidative damage in membrane lipids may alter the normal function of these enzymes. Indeed, previous study from our group showed that mild hyperhomocysteinemia increases thiobarbituric acid reactive substances (TBARS) levels, an index of lipid peroxidation (Halliwell, 2006), in cerebral cortex in rats (Scherer et al., 2011). Other study demonstrated that Hcy *in vitro* decreases ATP and ADP hydrolysis in rat platelets probably by oxidative stress since antioxidants prevented such effects (Zanin et al., 2010).

We also investigated whether the chronic mild hyperhomocysteinemia could alter ectonucleotidases gene expression through RT-PCR assays in cerebral cortex of rats. Interestingly, the gene expression pattern of ectonucleotidases presented an increase in mRNA levels of E-NTPDase1 and ecto-5′-nucleotidase. Our results demonstrated that the decrease in ATP, ADP and AMP hydrolysis caused by Hcy treatment was accomplished by an increase in E-NTPDase1 and ecto-5′-nucleotidase mRNA transcript levels. These data suggest that the effect of Hcy on expression of ectonucleotidases could be related to a compensatory mechanism due to decrease in nucleotide hydrolysis caused by mild hyperhomocysteinemia in cerebral cortex of rats.

In order to test whether Hcy could alter extracellular nucleotides and Ado levels, we measured the concentrations of ATP, ADP, AMP and Ado in CSF of adult rats by HPLC. Results showed that ATP was

significantly increased, while ADP and Ado levels were decreased in Hcy-treated rats. Since ATP exerts inhibitory effects on ecto-5′-nucleotidase (Cunha, 2001a), we suggest that the decrease in Ado levels observed in our study may be related to the increase in the ATP levels which may lead to ecto-5′-nucleotidase activity inhibition.

ATP, a common constituent of synaptic vesicles, which is released as a co-transmitter together with classical neurotransmitters, such as glutamate noradrenaline, and acetylcholine (Zimmermann, 1994; Burnstock, 2009) may be neurotoxic when released in high levels into the extracellular space by insults such as ischemia/hypoxia (Lutz and Kabler, 1997; Braun et al., 1998; Melani et al., 2005), inflammation, trauma and stress (Nieber et al., 1999; Le Feuvre et al., 2002). The stimulation of P2X7 receptor by ATP induces multiple cytokine pathways that may coordinate inflammatory responses (Le Feuvre et al., 2002), increases glutamate release (Gu and MacDermott, 1997) and promotes cell death (Adinolfi et al., 2005). Thus, the increase in ATP levels promoted by mild hyperhomocysteinemia in our study may be related to the toxic effects of Hcy on the brain.

At the intracellular level, the metabolism of Hcy and Ado is closely related. In normal conditions, the hydrolysis of SAH by SAH hydrolase produces Hcy and Ado. However, it has been proposed that the increase in Hcy levels promotes the opposite direction of reaction catalyzed by SAH hydrolase, resulting in a decrease in the intracellular Ado concentration and increase in transmembrane Ado concentration gradient. By the action of nucleoside transporter it leads to a decrease in the extracellular Ado levels (Riksen et al., 2003, 2005). Based on the above, we suggest that mild hyperhomocysteinemia may impair both sources of Ado production since the Hcy levels are increased and nucleotide degradation cascade is inhibited. These data corroborate with studies that show that the hyperhomocysteinemia decreases Ado concentration in plasma and renal tissue from rats (Chen et al., 2002) and that Hcy and Ado levels are negatively correlated in rat serum (Bohmer et al., 2010).

Ado regulates many physiological processes, particularly in excitable tissues such as brain (Cunha, 2001b). This nucleoside acts both as a homeostatic transcellular messenger and as a neuromodulator, controlling neurotransmitter release such as glutamate, and neuronal excitability (Barrie and Nicholls, 1993; Fredholm et al., 2005). Ado also presents other functions such as control of metabolism rate of neurons and astrocytes (Haberg et al., 2000; Hammer et al., 2001) vascular resistance (Shin et al., 2000), axonal growth (Rivkees et al., 2001), and analgesic (Johansson et al., 2001) and anticonvulsant properties (reviewed in Fredholm et al., 2005).

Studies have shown that the density of the different Ado receptors is ontogenetically regulated. A decrease in the density of A_1 receptors and an increase in the density of A_{2A} receptors in aged animals have been observed (Cunha et al., 1995, 2001; Lopes et al., 1999; Rebola et al., 2003). Furthermore, in some diseases, such as ischemia, the increase in Ado release could contribute to increase cerebral blood flow and protect cerebral tissue from ischemic insult (Coney and Marshall, 1998). Provided that elevated Hcy levels have been linked to cerebral vascular damage (Faraci and Lentz, 2004; Lee et al., 2005), and that glutamatergic excitotoxicity is associated with hyperhomocysteinemia (Zieminska and Lazarewicz, 2006) it is possible that the decrease in Ado levels could contribute to the cerebral alterations promoted by Hcy. Fig. 4 summarizes the effects of mild hyperhomocysteinemia on extracellular adenine nucleotides metabolism in rat brain, highlighting those processes that were quantified throughout the investigations.

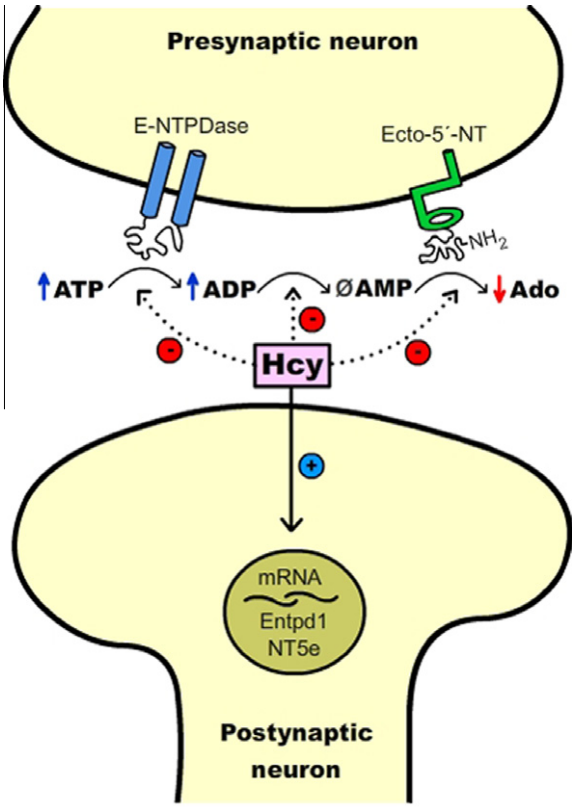

Fig. 4. Summary of the effects of mild hyperhomocysteinemia on extracellular adenine nucleotides metabolism in rat brain, highlighting those processes that were quantified throughout the investigations. Homocysteine (Hcy) inhibits E-NTPDase and ecto-5′-nucleotidase (Ecto-5'-NT) activities and consequently extracellular ATP levels were increased, while ADP and adenosine (Ado) concentrations were decreased. Moreover, Hcy increases E-NTPDase1 (Entpd1) and ecto-5′-nucleotidase (Nt5e) gene expression, suggesting a compensatory mechanism to the decrease in nucleotide hydrolysis.

Since the maintenance of adenine nucleotides and nucleosides levels in the CNS is extremely important to normal brain function since these molecules play a significant role in the pathophysiology of various neurological disorders (Di Virgilio et al., 2009) and that the mild hyperhomocysteinemia is a risk factor for neurodegenerative diseases (Obeid and Herrmann, 2006), our findings lead us to the hypothesis that the

alterations in the ectonucleotidases and the unbalance in ATP and Ado levels might contribute, at last in part, to cerebral damage caused by Hcy.

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