Role of antioxidants on Na$^+$,K$^+$-ATPase activity and gene expression in cerebral cortex of hyperprolinemic rats

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Abstract Considering that Na$^+$,K$^+$-ATPase is an embedded-membrane enzyme and that experimental chronic hyperprolinemia decreases the activity of this enzyme in brain synaptic plasma membranes, the present study investigated the effect of chronic proline administration on thiobarbituric acid-reactive substances, as well as the influence of antioxidant vitamins E plus C on the effects mediated by proline on Na$^+$,K$^+$-ATPase activity in cerebral cortex of rats. The expression of Na$^+$,K$^+$-ATPase catalytic subunits was also evaluated. Results showed that proline increased thiobarbituric acid-reactive substances, suggesting an increase of lipid peroxidation. Furthermore, concomitant administration of vitamins E plus C significantly prevented the increase of lipid peroxidation, as well as the inhibition of Na$^+$,K$^+$-ATPase activity caused by proline. We did not observe any change in levels of Na$^+$,K$^+$-ATPase mRNA transcripts after chronic exposure to proline and vitamins E plus C. These findings provide insights into the mechanisms through which proline exerts its effects on brain function and suggest that treatment with antioxidants may be beneficial to treat neurological dysfunctions present in hyperprolinemic patients.

Keywords hyperprolinemia · Na$^+$,K$^+$-ATPase · Catalytic subunits · Lipid peroxidation · Vitamin E and C · Antioxidant

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Introduction

Na$^+$,K$^+$-ATPase (EC 3.6.1.3) is a membrane-bound enzyme that plays an essential role in controlling neuronal excitability by maintaining electrochemical gradients, through active transport of Na$^+$ and K$^+$ ions across the cell membrane, at the expense of ATP (Kaplan 2002; Mobasheri et al. 2000). Structurally, the enzyme is composed of α- and β-subunits. The α-subunit is catalytic and contains the binding sites for the cations, ATP and ouabain (a cardiac glycoside and specific inhibitor of the enzyme); the β-subunit is regulatory and is responsible for proper trafficking of the complex and its correct insertion into the plasma membrane (Blanco and Mercer 1998; Geering 2001). Individual genes of four α-subunit isoforms (α$_1$, α$_2$, α$_3$ and α$_4$) and at least three β-subunit isoforms (β$_1$, β$_2$ and β$_3$) of Na$^+$,K$^+$-ATPase have been identified in mammals (Kaplan 2002; Serluca et al. 2001). A third subunit, named γ-subunit, has also been described in some tissues; it apparently modulates the enzyme activity (Dempski et al. 2009). The isoforms combine to form several Na$^+$,K$^+$-ATPase isozymes which show different tissue distribution patterns. In the brain, mainly three α isoforms (α$_1$, α$_2$, α$_3$)

and two β isoforms (β_1 and β_2) are expressed; β_3 is expressed but at very low level (Wetzel et al. 1999).

Considering that Na^+,K^+-ATPase is one of the most abundant brain enzyme, consuming about 40–50% of the ATP generated (Kaplan 2002), it is not surprising that alterations in its activity may cause a variety of abnormalities. Indeed, Na^+,K^+-ATPase has been suggested to play a central role in the pathogenesis of neurodegenerative diseases, such as Alzheimer, multiple sclerosis, Parkinson's disease and epilepsy (Grisar et al. 1992; Hattori et al. 1998; Rose and Valdes 1994). Mutations in catalytic subunits expressed in the brain can also give rise to diseases such as epilepsy and parkinsonism (Aperia 2007). Preclinical studies have also demonstrated that alterations of this enzyme activity is implicated in several conditions affecting the CNS, such as ischemia (Wyse et al. 2000), depression (de Vasconcellos et al. 2005; Gamaro et al. 2003), ovariectomy (Ben et al. 2009; Monteiro et al. 2005; 2007) and metabolic diseases (Pontes et al. 1999; 2001; Streck et al. 2002; Wyse et al. 2001).

Hyperprolinemia type II (HP II) is a metabolic disease caused by deficiency of Δ^1-pyrroline-5-carboxylic acid dehydrogenase activity characterized by tissue accumulation of proline (Pro). Affected patients usually present neurological symptomatology, including seizures and mental retardation; a relationship between high concentration of Pro and neurological symptoms has been established (Farrant et al. 2001; Flynn et al. 1989; Phang et al. 2001). Since the mechanisms underlying the HP II pathophysiology are still obscure, we have developed a chemical experimental model of hyperprolinemia in rats that mimics the tissue levels of Pro found in human HP II (Moreira et al. 1989; Phang et al. 2001). It has been reported that rats subjected to experimental hyperprolinemia present significant inhibition of Na^+,K^+-ATPase activity in cortical synaptic plasma membranes (Pontes et al. 1999) and that Pro elicits oxidative stress and decreases antioxidant defenses in rat brain (Delwing et al. 2003). Moreover, some biochemical and behavioral effects caused by experimental hyperprolinemia were prevented by the antioxidants vitamins E plus C, suggesting that free radicals formation may be involved in such effects (Delwing et al. 2005).

Considering that a variety of regulatory mechanisms assure appropriate Na^+,K^+-ATPase expression and activity adapted to changing physiological demands (Blanco and Mercer 1998; Geering 2001; Jorgensen et al. 2003; Kaplan 2002; Mobasheri et al. 2000), in the present study we investigated the possible mechanisms involved in the inhibition of Na^+,K^+-ATPase activity elicited by hyperprolinemia. A parameter of lipid peroxidation, namely thiobarbituric acid-reactive substances (TBARS) was studied, as well as the influence of antioxidant vitamins E plus C on the Pro-mediated effects on Na^+,K^+-ATPase activity.

The alterations on the Na^+,K^+-ATPase activity after chronic exposure to Pro and/or antioxidant vitamins were also investigated in association with changes in expression of Na^+,K^+-ATPase catalytic subunits (isoforms α_1, α_2 and α_3) in cerebral cortex of rats.

Experimental procedures

Animals

Wistar rats were obtained from the Central Animal House of the Department of Biochemistry at the Federal University of Rio Grande do Sul, Porto Alegre, Brazil. Animals were maintained on a 12 h light/12 h dark cycle at a constant room temperature (22±1°C), with free access to water and commercial protein chow. Animal care followed the NIH "Guide for the Care and Use of Laboratory Animals" (NIH publication no. 80–23, revised 1996) and was approved by the University Ethics Committee.

Proline and vitamins E plus C administration

Experimental chronic hyperprolinemia was chemically induced by daily subcutaneous administration of proline from the 6th to the 28th day of life as described by Pontes et al. (1999). Proline (Sigma Chemical Co., USA) was dissolved in 0.9% NaCl, buffered to pH 7.4 with NaOH and administered twice a day. During the first 8 days of treatment (6th–13th day of life) rats received 12.8 μmol Pro/g body weight, from the 14th to 17th day they received 14.6 μmol Pro/g body weight, from the 18th to 21th day they received 16.4 μmol Pro/g body weight and from the 22th to 28th day of life they received 18.2 μmol Pro/g body weight. Rats subjected to this treatment achieved plasma Pro levels between 1.0 and 2.0 mM, similar to those found in hyperprolinemic type II patients (Moreira et al. 1989; Phang et al. 2001). Control animals received saline injections in the same volumes as those applied to Pro-treated rats. Animals were killed 12 h after the last injection by decapitation without anesthesia; by that time, blood and brain levels of Pro had returned to normal (Moreira et al. 1989). Besides saline or Pro administration, rats received a single daily intraperitoneal injection of vitamins E (40 mg/kg) plus C (100 mg/kg) or vehicle (saline), according to the protocols previously described (Delwing et al. 2006; Fighera et al. 1999; Wyse et al. 2002).

Tissue and homogenate preparation

After decapitation, the brain was removed and cerebral cortex was dissected out and immediately frozen in liquid

nitrogen. To allow for the preparation of synaptic plasma membrane, cortical tissue was homogenized in 10 vol. 0.32 mM sucrose solution containing 5 mM HEPES and 1 mM EDTA, pH 7.4. After homogenization, synaptic plasma membranes were prepared for subsequent determination of Na^+,K^+-ATPase activity. For TBARS assays, tissue was homogenized in 10 volumes (1:10, w/v) of 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl. Homogenates were centrifuged at $750 \times g$ for 10 min at 4°C, the pellet was discarded and the supernatant was immediately separated and used for biochemical measurements.

Preparation of synaptic plasma membrane

Synaptic plasma membrane fraction from cerebral cortex was prepared according to the method of Jones and Matus (1974) with some modifications (Wyse et al. 1995). 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 $69,000 \times g$ for 2 h, the fraction between 0.8 and 1.0 sucrose interface was taken as the membrane enzyme preparation.

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 the 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 addition of 1.0 mM ouabain. Na^+,K^+-ATPase activity was calculated by the difference between the two assays (Tsakiris and Deliconstantinos 1984; Wyse et al. 2000). Released inorganic phosphate (Pi) was measured by the method of Chan et al. (1986) and enzyme specific activity was expressed as nmol Pi released per min per mg of protein.

Thiobarbituric Acid Reactive Species (TBARS)

TBARS levels were 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% 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 $1,000 \times g$ for 10 min, the supernatant was taken and the resulting pink-stained TBARS were determined spectrophotometrically at 535 nm; the results were reported as nmol of TBARS per mg protein.

Analysis of gene expression by semi-quantitative RT-PCR

The analysis of Na^+,K^+-ATPase catalytic subunits expression were carried out by semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR) assay. All chemicals for these experiments were purchased from Invitrogen, USA.

The cerebral cortex frozen in liquid nitrogen was submitted to 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

Table 1 Primer sequences and PCR amplification conditions

Na^+,K^+-ATPase catalytic subunits	GenBank accession number	Primers (5′–3′)	PCR product	T (°C)	Cycles
alpha 1	NM_012504	F-TCTATGGACGACCATAAACTCAGCCTGG 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
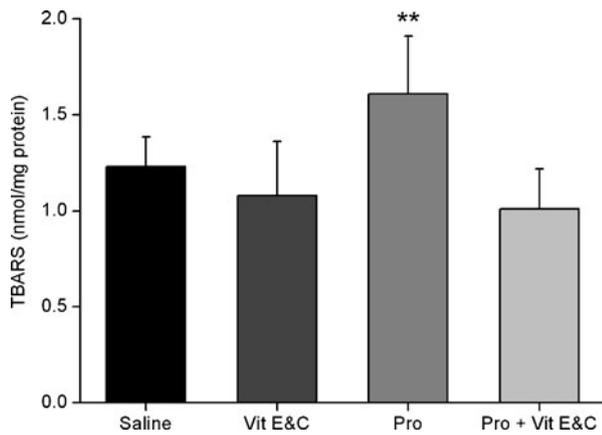
Fig. 1 Effect of proline, vitamins E&C, and proline plus vitamins E&C administration on thiobarbituric acid-reactive substances in cerebral cortex of rats. Data are mean ± S.D. for 7 animals per group. * Different from control group, $p < 0.01$ (Duncan multiple range test). *Pro, proline; Vit, vitamin*

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.
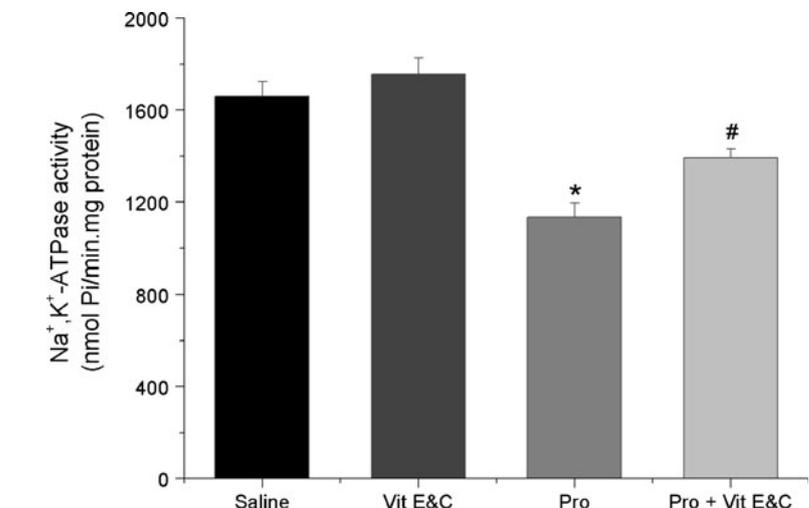
Protein determination

The protein content of tissue samples was determined using bovine serum albumin as standard, according to Lowry et al. (1951) or Bradford (1976).

Statistical analysis

Data were analyzed by one-way ANOVA followed by the Duncan's multiple range test when appropriate. All analy-

ses 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.
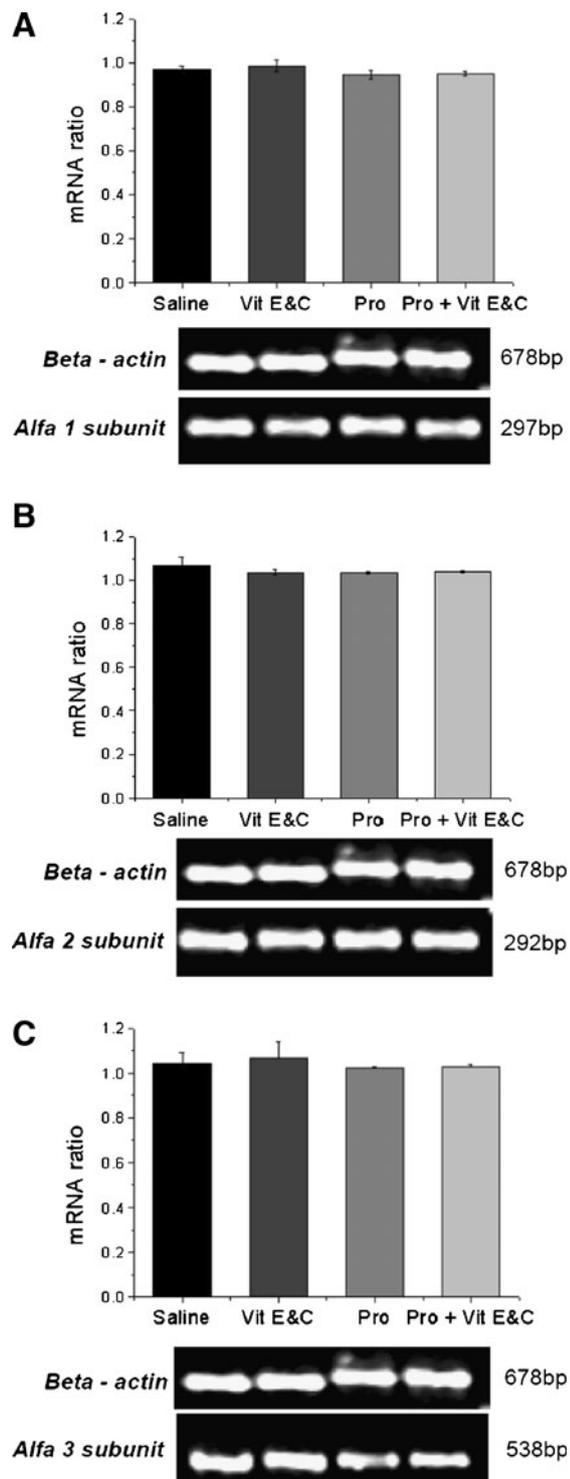
Results

Initially, the lipid peroxidation was measured by TBARS assay. Figure 1 shows that chronic Pro administration significantly increased TBARS (30%) in rat cerebral cortex, when compared to controls (saline-treated). Interestingly, the concomitant treatment with antioxidant vitamins E plus C prevented this effect [$F(3,24)=6.47$; $p < 0.01$].

Next, the influence of antioxidant vitamins E plus C administration on Na^+,K^+-ATPase activity in cortical synaptic plasma membranes of rats submitted to chronic hyperprolinemia was investigated. *Post hoc* analysis showed that treatment with vitamins *per se* did not alter enzyme activity, but significantly prevented the inhibition of Na^+,K^+-ATPase activity caused by chronic Pro administration [$F(3,12)=23.55$; $p < 0.001$] (Fig. 2).

Finally, we analyzed the relative expression of cortical Na^+,K^+-ATPase catalytic subunits after chronic exposure to Pro and vitamins E plus C by semi-quantitative RT-PCR. As shown in Fig. 3, the relative expressions of isoforms α_1, α_2 and α_3 of the Na^+,K^+-ATPase were not altered by Pro and/or vitamins treatment in cerebral cortex of rats ([$F(3,13)=1.06$; $p > 0.05$]; [$F(3,13)=0.654$; $p > 0.05$]; [$F(3,13)=0.26$; $p > 0.05$], respectively).

Discussion

It has been reported that experimental chronic hyperprolinemia significantly decreases Na^+,K^+-ATPase activity

Fig. 2 Effect of proline, vitamins E&C, and proline plus vitamins E&C administration on Na^+,K^+-ATPase activity in synaptic plasma membranes from cerebral cortex of rats. Data are mean ± S.D. for 4–5 animals per group. Results are expressed in nmol Pi/min mg protein. * Different from control group, # different from both control and proline groups, $p < 0.001$ (Duncan multiple range test). *Pro, proline; Vit, vitamin*

Fig. 3 Gene expression of α_1 (a), α_2 (b) and α_3 (c) subunits of Na$^+$, K$^+$-ATPase and β-actin after proline, vitamins E&C and proline plus vitamins E&C administration in cerebral cortex of rats. Electrophoresis data are representative of three individual experiments. Results are expressed as optical densitometry (O.D.) of the Na$^+$,K$^+$-ATPase subunits-related genes versus β-actin expression (mean $\pm$ S.D) of three independent replicates of RT-PCR experiments. *Pro, proline; Vit, vitamin*

in synaptic plasma membranes from rat cerebral cortex (Pontes et al. 1999). In the present study, we extended this investigation in an attempt to identify the possible mechanisms involved in enzyme inhibition. Since Na$^+$,K$^+$-ATPase is a membrane-bound enzyme, we first investigated the effect of Pro administration on a classic parameter of lipid peroxidation. Results showed an increase in TBARS levels in cerebral cortex from rats chronically treated with Pro. We also observed that concomitant administration of vitamin E plus C significantly prevented the increase on TBARS levels, as well as the inhibition of Na$^+$,K$^+$-ATPase activity promoted by this amino acid. Together, these findings support the involvement of reactive species and/or lipid peroxidation in the Pro-elicited effects.

It has been demonstrated that Na$^+$,K$^+$-ATPase is particularly susceptible to free radical attack since its inhibition has been associated with changes in plasma membrane lipid composition (Dencher et al. 2007), in the redox state of regulatory sulphydryl groups (Pari and Murugavel 2007) and in other amino acid residues caused by free radicals or lipid peroxidation (Potts et al. 2006; Siems et al. 1996). In addition, we have shown that Na$^+$,K$^+$-ATPase activity is inhibited in other animal models of inborn errors of metabolism and that oxidative stress was present in these conditions (Stefanello et al. 2005; Wyse et al. 2001; Wyse et al. 2002). Interestingly, in the present study we found that vitamins E and C partially prevented the inhibition of Na$^+$,K$^+$-ATPase activity promoted by Pro.

Considering that Na$^+$,K$^+$-ATPase is the target of multiple regulatory mechanisms activated in response to changing cellular requirements which modulate its activity and expression (Therien and Blostein 2000), we asked whether changes on Na$^+$,K$^+$-ATPase activity after chronic hyperprolinemia and antioxidants supplementation could be a consequence of alterations in the transcriptional control of enzyme. The relative expression of catalytic subunits, isoforms α_1, α_2 and α_3 of the Na$^+$,K$^+$-ATPase after chronic exposure to Pro and vitamins E plus C was then evaluated, however no changes in the levels of Na$^+$,K$^+$-ATPase mRNA transcripts were observed.

There is considerable evidence that regulatory post-translational mechanisms such as protein phosphorylation are essential to assure activity adapted to physiological demands of Na$^+$,K$^+$-ATPase (Blanco and Mercer 1998; Geering 2001; Jorgensen et al. 2003; Kaplan 2002; Mobasheri et al. 2000). In this sense, we performed computational analysis in NetPhosk, a kinase-specific prediction of protein phosphorylation site tool, which revealed that rat Na$^+$,K$^+$-ATPase presents a high score of possible PKC phosphorylation sites of the α_1 subunit (Ser23) and PKA phosphorylation sites of the α_2 and α_3 subunits (Ser940 and Ser933, respectively). Therefore, the decrease on Na$^+$,K$^+$-ATPase activity observed in present

work could be possibly attributed to changes in phosphorylation state of enzyme. In this context, it has been suggested that phosphorylation may occur either directly or as a result of secondary modulators such as nitric oxide (NO), which is also a potent mediator of oxidative stress (Therien and Blostein 2000). In fact, we cannot rule out that Pro elicits NO generation, since a previous work demonstrated that its administration inhibited acetylcholinesterase activity in rat brain and that this effect was prevented by administration of N^{ω}-nitro-L-arginine methyl ester (L-NAME), a potent nitric oxide sinthase inhibitor, suggesting that Pro affects this parameter through NO production and/or oxidative stress (Delwing et al. 2005). Altogether, these observations prompt us to hypothesize that Na^+,K^+-ATPase inhibition mediated by Pro may be attributed, at least in part, to changes in the enzyme phosphorylation state.

Another important modulator of Na^+,K^+-ATPase activity is the level of ATP into the cell. It has been reported that a reduction of intracellular ATP inhibits the activity of this enzyme (Therien and Blostein 2000) and it was recently shown that Pro disrupts energy metabolism such as creatine kinase, cytocrome c oxidase and succinate dehydrogenase possibly leading to limitation of enzymatic activities related to ATP production (Delwing et al. 2007; Ferreira et al. 2010; Kessler et al. 2003).

In summary, the present study demonstrated that hyperprolinemia increases TBARS levels and decreases the Na^+,K^+-ATPase activity; both effects are prevented by concomitant treatment with vitamins E plus C, thus suggesting the involvement of reactive oxygen species and/or lipid peroxidation in Pro-elicited effects. Therefore, it is reasonable to propose that mechanisms like lipid peroxidation, energy metabolism deficit and/or post-translational modifications may be interacting to promote Pro neurotoxicity. Additional studies in order to evaluate whether the administration of antioxidants may be beneficial in this pathology.

Conflict of interest The authors declare that they have no conflict of interest.

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