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Research Report

Creatine decreases convulsions and neurochemical alterations induced by glutaric acid in rats

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ABSTRACT

Glutaric acidemia type I (GA-I) is an inherited metabolic disease characterized by striatal degeneration, seizures, and accumulation of glutaric acid (GA). Considering that GA impairs energy metabolism and induces reactive species generation, we investigated whether the acute administration of creatine, an amino acid with antioxidant and ergogenic properties, protects against the seizures and neurochemical alterations (inhibition of Na^+,K^+-ATPase and increased protein carbonylation) induced by the intrastriatal injection of GA (4 μmol/striatum). We also investigated whether creatine protected against the GA-induced inhibition of glutamate uptake *in vitro*. Creatine administration (300 mg/kg, p.o.) decreased seizures (evidenced by electrographic changes), protein carbonylation and Na^+,K^+-ATPase inhibition induced by GA. However, creatine, at a dose capable of fully preventing GA-induced protein carbonylation (50 and 150 mg/kg, p.o.), did not prevent convulsions and Na^+,K^+-ATPase inhibition, suggesting that the anticonvulsant activity of creatine in this experimental model is not related to its antioxidant action. Creatine also protected against the GA-induced inhibition of L-[^{3}H]glutamate uptake in synaptosomes, suggesting that creatine may reduce the deleterious effects of GA by maintaining glutamate uptake in the synaptic cleft. Therefore, considering that creatine significantly attenuates the deleterious effects of GA assessed by behavioral and neurochemical measures, it is plausible to propose the use of this amino acid as an adjuvant therapy in the management of glutaric acidemia.

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1. Introduction

Glutaric acidemia type I (GA-I) is an inherited organic acid cerebral disorder caused by the impairment of the mitochondrial enzyme glutaryl-CoA dehydrogenase (GCDH; EC 1.3.99.7), which catalyzes the dehydrogenation and decarboxylation of glutaryl-CoA to crotonyl-CoA in the catabolic pathway of L-lysine, L-hydroxylysine and L-tryptophan (Goodman et al., 1977; Liesert et al., 1999). Deficient GCDH activity gives rise to an accumulation of organic acids and their derivatives in body fluids and brain tissue of affected patients, i.e. glutaric acid (GA), 3-hydroxyglutaric (3-OH-GA) and glutaconic acids (Baric et al., 1998; Goodman et al., 1977). Clinical manifestations of GA I are predominantly neurological, including generalized convulsions, which are accompanied by bilateral destruction of caudate and putamen (Hoffmann and Zschocke, 1999; Morton et al., 1991) and acute loss of motor skills and dystonia (Freudenberg et al., 2004). Furthermore, postmortem examination of the basal ganglia and cerebral cortex of patients with GA I show postsynaptic vacuolization characteristic of glutamate-mediated brain injury (Goodman et al., 1977). Recently, it has been proposed that GA and 3-OH-GA induce striatal degeneration by disrupting mitochondrial energy metabolism (Ullrich et al., 1999; Das et al., 2003; Ferreira et al., 2005), promoting oxidative stress (de Oliveira Marques et al., 2003; Latini et al., 2002, 2005), and increasing glutamatergic neurotransmission (Wajner et al., 2004), which ultimately causes secondary excitotoxicity (Kolker et al., 1999; 2000a,b, 2002a,b; Porciuncula et al., 2004; Rosa et al., 2004). In this context, it has been shown that the intrastriatal injection of GA causes convulsions, increases oxidative damage markers (total protein carbonyl and thiobarbituric acid-reactive substances) and decreases striatal Na^+,K^+-ATPase activity in rats, mimicking, in various aspects, the neurological alterations of the disease in humans (Lima et al., 1998; Fighera et al., 2006).

Creatine (*N*-[aminoiminomethyl]-*N*-methyl glycine) is an endogenous amino acid produced from glycine, methionine and arginine in the liver, kidney and pancreas (Wyss and Kaddurah-Daouk, 2000). Recent experimental findings have demonstrated that creatine affords significant neuroprotection against hypoxia, amyotrophic lateral sclerosis, ischemia, oxidative insults and excitotoxicity (Holtzman et al., 1998; Klivenyi et al., 1999; Wick et al., 1999; Michaels and Rothman, 1990; Malcon et al., 2000). It is interesting that creatine supplementation also restores creatine phosphate levels and decreases mitochondrial generation of reactive oxygen species (ROS) induced by 3-OH-GA (Kolker et al., 2001; Das et al., 2003), one of the metabolites that accumulates in glutaric acidemia.

Although creatine reduces 3-OH-GA-induced production of reactive species, little is known about the protective effects of this amino acid against the convulsions and the deleterious neurochemical alterations induced by GA. In this context, considering that ROS are involved in the convulsive behavior induced by GA, it is feasible to propose that drugs, such as creatine, which prevents phosphocreatine (PCr) depletion (Wyss and Kaddurah-Daouk, 2000) and acts as scavenger of ROS (Lawler et al., 2002), may protect against GA-induced behavioral, electrographic and neurochemical deleterious effects. Therefore, in the present study, we decided to investigate whether the administration of creatine protects against GA-induced seizures and some known deleterious neurochemical effects of GA, such as protein oxidative damage, Na^+,K^+-ATPase activity inhibition (Fighera et al., 2006) and synaptosomal L-[^{3}H] glutamate uptake inhibition (Porciuncula et al., 2000).

2. Results

In the present investigation, we showed that administration of creatine (300 mg/kg, p.o.) increased the latency for the first convulsive episode [$F(3,54)=16.89$; $P<0.05$; Fig. 1A] and decreased the duration of convulsive episodes [$F(3,54)=13.60$; $P<0.05$; Fig. 1B] induced by the unilateral (right) intrastriatal injection of GA (4 μmol/striatum). This result was confirmed by electrographic recordings (Fig. 2). GA-induced seizures were characterized by clonus of the left forelimb and/or hindlimb and head (myoclonic jerks), rotational behavior and full lateralization toward the left side of the body, which were accompanied by the occurrence of multispikes plus slow waves and major seizure activity in the EEG. Generalized convulsions appeared in the electroencephalographic recordings as the major seizure activity (2–3 Hz high-amplitude activity). After the ictal discharge, postictal EEG suppression and slow waves were observed, correlating with behavioral catalepsy. The intrastriatal injection of GA induced the appearance of high-voltage synchronic clusters in the EEG followed by increase of striatal discharges (basal: 161±26.9 μV; after GA injection: 537±41.2 μV). GA injection increased cortical discharges basal: 124±24.9 μV; after GA injection: 461±45.9 μV; Fig. 2B). In addition, electrographic recordings of the ipsilateral striatum and cerebral cortex revealed that the intrastriatal injection of GA induced the appearance of an epileptogenic focus in the right striatum, which spread to the ipsilateral cortex.

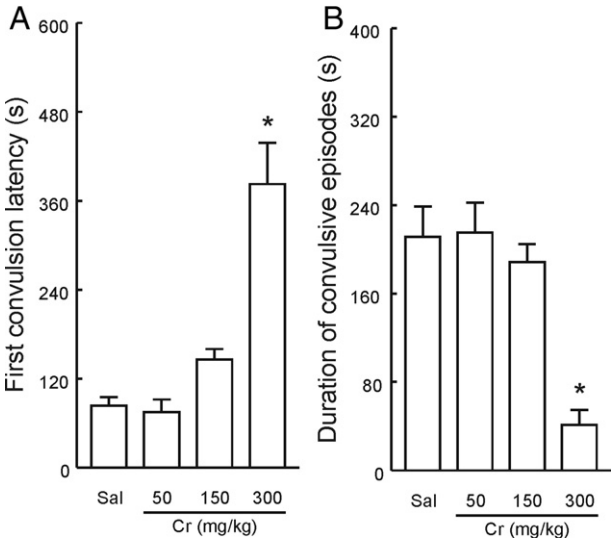

Fig. 1 – Creatine pretreatment (300 mg/kg, p.o.) increases the latency for the first convulsive episode (A) and decreases the duration (B) of convulsive episodes induced by GA (4 μmol/striatum). Data mean±S.E.M. for $n=8$–10 in each group. *$P<0.05$ compared with Vehicle-GA group (*F* test for simple effect).

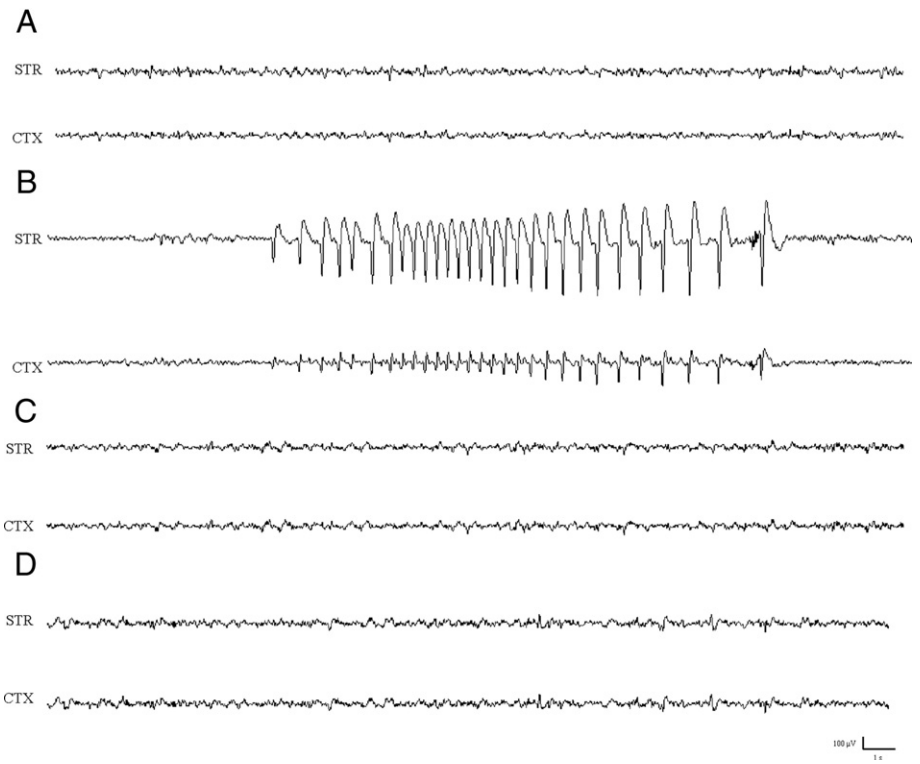

Fig. 2 – Representative electroencephalographic recordings before (A) and after intrastriatal GA administration (4 μmol/striatum; B) which were accompanied by pattern of continuous high-amplitude sharp spikes in low-frequency rhythmic spiking activity in ipsilateral cortex. Typical electroencephalographic recordings after injection of creatine (300 mg/kg, p.o.) (C) and after intrastriatal GA administration (D); STR, ipsilateral striatum; CTX, ipsilateral cortex. Scale bars, 100 μV and 1 s.

To quantify the epileptic effect of intrastriatal GA administration, we calculated the power densities of the different EEG frequencies. As shown in Figs. 3A and B, the field activity in control conditions or after creatine was similar and characterized by intermittent delta–beta oscillations. The injection of GA into striatum increased striatal (71%) and ipsilateral cortex (81%) delta frequency, and decreased beta oscillation in the striatum (37%) and ipsilateral cortex (50%), when compared with control conditions.

Electroencephalographic recordings also showed that creatine (300 mg/kg, p.o.) prevented ictal activity induced by intrastriatal injection of GA during all observation period, followed by a decrease of GA-induced spike amplitude between 65 and 70% with a mean of 151±25 μV (Fig. 2D). Quantitative analysis of EEG revealed that creatine pretreatment decreases striatal [$F(1,10)=9.81$; $P<0.03$; Fig. 3A] and ipsilateral cortex delta power density [$F(1,10)=6.42$; $P<0.05$; Fig. 3B]. In addition, creatine administration was effective against GA-induced striatal [$F(1,10)=20.90$; $P<0.05$; Fig. 3A] and ipsilateral cortex [$F(1,10)=24.88$; $P<0.05$; Fig. 3B] beta oscillation decrease.

The effects of GA and creatine injection on the total protein carbonylation in the injected and in the contralateral striatum were also determined. Statistical analysis revealed that intrastriatal injection of GA increased protein carbonyl content in the injected striatum [$F(1,59)=57.48$; $P<0.05$] and that creatine (50 to 300 mg/kg, p.o.) prevented the protein carbonylation increase induced by GA [$F(3,59)=18.69$; $P<0.05$; Fig. 4].

Fig. 5 shows the effect of creatine (50–300 mg/kg, p.o.) on GA-induced Na^+,K^+-ATPase activity decrease. Statistical analyses showed that the decrease in Na^+,K^+-ATPase activity [$F(1,59)=95.58$; $P<0.05$] induced by GA (4 μmol/striatum) was prevented by creatine at the dose of 300 mg/kg (p.o.). Creatine doses lower than 300 mg/kg did not alter GA-induced decrease in Na^+,K^+-ATPase activity.

Considering that acute creatine administration protected against GA-induced behavioral, electrographic and neurochemical deleterious effects, we decided to investigate whether creatine protects against GA-induced decrease of glutamate uptake in synaptosomes (Porciuncula et al., 2000). Statistical analysis revealed that creatine (10 μM; Fig. 6A) prevented GA-induced decrease of glutamate uptake [$F(3,16)=8.49$; $P<0.05$; Fig. 6B].

3. Discussion

In the present study, we confirm and extend our previous findings that GA elicits behavioral and electrographic seizures and increases reactive species generation *in vivo* (Fighera et al., 2006). In addition, we show, for the first time, that a single administration of creatine affords significant protection against the acute deleterious effects of GA: increase in protein carbonylation, decrease of Na^+,K^+-ATPase activity and electrographic convulsions *in vivo*. Creatine also protected against GA-induced decrease of L-[^{3}H] glutamate uptake in synaptosomes.

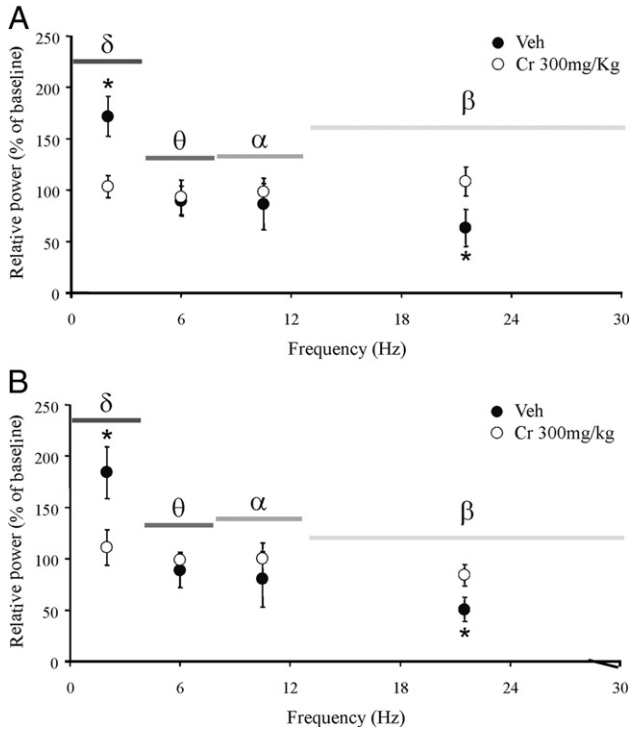

Fig. 3 – Quantitative analysis of EEG recording demonstrated that creatine pretreatment (300 mg/kg, p.o.) attenuated GA-induced delta increase and beta depression power density (4 μmol/2 μl) in the striatum (A) and ipsilateral cortex (B). Data mean ± S.E.M. for $n = 5–6$ in each group. *$P < 0.05$ when compared with basal conditions.

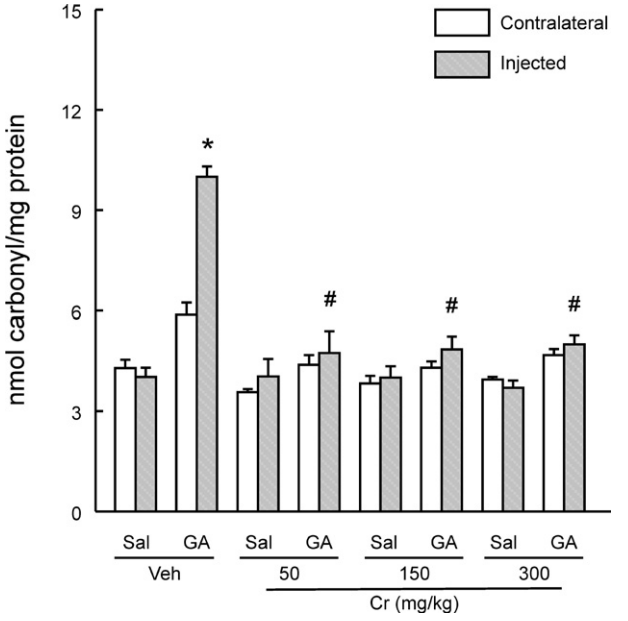

Fig. 4 – Creatine pretreatment (50 to 300 mg/kg, p.o.) prevents the striatal GA-induced protein carbonylation increase. Data are mean + S.E.M. for $n = 8–10$ in each group. *$P < 0.05$ compared with Vehicle-Sal group. #$P < 0.05$ compared with Vehicle-GA group (Student–Newman–Keuls test).

There is a significant body of evidence suggesting that GA accumulation may play a role in the convulsions and neurological impairment seen in patients with glutaric acidemia (Lima et al., 1998; Kolker et al., 1999, 2001, 2002b). Moreover, experimental findings *in vivo* and *in vitro* suggest that energy metabolism impairment and oxidative stress play an important role in the convulsive behavior elicited by GA (Flott-Rahmel et al., 1997; Lima et al., 1998; Frizzo et al., 2004; Porciuncula et al., 2000, 2004; Rosa et al., 2004, Ferreira et al., 2005). It is also worth remarking that a number of GA I patients excrete increased amounts of lactate, 3-hydroxybutyrate, acetoacetate and dicarboxylic acids, further indicating a mitochondrial dysfunction in this organic acidemia (Gregersen and Brandt, 1979; Floret et al., 1979).

In this context, the currently reported increase of striatal and cortical delta frequency induced by GA, to a certain degree, agrees with the study of Fujimoto et al. (2000), who have found periodic synchronic discharges, characterized by intermittent 4–6 Hz positive and 100–200 μV delta waves in cortical structure in a patient with glutaric aciduria type 1 during an episode of acute encephalopathy.

An impairment of glutamate metabolism and homeostasis has been suspected to underlie several brain diseases, including epilepsy (Kelly and Stanley, 2001). In fact, recent studies have demonstrated that inhibition of glutamate transporters leads to recurrent neuronal activity, characterized by periodic cell depolarization and bursts of action potentials (Demarque et al., 2004). This oscillatory effect alters the background pattern of bilateral recurrent paroxysmal bursts followed by cortical beta-gamma oscillations (Milh et al., 2007). In this context, the results presented in this report suggest that cellular glutamate uptake impairment elicited by GA (Porciuncula

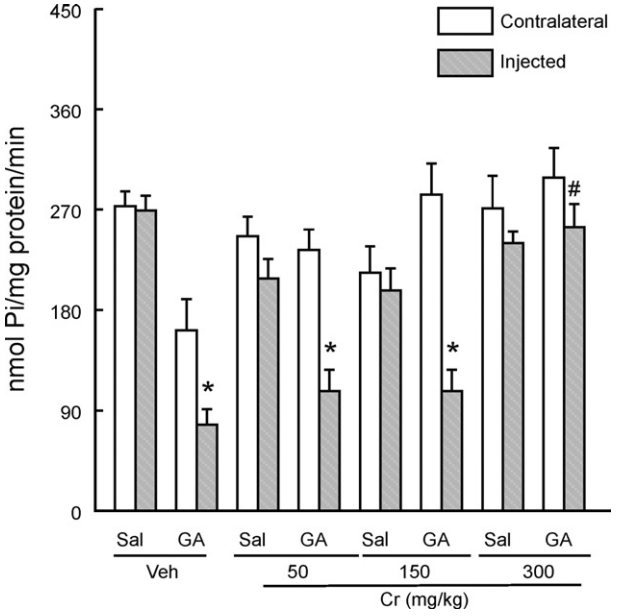

Fig. 5 – Effect of creatine pretreatment (50 to 300 mg/kg, p.o.) on the GA-induced Na^+,K^+-ATPase activity inhibition. Data are mean ± S.E.M. for $n = 8–10$ in each group. *$P < 0.05$ compared to contralateral striatum. #$P < 0.05$ compared with Vehicle-GA group.

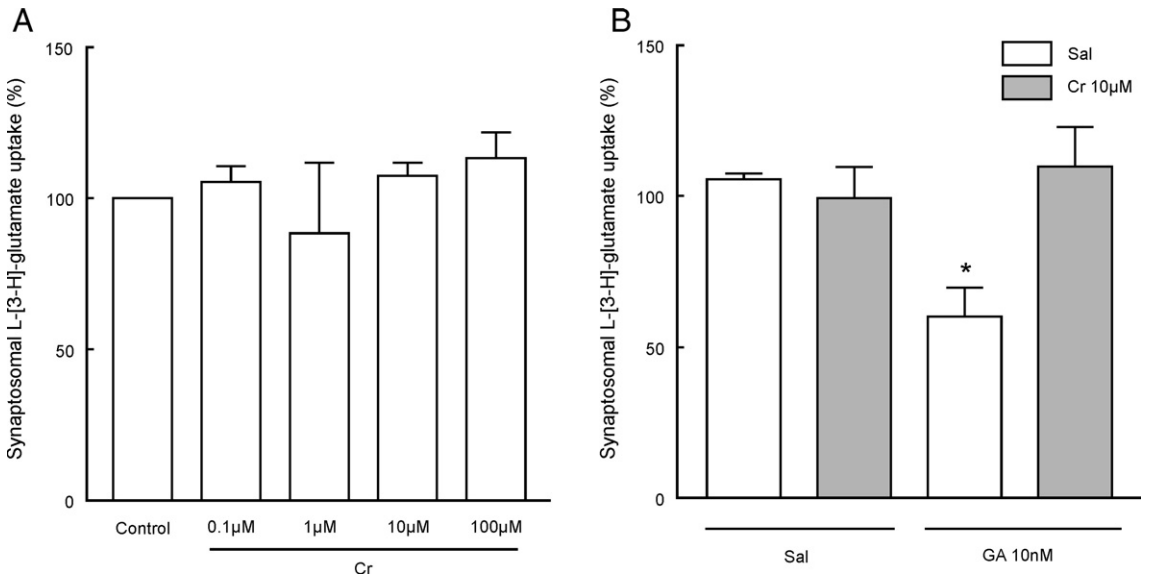

Fig. 6 – Effect of creatine on synaptosomal [^{3}H] glutamate uptake. Creatine (0.1 to 100 μM) addition to membrane preparation had not effect on L-[^{3}H] glutamate uptake (A). The incubation with creatine (10 μM) into synaptosomal membrane preparation reversed GA-induced L-[^{3}H] glutamate uptake inhibition (B). $^*P<0.05$ compared with control. $^\#P<0.05$ compared with GA group. Data are the means ± SEM of $n=6$–8 animals in each group.

et al., 2000, 2004) may lead increased excitatory activity, which ultimately results in electrographic and behavioral seizures (Fighera et al., 2006). Furthermore, since substantial evidence supports that mitochondrial dysfunction and consequent ATP depletion are a major cause of seizures and oxidative stress (Cassarino et al., 1999), it is plausible to propose that a disturbed energy metabolism elicited by this organic acid (Funk et al., 2005; Ferreira et al., 2005) leads to neuronal hyperexcitability, seizures and oxidative stress. In line with this view, and based in the previously reported positive correlation between duration of GA-induced seizures and inhibition of Na^+,K^+-ATPase activity (Fighera et al., 2006), we also suggest that an inhibition of selected targets to free radicals, such as Na^+,K^+-ATPase, may play an important role in the hyperexcitability and concomitant oxidative damage elicited by GA. On the other hand, it is critical to emphasize that, in this model of organic acidemia, a primary metabolic inhibition induced by intrastriatal injection of GA may alter ATP availability for several regulatory processes, disrupting normal Na^+,K^+-ATPase regulation. In this context, previous studies have proposed that nitric oxide, carbon monoxide and NMDA glutamate receptors modulate Na^+,K^+-ATPase activity through activation of regulatory proteins kinase G and C, in which free radicals play modulatory role (Nathanson et al., 1995; Petrushanko et al., 2006). However, further studies are necessary to clarify this point.

In the present study, we demonstrated that creatine pretreatment (300 mg/kg, p.o.) attenuated-GA induced convulsive behavior and protected against protein carbonylation and Na^+, K^+-ATPase activity inhibition. On the other hand, this report also revealed that creatine, at doses capable of preventing GA-induced protein carbonylation, does not prevent GA-induced seizures and Na^+,K^+-ATPase activity decrease, suggesting that protein carbonylation may occur separately from convulsive episodes (Fighera et al., 2006) and that the ability of creatine to reduce protein carbonylation is not related to its anticonvulsant action. However, one must also consider that selected targets, such Na^+,K^+-ATPase, which could not contribute significantly to the total protein carbonyl content, could be responsible for the GA-induced convulsions. Thus, if these targets were more sensitive to oxidative damage, they would require additional antioxidant protection, which could be afforded by increasing creatine doses. Therefore, it is not possible to rule out antioxidant mechanisms in the anticonvulsant action of creatine.

In agreement with this view, it has been claimed that the neuroprotective effect exerted by creatine in several neurodegenerative processes involves buffering of intracellular energy, preventing the increase of Ca^{2+} and ROS intramitochondrial levels, which lead to excitotoxic and cell death (O'Gorman et al., 1997; Leist and Nicotera, 1998; Dolder et al., 2003; Klivenyi et al., 2003; Andres et al., 2005). In addition, recent findings have indicated that creatine exerts cytoprotection via direct antioxidant activity (Sestili et al., 2006). Therefore, it is also possible that stabilization of buffering of intracellular energy and antioxidant properties may underlie its recurrent protection evidenced in this model of organic acidemia.

There is evidence that substances that increase GABAergic function and/or inhibit glutamatergic transmission may decrease convulsive episodes and oxidative stress (Karpiak et al., 1981; Amato et al., 1999; Fighera et al., 2003; Royes et al., 2003, 2006). In line of this view, many studies have investigated the role of glutamatergic and GABAergic neurotransmission on 3-OH-GA and glutaric acid-induced neurotoxicity (for a review see: Wajner et al., 2004). In the present study, we showed that GA inhibits glutamate uptake in striatal synaptosomes. Our results are in agreement with previous data from the literature, demonstrating that GA reduces glutamate uptake in forebrain synaptosomes or cultured astrocytes (Porciuncula et al., 2000, 2004).

Although tissue concentration of GA after intrastriatal administration is unknown, we may speculate that reduction of glutamate uptake by GA may facilitate the activation of excitatory amino acids receptors and, consequently, seizures. Of note, it has been demonstrated that glutamate transporters inhibitors do not produce seizures by themselves, but they are capable of facilitating seizures during energetic failure (Sepkuty et al., 2002; Demarque et al., 2004). Since GA induces cellular energy impairment and oxidative stress (Ferreira et al., 2005), creatine may have acted as an antioxidant by maintaining mitochondrial bioenergetics and protect neurons from excitotoxic damage caused by GA by increasing glutamate uptake and therefore, reducing the concentration of this amino acid in the synaptic cleft.

The exact mechanism by which GA reduces glutamate uptake is still unknown. It has been suggested that the inhibition of glutamate uptake could be due to a direct interaction of GA with glutamate transporters (Porciuncula et al., 2004). However, the activity of glutamate transporters can also be reduced by several indirect mechanisms, including reactive species formation and reduction in Na^+,K^+-ATPase activity (Volterra et al., 1994; Nanitsos et al., 2004). Thus, the reduction of glutamate uptake by GA could be related with its ability to induce oxidative stress and reduce Na^+,K^+-ATPase activity. Our results are in agreement with this view, since creatine reverted the oxidative stress and Na^+,K^+-ATPase activity reduction induced by GA.

In summary, the current study reports that striatal GA administration induces convulsive behavior, protein carbonylation and decrease of Na^+,K^+-ATPase activity, which are prevented by creatine. In addition, creatine protected against GA-induced synaptosomal glutamate uptake inhibition. Although the precise mechanism underlying the striatal degeneration and convulsive behavior of GAI patients is not known, it is plausible to propose that compounds, such as creatine, that decrease GA-induced toxicity assessed by behavioral and neurochemical parameters may be useful as adjuvant therapeutic measures against GA accumulation. However, clinical studies shall be conducted in order to evaluate its clinical efficacy in glutaric acidemic patients.

4. Experimental procedures

4.1. *Animal and reagents*

Adult male Wistar rats (270–300 g) maintained under controlled light and environment (12:12 h light–dark cycle, 24±1 °C, 55% relative humidity) with free access to food (Guabi, Santa Maria, Brazil) and water were used. Animal utilization protocols followed the Official Government Ethics guidelines and were approved by the University Ethics Committee. All reagents were purchased from Sigma (St. Louis, MO).

4.2. *Behavioral evaluation and surgical procedure*

Animals were anesthetized with Equitesin (1% phenobarbital, 2% magnesium sulfate, 4% chloral hydrate, 42% propylene glycol, 11% ethanol; 3 ml/kg, i.p.) and placed in a rodent stereotaxic apparatus. Under stereotaxic guidance, a cannula was inserted unilaterally into the striatum (coordinates relative to bregma: AP, 0 mm; ML, 3.0 mm; V, 3.0 mm from the dura). Chloramphenicol (200 mg/kg, i.p.) was administrated immediately before the surgical procedure. The experiments were performed 7–9 days after surgery when animals did not show any sign of pain, infection or discomfort.

Creatine (50, 150 or 300 mg/kg) or vehicle (0.1% carboxymethylcellulose) was infused by intragastric gavage (single administration) 45 min before the intrastriatal administration of GA (4 μmol/2 μl) or saline (5.5 μmol/2 μl). The intrastriatal administration (2 μl) was performed over a 2 min period using a 10 μl Hamilton syringe attached to a 30 gauge needle, whose tip protruded 2 mm from the cannula, allowing injection the dorsal striatum. All intrastriatal injections were made in unanesthetized rats and an additional minute was allowed to elapse before removal of needle to avoid backflow of drug through the cannula. Immediately after the intrastriatal injections, the animals were transferred to a round open field (54.7 cm in diameter) with a floor divided into 10 equal areas. During 20 min, the animals were video-monitored for the appearance convulsive episodes, according to (de Mello et al., 1996). Accordingly, clonic convulsions are episodes characterized by typical partial clonic activity affecting the face, head, vibrissae and forelimbs. Such clonic events are short, typically lasting 1–2 s and can occur either individually or in multiple discrete episodes before generalization and over time. Generalized convulsive episodes are characterized by generalized whole-body clonus involving all four limbs and tail, rearing, wild running and jumping, sudden loss of upright posture and autonomic signs, such as hypersalivation and defecation, respectively. Immediately after the behavioral evaluation, the animals were sacrificed for cannula placement or biochemical analysis.

4.3. *Placement of cannula and electrodes for EEG recordings*

A subset of animals (n=5–6) were anesthetized with Equitesin and surgically implanted with a cannula and electrodes under stereotaxic guidance. The guide cannula (27 gauge) was glued to a multipin socket and inserted into the right striatum through a previously opened skull orifice. One screw electrode was placed over the right parietal cortex (coordinates in mm: AP, −4.5; L, 2.5; V, 2) along with a ground lead positioned over the nasal sinus. Bipolar nichrome wire Teflon-insulated depth electrodes (100 μm) were implanted into the right striatum (coordinates in mm: AP, 0; L, 3; V, 4.2). The electrodes were connected to the multipin socket and, together with the injection cannula, were fixed to the skull with dental acrylic cement. Electroencephalographic recordings were performed for at least 7 days after the surgery.

4.4. *Intrastriatal injection of drugs and EEG recording*

Routinely, the animals were allowed to settle for habituation in a Plexiglas cage (25×25×60 cm) for at least 20 min. Baseline EEG recordings were obtained 10 min prior to drugs administration in order to establish an adequate control brain electrical activity. The drug injection protocol used in this set of experiments was the same used in those experiments that evaluated the effect of creatine on GA-induced behavioral seizures, except

that EEG was concomitantly recorded using digital encephalographic equipment (Neuromap EQSA260, Neuromap LTDA, Brazil). EEG signals were amplified, filtered (0.1 to 70.0 Hz, bandpass), digitalized (sampling rate 250 Hz) and stored in a PC for off-line analysis, as described below.

4.5. *EEG analyses*

In order to quantify the GA-induced seizure activity, as well as the possible anticonvulsant effect of creatine, we calculated the power densities of the different EEG frequencies obtained from freely moving rats. Digitalized data from basal, preinfusion and seizure periods were divided in 30 s segments and a 4 s sample from each segment was converted into frequency domain by fast Fourier transformation (FFT) method (Dringenberg et al., 2003). The resultant power values displayed for each frequency were grouped into 4 bands represented by delta (0–4 Hz), theta (>4–8 Hz), alpha (>8–13 Hz) and beta (>13–30 Hz). EEG recordings were visually analyzed for the appearance of seizure activity. Seizures were defined by the occurrence of episodes consisting of the following alterations in the recording leads: isolated sharp waves (≥1.5 × baseline); multiple sharp waves (≥2 × baseline) in brief spindle episodes (≥1 s≥5 s); multiple sharp waves (≥2 × baseline) in long spindle episodes (≥5 s); spikes (≥2 × baseline) plus slow waves; multispikes (≥2 × baseline, ≥3 spikes/complex) plus slow waves; major seizure (repetitive spikes plus slow waves obliterating background rhythm, ≥5 s). Data from seizure periods were expressed as percent of baseline values. Rhythmic scratching of the electrode headset by the animal rarely caused artifacts. These recordings were easily identified and discarded.

4.6. *Protein carbonyl assay*

Immediately after the behavioral evaluation, the animals were killed by decapitation and had their brain exposed by the removal of the parietal bone. A punch of the injected and noninjected striata was rapidly obtained. Striatal tissue was homogenized in 10 volumes (w/v) of 10 mM Tris–HCl buffer pH 7.4 using a glass homogenizer and its carbonyl protein content was determined by the method described by Levine et al. (1990) adapted for brain tissue (Schneider Oliveira et al., 2004).

4.7. *Na^+,K^+-ATPase activity measurement*

The measurement of Na^+,K^+-ATPase activity was performed in the same fresh, diluted, noncentrifuged homogenates used for determination of the striatal protein carbonyl content. Assay of enzyme activity was performed according by Wyse et al. (2000). Briefly, the incubation medium consisted of 30 mM Tris–HCl buffer, pH 7.4; 0.1 mM EDTA, 50 mM NaCl, 5 mM KCl, 6 mM $MgCl_2$ and 50 μg of protein in the presence or absence of ouabain (2 mM), in a final volume of 350 μl. The reaction was started by the addition of adenosine triphosphate to a final concentration of 5 mM. After 30 min at 37 °C, the reaction was stopped by the addition of 70 μl of trichloroacetic acid (TCA, 50%). Saturating substrate concentrations were used, and reaction was linear with protein and time. Appropriate controls were included in the assays for nonenzymatic hydrolysis of ATP. The amount of inorganic phosphate released was quantified by the colorimetric method described by Fiske and Subbarow (1925), and Na^+,K^+-ATPase activity was calculated by subtracting the ouabain-sensitive activity from the overall activity (in the absence of ouabain).

4.8. *Synaptosomal preparation*

The animals were sacrificed by decapitation and had their brain exposed by the removal of the parietal bone. A punch of the striatum was rapidly removed and synaptosomal preparation was obtained by isotonic Percoll/sucrose discontinuous gradients at 4 °C, as previously described (Dunkley et al., 1986). Briefly, homogenates (10%, w/v) from striatum were made in 5 mM HEPES and 320 mM sucrose (pH 7.4), and centrifuged twice at 1000×*g* for 5 min to produce a pellet (P1) and a supernatant (S1). The S1 were centrifuged twice at 10,000×*g* for 20 min to produce P2 and S2 that were discarded. P2 was resuspended in buffer HEPES/sucrose and was subjected to 16, 10 and 7.5% Percoll solution density gradient centrifugation at 15,000×*g* for 20 min. The synaptosomal fractions were isolated, suspended and homogenized in Krebs' buffer (pH 7.4), containing in mM 145 NaCl, 5 KCl, 1.2 $KH_2PO_4.2H_2O$, 1.3 $MgSO_4.6H_2O$, 20 HEPES, 10 glucose and 1.2 $CaCl_2$, and centrifuged twice at 15,000×*g* for 20 min. The supernatant was removed and the pellet resuspended in Krebs' buffer. The synaptosomal fraction used contained approximately 1 mg of protein/ml. This fraction also contained approximately 5% contamination with fragments of the inner and outer mitochondrial membranes, microsomes, myelin as well as neural and glial plasma membranes (Dunkley et al., 1986; Nagy et al., 1986; Migues et al., 1999). In order to evaluate the integrity of the synaptosomes, lactate dehydrogenase (LDH; EC 1.11.27) release was monitored by incubating the preparation with GA for 15 min. The LDH activity in the incubation medium was assayed spectrophotometrically at a wavelength of 340 nm (Labtest reagents, Brazil). Under the experimental conditions used, no changes in LDH were observed.

4.9. *Synaptosomal [^{3}H] glutamate uptake*

Experiments were performed in a final volume of 500 μl in a standard incubation medium composed of Krebs' buffer, and 5 μM (0.1 μCi) of L-[H^3]-glutamate (49 Ci/mmol, Amersham International, UK). Synaptosomal [^{3}H] glutamate uptake was measured in the presence of physiological concentrations of creatine (0.1 to 100 μM) (Ipsiroglu et al., 2001).

The effect of creatine (10 μM) (a concentration that had no effect *per se*) on synaptosomal [^{3}H]glutamate uptake was evaluated in the presence of GA (10 nM). This concentration of GA was previously reported to reduce [^{3}H] glutamate uptake in forebrain synaptosomes (Porciuncula et al., 2000). Controls did not contain glutaric acid or creatine. The uptake was carried out for 5 min at 37 °C after the addition of synaptosomes (100 μg of protein/tube) and stopped by centrifugation (16,000×*g* for 1 min at 4 °C). Radioactivity in the synaptosomal pellet was measure after the addition of scintillation liquid in a Packerd Model 1409 scintillation counter. The specific glutamate uptake was calculated as the difference between total uptake at 37 °C and the uptake at 4 °C (nonspecific uptake). Under our experimental conditions, the specific uptake was 95 ± 13 pmol/min/mg protein.

4.10. *Protein determination*

Protein content was measured colorimetrically by the method of Bradford (1976) using bovine serum albumin (1 mg/ml) as a standard.

4.11. *Statistics*

The latency for convulsion, total time spent convulsioning and electroencephalographic recordings were analyzed by one- or two-way ANOVA, depending on the experimental design, followed by a Student–Newman–Keuls test. Data from *in vivo* total carbonyl and Na^+,K^+-ATPase activity determinations were analyzed by a 2 (carboxymethylcellulose or creatine)×2 (saline or GA)×2 (injected or contralateral hemisphere) factorial ANOVA (analysis of variance), with the hemisphere factor treated as a within-subject factor. *Post hoc* analyses were carried out by the *F* test for simple effect or the Student–Newman–Keuls test, when appropriate. Data from *in vitro* Synaptosomal [^{3}H] glutamate uptake were analyzed by one-way ANOVA. All data are expressed as mean±S.E.M.. $P<0.05$ was considered significant.

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