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Acute and chronic hypermethioninemia alter Na^+,K^+-ATPase activity in rat hippocampus: prevention by antioxidants

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ABSTRACT

In the current study we initially investigated the influence of antioxidants (vitamins E plus C) on the effect mediated by acute and chronic administration of methionine (Met) on Na^+,K^+-ATPase activity in rat hippocampus. We also verified whether the alterations on the enzyme after administration of Met and/or antioxidants were associated with changes in relative expression of Na^+,K^+-ATPase catalytic subunits (isoforms α1, α2 and α3). For acute treatment, young rats received a single subcutaneous injection of Met or saline (control) and were sacrificed 12 h later. In another set of experiments, rats were pretreated for 1 week with daily intraperitoneal administration of vitamins E (40 mg/kg) and C (100 mg/kg) or saline. After that, rats received a single injection of Met or saline and were killed 12 h later. For chronic treatment, Met was administered to rats from the 6th to the 28th day of life; controls and treated rats were sacrificed 12 h after the last injection. In parallel to chronic treatment, rats received a daily intraperitoneal injection of vitamins E and C from the 6th to the 28th day of life and were killed 12 h after the last injection. Results showed that administration of antioxidants partially prevented the inhibition of enzyme activity caused by acute and chronic hypermethioninemia. Besides, we demonstrated that transcription of catalytic subunits of Na^+,K^+-ATPase was not altered by chronic and acute exposure to Met and/or vitamins E plus C. These data strongly suggest the oxidative damage as one possible mechanism involved in the reduction of Na^+,K^+-ATPase activity caused by hypermethioninemia and if confirmed in human beings, we might propose the use of antioxidants as an adjuvant therapy in hypermethioninemic patients.

1. Introduction

It has been shown that elevation of plasma methionine (Met) may occur in several genetic abnormalities, such as methionine adenosyltransferase activity deficiency, homocystinuria and glycine *N*-methyltransferase (Mudd et al., 2001; Augoustides-Savvopoulou et al., 2003). Some hypermethioninemic patients can present a variable degree of neurological dysfunction, including mental retardation, cognitive deficit and cerebral edema; however the exact mechanisms involved in these alterations remain elusive (Mudd et al., 2000, 2001).

Na^+,K^+-ATPase (EC 3.6.1.37), or sodium pump, is one of major membrane proteins that is responsible for generating the membrane potential through the active transport of Na^+ and K^+ ions in the central nervous system, necessary to maintain neuronal excitability and cellular volume control (Kaplan, 2002; Aperia, 2007), consuming about 40–60% of ATP produced in brain (Erecinska et al., 2004). This enzyme consists of α and β subunit, and in some tissues is associated with another protein, γ (Kaplan, 2002; Jorgensen et al., 2003; Geering, 2008). The α-subunit is catalytic and contains the binding sites for the cations, ATP and cardiac glycosides including ouabain (a specific inhibitor of the enzyme). The β-subunit has a crucial role in the structural and functional maturation of enzyme and modulates its transport properties (Jorgensen et al., 2003; Geering, 2008). Four α-subunit isoforms (α1, α2, α3 and α4) have been identified in mammals, and in brain, most neurons express

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α1 and α3, and astrocytes express α1 and α2 (Taguchi et al., 2007).

Disturbance in Na^+,K^+-ATPase density and/or activity could have significant implications on brain function. Studies show that the activity of this enzyme is altered in various disorders affecting the brain, such as inborn errors of metabolism (Matté et al., 2007; Zugno et al., 2007), ischemia (Wyse et al., 2000), neurodegenerative (Yu, 2003; Vignini et al., 2007) and neuropsychiatric disorders (Goldstein et al., 2006). In turn, mutations in the catalytic subunits can cause neural dysfunction, leading to seizures and neurodegeneration (Palladino et al., 2003). On the other hand, a decline of Na^+,K^+-ATPase activity has been attributed to change in lipid microenvironment and associated oxidative damage (Chakraborty et al., 2003; Dencher et al., 2007). Additionally, Met in vitro and in vivo reduces Na^+,K^+-ATPase activity probably by oxidative stress induction (Stefanello et al., 2005, 2007a).

Considering that Na^+,K^+-ATPase plays a central role in cellular ionic gradient maintenance and it is particularly sensitive to reactive oxygen species (Wang et al., 2003; Ullrich et al., 2007; Lima et al., 2008), and that enzyme is target of multiple regulatory mechanisms activated in response to changing cellular requirements which modulate its activity and expression (Therien and Blostein, 2000; Mobasheri et al., 2000; Geering, 2008), in the current study we examined the influence of antioxidants vitamins E plus C on the effects mediated by Met on Na^+,K^+-ATPase activity. We also verified whether the alterations on the Na^+,K^+-ATPase activity after acute and chronic exposure to Met and/or antioxidants were associated with specific changes in expression of Na^+,K^+-ATPase catalytic subunits (isoforms α1, α2 and α3) in hippocampus of rats.

2. Materials and methods

2.1. Animals and reagents

Wistar rats were obtained from the Central Animal House of the Departamento de Bioquímica, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil. The animals were maintained on a 12 h light/12 h dark cycle at a constant temperature (22 ± 1 °C), with free access to water and commercial protein chow. The NIH "Guide for the Care and Use of Laboratory Animals" (NIH publication No. 80-23, revised 1996) was followed in all experiments. The study was approved by the Ethics Committee of the Universidade Federal do Rio Grande do Sul, Brazil. All chemicals were purchased from Sigma Chemical Co., St. Louis, MO, USA.

2.2. Methionine and vitamins E plus C administration

For acute treatment, 29-day-old Wistar rats received a single subcutaneous injection of Met correspondent to 2.68 μmol Met/g body weight and control rats received an equivalent volume of saline (Stefanello et al., 2007b). The animals were killed 12 h after injection by decapitation without anaesthesia. Met was dissolved in 0.9% NaCl, buffered to pH 7.4.

For chronic treatment, Met was administered subcutaneously twice a day from the 6th to the 28th day of life as described by Stefanello et al. (2007b). Animals received 1.34 μmol Met/g body weight during the first 8 days of treatment, 2.01 μmol Met/g body weight from day 14th to 20th, and 2.68 μmol Met/g body weight from day 21st to 28th. Rats subjected to this treatment achieved plasma Met levels similar to those found in hypermethioninemic patients with some inherited pathological conditions (Mudd et al., 2001; Augoustides-Savvopoulou et al., 2003), maximal plasma levels were reached 15 min after Met injection (around to 2 mmol/L). Twelve hours after treatment, plasma Met concentrations returned to normal levels. Control animals received saline solution in the same volumes as those applied to Met-treated rats. The animals were sacrificed 12 h after the last injection for biochemical studies.

In another set of experiments, 22-day-old rats were pretreated for 1 week with daily intraperitoneal administration of saline (control) or vitamins E (40 mg/kg) and C (100 mg/kg). Twelve hours after the last injection, animals received one injection of Met (2.68 μmol/g body weight) or saline and were killed 12 h after the injection by decapitation without anaesthesia. Next, in parallel to chronic treatment, rats received a single daily intraperitoneal injection of vitamins E (40 mg/kg) plus C (100 mg/kg) or vehicle (saline) from the 6th to the 28th day of life. The animals were killed 12 h after the last injection by decapitation without anaesthesia. Vitamins doses and route of administration were chosen according to the protocols previously described by us and other investigators (Fighera et al., 1999; Wyse et al., 2002; Delwing et al., 2006).

2.3. Tissue and homogenate preparation

After decapitation, the brain was removed and the hippocampus was dissected out and immediately frozen in liquid nitrogen. For preparation of synaptic plasma membrane and determination of Na^+,K^+-ATPase activity, the hippocampus was homogenized in 10 volumes 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 for subsequent determination of Na^+,K^+-ATPase activity.

2.4. Preparation of synaptic plasma membrane

Synaptic plasma membrane from hippocampus was prepared according to the method of Jones and Matus (1974) with some modifications (Wyse et al., 2000). They were isolated using a discontinuous sucrose density gradient consisting of successive layers of 0.3, 0.8 and 1.0 mM. After centrifugation at 69,000 × *g* for 2 h, the fraction between 0.8 and 1.0 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. After 10 min of pre-incubation at 37 °C, the reaction was started by addition of ATP to a final concentration of 3.0 mM and was incubated for 5 min. 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. (2000). Released inorganic phosphate (Pi) was measured by the method of Chan et al. (1986). Specific activity of the enzyme was expressed as nmol Pi released per min per mg of protein.

2.6. Analysis of gene expression by semi-quantitative RT-PCR

The analysis of Na^+,K^+-ATPase catalytic subunits expression was carried out by a semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) assay. For this set of experiments, all chemicals were purchased from Invitrogen, USA.

The hippocampus from rats was isolated for total RNA extraction with TRIzol reagent in accordance with the manufacturer instructions. The cDNA species were synthesized with SuperScript First-Strand Synthesis System for RT-PCR from 2 μg of total RNA and oligo (dT) primer in accordance with the suppliers. RT reactions were performed for 50 min at 42 °C. cDNA (0.1 mL) was used as a template for PCR with the specific primers for Na^+,K^+-ATPase catalytic subunits (Table 1). β-Actin-PCR was carried out as an internal standard. PCR reactions were performed with a total volume of 25 μL using a final concentration of 0.08 μM of each primer indicated below, 1.6 mM of $MgCl_2$ and 1 U Taq Platinum Polymerase in the supplied reaction buffer. Conditions for Na^+,K^+-ATPase catalytic subunits PCR were as follows: initial 2 min denaturation step at 94 °C; 1 min at 94 °C, 1 min annealing step at 62 °C, 1 min extension step at 72 °C for 30 cycles and a final 10 min extension at 72 °C. Conditions for β-actin PCR were as follows: initial 1 min denaturation step at 94 °C, 1 min at 94 °C, 1 min annealing step at 54 °C, 1 min extension step at 72 °C for 35 cycles and a final 10 min extension at 72 °C. PCR products were submitted to electrophoresis using a 1% agarose gel with GelRed®. The fragments length of PCR reactions was confirmed with Low DNA Mass Ladder. The relative abundance of each mRNA versus β-actin was determined by densitometry using the freeware ImageJ 1.37 for Windows.

2.7. Homocysteine levels determination

Homocysteine (Hcy) levels were determined in plasma as described by Araki and Sako (1987), using high-performance liquid chromatography (HPLC) with fluorescence detection.

2.8. Protein determination

The protein content of hippocampus homogenates was determined by the method of Bradford (1976) using bovine serum albumin as standard.

2.9. Statistical analysis

Data were analyzed by one-way ANOVA followed by the Duncan multiple range test when the *F*-test was significant. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software in a PC compatible computer. Values of $P < 0.05$ were considered to be significant.

3. Results

First, we investigated the influence of administration of antioxidants vitamins E plus C on the Na^+,K^+-ATPase activity in synaptic plasma membranes from hippocampus of rats submitted to chronic and acute hypermethioninemia. *Post hoc* analysis showed that

Table 1
Primer sequences and PCR amplification products.

Na^+,K^+-ATPase catalytic subunits	GenBank accession number	Primers (5′–3′)	PCR product	T (°C)	Cycles
alpha 1	NM_012504	F-CTATGGACGACCATAAACTCAGCCTGG R-AGCAGACAGCACGACCCCGAGGTAC	297	62	30
alpha 2	NM_012505	F-ACCAAGTGGATCTGTCCAAGGGCCTC R-GCTTCCTGGTAGTAGGAGAAGCAGCCAG	292	62	30
alpha 3	NM_012506	F-AAAGATGACAAGAGCTCGCCCAAGAAG R-TGATCTCCACCAGGTCCCCGACCAC	538	62	30
β-actin	NP_742006	F-TATGCCAACACAGTGCTGCTGG R-TACTCCTGCTTCCTGATCCACAT	210	54	35

treatment with vitamins *per se* did not alter the activity of this enzyme, but partially prevented the inhibition of Na^+,K^+-ATPase activity caused by acute [$F(3,15) = 16.26$; $P < 0.001$] (Fig. 1A) and chronic [$F(3,16) = 14.73$; $P < 0.001$] (Fig. 1B) administration of Met.

To better understand the mechanisms involved in the effects elicit by Met and/or antioxidants on Na^+,K^+-ATPase activity, we analyzed the relative expression of hippocampal enzyme catalytic subunits after administration of Met and vitamins E plus C by semi-quantitative RT-PCR. As can be observed in Fig. 2, the relative expressions of isoforms α1, α2 and α3 of the Na^+,K^+-ATPase were not altered by acute Met and/or vitamins treatment in hippocampus of rats ([$F(3,15) = 1.345$; $P > 0.05$]; [$F(3,17) = 0.881$; $P > 0.05$]; [$F(3,17) = 0.428$; $P > 0.05$]), respectively. Fig. 3 shows that chronic hypermethioninemia and/or antioxidants did not change the expression of Na^+,K^+-ATPase subunits α1 [$F(3,13) = 1.29$; $P > 0.05$], α2 [$F(3,13) = 0.944$; $P > 0.05$] and α3 [$F(3,12) = 3.09$; $P > 0.05$].

Since Hcy, a metabolite of Met, has been shown to inhibit Na^+,K^+-ATPase activity (Matté et al., 2007), we also determined the levels of this amino acid in animals submitted to hypermethioninemia model. Results showed that chronic Met administration increased Hcy levels at 1 h (Control: 9.87 μmol/L ± 0.62, Met: 26.47 μmol/L ± 0.58; $P < 0.001$), but did not alter 12 h after injection (Control: 9.26 μmol/L ± 2.07, Met: 7.75 μmol/L ± 1.45; $P > 0.05$).

4. Discussion

Neurological dysfunction observed in some hypermethioninemic patients is still poorly understood (Mudd et al., 2000, 2001). However, there is a growing body of evidence suggesting that elevated Met concentrations and/or its metabolites are potentially toxic (Mudd et al., 2001; Garlick, 2006). In this context, using an experimental model developed in our laboratory, we showed that Met administration reduces Na^+,K^+-ATPase activity, induces lipid peroxidation, increases acetylcholinesterase in brain of rats, as well as, impairs memory (Stefanello et al., 2007a,c). In this model, plasma Met levels were about 20-fold higher than normal values 1 h after amino acid injection and returned to control values 12 h after injection (Stefanello et al., 2007b). On the other hand, since Hcy is produced by Met metabolism and has been shown to inhibit Na^+,K^+-ATPase, we measured plasma levels of Hcy after chronic hypermethioninemia and observed that this amino acid increased (approximately 3-fold) 1 h, but not 12 h, after Met administration. These data probably suggest a higher contribution of Met on hippocampal Na^+,K^+-ATPase reduction found in hypermethioninemic rats. However, we cannot discard that Met derivatives, such as Hcy, may be involved in such effect.

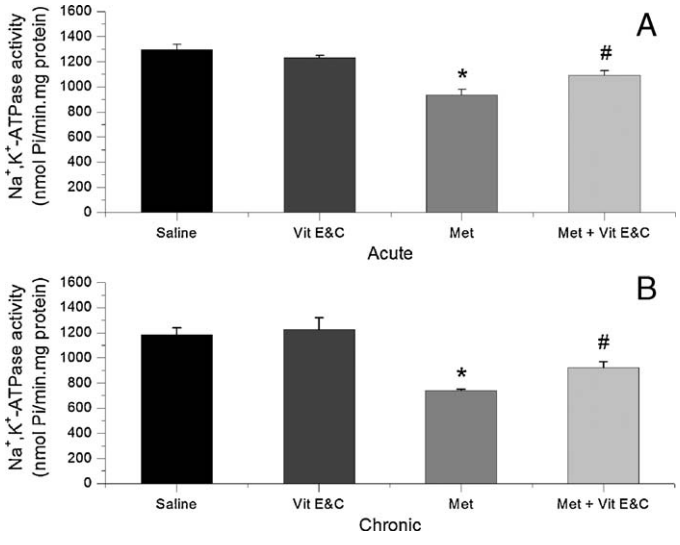

Fig. 1. Effect of acute (A) and chronic (B) administration of methionine, vitamins E plus C, and methionine and vitamins E plus C on Na^+,K^+-ATPase activity in synaptic plasma membranes from hippocampus of rats. Data are mean ± S.E.M. for 4–5 animals in each group. The results are expressed in nmol Pi/min mg protein. *Different from control groups, #different from both control and methionine groups, *$P < 0.001$ (Duncan multiple range test). Met, methionine; Vit, vitamin.

In the present study we initially evaluated the influence of antioxidants on the effects elicited by Met administration on Na^+,K^+-ATPase activity, in order to identify the possible mechanisms involved in the inhibition of this enzyme. It is known that vitamin E, a lipid-soluble vitamin, interacts with cell membranes, reportedly trapping free radicals and interrupting oxidative damage, preventing lipid peroxidation (Halliwell and Gutteridge, 2007). In addition, vitamin C is a water-soluble antioxidant that is highly concentrated in the brain (Lönnrot et al., 1996), where it acts as a scavenger of many deleterious reactive species and also be able to regenerate vitamin E (Halliwell and Gutteridge, 2007). Studies have suggested that antioxidants present neuroprotective properties in some experimental models of neurological disorders, such as Huntington's disease (Rebec et al., 2003), cerebral ischemia (MacGregor et al., 2003), metabolic diseases (Delwing et al., 2006, 2007; Zugno et al., 2007; Bavaresco et al., 2008).

Our results demonstrated that the administration of vitamins E plus C was able to partially prevent the inhibition of Na^+,K^+-ATPase activity caused by acute and chronic hypermethioninemia in rat hippocampus. Although we cannot precisely establish the exact pathway involved in such effect, we believe that treatment with vitamins could prevent the propagation of lipid peroxidation and/or also act as a stabilizer of the membranes in which Na^+,K^+-ATPase is inserted. In agreement with our hypothesis, it has been shown an association between Na^+,K^+-ATPase inhibition and alterations in plasma membrane lipid composition (Dencher et al., 2007), in the redox state of regulatory thiol groups (Pari and Murugavel, 2007) and in other amino acid residues caused by free radicals or lipid peroxidation (Siems et al., 1996; Potts et al., 2006). In addition, some data showed that the administration of vitamins E and C prevents the reduction of Na^+,K^+-ATPase promoted by high levels of homocysteine (Wyse et al., 2002), arginine (Bavaresco et al., 2003) and guanidinoacetate (Zugno et al., 2007). Since in our study vitamins E and C administration partially prevented Na^+,K^+-ATPase inhibition, we cannot rule out that other oxidative alteration, besides lipid peroxidation, could be involved in such effect.

Considering that enzyme activity is subject of long-term regulation by a variety physiologically relevant stimuli that ultimately affect Na^+-K^+-ATPase synthesis or degradation (Therien

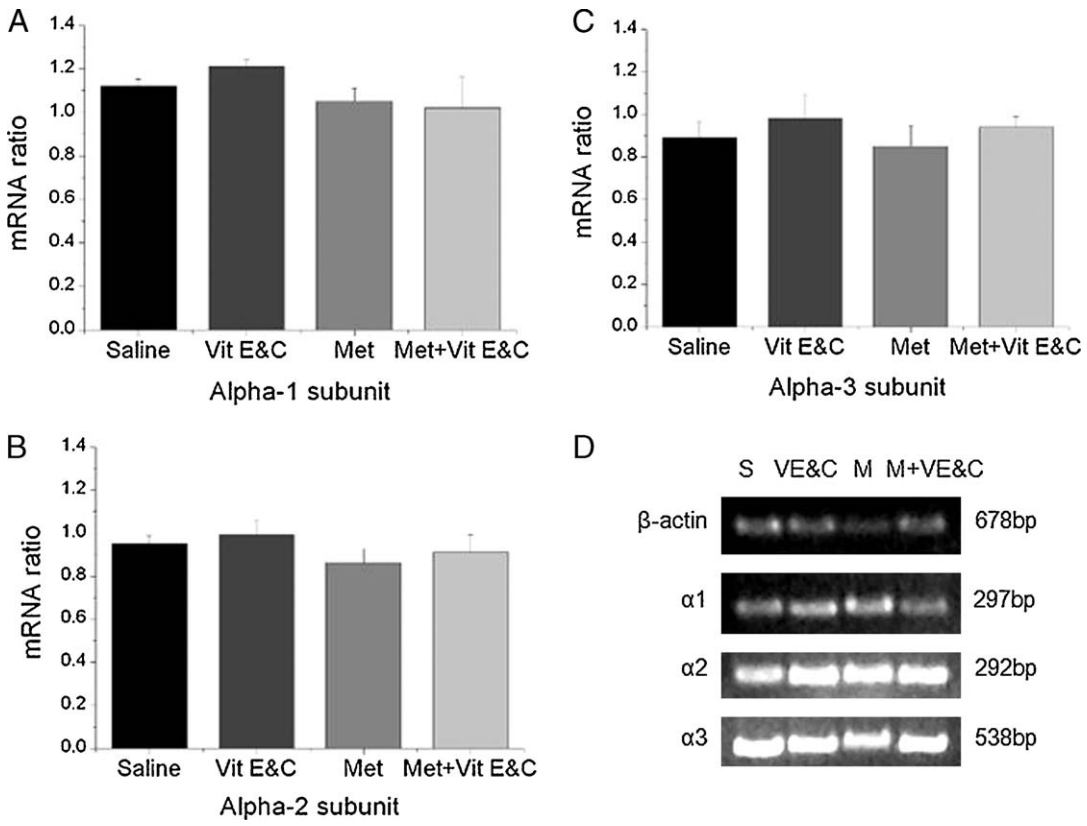

Fig. 2. Gene expression patterns of Na^+,K^+-ATPase subunits α1 (A), α2 (B), α3 (C) and β-actin after acute administration of methionine, vitamins E plus C, and methionine and vitamins E plus C in hippocampus of rats. Electrophoresis data are representative of three individual experiments (D). The results in the graphs were expressed as optical densitometry (O.D.) of the Na^+,K^+-ATPase subunits-related genes versus β-actin expression (mean ± S.E.M.) of three independent replicate RT-PCR experiments. Met, methionine; Vit, vitamin.

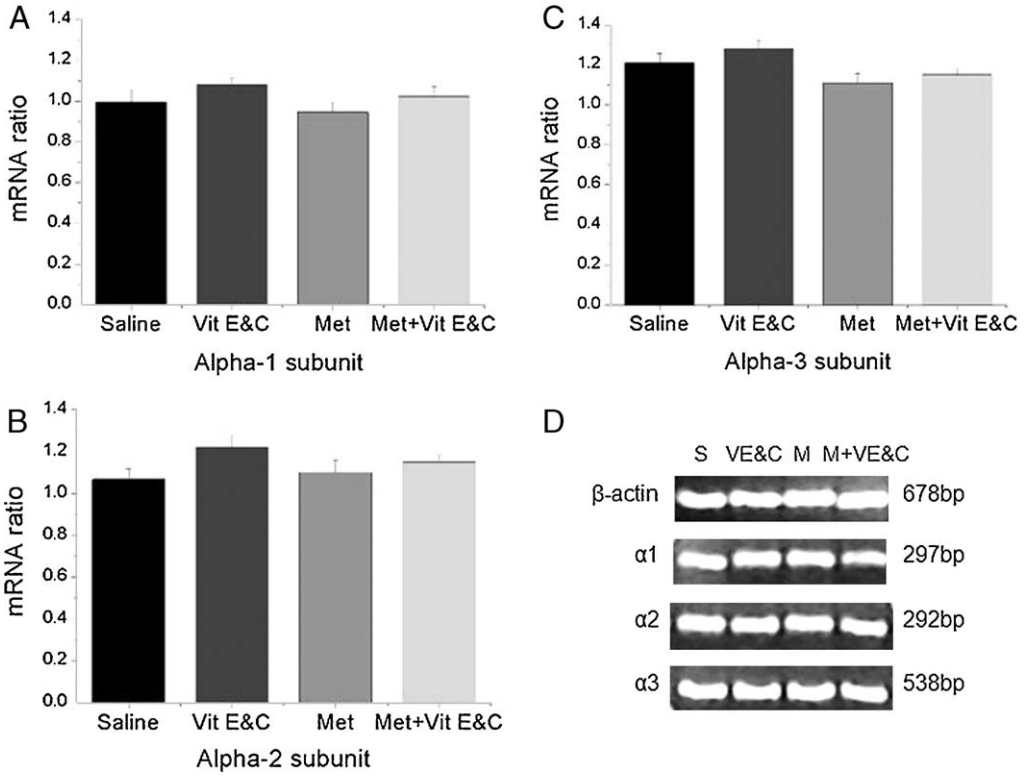

Fig. 3. Gene expression patterns of Na^+,K^+-ATPase subunits α1 (A), α2 (B), α3 (C) and β-actin after chronic administration of methionine, vitamins E plus C, and methionine and vitamins E plus C in hippocampus of rats. Electrophoresis data are representative of three individual experiments (D). The results in the graphs were expressed as optical densitometry (O.D.) of the Na^+,K^+-ATPase subunits-related genes versus β-actin expression (mean ± S.E.M.) of three independent replicate RT-PCR experiments. Met, methionine; Vit, vitamin.

and Blostein, 2000), we also investigate whether the alterations observed on Na^+,K^+-ATPase activity after Met treatments and/or antioxidants could be a consequence of changes in the transcriptional control. Results showed that the relative expression of catalytic subunits, isoforms α1, α2 and α3 of the Na^+,K^+-ATPase was not altered by acute and prolonged exposure to Met and vitamins E plus C. Since expression of enzyme was measured at the mRNA level, we cannot rule out that the amount of Na^+,K^+-ATPase protein was affected by hypermethioninemia.

Furthermore, there are considerable reports showing that mechanisms posttranslational such as phosphorylation, an example of short-term regulation, are essential to assure enzyme activity (Therien and Blostein, 2000; Geering, 2008; Poulsen et al., 2010). At this point, using NetPhosK, a tool for the kinase-specific prediction of protein phosphorylation sites, we found that the Na^+,K^+-ATPase sequence contains sites with a high possibility of phosphorylation by protein kinase C located at α1 subunit (Ser23) and cAMP/protein kinase A phosphorylation sites at α2 and α3 subunits (Ser940 and Ser933, respectively). These data lead us to speculate that the reduction of Na^+,K^+-ATPase activity provoked by Met may be attributed to changes in phosphorylation state of enzyme.

In summary, the present data demonstrated that high levels of Met and/or its metabolites decreased Na^+,K^+-ATPase activity in the hippocampus of rats and that the treatment with vitamins E plus C partially prevented this effect. We also showed that any modification on the level of Na^+,K^+-ATPase mRNA transcripts was observed after acute and chronic exposure to Met and vitamins E plus C, suggesting that several mechanisms as oxidative damage and/or posttranslational modifications could contribute to the effects caused by Met and/or its metabolites and vitamins. However, further research is required to investigate additional mechanisms involved in severe hypermethioninemia and the use of antioxidants as an adjuvant therapy for hypermethioninemic patients.

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