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Int. J. Devl Neuroscience 24 (2006) 3–8

INTERNATIONAL JOURNAL of DEVELOPMENTAL NEUROSCIENCE

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Folic acid pretreatment prevents the reduction of Na^+,K^+-ATPase and butyrylcholinesterase activities in rats subjected to acute hyperhomocysteinemia

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Received 11 November 2005; received in revised form 9 December 2005; accepted 12 December 2005

Abstract

The main objective of the present study was to evaluate the effect of folic acid pretreatment on parietal cortex Na^+,K^+-ATPase and serum butyrylcholinesterase activities in rats subjected to acute hyperhomocysteinemia. Animals were pretreated daily with an intraperitoneal injection of folic acid (5 mg/kg) or saline from the 22th to the 28th day of age. Twelve hours after the last injection of folic acid or saline, the rats received a single subcutaneous injection of homocysteine (0.6 μmol/g of weight body) or saline and were killed 1 h later. Serum was collected and the brain was quickly removed and parietal cortex dissected. Results showed that acute homocysteine administration significantly decreased the activities of Na^+,K^+-ATPase and butyrylcholinesterase on parietal cortex and serum, respectively. Furthermore, folic acid pretreatment totally prevented these inhibitory effects. We also evaluated the effect of acute homocysteine administration on some parameters of oxidative stress, namely thiobarbituric acid-reactive substances and total thiol content in parietal cortex of rats. No alteration of these parameters were observed in parietal cortex of homocysteinemic animals, indicating that these oxidative stress parameters were probably not responsible for the reduction of Na^+,K^+-ATPase and butyrylcholinesterase activities. The presented results confirm previous findings that acute hyperhomocysteinemia produces an inhibition of Na^+,K^+-ATPase and butyrylcholinesterase activities and that pretreatment with folic acid prevents such effects. Assuming that homocysteine might also reduce the activities of these enzymes in human beings, our results support a new potential therapeutic strategy based on folic acid supplementation to prevent the neurological damage found in hyperhomocysteinemia.

Keywords: Homocystinuria; Metabolic disease; Homocysteine; Folic acid; Na^+,K^+-ATPase; Butyrylcholinesterase

1. Introduction

Folic acid is a cofactor of the enzymatic reaction that promotes the remethylation of homocysteine (Hcy), a toxic sulfur-containing amino acid that can induce neuronal dysfunction and cell death (Mattson and Shea, 2003). This vitamin is also essential to DNA synthesis and repair, as well as to amino acid synthesis (Mattson and Haberman, 2003). Folic acid deficiency results in DNA strand breaks (Pogribny et al., 1995), DNA hypomethylation (Pogribny et al., 1997) and abnormal gene expression (Wainfan and Poirier, 1992). Furthermore, emerging evidence links elevated levels of Hcy and/or low serum folic acid levels with higher rates of vascular, neurodegenerative and neuropsychiatric diseases, as well as with neural tube defects, Down syndrome, cancer and homocystinuria (Brattstrom et al., 1988; Daly et al., 1995; Boushey et al., 1995; Rydlewicz et al., 2002; Mattson et al., 2002; Mattson, 2003; Fillon-Emery et al., 2004; Lamers et al., 2004; Verhoef and de Groot, 2005; Bottiglieri, 2005). On the other hand, it has been shown that folic acid supplementation can significantly reduce the risk of these disorders (Botez et al., 1982; Daly et al., 1995; Kim, 1999; Mattson et al., 2002; Verhaar et al., 2002; Smith and Bodamer, 2002; Mattson and Shea, 2003; Bottiglieri, 2005; Moore, 2005; Eskes, in press).

Homocystinuria is an inborn error of metabolism caused by cystathionine-β-synthase severe deficiency, leading to Hcy

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0736-5748/$30.00
doi:10.1016/j.ijdevneu.2005.12.003

accumulation in tissues. Affected patients present mental retardation, seizures and are more susceptible to heart disease and stroke (Mudd et al., 2001). Although neurological and vascular dysfunction is commonly found in homocystinuric patients, the exact mechanisms involved remain poorly understood. We have recently developed a chemically experimental model of hyperhomocysteinemia and found that rats subjected to this model present a reduction of brain Na^+,K^+-ATPase, serum butyrylcholinesterase (BuChE) and an impairment of memory (Wyse et al., 2002; Reis et al., 2002; Streck et al., 2004; Matté et al., 2004; Stefanello et al., 2005). Furthermore, it was shown that acute hyperhomocysteinemia cause a decrease of Na^+,K^+-ATPase activity and of the antioxidant defenses in rat hippocampus, which were prevented by Vitamins E and C pretreatment (Wyse et al., 2002). We have also shown that Hcy induces oxidative stress in vitro in parietal cortex, by increasing lipid peroxidation and reducing the total antioxidant defenses (Matté et al., 2004).

The present work extended previous studies from our laboratory and investigated whether folic acid pretreatment could affect the inhibition of Na^+,K^+-ATPase and BuChE activities in parietal cortex of young rats subjected to acute hyperhomocysteinemia. We also evaluated here the effect of acute hyperhomocysteinemia on some parameters of oxidative stress, namely thiobarbituric acid-reactive substances (TBARS) and total thiol content in this cerebral structure in order to test whether oxidative stress could be elicited in vivo by acute hyperhomocysteinemia.

2. Experimental procedure

2.1. Animals and reagents

Twenty-two-day-old Wistar rats (total number of rats = 57) were obtained from the Central Animal House of the Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil. Animals were maintained on a 12 h light/12 h dark cycle in an air-conditioned constant temperature (22 ± 1 °C) colony room. Rats had free access to a 20% (w/w) protein commercial chow and water. Animal care followed the official governmental guidelines in compliance with the Federação das Sociedades Brasileiras de Biologia Experimental and was approved by the Ethics Committee of the Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil.

All chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2.2. Drug administration procedure

Folic acid and D,L-Hcy were dissolved in 0.85% NaCl solution and buffered to pH 7.4. Animals were pretreated daily with an intraperitoneal injection of folic acid (5 mg/kg) (Lalonde et al., 1993) or saline (0.85% NaCl) from the 22th to the 28th day of age. Twelve hours after the last injection (folic acid or saline), the rats received a single subcutaneous injection of Hcy (0.6 μmol/g of weight body) or saline and were killed by decapitation without anesthesia 1 h later (Streck et al., 2002). Serum was collected and the brain was quickly removed and parietal cortex dissected.

2.3. Tissue and homogenate preparation

For preparation of synaptic plasma membrane and determination of Na^+,K^+-ATPase activity, the parietal cortex was homogenized in 10 volumes (1:10, w/v) of 0.32 mM sucrose solution containing 5.0 mM HEPES and 1.0 mM EDTA, pH 7.4. After homogenization, synaptic plasma membranes were prepared and the activity of Na^+,K^+-ATPase was determined.

For BuChE activity determination, the serum was diluted in 10 volumes (1:10, v/v) of 500 mM potassium phosphate buffer, pH 7.5.

For TBARS and total thiol content assays, parietal cortex was homogenized in 10 volumes (1:10, w/v) of 1.15% KCl or in phosphate buffer saline (PBS), pH 7.5, containing 1 mM EDTA, respectively.

2.4. Preparation of synaptic plasma membrane from parietal cortex

Synaptic plasma membrane from parietal cortex was prepared according to the method of Jones and Matus (1974) with some modifications (Wyse et al., 1998). The homogenate was centrifuged at 1000 × *g* for 10 min and the supernatant removed and centrifuged at 12,000 × *g* for 20 min. The pellet was then resuspended in hypotonic buffer (5.0 mM Tris–HCl buffer, pH 8.1) at 0 °C for 30 min, and applied on a discontinuous sucrose density gradient consisting of successive layers of 0.3, 0.8 and 1.0 M. After centrifugation at 69,000 × *g* for 120 min, the fraction between 0.8 and 1.0 M sucrose interface was taken as the membrane enzyme preparation.

2.5. Na^+,K^+-ATPase activity assay

The reaction mixture for Na^+,K^+-ATPase activity assay contained 5.0 mM $MgCl_2$, 80.0 mM NaCl, 20.0 mM KCl and 40.0 mM Tris–HCl, pH 7.4, in a final volume of 200 μL. The reaction was initiated by ATP addition. Controls were carried out under the same conditions with the addition of 1.0 mM ouabain. Na^+,K^+-ATPase activity was calculated by the difference between the two assays, as described by Wyse et al. (1998). Released inorganic phosphate (Pi) was measured by the method of Chan et al. (1986). Specific enzyme activity was expressed as nmol Pi released per min per mg of protein. All samples were run in duplicate.

2.6. BuChE activity assay

BuChE activity was determined by the method of Ellman et al. (1961) with some modifications. Hydrolysis rate was measured at acetylthiocholine concentration of 0.8 mM in 1 mL assay solutions with 100 mM potassium phosphate buffer pH 7.5 and 1.0 mM 5,5′-dithiobis (2-nitrobenzoic acid) (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 duplicate. Specific enzyme activity was expressed as μmol acetylthiocholine per hour per milligrams of protein.

2.7. TBARS assay

TBARS, an index of lipid peroxidation, was determined according to the method described by Ohkawa et al. (1979). Briefly, 50 μL of 8.1% sodium dodecyl sulfate, 1.5 mL of 20% acetic acid solution adjusted to pH 3.5 and 1.5 mL of 0.8% aqueous solution of thiobarbituric acid were added to 500 μL of tissue homogenate in a Pyrex tube, and then heated in a boiling water bath for 60 min. After cooling with tap water, the mixture was centrifuged at 1000 × *g* for 10 min. The organic layer was taken and the resulting pink color was determined in a spectrophotometer at 535 nm. The acid did not produce color when tested without the addition of the homogenate, demonstrating the absence of a direct reaction with thiobarbituric acid. Calibration curve was performed using 1,1,3,3-tetramethoxypropane and each curve point was subjected to the same treatment as that of the homogenate. The results were reported as nmol of malondialdehyde per mg protein. All samples were run in triplicate.

2.8. Total thiol content assay

Total thiol content was determined using the DTNB method, as described by Aksenov and Markesbery (2001) with some modifications. Briefly, 50 μL of the sample was mixed with 980 μL of PBS, pH 7.5, containing 1 mM EDTA. The reaction was started by the addition of 30 μL of 10 mM DTNB stock solution in PBS. After 30 min of incubation at room temperature, the absorbance at 412 nm