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Creatine prevents the inhibition of energy metabolism and lipid peroxidation in rats subjected to GAA administration

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Abstract Guanidinoacetate methyltransferase (GAMT) deficiency is an inherited neurometabolic disorder, biochemically characterized by the tissue accumulation of guanidinoacetate (GAA). Affected patients present epilepsy and mental retardation whose etiopathogeny is unclear. Previous reports have shown that GAA alters brain energy metabolism and that creatine, which is depleted in patients with GAMT deficiency, can act as a neuroprotector; as such, in the present study we investigated the effect of creatine administration on some of the altered parameters of energy metabolism (complex II, Na$^+$,K$^+$-ATPase and creatine kinase) and lipid peroxidation caused by intrastriatal administration of GAA in adult rats. Animals were pretreated for 7 days with daily intraperitonial administrations of creatine. Subsequently, these animals were divided into two groups: Group 1 (sham group), rats that suffered surgery and received saline; and group 2 (GAA-treated). Thirty min after GAA or saline, the animals were sacrificed and the striatum dissected out. Results showed that the administration of creatine was able to reverse the activities of complex II, Na$^+$,K$^+$-ATPase and creatine kinase, as well as, the levels of thiobarbituric acid reactive substances (TBARS), an index of lipid peroxidation. These findings indicate that the energy metabolism deficit caused by GAA may be prevented by creatine, which probably acts as an antioxidant since it was able to prevent lipid peroxidation. These data may contribute, at least in part, to a better understanding of the mechanisms related to the energy deficit and oxidative stress observed in GAMT deficiency.

Keywords Creatine · Guanidinoacetate · GAMT-deficiency · Metabolic disease

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Introduction

Guanidinoacetate (GAA), the immediate precursor of creatine, is predominantly synthesized in the kidney from arginine and glycine by the enzyme, glycine amidinotransferase (Takeda et al. 1992). After synthesis, it is then transported by creatine transporters to various tissues, including brain and liver, where it is methylated to creatine by guanidinoacetate methyltransferase (GAMT). GAMT deficiency was the first of the inborn errors of metabolism to be detected among the creatine deficiency syndromes. This disorder is biochemically characterized by the tissue accumulation of GAA and depletion of creatine. Affected patients present neurological symptoms, including muscular hypotonia, involuntary extrapyramidal movements and epilepsy (Von Figura et al. 2001), whose pathophysiology is unknown.

The brain is one of the tissues that depends most on a continuous supply of energy and, therefore, one of the

tissues most vulnerable to a lack of energy, and thus extremely susceptible to damage under situations of reduced energy metabolism (Bolaños et al. 2009). Mitochondria represent the main source and target of reactive oxygen species (ROS) (Milner et al. 2007). In this context, it has been shown that energy metabolism impairment seems to be implicated in the pathogenesis of a number of neurological conditions, including neurodegenerative diseases (Chinta and Andersen 2006; Mattson et al. 2008), metabolic diseases (Bavaresco et al. 2006; Delwing et al. 2003, 2007; Matté et al. 2007) psychiatric diseases (Freitas et al. 2010), as well as, ageing (Barja 2004; Linford et al. 2006). In this context, we have shown that GAA decreases complex II of the respiratory chain in the striatum of rats (Zugno et al. 2007).

Na^+, K^+-ATPase is an enzyme embedded in the plasma membrane that catalyzes the active transport of monovalent cations inside and outside the cell membrane at the expense of ATP hydrolysis, thereby maintaining the membrane potential necessary for neuronal excitability (Mobasheri et al. 2000). There are reports showing that the Na^+, K^+-ATPase is reduced in epilepsy (Grisar et al. 1992), depressive disorders (Goldstein et al. 2006), in an experimental model of cerebral ischemia (Wyse et al. 2000), neurodegenerative diseases (Hattori et al. 1998; Vignini et al. 2007) and in metabolic disease (Stefanello et al. 2007; Bavaresco et al. 2008). In addition, recent reports from our laboratory have shown that intrastriatal administration of GAA inhibits Na^+, K^+-ATPase activity in the rat striatum (Zugno et al. 2006).

Creatine kinase has an important role in the homeostasis of cells with a high energy demand, such as skeletal and cardiac muscle and neuronal tissues such as brain and retina (Wallimann et al. 1998), since the isoforms of this enzyme are present in locations strategic for energy demand and production. The decreased activity of this enzyme has been associated with neuronal loss after ischemia and neurodegenerative diseases (Tomimoto et al. 1993, David et al. 1998; Aksenov et al. 2000). It has been shown that GAA inhibits creatine kinase, probably by oxidative stress (Zugno et al. 2007).

Creatine, a nitrogenous guanidine compound, plays a central role in energy provision through a reaction catalyzed by creatine kinase. In the form of phosphocreatine, it becomes essential for several metabolic processes, functioning as an energy reserve (Schulze et al. 1997; Schulze 2003). Clinical studies demonstrate that creatine supplementation has a marked neuroprotective effect on neurodegenerative diseases (Wyss and Schulze 2002; Bender et al. 2006; Hersch et al. 2006; Tarnopolsky 2007; Bolaños et al. 2009). It has also been shown that creatine presents antioxidant properties per se (Lawler et al. 2002; Sestili et al. 2006).

Since GAA inhibits the activities of complex II of the respiratory chain, Na^+, K^+-ATPase and creatine kinase, as well as, increases levels of TBARS in rat striatum, whilst in turn, creatine has been suggested to act as a neuroprotector and is decreased in tissues of individuals with GAMT deficiency, this study aimed to determine whether creatine administration could prevent the alterations in energy metabolism caused by GAA. Striatum was used because patients with GAMT deficiency may present basal ganglia abnormalities (Von Figura et al. 2001).

Materials and methods

Subjects and reagents

Wistar rats weighing 180–200 g were obtained from the Central Animal House of the Department of Biochemistry, Institute of Basic Health Sciences, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil. Animals were maintained on a 12/12 h light/dark cycle in an air-conditioned constant temperature ($22\pm1^\circ$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 Federation of Brazilian Societies for Experimental Biology and was approved by the Ethics Committee of the Federal University of Rio Grande do Sul, Brazil and followed the NIH *Guide for the Care and Use of Laboratory Animals* (NIH publication 85–23, revised 1985). All efforts were made to minimize the number of animals used and their suffering. The chemicals were purchased from Sigma Chemical Co., St. Louis, MO, USA.

Pretreatment with creatine

The animals used for experiments in this study were subjected to a pretreatment for 7 days, receiving a daily intraperitoneal injection of creatine (50 mg/kg) (Ribeiro et al. 2006). During this pretreatment, stereotaxic surgery was performed in the animals in order to facilitate the administration of GAA, as described below.

Surgery and intrastriatal administration

Surgery and intrastriatal infusion were performed, according to Folbergrova et al. (2001). Animals were anesthetized by intraperitoneal injection of ketamine and xylazine (100 mg/kg and 14 mg/kg, respectively). The heads of the animals were fixed in a stereotaxic apparatus, the skin of the skull was removed and a 27-gauge 9-mm guide cannula was then placed above the striatum (AP: −0.5 mm; L: −2.5 mm; DV: −2.5 mm). The cannula was fixed with acrylic cement.

Experiments were performed at 48 h after surgery. A 30-gauge cannula was fitted into the guide cannula and connected by a polyethylene tube to a $5\,\mu L$ Hamilton microsyringe. The tip of the infusion cannula protruded 1.0 mm beyond the guide cannula towards the striatum.

The animals were divided into three groups: Group 1 (sham group), rats that suffered surgery and received saline; and group 2 (GAA-treated), rats that received $10\,\mu M$ of GAA solution (0.02 nmol/striatum). The volume administered intrastriatally (saline or GAA solution) was $2\,\mu L$. The animals were killed by decapitation without anesthesia at 30 min after insertion.

Tissue and homogenate preparation

Animals were killed by decapitation without anesthesia, the brain was quickly removed on a Petri dish placed on ice and the striatum was dissected, weighed and kept chilled until homogenization with a ground glass type Potter–Elvehjem homogenizer in the specific buffer used for each technique. For the determination of complex II activity, the striatum was homogenized 1:10 (w/v) in SETH buffer pH 7.4 (250 mM sucrose, 2 mM EDTA, 10 mM Trizma base and 50 UI.mL^{-1} heparin). For preparation of synaptic plasma membrane and determination of Na$^+$,K$^+$-ATPase activity, striatum was homogenized in 10 volumes of 0.32 mM sucrose solution containing 5.0 mM HEPES and 1.0 mM EDTA, pH 7.4.

To determine the activity of creatine kinase in the cytosolic and mitochondrial fractions, samples of striatum were prepared according to Ramirez and Jiménez (2000). The striatum was homogenized 1:20 (w/v) in buffered sucrose/EGTA, and then centrifuged at 800 × g for 10 min, and the pellet discarded. The supernatant was then centrifuged at 27000 × g for 30 min in a Sorval DC-2B centrifuge. The resulting supernatant contained the cytosolic fraction of the sample. The pellet containing mitochondria was resuspended in 1 ml of sucrose buffer, centrifuged again for 10 min, and the supernatant discarded and resuspended in 1 ml of 15 mM Trizma MgSO$_4$, resulting in the mitochondrial fraction. The cytosolic and mitochondrial fractions were prepared on the day of the experiment and immediately used for the determination of enzyme activity. Briefly, to determine the levels of thiobarbituric acid reactive substances (TBARS), 500 µL of tissue were homogenized in a Pyrex tube striatum with 10 volumes of 1.15% KCl.

Determination of complex II activity

The activity of complex II was determined according to the method of Fischer et al. (1985), by the decrease in absorbance due to reduction of 2,6-dicloroindofenol (DCIP) at 600 nm, with 700 nm wavelength as reference ($\varepsilon=19.1$ mM^{-1}cm^{-1}), in the presence of phenazine metassulfato (PMS). The reaction mixture (40 mM potassium phosphate buffer, pH 7.4; 16 mM succinate and 8 µM DCIP) was pre-incubated with 40–80 µg of protein at 30°C for 20 min. Subsequently, to determine the activity of complex II, 4 mM sodium azide and 7 µM rotenone were added. The reaction was initiated by the addition of 40 µM DCIP and monitored for 5 min. Results are expressed as nmol/min.mg protein.

Preparation of synaptic plasma membrane from striatum

Synaptic plasma membranes were prepared according to the method of Jones and Matus (1974), with some modifications (Wyse et al. 1995). The homogenate was centrifuged at 1 000×g for 20 min and the supernatant removed and centrifuged at 12 000×g for a further 20 min. The pellet was then resuspended in hypotonic buffer (5.0 mM Tris–HCl buffer, pH 8.1), incubated 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 2 h, the fraction at the 0.8–1.0 M sucrose interface was taken as the membrane enzyme preparation.

Na$^+$,K$^+$-ATPase activity assay

The reaction mixture for the Na$^+$,K$^+$-ATPase 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 $200\,\mu L$. The reaction was initiated by the addition of ATP to a final concentration of 3.0 mM. 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, according to the method of 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.

Determination of creatine kinase activity

The reaction mixture for creatine kinase assay contained the following final concentrations: 60 mM Tris–HCl buffer, pH 7.5, 7 mM phosphocreatine, 9 mM MgSO$_4$, and approximately $0.4–1.2\,\mu g$ protein in a final volume of 0.1 mL. For enzymatic analysis in mitochondrial fractions, 0.625 mM lauryl maltoside was added to the medium. After 5 min of pre-incubation at 37°C, the reaction was started by the addition of 3.2 mM ADP plus 0.8 mM reduced glutathione. After 10 min of incubation at 37°C, the reaction was stopped by the addition of $1\,\mu mol$ *p*-hydroxymercuribenzoic acid. The reagent concentrations

and the incubation time were chosen to assure linearity of the enzymatic reaction. Appropriate controls containing all components of the incubation medium except homogenates, were carried out to measure chemical hydrolysis of phosphocreatine. The creatine formed was estimated according to the colorimetric method of Hughes (1962). The color was developed by the addition of 0.1 mL 2% α-naphthol and 0.1 mL 0.05% diacetyl in a final volume of 1 mL and read at 540 nm after 20 min. Results were expressed as μmol of creatine formed per min per mg protein.

TBARS assay

The TBARS assay is a measurement of lipid peroxidation and was carried out according to Ohkawa et al. (1979). Briefly, homogenates in 1.15% KCl were mixed with 20% trichloroacetic acid and 0.8% thiobarbituric acid and heated in a boiling water bath for 60 min. TBARS were determined by the absorbance at 535 nm. The results were reported as nmol of malonaldehyde per mg protein.

Protein determination

Protein was measured by the method of Lowry et al. (1951) or Bradford (1976) using bovine serum albumin as standard.

Statistical analysis

Data were analyzed by the Kruskal-Wallis 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

Figure 1 shows the effect of intrastriatal administration of GAA, creatine and GAA plus creatine on the complex II activity of the respiratory chain in the striatum of adults rats sacrificed at 30 min after injection. It can be observed that the intrastriatal administration of GAA significantly decreased the activity of this complex [$p<0.05$], when compared to sham groups. Creatine administration per se did not alter the activity of this complex, but was able to reverse the effect caused by the GAA.

Figure 2 shows the effect of intrastriatal injection of GAA, creatine and GAA plus creatine on the Na$^+$,K$^+$-ATPase activity in the synaptic plasma membranes of the striatum of adult rats. Results showed that intrastriatal administration of GAA significantly decreased Na$^+$,K$^+$-ATPase activity [$p<0.05$], when compared to sham groups.
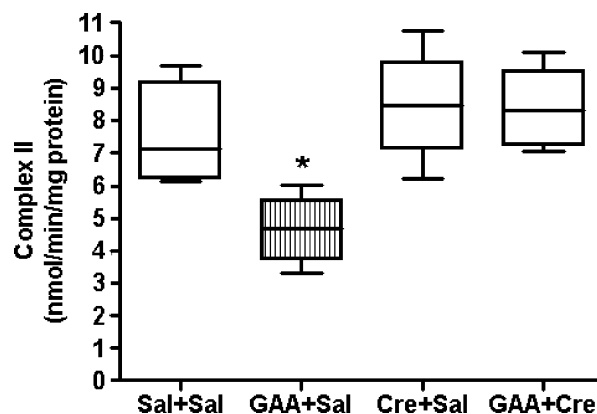

Fig. 1 Effect of GAA, creatine and GAA plus creatine on the activity of complex II of the respiratory chain in the striatum of adult rats. Box-plot representation: The *horizontal line* inside the box represents the median; the *lower and upper borders* of the box represent the 25th and 75th percentiles, respectively; the whiskers correspond to the extension of the box width by 1.5 fold (i.e., the IQR) from both ends of the box. Whenever the minimum or maximum value observed is within the whisker interval, the alternative limit of the corresponding whisker becomes the corresponding minimum or maximum observed value. Data represent results of 4–5 animals in each group. *GAA* Guanidinoacetate; *Cre* Creatine. (*$p<0.05$)

Creatine per se did not alter the activity of this enzyme, but was able to reverse the effect caused by the GAA.

We also examined the effect of intrastriatal injection of GAA, creatine and GAA plus creatine on creatine kinase activity. As can be observed in Fig. 3, GAA significantly decreased the activity of creatine kinase in the mitochondrial fraction [$p<0.05$] in the striatum of adult rats at 30 min after injection, when compared to sham groups; creatine was able to reverse the inhibitory effect of GAA on creatine kinase.
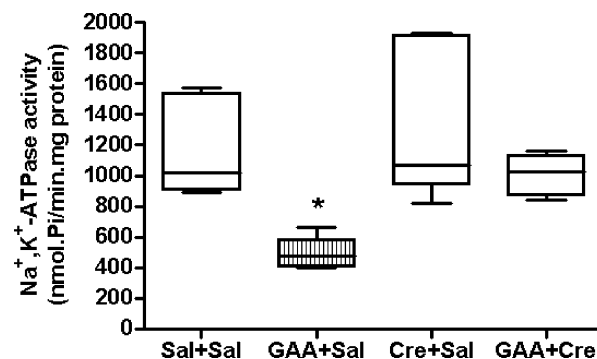

Fig. 2 Effect of GAA, creatine and GAA plus creatine on the Na$^+$,K$^+$-ATPase activity in the synaptic plasma membrane of adult rat striatum. Box-plot representation: The *horizontal line* inside the box represents the median; the *lower and upper borders* of the box represent the 25th and 75th percentiles, respectively; the whiskers correspond to the extension of the box width by 1.5 fold (i.e., the IQR) from both ends of the box. Whenever the minimum or maximum observed value is within the whisker interval, the alternative limit of the corresponding whisker becomes the corresponding minimum or maximum observed value. Data represent the results of 5 animals in each group. *GAA* Guanidinoacetate; *Cre* Creatine. (*$p<0.05$)
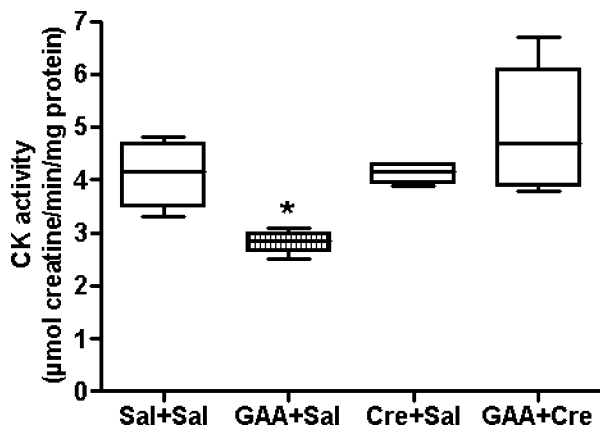
Fig. 3 Effect of GAA, creatine and GAA plus creatine on the creatine kinase activity in the mitochondrial fraction of the adult rat striatum. Box-plot representation: The *horizontal line* inside the box represents the median; the *lower and upper borders* of the box represent the 25th and 75th percentiles, respectively; the whiskers correspond to the extension of the box width by 1.5 fold (i.e., the IQR) from both ends of the box. Whenever the minimum or maximum value observed is within the whisker interval, the alternative limit of the corresponding whisker becomes the corresponding minimum or maximum observed value. Data represent the results of 4 to 6 animals in each group. *GAA* Guanidinoacetate; *Cre* Creatine. (*$p < 0.05$)

Since complex II, Na^+,K^+-ATPase and creatine kinase activities can be inhibited by free radicals, we tested the effect of the intrastriatal injection of GAA, creatine and GAA plus creatine on TBARS levels in the striatum of adult rats, sacrificed at 30 min after injection. Figure 4 shows that GAA significantly increased TBARS [$p < 0.05$] when compared to sham groups. Creatine administration
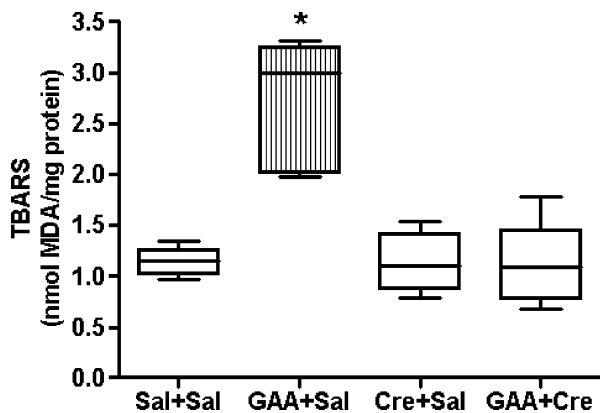

Fig. 4 Effect of GAA, creatine and GAA plus creatine on the levels of thiobarbituric acid reactive substances (TBARS) in the striatum of adult rats. Box-plot representation: The *horizontal line* inside the box represents the median; the *lower and upper borders* of the box represent the 25th and 75th percentiles, respectively; the whiskers correspond to the extension of the box width by 1.5 fold (i.e., the IQR) from both ends of the box. Whenever the minimum or maximum value observed is within the whisker interval, the alternative limit of the corresponding whisker becomes the corresponding minimum or maximum observed value. Data represent the results for 5 animals in each group. *GAA* Guanidinoacetate; *Cre* Creatine. (*$p < 0.05$)

per se did not alter this parameter, but reversed the increase in TBARS caused by GAA.

Discussion

The creatine deficiency syndromes consist of three disorders in the biosynthesis and transport of creatine, which result in absent or severely decreased creatine concentrations in the brain. GAMT deficiency is an inherited metabolic disorder, classified as one of the creatine deficiency syndromes; it is biochemically characterized by the tissue accumulation of GAA. Affected patients present neurological damage, the underlying mechanisms of which are poorly understood (Von Figura et al. 2001).

We have previously reported that GAA administration reduces complex II, Na^+,K^+-ATPase and creatine kinase activities and increases TBARS, an index of lipid peroxidation (Zugno et al. 2006, 2007). In the present study, we extended our investigation to verify the influence of creatine on these effects. Results showed that creatine administration prevented the impairment of energy metabolism and the increase in lipid peroxidation caused by GAA.

It has been demonstrated that GAA is a compound that induces oxidative stress by increasing free radical formation (Hiramatsu 2003; Zugno et al. 2004) and decreasing brain antioxidant defenses (Zugno et al. 2008). On the other hand, complex II, Na^+,K^+-ATPase and creatine kinase, enzymes crucial for the maintenance of normal brain function, are vulnerable to free radical attack (Wolosker et al. 1996; Rustin and Rötig 2002; Yousef et al. 2002). The increase in reactive species generation, promoted by GAA, can alter groups that are essential for enzymatic activity, such as sulphydryl groups, or components of the plasma membrane, where enzymes are anchored (Wolosker et al. 1996).

Creatine is a critical component in maintaining cellular energy homeostasis. While the role of creatine in the muscular system is well recognized, there is growing evidence that it also plays an important role in the central nervous system (CNS) (Andres et al. 2008). Although many of the molecular mechanisms are not well understood, creatine supplementation has been effective in a variety of animal/cellular models of neurodegenerative diseases such as Alzheimer's, Parkinson's and Huntington's (Klein and Ferrante 2007). The neuroprotective effect of creatine has been attributed to its antioxidant properties, which may result from different mechanisms of action: direct scavenging of radical species, iron chelation (Azzi et al. 2004), and amelioration of cellular energetics (Persky and Brazeau 2001; Wyss and Schulze 2002).

Considering that complex II, Na^+,K^+-ATPase and creatine kinase activities are susceptible to free radicals and that GAA induces oxidative stress (Wolosker et al. 1996; Rustin and

Rötig 2002; Yousef et al. 2002), we suggest that the ability of creatine to prevent such changes, observed in our study, may be mediated by its antioxidant capacity. The fact that arginine, the amino acid precursor of creatine, has antioxidant properties suggests that creatine may also be an antioxidant (Lawler et al. 2002). Several guanidino compounds that perform the transfer between the N-methyl group and guanidino (as in creatine), act as scavengers of hydroxyl groups, and are also considered to be antioxidants (Yildiz et al. 1998). Sestili et al. (2006) showed that creatine exerts antioxidant activity in cultured mammalian cells exposed to various oxidative agents, demonstrating the potential of this amine to directly neutralize reactive oxygen species in living cells.

It has been reported that creatine supplementation increases intracellular concentrations of phosphocreatine, which may bind to phospholipids in the cell membrane, reducing its permeability and therefore the loss of intracellular elements, such as enzymes, promoting stabilization of membranes (Persky and Brazeau 2001; Wyss and Schulze 2002). Moreover, the increased concentrations of phosphocreatine may facilitate the generation of ATP, and promote the maintenance of homeostasis of intracellular Ca^{2+}, preventing cell damage and energy impairment in some neurodegenerative diseases (Wyss and Schulze 2002). However, the decrease in Na^+,K^+-ATPase activity, observed in our study, was probably not due to the ATP deficit, as ATP was employed in the assay medium.

Studies have shown that creatine administration may reduce the frequency of epileptic seizures. Pretreatment of rats with creatine (10-days old), 3 days before exposure to hypoxia, has been shown to lead to a recovery in the levels of ATP and phosphocreatine and decrease convulsions induced by hypoxia (Holtzman et al. 1998). Magni et al. (2007), showed that creatine administration (300 mg/kg, p.o.) decreased seizures induced by glutaric acid in rats, increasing the latency for the first convulsive episode and decreasing the duration of convulsive episodes.

In addition to its functions in the buffering and transport of high energy phosphates, new roles for creatine have recently been suggested in CNS, such as direct anti-apoptotic effects (O'Gorman et al. 1997), alterations in vasculature, leading to improved circulation in the brain (Prass et al. 2007) and beneficial effects on cognitive processes (Rae et al. 2003).

In summary, in the present study we demonstrate that creatine was able to prevent bioenergetic disturbances and oxidative stress, suggesting that this compound may have a protective role in the brain alterations caused by GAA. These data are very encouraging, since creatine supplementation may constitute a good alternative therapeutic for patients with GAMT deficiency. However, more studies are needed to elucidate other processes and mechanisms involved in the neurological dysfunction presented by these patients.

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