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Effectiveness of creatine monohydrate on seizures and oxidative damage induced by methylmalonate ☆

Luiz Fernando Freire Royes, Michele Rechia Fighera, Ana Flávia Furian, Mauro Schneider Oliveira, Jociane de Carvalho Myskiw, Natália Gindri Fiorenza, João Carlos Petry, Rafael Correa Coelho, Carlos Fernando Mello *

Departamento de Fisiologia e Farmacologia, Universidade Federal de Santa Maria, 97105-900 Santa Maria, RS, Brazil

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Abstract

Methylmalonic acidemias are metabolic disorders caused by a severe deficiency of methylmalonyl CoA mutase activity, which are characterized by neurological dysfunction, including convulsions. It has been reported that methylmalonic acid (MMA) accumulation inhibits succinate dehydrogenase (SDH) and β-hydroxybutyrate dehydrogenase activity and respiratory chain complexes in vitro, leading to decreased CO_2 production, O_2 consumption and increased lactate production. Acute intrastriatal administration of MMA also induces convulsions and reactive species production. Though creatine has been reported to decrease MMA-induced convulsions and lactate production, it is not known whether it also protects against MMA-induced oxidative damage. In the present study we investigated the effects of creatine (1.2–12 mg/kg, i.p.) and MK-801 (3 nmol/striatum) on the convulsions, striatal content of thiobarbituric acid reactive substances (TBARS) and on protein carbonylation induced by MMA. Moreover, we investigated the effect of creatine (12 mg/kg, i.p.) on the MMA-induced striatal creatine and phosphocreatine depletion. Low doses of creatine (1.2 and 3.6 mg/kg) protected against MMA-induced oxidative damage, but did not protect against MMA-induced convulsions. A high dose of creatine (12 mg/kg, i.p.) and MK-801 (3 nmol/striatum) protected against MMA-induced seizures (evidenced by electrographic recording), protein carbonylation and TBARS production ex vivo. Furthermore, acute creatine administration increased the striatal creatine and phosphocreatine content and protected against MMA-induced creatine and phosphocreatine depletion. Our results suggest that an increase of the striatal high-energy phosphates elicited by creatine protects not only against MMA-induced convulsions, but also against MMA-induced oxidative damage. Therefore, since NMDA antagonists are limited value in the clinics, the present results indicate that creatine may be useful as an adjuvant therapy for methylmalonic acidemic patients.
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Keywords: Creatine; Convulsion; TBARS; Protein carbonylation; L-methylmalonic acid; *N*-methyl-D-aspartate; Striatum; EEG

1. Introduction

Methylmalonic acidurias comprise a group of inherited metabolic disorders caused by a deficiency of the mitochondrial enzyme methylmalonyl CoA mutase (MCM, EC 5.4.99.2), or by defects in the synthesis of 5′-deoxyadenosylcobalamin, the cofactor of MCM. Deficient MCM, which physiologically catalyses the reaction of methylmalonyl CoA to succinyl CoA, leads to the primary accumulation of methylmalonyl CoA, and to the secondary accumulation of other metabolites, such as propionate, 3-hydroxypropionate and 2-methylcitrate (Fenton et al., 2001). The affected infants present a variable degree of mental retardation and severe neurological dysfunction, such as delayed development, seizures, demyelination and cerebral edema of the white matter (Roodhooft et al., 1990; Brismar and Ozand, 1994; Fenton et al., 2001). Histopathology and neuroimaging studies revealed severe necrosis as well as symmetric degeneration of the basal ganglia in these patients (Brismar and Ozand, 1994). Furthermore, it has been shown that patients with methylmalonic acidemia, during acute metabolic crises, present elevated amounts of lactate in globus pallidus suggesting neuronal damage, via inhibition of mitochondrial energy metabolism (Trinh et al., 2001). In this context, MMA seems to generate other neurotoxins, such as malonic acid, an

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* Corresponding author. Tel.: +55 55 3220 9378; fax: +55 55 3220 8241.
E-mail address: cf.mello@smail.ufsm.br (C.F. Mello).

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inhibitor of complex II, and 2-methylcitrate, a compound with multiple inhibitory effects on the tricarboxylic acid cycle (Okun et al., 2002), which might contribute for the metabolic collapse and secondary excitotoxic mechanism induced by MMA exposure. Interestingly, the SDH substrate, succinate, has been recently proposed to play a neurotoxic role in methylmalonic acidemia (Roehrs et al., 2004). In fact, there is a considerable body of evidence suggesting that MMA impairs mitochondrial function, since it increases lactate production ex vivo and in vitro (Wajner et al., 1992; Greenamyre et al., 1994; Royes et al., 2003), decreases ATP (McLaughlin et al., 1998) and phosphocreatine levels (Royes et al., 2003), CO_2 production (Wajner et al., 1992) and O_2 utilization (Toyoshima et al., 1995).

Acute creatine administration increases striatal phosphocreatine levels and protects against MMA-induced convulsions and phosphocreatine depletion (Royes et al., 2003). In line with this view, creatine protects against MMA-induced neurotoxicity in primary neuron cultures, probably by increasing energy phosphates (Kölker et al., 2000).

Besides its inhibitory role on the energetic metabolism, an excitatory role for MMA was demonstrated. Accordingly, it has been shown that MMA causes convulsive behavior through glutamatergic mechanisms (de Mello et al., 1996; Malfatti et al., 2003), and striatal degeneration (Narasimhan et al., 1996). The depolarizing effect of MMA on isolated neurons and astrocytes, has also been demonstrated (McLaughlin et al., 1998), as well as its ability to induce LTP in the striatum (Calabresi et al., 2001). Therefore, excessive glutamate receptor stimulation, in particular the NMDA receptor, has been implicated as a major pathway that leads to MMA-induced convulsions (de Mello et al., 1996; Royes et al., 2003). More recently, reactive oxygen species (ROS) have been implicated in the convulsive behavior elicited by MMA, since it has been shown that intrastriatal MMA administration, besides causing convulsive behavior, increases local thiobarbituric acid reacting substances (TBARS) content and inhibits Na^+,K^+-ATPase activity (Malfatti et al., 2003). Moreover, while the systemic administration of antioxidants, such ascorbic acid, α-tocopherol and GM1 (Fighera et al., 1999, 2003) attenuate, ammonia, a pro-oxidant agent, increases MMA-induced convulsions (Marisco et al., 2003). However, until the present moment, it is not known if the ergogenic compound creatine, which has been proposed as a possible adjunct treatment for methylmalonic acidemic patients, also protects against MMA-induced oxidative damage. Therefore, in the present study we decided to investigate whether creatine protects against the behavioral, electrographic and oxidative effects of the intrastriatal injection of MMA.

2. Materials and methods

2.1. Animals 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. All experimental protocols were designed aiming to keep the number of animals used to a minimum, as well as their suffering. All experimental protocols were conducted in accordance with national and international legislation (guidelines of Brazilian College of Animal Experimentation (COBEA) and of U.S. Public Health Service's Policy on Humane Care and Use of Laboratory Animals — PHS Policy), and with the approval of the Ethics Committee for Animal Research of the Federal University of Santa Maria. All reagents were purchased from Sigma (St. Louis, USA), except thiobarbituric acid (TBA), which was obtained from Merck (Darmstadt, Germany).

2.2. Surgical procedure and drug administration protocol

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, DV 4.2 mm for the striatum) (Paxinos and Watson, 1986). Chloramphenicol (200 mg/kg, i.p.) was administrated immediately before the surgical procedure. Three days after the surgery, the animals were injected with creatine (1.2, 3.6 or 12 mg/kg, i.p.) or saline (0.9% NaCl; 10 ml/kg, i.p.) 30 min before the intrastriatal administration of MMA (6 μmol/2 μl) or saline (9 μmol/2 μl). The involvement of NMDA receptor activation on the convulsions as well as alterations of striatal TBARS and carbonyl protein content induced by MMA was investigated by intrastriatally injecting the animals with 0.5 μl of saline (0.9% NaCl) or MK-801 (3 nmol) 30 min before the intrastriatal administration of MMA (4.5 μmol/1.5 μl) or saline (6.7 μmol/1.5 μl).

Immediately after the injections the animals were transferred to a round open field (54.7 cm in diameter) with a floor divided into 10 equal areas. The open field session lasted 15 min, and during this time the animals were observed for the appearance of convulsions. The number and duration of convulsive episodes were recorded (de Mello et al., 1996).

2.3. Placement of cannula and electrodes for EEG recordings

Rats were surgically implanted with a cannula and electrodes under stereotaxic guidance. In brief, rats were anesthetized with Equitesin and two screw electrodes were placed bilaterally over the parietal cortex along with a ground lead positioned over the nasal sinus. Bipolar nichrome wire Teflon-insulated depth electrodes (100 μm) were implanted ipsilaterally into striatum. For intrastriatal infusion of drugs, a guide cannula (27 gauge) was glued to a multipin socket and inserted through a previously opened skull orifice. The coordinates from bregma for implantation of the electrodes were (in mm): AP, −4.5; L, 2.5; and DV, 2 for the cortex and AP, 0; L, 3; DV, 4.2 for the striatum (Paxinos and Watson, 1986). The electrodes were connected to a multipin socket and, together with the injection cannula, were fixed to the skull with dental acrylic cement. The experiments were performed 7–9 days after surgery.

2.4. EEG recordings and intrastriatal injection of drugs

The procedures for EEG recording and intracerebral injection of drugs were previously described (Cavalheiro et al., 1992). Briefly, the animals were allowed to habituate to a Plexiglass cage (25 × 25 × 60 cm) for at least 10 min before the EEG recording. The rats were then connected to the lead socket in a swivel inside a Faraday's cage. Routinely, a 10 min baseline recording was obtained to establish an adequate control period. The drug injection protocol used in this set of experiments was the same used in those experiments that evaluated the possible protective effect of creatine on MMA-induced behavioral convulsions, except that EEG was concomitantly recorded. EEG signals were amplified, filtered (0.1 to 50.0 Hz, bandpass) and recorded using an analogical electroencephalographer (Berger TP 119). Several 30-s epoch were selected during preinfusion (10 min) and postinfusion (45 min) periods to determine significant EEG changes. The preinfusion segment was defined as ending immediately before the beginning of infusion. The postinfusion epochs started immediately after the end of creatine (12 mg/kg, i.p.) or saline (0.9%; 10 ml/kg, i.p.); MMA (6 μmol/2 μl) or saline (9 μmol/2 μl); MK-801 (3 nmol/0.5 μl) and MMA (4.5 μmol/1.5 μl) or saline (6.7 μmol/1.5 μl).

All intrastriatal injections were made in unanesthetized rats by using a needle (30 gauge) protruding 1 mm below the guide cannula. All drugs were injected over 1-min period by using a Hamilton syringe, and an additional minute was allowed to elapse before removal of needle to avoid backflow of drug through the cannula.

2.5. Colorimetric determination of TBARS content

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 striatum was rapidly obtained using a stainless steel puncher (5 mm in internal diameter) around the site of cannula placement. The contralateral hemisphere was subjected to the same procedure. Tissues were homogenized in 10 volumes (w/v) of 10 mM Tris–HCl (pH 7.4), containing sodium dodecyl sulfate (SDS, 0.10% — final concentration) using a glass homogenizer and TBARS content was estimated in a medium containing 0.2 ml of brain homogenate, 0.1 ml of 8.1% SDS, 0.4 ml of acetic acid buffer (500 mM, pH 3.4), and 0.75 ml of 0.81% TBA. The mixture was finally made up to 2 ml with type I ultrapure water, and heated at 95 °C for 90 min in a water bath using a glass ball as a condenser. After cooling to room temperature, absorbance was measured in the supernatant at 532 nm (Ohkawa et al., 1979).

2.6. Colorimetric determination of the protein carbonyl content

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 contralateral striata was rapidly obtained, as described above.

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 Yan et al. (1995), adapted for brain tissue by Oliveira et al. (2004). Briefly, homogenates were diluted to 750–800 μg/ml of protein in each sample, and 1 ml aliquots were mixed with 0.2 ml of 2,4-dinitrophenylhydrazine (DNPH, 10 mM) or 0.2 ml HCl (2 M). After incubation at room temperature for 1 h in a dark ambient, 0.6 ml of denaturing buffer (150 mM sodium phosphate buffer, pH 6.8, containing 3% SDS), 1.8 ml of heptane (99.5%) and 1.8 ml of ethanol (99.8%) were added sequentially, mixed with vortex agitation for 40 s and centrifuged for 15 min. Next, the protein isolated from the interface was washed two times with 1 ml of ethyl acetate/ethanol 1 : 1 (v/v) and suspended in 1 ml of denaturing buffer. Each DNPH sample was read at 370 nm in a Hitachi U-2001 spectrophotometer against the corresponding HCl sample (blank), and total carbonylation calculated using a molar extinction coefficient of 22,000 M^{-1} cm^{-1}, as described by Levine et al. (1990).

2.7. Creatine and phosphocreatine determination

After behavioral evaluation the animals were sacrificed, as described above, and the striata were homogenized in 1 ml of 3 mM perchloric acid. The samples were centrifuged at 12 000 ×*g* for 5 min (−7 °C). After centrifugation, the supernatant was separated and the pH adjusted to pH 7.4 with KOH (5 M) and KH_2PO_4 (25 mM). Creatine (Cr) and phosphocreatine (PCr) content were assayed in the neutralized supernatants by HPLC at controlled room temperature (20 °C), under isocratic conditions using a reversed-phase 125 × 4 mm analytical column protected by a 4 × 4 mm guard cartridge (5 μm particle size, RP-18 LiChrosphere 100, Hewlett Packard, Netherlands). The mobile phase was pumped at a flow-rate of 1 ml/min and consisted of 14.7 mM KH_2PO_4 in 1.15 mM Tetra-*n*-buthylammonium hydrogen malate aqueous solution adjusted to pH 5.3 with 5 mM KOH, and the creatine and phosphocreatine peaks were identified by UV detection at 214 nm, as described by Karatzaferi et al. (1999).

2.8. Protein determination

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

2.9. Statistical analysis

Data from ex vivo TBARS, total carbonyl, creatine and phosphocreatine determinations were analyzed by a 2 (saline or creatine) × 2 (saline or MMA) × 2 (injected or contralateral hemisphere) factorial ANOVA, 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. $P < 0.05$ was considered significant. The number of convulsive episodes and total time spent convulsioning were analyzed by one-way ANOVA, followed by a Student-Newman-Keuls test. All data are expressed as mean + S.E.M.

3. Results

The effect of the systemic creatine administration on MMA-induced increase of striatal TBARS content and total protein carbonylation ex vivo is shown in Figs. 1 and 2, respectively. Statistical analysis considering the hemispheres as a within subject factor revealed a significant drug (saline or MMA) by hemisphere (injected or contralateral) interaction for TBARS [$F(1,42)=6.18$; $P<0.05$ — Fig. 1], indicating that MMA injection caused a significant increase of TBARS content along the hemispheres. Tests of between subject effects revealed a significant pretreatment (0.9% saline or 1.2, 3.6 or 12 mg/kg creatine) by drug (saline or MMA) interaction [$F(3,42)=7.17$; $P<0.001$]. Post hoc analysis revealed that MMA injection increased TBARS content in both striata and that systemic creatine administration prevented MMA-induced increase of TBARS production.

The effects of the systemic creatine administration and unilateral MMA injection on the total protein carbonylation of the injected and of the non-injected striata were also determined. Statistical analysis of carbonyl content data considering the hemispheres as a within subject factor revealed a significant pretreatment (0.9% saline or 1.2, 3.6 or 12 mg/kg creatine) by drug (saline or MMA) by hemisphere (injected or contralateral) interaction [$F(3,46)=3.44$; $P<0.05$ — Fig. 2]. Post hoc analysis showed that the intrastriatal administration of MMA increased the total carbonyl protein content of the injected striatum, and that systemic injection of creatine protected against MMA-induced protein carbonylation.

The systemic injection of creatine also decreased the number [$F(3,47)=4.61$; $P<0.05$] and the duration of convulsive episodes [$F(3,47)=3.86$; $P<0.05$ — Table 1] induced by MMA (6 µmol/2 µl) linearly with its dose [significant linear trend for number $F(1,25)=12.13$; $P<0.002$ and duration $F(1,25)=11.32$; $P<0.002$ of convulsive episodes]. Electrographic recording of the ipsilateral striatum and cerebral cortex revealed that intrastriatal MMA injection caused the appearance of an epileptogenic focus in the injected striatum and spread to the ipsi and contralateral cortex (Fig. 3C). The injection of creatine (12 mg/kg, i.p.) protected against MMA-induced seizures evidenced by electrographic recording (Fig. 4C).

The intrastriatal injection of MK-801 protected against MMA-induced striatal TBARS [significant pretreatment (saline or MK-801) by treatment (saline or MMA) interaction: $(1,28)=5.25$; $P<0.05$ — Fig. 1] and protein carbonylation increase [$F(1,21)=11.10$; $P<0.05$ — Fig. 2]. In addition, MK-801 attenuated MMA-induced seizures measured by

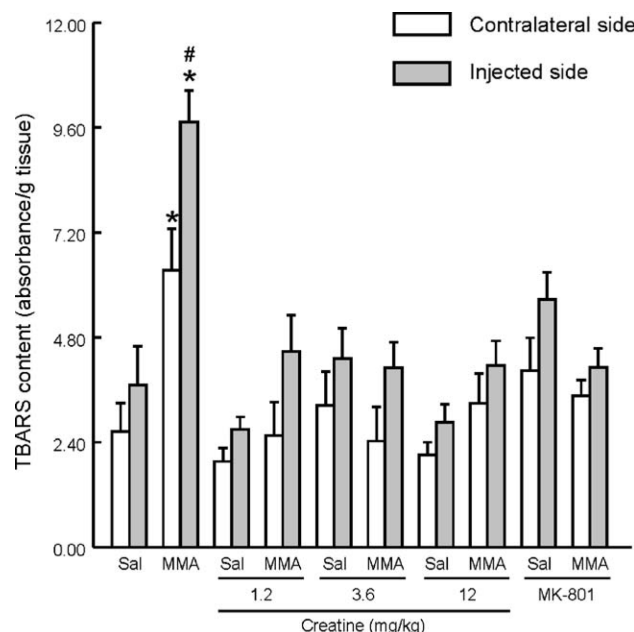

Fig. 1. Creatine (1.2, 3.6 or 12 mg/kg, i.p.) and MK-801 (3 nmol/0.5 µl) prevent the striatal MMA-induced TBARS increase (6 µmol/2 µl; 4.5 µmol/1.5 µl, respectively) ex vivo. Data mean+S.E.M. for $n=6$–8 in each group. *$P<0.05$ compared with Saline group. $^{\#}P<0.05$ compared with respective control hemisphere (Student-Newman-Keuls test).

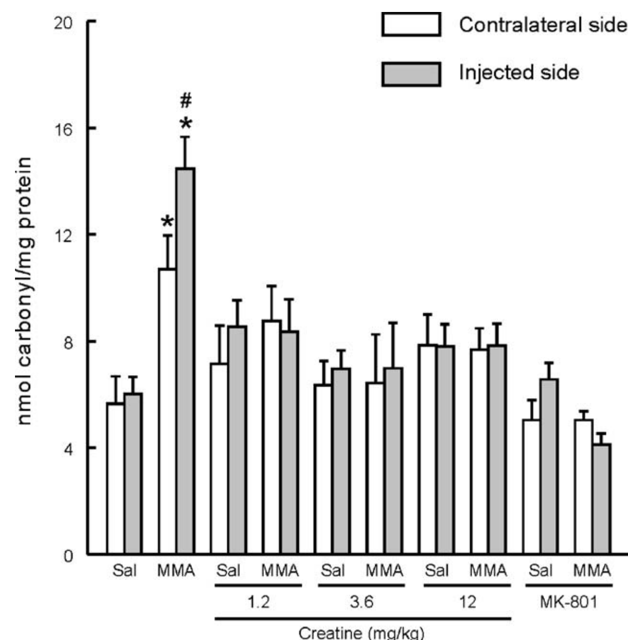

Fig. 2. Creatine (1.2, 3.6 and 12 mg/kg, i.p) and MK-801 (3 nmol/0.5 µl) prevent the striatal MMA-induced protein carbonylation increase (6 µmol/2 µl; 4.5 µmol/1.5 µl, respectively) ex vivo. Data are mean+S.E.M. for $n=6$–8 in each group. *$P<0.05$ compared with Saline group. $^{\#}P<0.05$ compared with respective control hemisphere (Student-Newman-Keuls test).

Table 1
Effect of creatine and of MK-801 on the number and duration of convulsive episodes induced by MMA

	Number of convulsive episodes	Time spent convulsioning (s)
Saline–MMA	11±0.88	291.25±23.9
Creatine (1.2 mg/kg) – MMA	8±1.70	200.71±51.9
Creatine (3.6 mg/kg) – MMA	11±1.60	159.33±16.8
Creatine (12 mg/kg) – MMA	3±1.28*	66.2±17.1*
MK-801–MMA	3±0.90*	39.0±6.1*

Data are mean+S.E.M. for $n=8$–10 in each group (Student-Newman-Keuls test). *$P<0.05$ compared with Saline–MMA group.

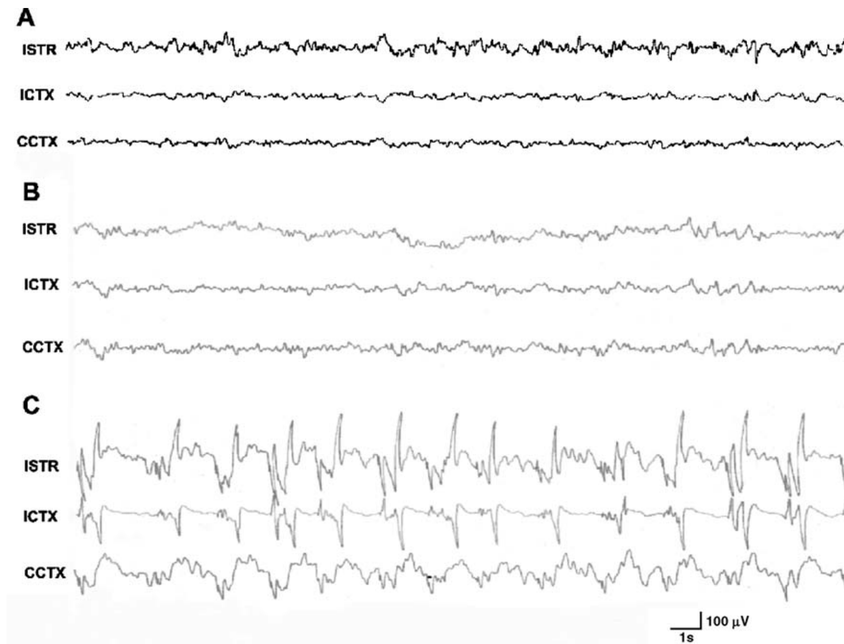

Fig. 3. Electroencephalographic recordings before (A) and after (B) saline (0.9% NaCl, i.p.) administration; and typical seizure sequence observed after intrastriatal injection of MMA (6 µmol/2 µl) (C), which were accompanied by the behavioral alterations described in the Results section. ISTR, ipsilateral striatum; ICTX, ipsilateral cortex and CCTX, contralateral cortex. Calibration bars, 100 µV and 1s.

number [$F(1,28)=5.45$; $P<0.05$], duration of convulsive episodes [$F(1,28)=17.24$; $P<0.05$ — Table 1] and electrographic recording (Fig. 5C), confirming previous results from our group (de Mello et al., 1996; Royes et al., 2003).

The effect of creatine (12 mg/kg, i.p.) and MMA administration (6 µmol/2 µl) on the creatine and phosphocreatine content of the injected and contralateral striata was also determined and is shown in Table 2. Statistical analysis (three-way ANOVA, with the hemispheres treated as a within subject factor) revealed a significant pretreatment (0.9% saline or creatine) by treatment (9 µmol saline or 6 µmol MMA) by hemisphere (contralateral or injected) interaction [$F(1,20)=5.90$; $P<0.05$] and [$F(1,20)=4.92$; $P<0.05$] a significant main effect of pretreatment (0.9% saline or creatine) [$F(1,20)=8.73$;

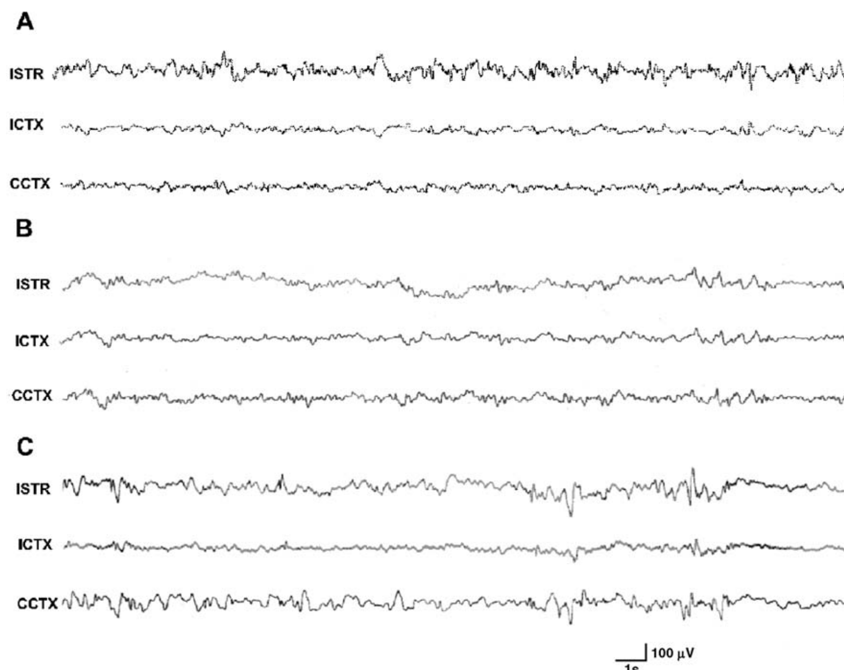

Fig. 4. Electroencephalographic recordings before (A) and after (B) creatine (12 mg/kg, i.p.) administration; and after intrastriatal injection of MMA (6 µmol/2 µl) (C). ISTR, ipsilateral striatum; ICTX, ipsilateral cortex and CCTX, contralateral cortex. Calibration bars, 100 µV and 1s.

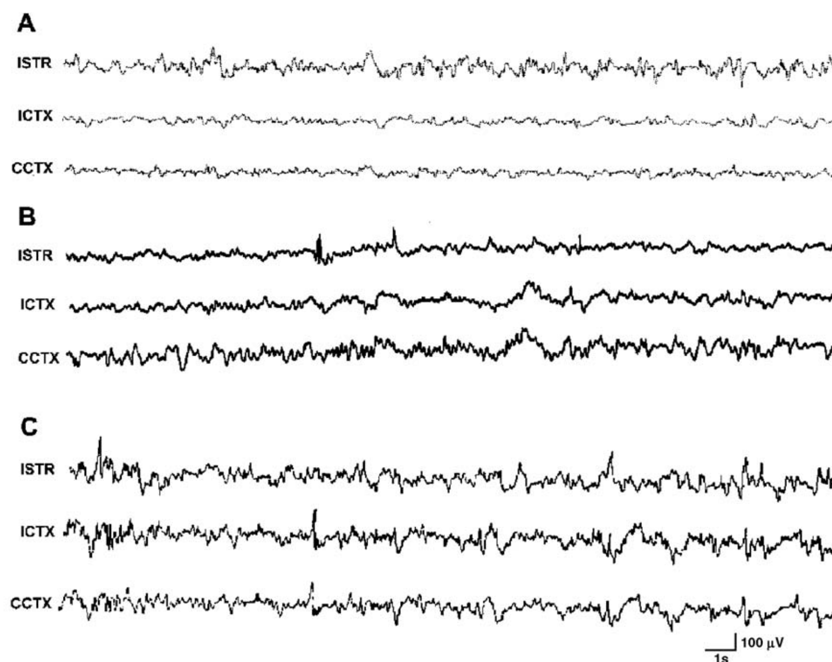

Fig. 5. Electroencephalographic recordings before (A) and after (B) MK-801 (3 nmol/0.5 µl) administration; and after intrastriatal injection of MMA (6 µmol/1.5 µl) (C). ISTR, ipsilateral striatum; ICTX, ipsilateral cortex and CCTX, contralateral cortex. Calibration bars, 100 µV and 1s.

$P<0.05$] and [$F(1,20)=4.67$; $P<0.05$], indicating that creatine administration caused a general increase in the striatal content of creatine and phosphocreatine, respectively. Post hoc analysis (F test on simple effect) revealed that creatine administration altered the creatine [$F(1,20)=5.80$; $P<0.05$] and phosphocreatine content [$F(1,20)=7.11$; $P<0.05$] among the hemispheres, only in those animals injected with MMA compared to Saline–MMA group, indicating that creatine administration also protected against MMA-induced creatine and phosphocreatine depletion.

4. Discussion

In the present study we replicate previous findings that MMA induces convulsions and increases the formation of reactive species reaction products ex vivo and show, for the first time, that: a) MMA-induced TBARS production and protein carbonylation ex vivo is significantly attenuated by MK-801 and b) creatine affords significant protection against MMA-induced TBARS production, protein carbonylation and electrographic convulsions. The currently reported protective effect of MK-801 against MMA-induced oxidative damage has interesting implications. Since MMA does not directly interact with glutamate receptors (de Mello et al., 1997), it is possible that oxidative damage may result from an indirect stimulation of NMDA receptors by MMA, as previously suggested (de Mello et al., 1996). This would imply the NMDA receptor activation as a convergent pathway for the convulsions and oxidative damage induced by this organic acid. On the other hand, it is well known that convulsive activity increases cerebral oxidative damage markers (Hiramatsu et al., 1996; Patsoukis et al., 2004a,b, 2005). Since MK-801 prevented behavioral (Table 1) and electrographic convulsions (Fig. 5C) and reactive species generation induced by MMA (Figs. 1 and 2), it is also possible that MK-801 prevented MMA-induced oxidative damage by simply inhibiting convulsive activity (de Mello et al., 1996; Royes et al., 2003). Therefore it is not possible to define if the currently reported decrease of oxidative damage markers is due to a specific decrease in NMDA-mediated mechanisms or a consequence of decreased convulsive activity.

The systemic administration of creatine increased striatal creatine and phosphocreatine content, and protected against the convulsions and lactate increase induced by the intrastriatal administration of MMA, suggesting that such a protective effect of creatine may be related to an improvement of the energy status of creatine-treated animals (Royes et al., 2003). Our results contrast with those from Perasso et al. (2003), who have shown that a single dose of creatine (160 mg/kg, i.p.) causes limited

Table 2
Effect of creatine and MMA administration on striatal creatine and phosphocreatine content

Treatment	Creatine (Cr)		Phosphocreatine (Pcr)	
	Contralateral striatum	Injected striatum	Contralateral striatum	Injected striatum
Sal–Sal	130.5±26.8	106.4±30.8	52.5±3.7	34.8±3.7
Sal–MMA	112.8±36.0	62.1±21.4#	42.0±6.0	12.5±2.4#
Cr (12 mg/kg)–Sal	194.7±19.5	112.3±24.8#	110.8±11.8	52.1±4.6#
Cr (12 mg/kg)–MMA	160.6±21.4*	133.4±15.3*	99.1±14.5*	74.4±5.6*

Values of creatine and phosphocreatine are expressed in nmol/mg protein. Data are mean±SEM for $n=6$ per group. # $P<0.05$ compared with contralateral striatum and * $P<0.05$ compared with Sal–MMA by F test for simple effect.

cerebral creatine increase, with almost no effect on cerebral phosphocreatine levels. It is possible that the discrepancy between our results has emerged due to a blood–brain barrier damage caused by the cannula insertion into the striatum in our study, which may have facilitated creatine diffusion into the brain. Facilitating creatine entry into the CNS would also facilitate phosphocreatine production.

We also showed in the present study that NaCl (9 μmol/striatum) injection caused a significant decrease in the striatal creatine and phosphocreatine (see Table 2) content, compared with the respective non-injected hemisphere. We attribute this effect of NaCl administration to a possible Na^+,K^+-ATPase activation by Na^+ overload in the injected structure. Since a substantial body of evidence supports a direct functional coupling of creatine kinase and Na^+,K^+-ATPase (Hemmer and Walliman, 1993; Wyss and Daddurah-Daouk, 2000), it is possible that sodium-pump activation may underlie the presently described decrease in the striatal phosphocreatine content by NaCl.

Although the main known effect of creatine in the literature is increase of high-energy phosphate levels, it has also other effects that include a direct antioxidant effect (Lawler et al., 2002), interaction with the benzodiazepine receptor (Kawasaki et al., 2001) and depression of neuronal excitability (Parodi et al., 2003). All these effects could in theory explain, at least in part, the observed protection of creatine, particularly the radical scavenging action and the anticonvulsant activity. These considerations are particularly worth of remark, because antioxidant agents such as α-tocopherol, ascorbate (Fighera et al., 1999) and GM1 ganglioside (Fighera et al., 2003) decrease MMA-induced convulsions.

In this study we also show that creatine, at doses capable of preventing MMA-induced lipoperoxidation (Fig. 1) and protein carbonyl formation (Fig. 2) did not prevent MMA-induced convulsions, and that a high dose of creatine (12 mg/kg; i.p.) effectively attenuated MMA-induced convulsions and oxidative damage. These results suggest that selected targets, which could not contribute significantly to the total protein carbonyl and lipoperoxidation, could be responsible for the MMA-induced convulsions. Furthermore, if these targets were more sensitive to oxidative damage, they would require additional antioxidant protection, which could be afforded by increasing creatine doses.

In this context, it has been reported that protein modifications elicited by direct oxidative attack on lysine, arginine, proline or threonine or by secondary reaction of cysteine, histidine or lysine can lead to the formation of protein carbonyl in several diseases (Dean et al., 1997) but that, in some cases, only selected proteins show increases in the levels of carbonylation (Castegna et al., 2002).

Notwithstanding, it has been suggested that creatine may exert a selective antioxidant role by maintaining mitochondrial bioenergetics. Indeed, it has been claimed that the neuroprotective effect exerted by creatine in several neurodegenerative processes involves buffering of intracellular energy reserves and preventing the opening of the mitochondrial permeability transition pore (MTP), which ultimately increases Ca^{2+} and ROS intramitochondrial levels and causes excitotoxic and apoptotic cell death (O'Gorman et al., 1997; Leist and Nicotera, 1998; Lipton and Nicotera, 1998; Stachowiak et al., 1998). This fits well with the recent findings that MMA opens MTP, and that catalase prevents MMA-induced MTP opening and citotoxicity (Maciel et al., 2004). Therefore, one might also argue that creatine may decrease total protein carbonylation and lipoperoxidation by attenuating MMA-induced ROS production by mitochondria. Accordingly, Wyse et al. (2000) have demonstrated that MMA-induced lipoperoxidation in vitro depends on the presence of mitochondria, further indicating that this organelle is primarily involved in MMA-induced lipoperoxidation, and probably in protein carbonylation. Consequently, it is also possible that stabilization of mitochondrial function may underlie the currently reported antioxidant action exerted by creatine. Since phosphocreatine/creatine/creatine kinase system is fundamental for normal energy homeostasis and considering that inhibition of mitochondrial creatine kinase (Mi-CK) activity by MMA plays an important role in the MMA-induced toxicity (Schuck et al., 2004), it seems reasonable to suggest that stabilization of the octameric structure of Mi-CK and maintenance of local energy buffered system induced by creatine prevents oxidative stress and excitotoxicity induced by MMA.

We do not know the reason why MMA concentrations within the molar range are necessary to cause significant behavioral or morphological effects when injected into the striatum of animals (Narasimhan et al., 1996; de Mello et al., 1996; Marisco et al., 2003; Fleck et al., 2004), while concentrations within the millimolar range cause neurochemical and morphological alterations in vitro, and are supposed to cause the neurological alterations in patients (Wajner et al., 1992; Toyoshima et al., 1995; Fontella et al., 2000; Kölker et al., 2000; Wyse et al., 2000; Brusque et al., 2001, 2002). However, we may speculate that this phenomenon may be a related to the model itself, since it occurs with a wide range of drugs or neurotoxins, including malonic, succinic, pyroglutamic and glutamic acid (Mangano and Schwarcz, 1983; Rieke et al., 1984; Davolio and Greenamyre, 1995; Malcon et al., 2000; Moy et al., 2000; Zeevalk et al., 2002; Maragos et al., 2002; Maciel et al., 2004; Roehrs et al., 2004; Fleck et al., 2004; Fernández-Gomez et al., 2005; Goni-Allo et al., 2005).

In fact, many studies have shown that drug concentrations necessary to cause neurotoxicity (or a behavioral correlate, such as convulsions) in vivo are much higher than those used to cause neurotoxicity or electrophysiological alterations in vitro. A possible explanation for such a difference may be a more effective diffusion, compartmentalization and metabolization of these compounds in vivo, which could rapidly decrease their concentration in the injected tissue. Regardless of the cause for this discrepancy, the results obtained with these models agree with the vast majority of the in vitro studies (Wajner et al., 1992). Even considering the significant agreement between these models, caution regarding the interpretation of these studies in which high concentrations of neurotoxins are injected into the brain is advisable.

In summary, although further in-depth studies are necessary to definitely establish a role for ROS in the development of

MMA-induced convulsions, the currently reported antioxidant action of creatine reinforces a neuroprotective role for this amino acid against oxidative insults and excitotoxicity (Matthews et al., 1998; Klivenyi et al., 1999; Kölker et al., 2000; Ferrante et al., 2000). Due to the lack of significant adverse effects of creatine supplementation (Stöckler et al., 1996; Volek and Rawson, 2004), and to the striking neuroprotective actions of this amino acid against several MMA-induced effects (Royes et al., 2003), the authors suggest, in an empirical basis, that creatine supplementation may be useful as an adjuvant therapeutic measure for methylmalonic acidemic patients. However, further studies shall be conducted to investigate whether creatine supplementation benefits these patients.

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