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Mild Hyperhomocysteinemia Increases Brain Acetylcholinesterase and Proinflammatory Cytokine Levels in Different Tissues

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Abstract Mild hyperhomocysteinemia is considered to be a risk factor for cerebral and cardiovascular disorders and can be modeled in experimental rats. Inflammation has been implicated in the toxic effects of homocysteine. Cholinergic signaling controls cytokine production and inflammation through the "cholinergic anti-inflammatory pathway," and brain acetylcholinesterase activity plays a role in this regulation. The aim of this present study is to investigate the effect of mild chronic hyperhomocysteinemia on proinflammatory cytokine levels in the brain, heart, and serum of rats. Activity, immunocontent, and gene expression of acetylcholinesterase in the brain and butyrylcholinesterase activity in serum were also evaluated. Mild hyperhomocysteinemia was induced in Wistar rats by homocysteine administration (0.03 μmol/g of body weight) twice a day, from the 30th to the 60th days of life. Controls received saline in the same volumes. Results demonstrated an increase in tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), and the chemokine monocyte chemotactic protein-1 (MCP-1) in the hippocampus, as well as an increase in IL-1β and IL-6 levels in cerebral cortex. Acetylcholinesterase activity was increased in rats subjected to mild hyperhomocysteinemia in both cerebral structures tested; the immunocontent of this enzyme was also increased in the cerebral cortex and decreased in the hippocampus. Levels of acetylcholinesterase mRNA transcripts were not altered. Peripherally, homocysteine increased TNF-α, IL-6, and MCP-1 levels in the heart and IL-6 levels in serum. Taken altogether, these findings suggest that homocysteine promotes an inflammatory status that can contribute, at least in part, to neuronal and cardiovascular dysfunctions observed in mild hyperhomocysteinemia.

Keywords Mild hyperhomocysteinemia · Cytokines · Gene expression · Acetylcholinesterase · Butyrylcholinesterase

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Introduction

Homocysteine (Hcy), an endogenous sulfur-containing amino acid produced by the methionine metabolism, has recently been recognized as one of the most potent excitatory agents in the central nervous system (CNS) [1, 2]. Elevated circulating levels of Hcy (hyperhomocysteinemia) can be classified according the severity: mild (15–30 μmol/L), moderate (31–100 μmol/L), and severe (>100 μmol/L) [3, 4]. Although severe hyperhomocysteinemia is rare, about 1 in 20 individuals presents mild hyperhomocysteinemia [5], it is a well-established risk factor for several cerebral and cardiovascular disorders, such as epilepsy [6, 7], stroke [8], neurodegenerative and neuropsychiatric diseases [9–12], atherosclerosis, and brain ischemia [13, 14].

The underlying mechanism by which Hcy exerts its toxic effects remains unexplained. In order to research into that mechanism, an experimental model of chronic mild hyperhomocysteinemia in adult rats was developed [15] aiming to

produce plasma levels of Hcy similar to those considered as a risk factor for human cardiovascular and cerebral diseases [16]. Rats submitted to such mild hyperhomocysteinemia presented oxidative damage in cerebral cortex and plasma [15], as well as a decrease in nucleotide hydrolysis in the brain accompanied by an increase in the ectonucleotidase gene expression in the cerebral cortex. Accordingly, ATP levels were significantly increased, while adenosine (Ado) decreased in cerebrospinal fluid of Hcy-treated rats [17]. Since ATP shows proinflammatory properties and Ado is considered an anti-inflammatory compound [18], these results suggest that inflammation might be associated to the adverse effects of mild hyperhomocysteinemia.

Inflammation has been associated with the physiopathology of many neurodegenerative and cardiovascular diseases. An increase in levels of inflammatory proteins in the brain and plasma of Alzheimer's disease and vascular dementia patients has been described [19], and recent studies have shown that inflammatory cytokines play a central role in the pathogenesis of atherosclerosis [20]. Platelet-activating factors (PAFs), reactive oxygen species (ROS), nitric oxide (NO), and various proinflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6), are some of the important inflammation mediators [21, 22], and recent studies of acute and chronic administration of Hcy producing plasma levels similar to those found in severe hyperhomocysteinemia showed greater concentration of cytokines (TNF-α, IL-1β, and IL-6) and chemokine monocyte chemoattractant protein-1 (MCP-1) in serum, hippocampus, and cerebral cortex [23, 24].

The brain cholinergic system is known to modulate several important functions such as learning, memory, cortical organization of movement, and the control of cerebral blood flow [25–28]. Recent studies also suggest the involvement of acetylcholine (ACh) in the inflammation through the "cholinergic anti-inflammatory pathway," defined as neural signals transmitted via the vagus nerve to inhibit the release of peripheral cytokines [29–31]. Along with that, both enzymes that hydrolyze ACh, Acetylcholinesterase (AChE, EC 3.1.1.7) which are highly present in the brain and butyrylcholinesterase (BuChE, EC 3.1.1.8) found in blood serum, pancreas, liver, and SNC [21, 32], have been considered regulators of inflammation since they control ACh action [21].

In the present study, we decided to investigate the effect of chronic mild hyperhomocysteinemia on the proinflammatory cytokine levels in the brain, heart, and serum of rats. The activity, immunocontent, and gene expression of AChE in the cerebral cortex and hippocampus, as well as serum BuChE activity were also evaluated in order to better understand the association between mild hyperhomocysteinemia and inflammation.

Material and Methods

Animals and Reagents

Female Wistar rats (30 days old) were obtained from the Central Animal House of Biochemistry Department, Institute of Basic Health Sciences at the Universidade Federal do Rio Grande do Sul, in Porto Alegre, Brazil. They were maintained on a 12:12 h light/dark cycle (lights on 07:00–19:00 h) in an 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 the experimental protocol was approved by the University's Ethics Committee (#19634).

Chronic Mild Hyperhomocysteinemia

DL-Hcy (Sigma-Aldrich®) (0.03 μmol/g of body weight) was administered subcutaneously, twice a day, from the 30th to the 60th day of the life of rats; controls received the same volume of saline solution (0.5 mL/100 g of body weight). Plasma Hcy concentration in rats subjected to such treatment achieved levels similar to those described for the plasma of patients with mild hyperhomocysteinemia (30 μM) [15]. The rats were decapitated 12 h after the last Hcy injection. The cerebral cortex, hippocampus, heart, and serum were used for the biochemical analysis.

Cytokines (TNF-α, IL-1β, and IL-6) and Chemokine CCL2 (MCP-1) Assay

For the acquisition of serum, the whole blood was centrifuged at 1,000×*g* for 5 min, and serum was immediately removed. The cerebral cortex, hippocampus, and heart were homogenized in 1:5 (*w*/*v*) saline solution (0.9 % NaCl). The homogenate was centrifuged at 800×*g* for 10 min at 4 °C, and the supernatant was used in the assays.

TNF-α, IL-1β, IL-6, and MCP-1 levels in the cerebral cortex, hippocampus, heart, and serum were quantified by rat high-sensitivity enzyme-linked immunoabsorbent assays (ELISA) with commercially available kits (Invitrogen®).

AChE Activity Assay

For the AChE assay, the cerebral cortex and hippocampus were homogenized in ten volumes of 0.1 mM potassium phosphate buffer, pH 7.5, and centrifuged for 10 min at 1,000×*g*. The supernatants were used for the enzymatic AChE analyses. AChE activity was determined according to the method of Ellman and colleagues [33], with some modifications [34]. Hydrolysis rates were measured at ACh concentration of 0.8 mM in 300 μL assay solution with 30 mM

phosphate buffer, pH 7.5, and 1.0 mM 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) at 25 °C. About 15 μL of cerebral cortex and hippocampus supernatant was added to the reaction mixture and preincubated for 3 min. The hydrolysis was monitored by formation of the thiolate dianion of DTNB at 412 nm for 2–3 min (intervals of 30 s). All samples were run in triplicate.

Western Blot Analysis of AChE

Cerebral cortex and hippocampus of rats were homogenized in a lysis solution containing 2 mM EDTA, 50 mM Tris–HCl, pH 6.8, and 4 % sodium dodecyl sulphate (SDS). For electrophoresis analysis, samples were dissolved in 25 % (*v*/*v*) of a solution containing 40 % glycerol, 5 % 2-mercaptoethanol, and 50 mM Tris–HCl, pH 6.8, and boiled for 5 min. Total protein homogenate were separated by 10 % SDS–PAGE (50 μg/lane of total protein) and transferred (Trans-Blot SD Semi-Dry Transfer Cell, Bio-Rad) to nitrocellulose membranes for 1 h at 15 V in transfer buffer (48 mM Trizma, 39 mM glycine, 20 % methanol, and 0.25 % SDS). The blot was then washed for 10 min in Tris-buffered saline (TBS) (0.5 M NaCl, 20 mM Trizma, pH 7.5), followed by a 2-h incubation in blocking solution (TBS plus 5 % defatted dry milk). After incubation, the blot was washed twice for 5 min with blocking solution plus 0.05 % Tween 20 (T-TBS) and then incubated overnight at 4 °C in blocking solution containing AChE antibody (rabbit polyclonal IgG, AChE (H-134), Santa Cruz Biotechnology) diluted 1:1000. The blot was then washed twice for 5 min with T-TBS and incubated for 2 h in antibody solution containing peroxidase-conjugated anti-rabbit IgG diluted 1:2000. The blot was washed twice again for 5 min with T-TBS and twice for 5 min with TBS. The blot was developed using a chemiluminescence kit (Immobilon Western Chemiluminescent HRP Substrate, Millipore).

Gene Expression Analysis by Semiquantitative RT-PCR

Analysis of the ache gene expression was performed by a semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) assay [35]. Twelve hours after the last injection of Hcy, the cerebral cortex and hippocampus of rats were isolated for total RNA extraction with the Trizol® Reagent (Invitrogen) in accordance with the manufacturer's instructions. Complementary DNA (cDNA) species were synthesized with the ImProm-II™ Reverse Transcription System (Promega) from 1 μg of total RNA, following suppliers. cDNA (1 μL) was used as a template for PCR with specific primer for ache. β-actin was used for normalization as a constitutive gene. PCR reactions for ache and β-actin genes were performed in a total volume of 20 μL, containing 0.1 μM primers, 0.2 mM DNTP, 2 mM $MgCl_2$, and 0.5 U Taq DNA Polymerase® (Invitrogen). The following conditions were used for the PCR reactions: 1 min at 94 °C, 1 min at the annealing temperature (ache: 55 °C, β-actin: 58.5 °C), and 1 min at 72 °C for 35 cycles. Postextension at 72 °C was performed for 10 min. The amplification products were ache 785 bp and β-actin 210 bp. For each set of PCR reactions, negative control was included. PCR products were analyzed on a 1 % agarose gel containing GelRed® (Biotium) 10× and photographed under UV light. The band intensities were measured by optical densitometry using the freeware ImageJ 1.37 for Windows, and the relative gene expression was determined through the band intensities of ache genes compared to β-actin (ache/β-actin).

BuChE Activity Assay

BuChE activity was determined by the method of Ellman and colleagues [33] with some modifications. Hydrolysis rates were measured at ACh concentration of 0.8 mM in 1 mL assay solution with 100 mM potassium phosphate buffer, pH 7.5, and 1.0 mM DTNB. Fifty microliters of rat-diluted serum was added to the reaction mixture and preincubated for 3 min. The hydrolysis was monitored by formation of the thiolate dianion of DTNB at 412 nm for 2 min (intervals of 30 s) at 25 °C. All samples were run in triplicate.

Protein Determination

Protein was measured by the Coomassie Blue method according to Bradford [36] or Lowry and colleagues [37] using bovine serum albumin as the 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 whenever $p<0.05$.

Results

The effect of chronic Hcy administration on cytokine levels (TNF-α, IL-1β, and IL-6) and chemokine CCL2 (MCP-1) in the cerebral cortex and the hippocampus of rats is depicted in Fig. 1. Hcy significantly increased the levels of IL-1β ($p<0.05$) and IL-6 ($p<0.01$) but did not alter TNF-α ($p>0.05$) or MCP-1 levels ($p>0.05$) in the cerebral cortex. However, in the hippocampus, a significant increase in the levels of all cytokines tested (TNF-α ($p<0.01$), IL-1β ($p<0.01$), IL-6 ($p<0.01$), and MCP-1 ($p<0.01$) was observed.

In order to investigate whether mild hyperhomocysteinemia would affect AChE function, we evaluated this enzyme

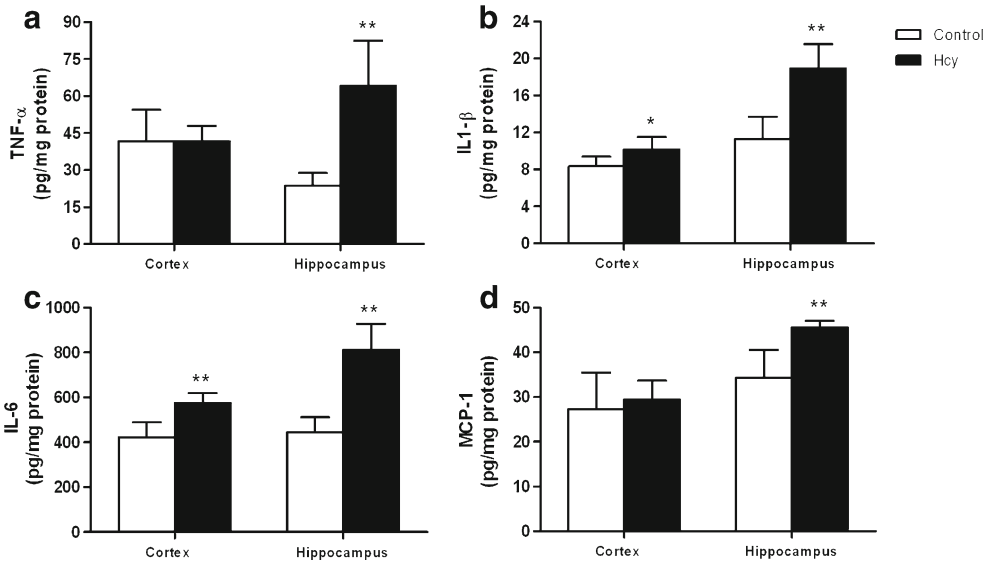

Fig. 1 Effect of chronic homocysteine administration on cytokines (TNF-α, IL-1β, and IL-6) and chemokine CCL2 (MCP-1) levels in the cerebral cortex and hippocampus of rats. Results are expressed as mean±SD for four to five animals in each group. Different from control, *$p<0.05$, **$p<0.01$ (Student's *t* test). *Hcy* homocysteine, **a** *TNF-α* tumor necrosis factor alpha, **b** *IL-1β* interleukin-1β, **c** *IL-6* interleukin-6, **d** *MCP-1* monocyte chemoattractant protein-1

activity, its immunocontent, and gene expression in the cerebral cortex and hippocampus. The chronic exposure to Hcy caused a significant increase of AChE activity in the cerebral cortex ($p<0.01$) and the hippocampus of rats ($p<0.05$) (Fig. 2). Mild hyperhomocysteinemia also increased the immunocontent of AChE in the cerebral cortex ($p<0.05$) but decreased in the hippocampus ($p<0.01$) (Fig. 3). Quantitative RT-PCR analysis showed that ache gene expression was not altered by Hcy in both brain structures tested ($p>0.05$) (Fig. 4).

The effects of mild hyperhomocysteinemia on proinflammatory cytokine levels in the heart and serum, as well as on BuChE activity in the serum of rats, were also evaluated. It was observed that Hcy significantly increased TNF-α ($p<0.01$), IL-6 ($p<0.05$), and MCP-1 ($p<0.001$) levels in the heart (Fig. 5). Hcy also increased IL-6 levels ($p<0.001$) (Fig. 6) but did not alter TNF-α ($p>0.05$), IL-1β ($p>0.05$), and MCP-1 ($p>0.05$) levels or BuChE activity ($p>0.05$) in the serum of treated rats.

Discussion

Mild hyperhomocysteinemia and inflammation are associated with the development and progression of several cerebral and cardiovascular diseases; however, the mechanism of the relationship between both conditions is unclear. Since CNS interacts dynamically with the immune system to modulate inflammation and ACh has been considered an anti-inflammatory molecule [31], the aim of the present study was to evaluate the proinflammatory cytokines levels in the brain, heart, and serum of rats, as well as the activity, immunocontent, and gene expression of AChE in the cerebral cortex and hippocampus, and BuChE activity in the serum of rats subjected to a model of mild hyperhomocysteinemia.

The proinflammatory cytokines TNF-α, IL-1β, and IL-6 participate prominently in the immune response to pathogens in the periphery and have unique and specific actions on neurons and circuits within the brain [38], being released by macrophages and microglia as part of the early acute phase

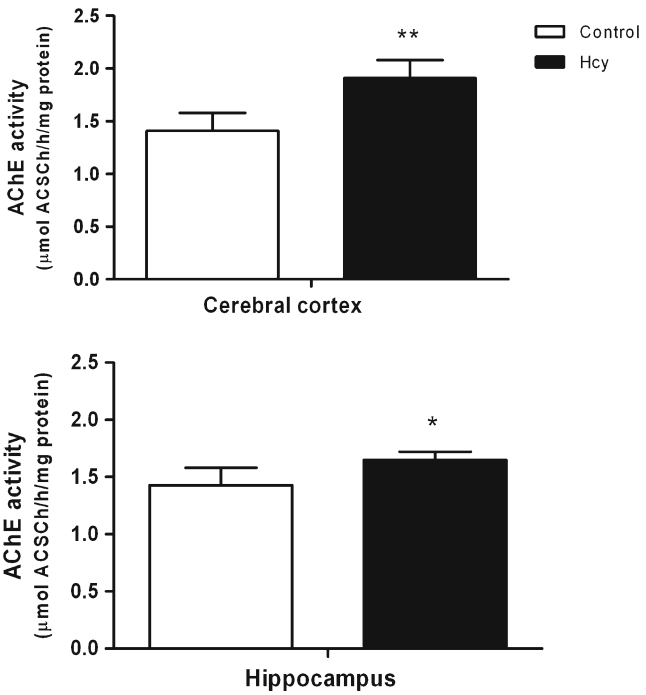

Fig. 2 Effect of chronic homocysteine administration on acetylcholinesterase activity in the cerebral cortex and hippocampus of adult rats. Results are expressed as mean±SD for five to six animals in each group. Different from control, *$p<0.05$, **$p<0.01$ (Student's *t* test). *Hcy* homocysteine, *AChE* acetylcholinesterase

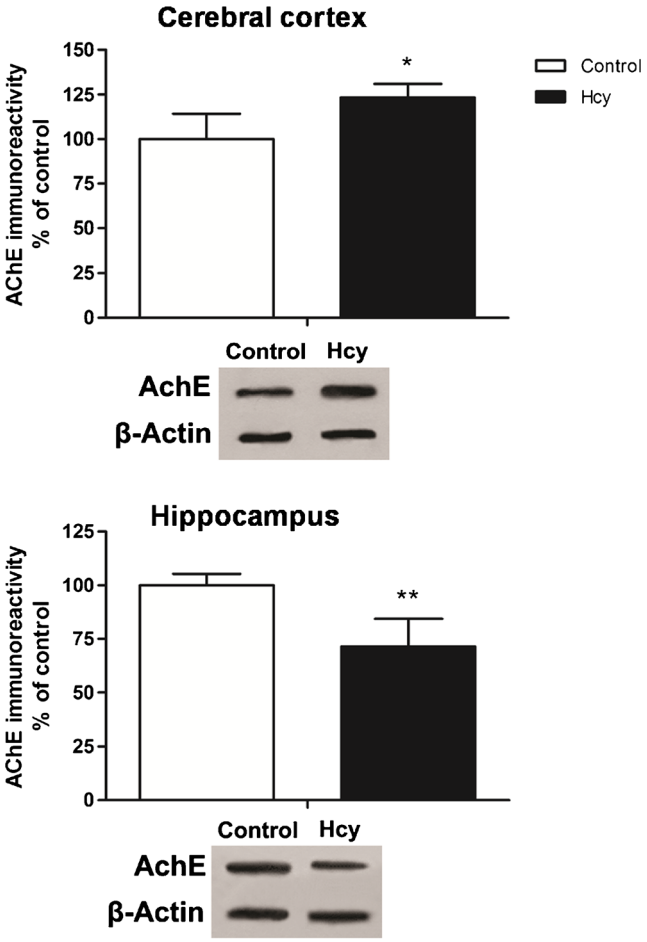

Fig. 3 Effect of chronic homocysteine administration on immunocontent of the acetylcholinesterase in the cerebral cortex and hippocampus of rats. All lanes received equivalent amounts (50 μg) of total protein from cell extract. The blots were developed using an ECL kit. Uniformity of gel loading was confirmed with β-actin as the standard, since its level is not affected by the experimental treatment. Representative immunological reactions are shown below. Results are expressed as mean±SD for five to six animals in each group. Different from control, *$p<0.05$, **$p<0.01$ (Student's *t* test). *Hcy* homocysteine, *AChE* acetylcholinesterase

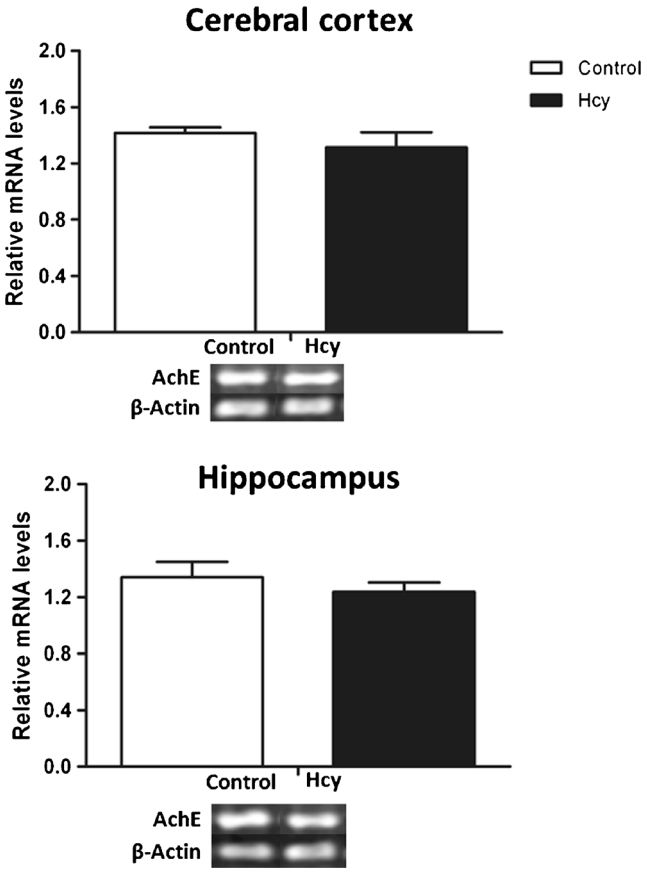

Fig. 4 Gene expression of ache after homocysteine administration in the cerebral cortex and hippocampus of rats. Results are expressed as mean± SD for four to five animals in each group. *Hcy* homocysteine

reaction [39]. TNF-α is the key initiator of immune-mediated inflammation in multiple organ systems including the brain [40], acting synergistically with IL-1β to induce IL-6 expression [41]. In addition to the immune response, these cytokines are suggested to affect neurotransmission, memory modulation, and glucocorticoid function, as well as animal behavior and fear conditioning [38].

Present results show that mild hyperhomocysteinemia increased TNF-α, IL-1β, and IL-6, as well as the chemokine CCL2 (MCP-1) in the hippocampus of rats. In the cerebral cortex, we observed an increase in IL-1β and IL-6 levels. In this context, we previously demonstrated that ATP levels were increased and adenosine was decreased in cerebrospinal fluid of hyperhomocysteinemic rats [17]. ATP released during inflammation presents proinflammatory properties while adenosine, produced by catabolism of ATP, is an anti-inflammatory compound [18]. In addition to that, it has been reported that the stimulation of P2X7 receptor by ATP induces multiple cytokine pathways that may coordinate inflammatory responses [42]. These data suggest that the mild hyperhomocysteinemia promotes a proinflammatory state in the brain.

The "cholinergic anti-inflammatory pathway" regulates the peripheral cytokine response through neural signals transmitted via the vagus nerve [29, 43], and the degeneration of cholinergic neurons may increase the release of proinflammatory cytokine in the periphery system [44]. A similar pathway has been found in the brain of mice, since microglial activation was shown to be regulated by ACh nicotinic receptors [45].

Our results show that chronic mild hyperhomocysteinemia enhances AChE activity in the cerebral cortex and hippocampus of adult rats, suggesting a reduction in the ACh levels leading to a proinflammatory state. Along with that, it has been reported that AChE inhibition reduces microglial production of TNF-α in a hypoxia model [46] and that galantamine, an AChE inhibitor used in the treatment of cholinergic insufficiency and memory loss in Alzheimer's disease, suppresses systemic cytokine levels during endotoxemia [43]. Thus, the increase in the proinflammatory cytokine levels in

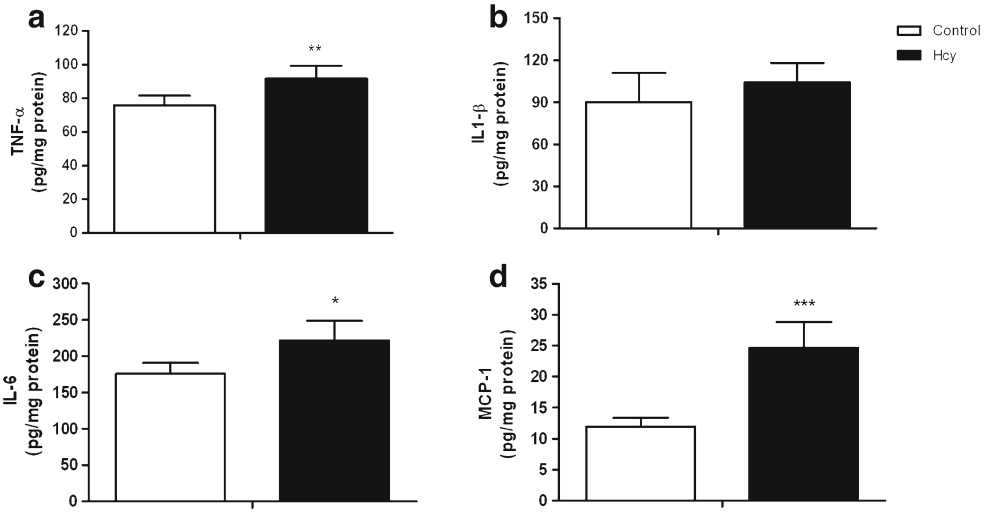

Fig. 5 Effect of chronic homocysteine administration on cytokines (TNF-α, IL-1β, and IL-6) and chemokine CCL2 (MCP-1) levels in the heart of rats. Results are expressed as mean± SD for four to six animals in each group. Different from control, *$p<0.05$, **$p<0.01$, ***$p<0.001$ (Student's *t* test). *Hcy* homocysteine, **a** *TNF-α* tumor necrosis factor alpha, **b** *IL-1β* interleukin-1β, **c** *IL-6* interleukin-6, **d** *MCP-1* monocyte chemoattractant protein-1

the cerebral cortex and hippocampus caused by mild hyperhomocysteinemia can be associated, at least in part, with the enhancement of AChE activity, since this enzyme hydrolyzes ACh, which is considered an anti-inflammatory molecule that can act by inhibiting the production of proinflammatory mediators [21, 47].

Considering that chronic mild hyperhomocysteinemia increases the AChE activity, the immunocontent and gene expression of this enzyme in the cerebral cortex and hippocampus of adult rats were further studied. Results demonstrate that the immunocontent of AChE was significantly increased in the cerebral cortex and decreased in hippocampus, while the enzyme gene expression pattern was not affected in both cerebral structures tested.

It has been reported that AChE levels are controlled by post-transcriptional regulation [48, 49]. The mechanism by which Hcy changes the AChE immunocontent in the cortex and hippocampus in different forms (increasing the former while decreasing the latter) is not clear. However, it is known that AChE isoforms respond differently under toxicological and pathological stress. AChE variants that show indistinguishable enzymatic activity differ in their multimeric assembly and membrane association patterns [49, 50].

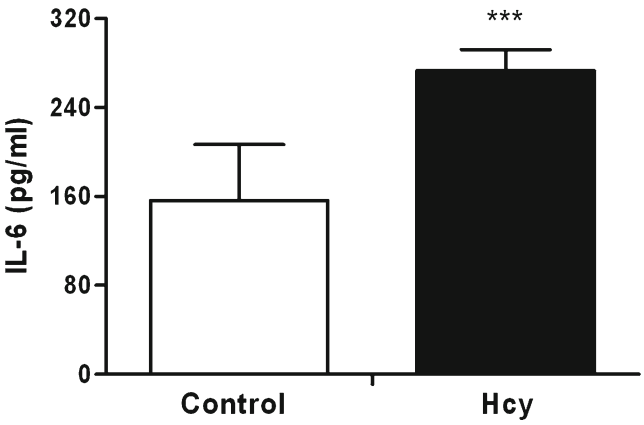

Fig. 6 Effect of chronic homocysteine administration on IL-6 levels in the serum of rats. Results are expressed as mean±SD for five to seven animals in each group. Different from control, ***$p<0.001$ (Student's *t* test). *Hcy* homocysteine, *IL-6* interleukin-6

It is so plausible to suggest that the stimulation of AChE activity in cerebral cortex can be due to an increment in the content of catalytic subunits, increasing the levels of available enzyme molecules. On the other hand, the decrease of AChE immunocontent in hippocampus could be related to a compensatory response to such augmented enzyme activity.

Studies show that Hcy may promote endothelial dysfunction, leading to the activation of proinflammatory pathways in the vasculature [51, 52]. Hcy stimulates the IL-6 expression in cultured rat aorta vascular smooth muscle cells [53] and induces both mRNA expression and protein secretion of MCP-1 and IL-8 in cultured human aortic endothelial cells [54]. Elevation in the IL-6 levels is closely related to an increased risk of myocardial infarction and coronary diseases [55–58]. Holven et al. [59] showed that the IL-6 levels are increased in hyperhomocysteinemic subjects. Our results are in agreement with these studies, since we observed that mild hyperhomocysteinemia promotes an increase in TNF-α, IL-6, and MCP-1 levels in the heart, as well as increased IL-6 levels in the serum. Taken altogether, these findings support the hypothesis that elevated Hcy levels promote systemic inflammation, which is considered a risk factor to cardiovascular diseases.

Although the physiological role of BuChE is unclear, this enzyme catalyzes the hydrolysis of ACh and thus serves as a coregulator of cholinergic transmission, maintaining the structural and functional integrity of cholinergic pathways [60, 61]. Studies also suggest a relationship between BuChE activity and risk factors for coronary artery disease [62]. In the present study, we observed that BuChE activity was not affected by Hcy treatment in the serum. On the other hand, it is known that BuChE and AChE differ in their kinetic response to

concentrations of ACh. BuChE is less efficient in ACh hydrolysis at low concentrations, but highly efficient at high-substrate concentration when AChE activity is decreased [21]. Thus, the lack of Hcy effect on BuChE activity in the serum can be related to a decrease in the ACh levels by AChE stimulation in the brain, since studies suggest that the peripheral cholinergic system may communicate with the CNS via the vagus nerve [31].

In summary, in the present study, we demonstrated that adult rats submitted to mild hyperhomocysteinemia model presented an increase in the cytokine levels in the brain, heart, and serum. Brain AChE function was also altered by Hcy, suggesting that mild hyperhomocysteinemia may promote a proinflammatory status. These findings provide new basis for the understanding of toxicity mechanisms of Hcy in cerebral, cardiovascular, and peripheral tissues.

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