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Involvement of NO in the convulsive behavior and oxidative damage induced by the intrastriatal injection of methylmalonate

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Abstract

Acute intrastriatal administration of methylmalonic acid (MMA) induces convulsions through NMDA receptor-mediated mechanisms and increases production of end products of oxidative damage. Although it has been demonstrated that nitric oxide (NO) production increases with NMDA receptor stimulation and contributes to the oxidative damage observed in several neurodegenerative disorders, the role of NO in MMA-induced convulsions has not been investigated to date. In the present study we investigated the effects of the intrastriatal injection of N^{ω}-nitro-L-arginine methyl ester (L-NAME: 10^{-4} to 10^{0} nmol/0.5 μl) on the convulsions and striatal protein carbonylation induced by the intrastriatal injection of MMA (4.5 μmol/1.5 μl). L-NAME (10^{-3} to 10^{-1} nmol) protected against MMA-induced convulsions and protein carbonylation ex vivo. These results suggest the involvement of NO in the convulsive behavior and protein carbonylation elicited by MMA.

Keywords: Convulsion; Protein carbonylation; L-methylmalonic acid; Nitric oxide; Methylmalonic acidemia; L-NAME

Methylmalonic acidurias 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 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 [10,25,17]. A large percentage of affected infants die during the first weeks or month of life, and those who survive longer present a variable degree of mental retardation and other neurological abnormalities, like delayed development and seizures [29,4].

Experimental findings in vitro and ex vivo have shown that methylmalonic acid (MMA) inhibits succinate dehydrogenase (SDH) and β-hydroxybutyrate dehydrogenase [9,36,13] impairs mitochondrial function [6,25,36], increases lactate production [31,38], and decreases ATP levels [23], CO_2 production [38] and O_2 utilization [36]. In addition, we have demonstrated that intrastriatal injection of MMA induces convulsive behavior and excitotoxicity though NMDA receptor-mediated mechanisms [8,31].

A significant amount of work has suggested that NMDA receptors and reactive oxygen species (ROS) generation may underlie the neurotoxic effects of SDH inhibitors, such as 3-nitropropionate (3-NP) and malonate [14,33]. In this context, we have demonstrated that intrastriatal MMA administration, besides causing convulsive behavior, increases local thiobarbituric acid reactive substances (TBARS) and protein carbonylation [21,30]. Moreover, while the systemic administration of antioxidants, such GM1, ascorbic acid and α-

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tocopherol attenuate MMA-induced convulsions [11,12], the administration of ammonia, a substance that increases reactive species production, potentiates the convulsive behavior elicited by MMA [22]. These results suggest the involvement of reactive species, in addition to NMDA receptors [8], in the convulsive phenomenon elicited by MMA.

It has been suggested that a great deal of NMDA receptor activation-induced excitotoxicity is due to NO generation [18]. Accordingly, excessive production of nitric oxide (NO), a highly reactive species produced in tissues from L-arginine by the enzyme NO synthase (NOS), inhibits the mitochondrial respiratory chain, leading to mitochondrial damage [35]. In fact, several proteins involved in the energetic metabolism such as aconitase, SDH and creatine kinase (CK), are particularly sensitive to reactive species-induced damage [5,35] and to MMA [6].

Although there is convincing evidence of the participation of NO in the toxicity of glutamate, the role of NO in the pathophysiology of convulsive disorders are not completely defined. While some authors suggest an anticonvulsant role for NO [20], others suggest that it may be a proconvulsant agent [37]. The determining factor for such a discrepancy is not known, but one might argue that methodological differences may account for it. One interesting possibility is that the effect of NO on convulsions may vary with the model of seizure employed. As a matter of fact, it has been demonstrated that inhibition of NOS potentiates seizures induced by kainic acid and pilocarpine in rats [20,24,27]. Furthermore, the inhibition of NOS increases the convulsive behavior and susceptibility to seizures induced by NMDA [7,28]. Conversely, a decrease in tissue NO levels results in suppression of convulsions induced by pentylenetetrazol (PTZ) in rats [26]. Since the role of NO in the toxicity induced by MMA has been almost neglected in the literature, we decided to investigate the effect of N^{ω}-nitro-L-arginine methyl ester (L-NAME) administration, an inhibitor of NOS, on the MMA-induced convulsive behavior and striatal protein carbonylation ex vivo.

Adult male Wistar rats (270–300 g; $n = 8$–10 in each group) maintained under controlled light and environment (12 h light/12 h dark cycle, $24 \pm 1\,^{\circ}C$, 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 Ethics Committee of the Federal University of Santa Maria. All reagents were purchased from Sigma (St. Louis, MO, USA) and all solutions were prepared with type I ultra pure water. L-NAME was prepared in 100 mM phosphate buffered saline (pH 7.4, PBS).

The 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. Three days after the cannula placement, the animals received an intrastriatal injection of L-NAME (10^{-4} to 1 nmol/0.5 μl) or vehicle (PBS 100 mM/ pH 7.4) 30 min before administration of MMA (4.5 μmol/1.5 μl) or saline (6.7 μmol/1.5 μl).

After the injections, the animals were transferred to a round open field (54.7 cm in diameter) with a floor divided into 11 equal areas. The open field sessions lasted 15 min, and during this time the animals were observed for the appearance of convulsive behavior (myoclonic jerks and clonic movements involving hindlimbs and forelimbs contralateral to the injected striatum). The latency for the first convulsive episode and the total time spent convulsing were recorded [8].

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 striatum was rapidly obtained using a stainless steel puncher (5 mm in internal diameter) around the site of cannula placing. Tissues were 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 [39], adapted for brain tissue, as below. Briefly, homogenates were diluted to 750–800 (g/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, and 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. [19]. Protein content for biochemical experiments was measured colorimetrically by the method of Bradford [3] by using bovine serum albumin (1 mg/ml) as standard.

Data from ex vivo total carbonyl determinations were analyzed by a 2 (PBS or L-NAME) × 2 (saline or MMA) × 2 (injected or non-injected 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 latency for the first convulsive episode and total time spent convulsioning induced by MMA were analyzed by one-way ANOVA, followed by a Student–Newman–Keuls test. All data are expressed as mean + S.E.M.

The involvement of NO in the convulsant effects of MMA was investigated by intrastriatally injecting the animals with L-NAME (10^{-4} to 10^{0} nmol/0.5 μl). The striatal injection of L-NAME had no effect per se on behavioral parame-