



Methylmalonate-induced seizures are attenuated in inducible nitric oxide synthase knockout mice

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ABSTRACT

Methylmalonic acidemias consist of a group of inherited neurometabolic disorders caused by deficiency of methylmalonyl-CoA mutase activity clinically and biochemically characterized by neurological dysfunction, methylmalonic acid (MMA) accumulation, mitochondrial failure and increased reactive species production. Although previous studies have suggested that nitric oxide (NO) plays a role in the neurotoxicity of MMA, the involvement of NO-induced nitrosative damage from inducible nitric oxide synthase (iNOS) in MMA-induced seizures are poorly understood. In the present study, we showed a decrease of time spent convulsing induced by intracerebroventricular administration of MMA (2 $\mu\text{mol}/2 \mu\text{L}$; i.c.v.) in iNOS knockout (iNOS^{-/-}) mice when compared with wild-type (iNOS^{+/+}) littermates. Visual analysis of electroencephalographic recordings (EEG) showed that MMA injection induced the appearance of high-voltage synchronic spike activity in the ipsilateral cortex which spreads to the contralateral cortex while quantitative electroencephalographic analysis showed larger wave amplitude during MMA-induced seizures in wild-type mice when compared with iNOS knockout mice. We also report that administration of MMA increases NOx (NO₂ plus NO₃ content) and 3-nitrotyrosine (3-NT) levels in a greater extend in iNOS^{+/+} mice than in iNOS^{-/-} mice, indicating that NO overproduction and NO-mediated damage to proteins are attenuated in iNOS knockout mice. In addition, the MMA-induced decrease in Na⁺, K⁺-ATPase activity, but not in succinate dehydrogenase (SDH) activity, was less pronounced in iNOS^{-/-} when compared with iNOS^{+/+} mice. These results reinforce the assumption that metabolic collapse contributes for the secondary toxicity elicited by MMA and suggest that oxidative attack by NO derived from iNOS on selected target such as Na⁺, K⁺-ATPase enzyme might represent an important role in this excitotoxicity induced by MMA. Therefore, these results may be of value in understating the pathophysiology of the neurological features observed in patients with methylmalonic acidemia and in the development of new strategies for treatment of these patients.

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1. Introduction

Methylmalonic acidemia comprise a group of inherited metabolic disorders caused by either a deficiency of the mitochondrial enzyme methylmalonyl CoA mutase (MCM, EC 5.4.99.2), or defects

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in the synthesis of 5'-deoxyadenosylcobalamin, the cofactor of MCM. Deficient MCM activity, which physiologically catalyses the reaction of methylmalonyl CoA to succinyl CoA, leads to the primary accumulation of methylmalonyl CoA, and a secondary accumulation of other metabolites, such as succinate, propionate, 3-hydroxypropionate, and 2-methylcitrate (Fenton and Rosenberg, 1995; Okun et al., 2002; Kolker et al., 2003). The major long-term complications are chronic renal failure, cardiomyopathy and neurological deficits, including lethargy, hypotonia/hypertonia, myoclonus, psychomotor delay/mental retardation (Fenton and Rosenberg, 1995; Touati et al., 2006; Morath et al., 2007). Furthermore, infants with this inborn error of metabolism may become debilitated and septic rather quickly, however, the presence of sepsis not exclude consideration of other possibilities such development of convulsion (Burton, 1998).

In this context, it has been shown that intrastriatal administration of MMA besides causing convulsive behavior, increases protein carbonylation and thiobarbituric acid reacting substances (TBARS) (Malfatti et al., 2003; Royes et al., 2006) indicating the involvement of reactive species in the genesis and/or propagation of convulsions elicited by this organic acid. Accordingly, MMA-induced convulsions are exacerbated by ammonia (Marisco et al., 2003), which also increases tissue lipoperoxidation. These data are corroborated by findings that MMA induces dose-dependent lipoperoxidation *in vitro* (Fontella et al., 2000) and *ex vivo* (Fontella et al., 2000; Malfatti et al., 2003; Marisco et al., 2003; Figuera et al., 2003) following by impairment of Na⁺, K⁺-ATPase activity (Wyse et al., 2000; Royes et al., 2006), a key enzyme activity in the maintenance of ionic gradients.

Furthermore, recent findings from our group have suggested a differential involvement of nitric oxide synthase (NOS) on convulsive behavior and oxidative damage to proteins elicited by MMA. While the intrastriatal injection of NG-Nitro-L-arginine methyl ester (L-NAME), a non-selective NOS inhibitor, exerts a biphasic modulation of MMA-induced convulsive activity and protein carbonylation (Royes et al., 2005), a striatal NO depletion elicited by 7-nitroindazol (7-NI) exacerbates seizures, protein carbonylation and Na⁺, K⁺-ATPase activity inhibition induced by MMA (Royes et al., 2007).

Inducible nitric oxide synthase (iNOS, EC 1.14.13.39) is one of three NOS isoforms generating NO by conversion of L-arginine to L-citrulline (Pacher et al., 2007). iNOS is the isoform which contributes to exacerbation of inflammatory and degenerative conditions thought the excessive NO production and consequent reactive nitrogen species generation (RNS) (Madrigal et al., 2006; Pacher et al., 2007). In line of this view, genetic animal models have contributed significantly to understand aetiopathologies of epilepsies (Buchhalter, 1993; Burgess and Noebels, 1999). Recently, it has been demonstrated that iNOS knockout mice reach the kindled status induced by PTZ more slowly when compared to wild-type mice and it is also different from other mice strains (De Sarro et al., 1996; De Luca et al., 2005, 2006).

Since pharmacological and neurochemical evidence support that inflammation may be a common factor contributing or predisposing, to occurrence of seizures in various forms of epilepsy of different etiologies (Vezzani and Granata, 2005) it is rather possible that iNOS knockout mice present decreased MMA-induced seizure and oxidative stress when compared with wild-type littermates. Therefore, the current study was designed to investigate the possible mechanisms involved in the toxicity induced by MMA in iNOS^{-/-} versus iNOS^{+/+} mice.

2. Experimental procedures

2.1. Animal and reagents

Experiments were conducted using iNOS^{+/+} and iNOS^{-/-} mice, kept in a controlled room temperature (22 ± 2 °C) and humidity (60–80%) under a 12 h light/

dark cycle (lights on 6:00 A.M.). iNOS knock-out mice were on the C57BL/6 background, constructed as described previously (MacMicking et al., 1995). The mice used at the beginning of this study were male and female from 63 to 80 days old and weighted 24–30 g. Animal utilization reported in this study have been conducted in accordance with the policies of the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80–23) revised in 1996. All efforts were made to reduce the number of animals used, as well as minimize their suffering. All reagents were purchased from Sigma (St. Louis, MO, USA).

2.2. Placement of cannula and behavioral evaluation

Wild-type and iNOS knockout mice ($n = 8–10$ in each group) were anesthetized with ketamine (100 mg/kg, *i.p.*) and xilazine (30 mg/kg, *i.p.*) and placed in a rodent stereotaxic apparatus. Under stereotaxic guidance, a cannula was inserted into the right lateral ventricle (coordinates relative to bregma: AP 0 mm, ML 0.9 mm, V 1.8 mm from the dura). Chloramphenicol (200 mg/kg, *i.p.*) was administered immediately before the surgical procedure.

After a recovery period of three days, animals received intracerebroventricular injection of NaCl (2 μmol/2 μL) or MMA (2 μmol/2 μL). All intracerebroventricular injections were performed by using a needle (30 gauge) protruding 1 mm below a 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. The dose of MMA used in the present study was selected based on pilot dose–response experiments.

Immediately after the NaCl or MMA 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 20 min and during this time the mice were observed for the appearance of convulsive behavior, defined by the occurrence of myoclonic jerks and clonic movements involving hindlimbs and forelimbs contralateral to the injected site. In addition the animals were observed for appearance of generalized tonic-clonic convulsive episodes characterized by whole-body clonus involving all four limbs and tail followed by sudden loss of upright posture and autonomic signs, such hyper-salivation and defecation respectively. The onset time for the first convulsive episode (characterized by appearance of myoclonic jerks and clonic movements) and the sum of the duration of all convulsions presented by mice during the behavioral evaluation period (total time spent convulsing) was recorded using a stopwatch according de Mello et al. (1996).

2.3. Placement of cannula and electrodes and EEG recordings

A subset of animals ($n = 6$ in each group) were anesthetized with ketamine (100 mg/kg, *i.p.*) and xilazine (30 mg/kg, *i.p.*) and surgically implanted with a cannula and electrodes under stereotaxic guidance for the purpose of EEG recording. The guide cannula was glued to a multipin socket and inserted into the right ventricle through a previously opened skull orifice. Two screw electrodes were placed over the right (ipsilateral) and left (contralateral) parietal cortices (coordinates in mm: AP -4.5 and L 2.5), along with a ground lead positioned over the nasal sinus. The electrodes were connected to a multipin socket and fixed to the skull with dental acrylic cement. The EEG recordings were performed 7 days after surgery.

The procedures for EEG recording were carried out as previously described by Cavalheiro et al. (1992). Briefly, the animals were allowed to habituate to a Plexiglas cage (25 cm × 25 cm × 60 cm) for at least 30 min before the EEG recordings. Animals were then connected to the lead socket in a swivel inside a Faraday's cage. EEG was recorded using a digital encephalographer (Neuromap EQSA260, Neurotec LTDA, Itajubá, MG, Brazil). EEG signals were amplified, filtered (0.1–70.0 Hz, bandpass), digitalized (sampling rate 256 Hz) and stored in a PC for off-line analysis. Routinely, a 10 min baseline recording was obtained to establish an adequate control period. After baseline recording, NaCl or MMA were administered and mice were observed for 30 min for the appearing of behavioral convulsions, as described above. EEG recordings were visually analyzed for seizure activity, which were defined by isolated sharp waves (≥1.5 X baseline); multiple sharp waves (≥2 X baseline) in brief spindle episodes (≥1 s, ≥5 s); multiple sharp waves (≥2 X baseline) in long spindle episodes (≥5 s); spikes (≥2 X baseline) plus slow waves; multispikes (≥2X baseline, ≥3 spikes/complex) plus slow waves; major seizure (repetitive spikes plus slow waves obliterating background rhythm, ≥5 s). EEG spikes amplitude was calculated as variations of values (μV) before and after drug administration. Rhythmic scratching of the electrode headset by the animal rarely caused artifacts. These recordings were easily identified and discarded.

2.4. Tissue processing for neurochemical analyses

Immediately after the behavioral evaluation, the animals were killed by decapitation and had their brain exposed by the removal of the parietal bone. Cerebral cortex was dissected on an inverted ice-cold Petri dish and homogenized in cold 10 mM Tris-HCl buffer (pH 7.4) containing 0.5 mM EDTA and 320 mM sucrose. The homogenized was then divided in aliquots for subsequent neurochemical analyses, as described below.

2.5. Assay of NOx (NO₂ plus NO₃) as a marker of NO synthesis

For NOx determination, an aliquot (200 μ L) was homogenized in 200 mM Zn₂SO₄ and acetonitrile (96%, HPLC grade). After, the homogenate was centrifuged at 16,000 \times g for 20 min at 4 °C and supernatant was separated for analysis of the NOx content as described by Miranda et al. (2001). The resulting pellet was suspended in NaOH (6 M) for protein determination.

2.6. Slot blot assay for 3-nitrotyrosine

3-Nitrotyrosine immunoreactivity is a marker of oxidative nitric oxide damage and was determined as previously described by Joshi et al. (2006). Briefly, sample (5 μ L) (normalized to 4 μ g/mL), 5 μ L of 12% SDS and 5 μ L of modified Laemmli buffer containing 0.125 M Tris base pH 6.8, 4% (v/v) SDS, and 20% (v/v) glycerol were incubated for 20 min at room temperature, and the membranes were developed as described above except a 1:2000 dilution of anti-3-NT polyclonal antibody was used. Blots were dried, scanned with Adobe Photoshop, and quantified with Scion Image (PC version of Macintosh compatible NIH image). The 3-NT blot had a faint background that was corrected in image analysis.

2.7. Na⁺, K⁺-ATPase activity measurements

Assay of Na⁺, K⁺-ATPase activity was performed according Wyse et al. (2000). Briefly, the reaction medium consisted of 30 mM Tris-HCl buffer (pH 7.4), 0.1 mM EDTA, 50 mM NaCl, 5 mM KCl, 6 mM MgCl₂, and 50 μ g of protein in the presence or absence of the Na⁺, K⁺-ATPase inhibitor ouabain (1 mM), in a final volume of 350 μ L. The reaction was started by the addition of adenosine triphosphate (ATP) to a final concentration of 5 mM. After 30 min at 37 °C, the reaction was stopped by the addition of 70 μ L 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 non-enzymatic 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).

2.8. Succinate dehydrogenase (SDH) activity measurements

For SDH activity assay, a sample (500 μ L) was centrifuged at 1000 \times g for 10 min and the resulting supernatant was centrifuged at 12,000 \times g for 20 min. All procedures were performed at 4 °C. The pellet was suspended in 270 mM potassium PO₄ buffer, pH 7.2, containing 250 mM sucrose, 5 mM MgCl₂, 20 mM glucose, and 0.85% NaCl (buffer B) and frozen for 24 h. The protein content was adjusted to 1 mg/mL with buffer B. Succinate dehydrogenase activity was assayed as previously described by Dutra et al. (1993) using 2,6-dichlorophenolindophenol (DCIP) as the electron acceptor in the presence of phenazine methosulfate. The reaction mixture (1500 μ L) contained 50 mM potassium PO₄ buffer, pH 7.5, 1.5 mM KCN, 30 μ M DCIP, 3 μ g rotenone, 5 mM sodium succinate, 0.5 mM phenazine methosulfate. The mixture was preincubated for 10 min at 37 °C and the reaction started by the addition of the mitochondrial fraction (50 μ g of protein). The reduction of DCIP was measured spectrophotometrically by monitoring the fall of absorbance at 600 nm for 30 s.

2.9. Protein determination

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

2.10. Statistical analysis

Statistical analysis was carried out by one- or two-way analysis of variance (ANOVA) when appropriated. Post hoc analysis was carried out, when appropriate, by the Student–Newman–test. *P* and *F* values are presented only if *P* < 0.05.

3. Results

The effect of intracerebroventricular administration of MMA on behavioral convulsions in iNOS^{+/+} and iNOS^{-/-} mice is shown in Fig. 1. Behavioral and statistical analysis revealed that iNOS knockout (iNOS^{-/-}) had not effect on latency for the first convulsion induced by MMA when compared with wild-type littermates (Fig. 1A). However, the time spent in convulsive episodes in iNOS^{-/-} mice was significant lower when compared with wild-type littermates [*F*(1,37) = 6.29; *P* < 0.05, Fig. 1B]. Furthermore, behavioral and EEG recordings revealed a similar convulsive behavior after administration MMA between male and female group of animals (*data not shown*), suggesting that possible changes in

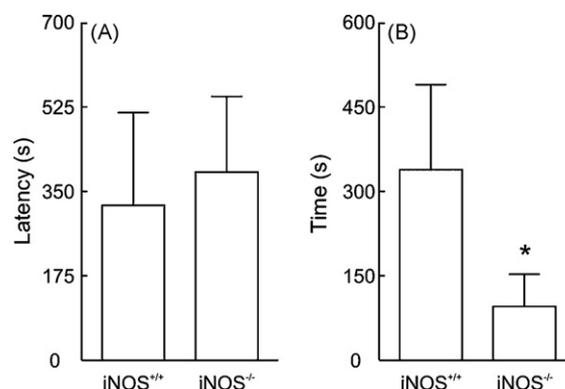


Fig. 1. (A) Latency for onset and (B) total time spent in seizures induced by MMA administration (2 μ mol/2 μ L; i.c.v.) in iNOS^{+/+} and iNOS^{-/-} mice. **P* < 0.05 compared with iNOS^{+/+} mice. Data are mean \pm S.E.M. for *n* = 8–10 in each group.

hormonal secretion at all levels of the reproductive neuroendocrine axis in both groups of animals had not effect on convulsive episodes induced by this organic acid. EEG recordings also confirmed behavioral seizures elicited by MMA in iNOS^{-/-} and iNOS^{+/+} mice.

The EEG recordings before and after MMA injection (2 μ mol/2 μ L; i.c.v.) in iNOS^{+/+} mice is shown in Fig. 2A and B. The following

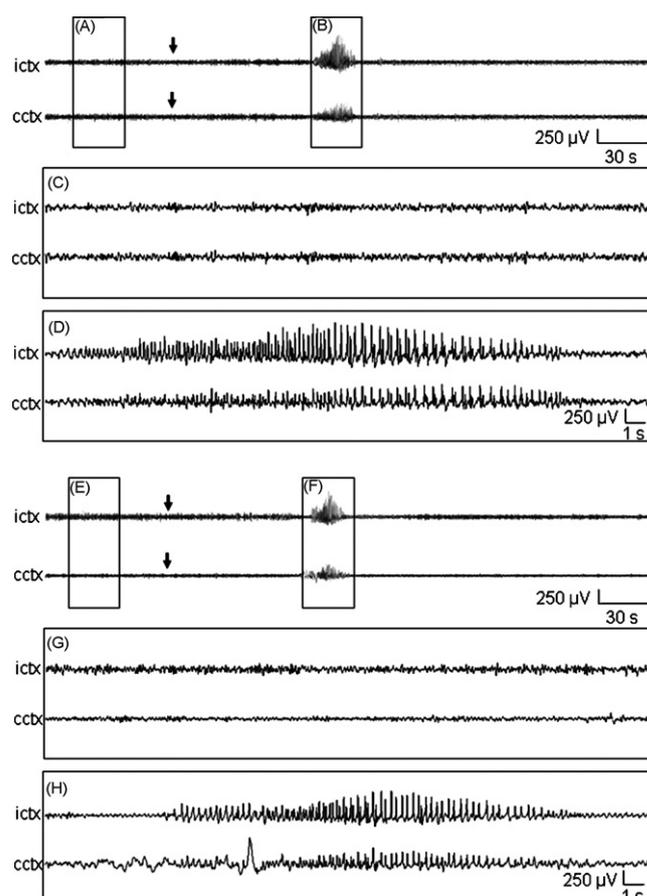


Fig. 2. Representative electroencephalographic recordings obtained in ipsi (ictx) and contralateral cortex (cctx) before (A) and after intracerebroventricular of MMA (2 μ mol/2 μ L) in iNOS^{+/+} mice (B). The arrow indicates MMA administration and the expanded waveforms from the EEG recording outline by boxes (A and B) are shown in (C and D) respectively. Representative electroencephalographic recordings obtained before (E) and after (F) the intracerebroventricular of MMA (2 μ mol/2 μ L) in iNOS^{-/-} mice. The typical seizure sequences observed after MMA injection were accompanied by the behavioral alterations described in the Results section. The arrow indicates MMA administration and the expanded waveforms from the EEG recording outline by boxes (E) and (F) are shown in G and H respectively.

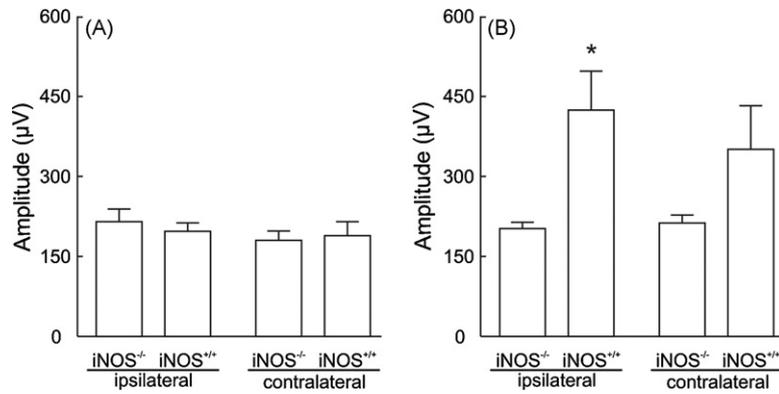


Fig. 3. Quantitative analysis of EEG recordings for wave amplitude in ipsilateral and contralateral cortex of iNOS^{+/+} and iNOS^{-/-} mice (A) before and (B) after MMA injection (2 μmol/i.c.v.). **P* < 0.05 compared with wild-type (iNOS^{-/-}) mice. Data mean + S.E.M. for *n* = 6 in each group.

behavioral repertoire observed in iNOS^{+/+} mice occurred concomitantly with electrographic recorded seizures: generalized seizures were characterized by the appearance of 2–3 Hz high-amplitude activity. These epileptic discharges (interictal spikes) were defined as abnormal paroxysmic in the cerebral cortex and consisted of high-amplitude biphasic sharp transients. Furthermore, EEG recordings revealed that MMA induced the appearance of high-voltage synchronic spike clusters in the ipsilateral cortex which spread to the contralateral cortex in wild-type mice (Fig. 2D).

The Fig. 2E and F showed the representative EEGs before and after MMA injection in iNOS^{-/-} mice, respectively. EEG recordings confirmed previous behavioral analysis since showed a similar latency for the first convulsion between iNOS^{+/+} and iNOS^{-/-} mice (Fig. 1A). On the other hand, EEG recordings revealed a decrease of ictal activity in iNOS^{-/-} mice (Fig. 2H) when compared with wild-type littermates (Fig. 2D). This wave pattern alteration observed in iNOS knockout mice corroborated with quantitative analyses of EEG recordings that showed a significant decrease in the amplitude of seizure spikes in ipsilateral cortex of iNOS^{-/-} mice (202 μV) when compared with ipsilateral cortex of iNOS^{+/+} mice (424 μV) after period of observation (20 min) [*F*(1,11) = 9.14; *P* < 0.05, Fig. 3B].

Fig. 4 shows the effect of intracerebroventricular injection of MMA on cerebral NO_x production in iNOS^{+/+} and iNOS^{-/-} mice. Statistical analysis revealed that MMA increased NO_x levels in cerebral cortex of wild-type iNOS^{+/+}, but not in iNOS^{-/-} mice [*F*(1,37) = 19.24; *P* < 0.05; Fig. 4A]. In addition, quantitative image analysis of NO-mediated nitrative damage to proteins (3-NT) revealed a significant increase of 3-NT immunoreactivity in iNOS^{+/+}

when compared with iNOS^{-/-} mice [*F*(1,37) = 8.69 *P* < 0.05, Fig. 4B] after MMA injection.

Considering that Na⁺, K⁺-ATPase activity correlates with time spent in MMA-induced convulsions (Royes et al., 2006) and this enzyme is also sensitive to NO overproduction (Moro et al., 2005), we also investigated whether there are differences in MMA-induced Na⁺, K⁺-ATPase activity inhibition in iNOS^{+/+} and iNOS^{-/-} mice. Statistical analysis showed that intracerebroventricular injection of MMA decreased Na⁺, K⁺-ATPase activity in iNOS^{+/+} and iNOS^{-/-} mice [*F*(1,37) = 22.42; *P* < 0.05, Fig. 5A]. In addition, statistical comparison between groups showed a higher inhibition of Na⁺, K⁺-ATPase activity in iNOS^{+/+} mice when compared with iNOS^{-/-} mice, reinforcing the idea that oxidative attack of select target such Na⁺, K⁺-ATPase represents a important role in the propagation of MMA-induced convulsive behavior (Royes et al., 2006).

Since it has been suggested that MMA induces convulsions through impairment of mitochondrial function (de Mello et al., 1996; Royes et al., 2003) and considering that SDH activity is especially sensitive to NO overproduction (Giulivi, 2003; Guix et al., 2005) we investigate whether iNOS knockout mice present altered sensitivity to SDH inhibition by MMA. Statistical analysis revealed that MMA injection induced a significant decrease in SDH activity of similar magnitude in both groups of mice [*F*(1,37) = 5.96; *P* < 0.05, Fig. 5B].

4. Discussion

In the present study we show that iNOS knockout mice present decreased MMA-induced seizure susceptibility compared with

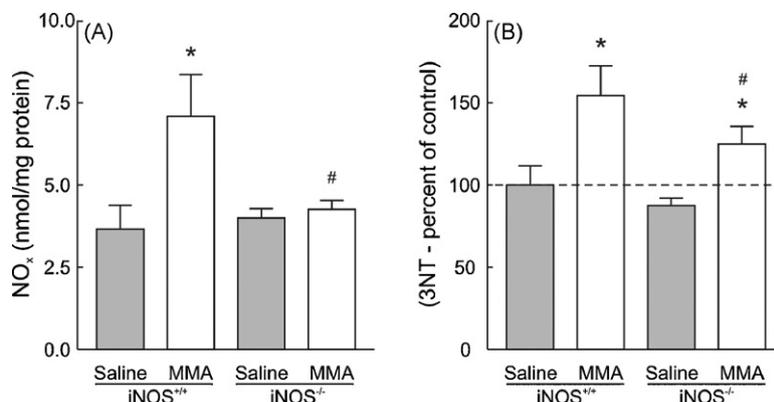


Fig. 4. The effect of MMA injection (2 μmol/2 μl; i.c.v.) on NO_x content (NO₂ plus NO₃ levels; A) and 3-Nitrotyrosine immunoreactivity (B) from cerebral cortex of iNOS^{+/+} and iNOS^{-/-} mice. **P* < 0.05 compared with wild-type mice treated with saline; #*P* < 0.05 compared with wild-type mice treated with MMA. Data are mean + S.E.M. for *n* = 8–10 in each group.

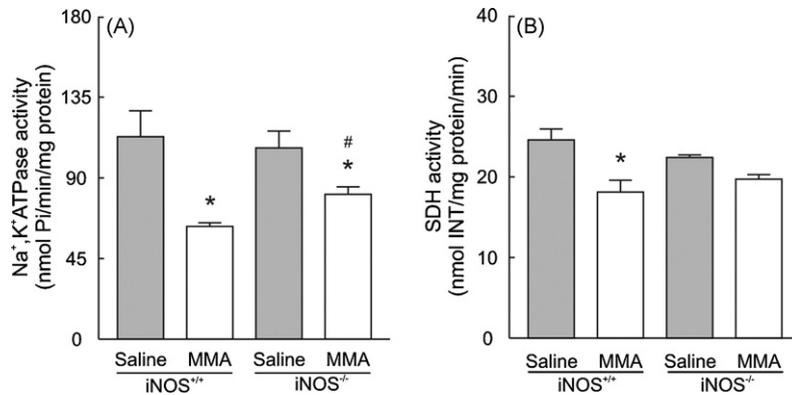


Fig. 5. The effect of MMA injection (2 μ mol/2 μ l; i.c.v.) on Na⁺, K⁺-ATPase activity (A) and SDH activity (B) from cerebral cortex of iNOS^{+/+} and iNOS^{-/-} mice. * $P < 0.05$ compared with wild-type mice treated with saline; # $P < 0.05$ compared with wild-type mice treated with MMA. Data are mean \pm S.E.M. for $n = 8$ –10 in each group.

wild-type littermates, suggesting the participation of iNOS in the convulsive behavior elicited by this organic acid. We also report that intracerebroventricular administration of MMA increases NOx and 3-NT levels in a greater extent in iNOS^{+/+} mice than in iNOS^{-/-} mice, indicating that NO overproduction and NO-mediated damage to proteins are attenuated in iNOS knockout mice. In addition, we show that MMA-induced decrease in Na⁺, K⁺-ATPase activity, but not in SDH activity, is less pronounced in iNOS^{-/-} mice when compared with wild-type littermates.

Recently, experimental findings from our group have evidenced the participation of NO in MMA-induced seizures and oxidative damage to proteins (Royes et al., 2005, 2007). In this context, the administration of low doses of the non-selective NOS inhibitor NG-Nitro-L-arginine methyl ester attenuates MMA-induced convulsions and protein carbonylation in rat striatum, while high doses of L-NAME have no effect on these parameters (Royes et al., 2005). Moreover, the administration of 7-nitroindazol, a preferential neuronal NOS inhibitor, increased seizures and protein carbonylation induced by MMA (Royes et al., 2007), suggesting a differential contribution of NOS isoforms to MMA-induced seizures and protein carbonylation.

Although it is believed that NO exerts an important role on neuronal hyperexcitability evidenced in several seizure disorders, (De Sarro et al., 1996; Paoletti et al., 1998; Borowicz et al., 2000; Itoh et al., 2004; de Vasconcelos et al., 2004; Kato et al., 2005), it is difficult to make a clear conclusion on the involvement of this free radical in epileptiform activity. The determining factor for such a discrepancy is not known, but one might argue that methodological differences may account for it. Another interesting possibility is that the effect of NO on convulsions may vary with the model of seizure employed and/or particular brain structures studied (Libri et al., 1997). Thus, since the role of NO in the pathophysiology of convulsions induced by MMA is not completely defined, the genetic animals models as iNOS^{-/-} mice may be considered a valid genetic animal model to investigate the role of iNOS in the convulsive behavior elicited by this organic acid. In this context, experimental findings described by De Luca et al. (2006) demonstrated that iNOS^{-/-} mice reach the kindled status induced by pentylentetrazole (PTZ) more slowly and presented lower levels of glutamate and higher levels of GABA when compared than iNOS^{+/+} after PTZ-induced kindling.

In the present study, we show novel data indicating a role for iNOS-derived NO in the convulsive episodes induced by MMA, since the total time spent in MMA-induced seizures was significantly shorter in iNOS^{-/-} when compared with iNOS^{+/+} mice. These results indicate that there are clear differences in the relative contribution of NOS isoforms to MMA-induced seizures,

and, in light of these results, one may suggest that nNOS-derived NO may be protective, while iNOS-derived NO may be pro-convulsant. Although a number of studies have shown that there is no constitutive expression of iNOS in brain (Zheng et al., 1993; Campbell et al., 1994; Iadecola et al., 1995), other studies have suggested that iNOS is not only inducible, but also expressed constitutively on several cell types and tissues, including the brain (Park et al., 1996; Starkey et al., 2001; Buskila et al., 2005). In fact, in the cerebral cortex, a brief application of glutamate triggered a rapid (1–2 min) and massive iNOS-dependent NO production, which may suggest that constitutively expressed iNOS in the brain may contribute to physiological and pathological processes in this tissue (Buskila et al., 2005).

On the other hand, although genetic animals have contributed significantly to our understanding of the aetiopathologies of epilepsy (Buchhalter, 1993; Burgess and Noebels, 1999), the exact underlying mechanism involving iNOS-dependent NO production in this model of neurological disease are poorly known. Recently, de Luca and colleagues (2006), have suggested that the inability of iNOS^{-/-} mice to increase the NO levels following PTZ administration indicate that this free radical plays a pro-epileptogenic role of some types of epilepsy.

Therefore, since the neurons are capable of rapid release of small amounts of NO serving as neurotransmitter and astrocytic NO production has been demonstrated mainly as slow reaction to various stress stimuli (Mander et al., 2005), it is reasonable to propose that initial NO production might be a counteracting response to convulsive episodes, while a massive iNOS-dependent NO production by astrocytes may bear important implications for maintenance of convulsive behavior evidenced in this model of organic aciduria. In agreement of this view, recent experimental findings from our group have demonstrated that while striatal NO depletion exacerbates seizures, protein carbonylation and Na⁺, K⁺-ATPase activity inhibition, the increase of NO production induced by L-arginine injection attenuates MMA-induced behavioral, electroencephalographic and neurochemical deleterious effects (Royes et al., 2007).

The present study also revealed a role for NO derived from iNOS in MMA-induced decrease in Na⁺, K⁺-ATPase activity. This enzyme has been considered a target especially sensitive to free radical damage (Jamme et al., 1995; Morel et al., 1998), including NO-mediated damage (Moro et al., 2005), and a decrease in its activity has been associated with the appearance and/or propagation of seizures induced by MMA (Malfatti et al., 2003; Royes et al., 2007). In fact, recent studies from our group have demonstrated that duration of convulsive episodes induced by injection intra-striatal of MMA and glutaric acid (GA) correlates with Na⁺, K⁺-ATPase

activity inhibition (Royes et al., 2006; Figuera et al., 2006). Therefore, since the MMA-induced increase in NOx and 3-NT levels and the decrease in Na⁺, K⁺-ATPase activity was larger in iNOS^{+/+} mice than in iNOS^{-/-} mice, we suggest that nitrosative attack by iNOS-derived NO play a role, at least in part, in MMA-induced decrease in Na⁺, K⁺-ATPase activity. Moreover, it is plausible to propose that iNOS knockout mice presented less severe seizures than wild-type mice because MMA-induced decrease in Na⁺, K⁺-ATPase activity was smaller in this mice cohort.

A significant body of evidence has demonstrated that MMA compromises mitochondrial functions (Dutra et al., 1993; Fleck et al., 2004; Maciel et al., 2004), leading to decreased CO₂ production (Wajner et al., 1992) and O₂ consumption (Toyoshima et al., 1995), decreased ATP/ADP ratio (McLaughlin et al., 1998), phosphocreatine content (Royes et al., 2003) and succinate-supported O₂ consumption (Maciel et al., 2004; Kowaltowski et al., 2006). Furthermore, brain mitochondrial swelling experiments demonstrate that MMA is an important inhibitor of succinate transport by dicarboxylate carriers (Mirandola et al., *in press*), suggesting that mitochondrial dicarboxylate carrier inhibition by MMA has important physiopathological implications, such impairment of neuronal energy metabolism and mitochondria-derived reactive species. In the line of this view, the results presented in this report revealed that extend of MMA-induced SDH activity inhibition was similar in both wild-type and iNOS knockout mice. In addition, these results suggest that the differences found in MMA-induced seizures in iNOS^{+/+} and iNOS^{-/-} mice are not due differences in SDH inhibition and reinforce the assumption that MMA-induced convulsive behavior is mediated by generation of reactive species (Figuera et al., 1999, 2003; Marisco et al., 2003; Malfatti et al., 2003) and secondary excitotoxicity (de Mello et al., 1996; Royes et al., 2003). In line of this view, considering that hyperactivation of glutamate receptors, especially the NMDA subtype, are involved in the convulsive behavior elicited by MMA (de Mello et al., 1996; Royes et al., 2003) and stimulates iNOS enzyme (Iravani et al., 2004; Mander et al., 2005), it might be possible that the activation of iNOS regulates somehow synaptic activity facilitating the propagation of convulsive episodes induced by MMA. On the other hand, it is important to point out that the presently observed MMA-induced convulsive state might also be interpreted as a consequence of oxidative-stress-induced by iNOS pathways after MMA injection, since the induction of enzymes such iNOS and cyclooxygenase-2 (COX-2) are responsible for a great portion of the neurological damage produced in several models of stress and epilepsy (Vezzani and Granata, 2005; Madrigal et al., 2006; Kamida et al., 2007). However, further studies are needed to clarify this point.

In summary, the present study reinforces the significant participation of NO in the excitotoxicity induced by MMA and reports novel data not only about the role of iNOS-derived NO in MMA-induced seizures and concomitant nitrosative damage elicited by this organic acid, but also show that there is a role for iNOS in acute exposure to excitotoxic agents. We think that these results may be of value in understating the pathophysiology of the neurological features observed in patients with methylmalonic acidemia and in the development of new strategies for treatment of these patients.

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