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ORIGINAL RESEARCH

Experimental Evidence that Methylmalonic Acid Provokes Oxidative Damage and Compromises Antioxidant Defenses in Nerve Terminal and Striatum of Young Rats

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Abstract Methylmalonic acidemia and propionic acidemia are organic acidemias biochemically characterized by predominant tissue accumulation of methylmalonic acid (MMA) and propionic acid (PA), respectively. Affected patients present predominantly neurological symptoms, whose pathogenesis is not yet fully established. In the present study we investigated the in vitro effects of MMA and PA on important parameters of lipid and protein oxidative damage and on the production of reactive species in synaptosomes from cerebrum of developing rats. Synaptosomes correspond to nerve terminals that have been used to investigate toxic properties of compounds on neuronal cells. The in vivo effects of intrastriatal injection of MMA and PA on the same parameters and on enzymatic antioxidant defenses, were also studied. MMA-induced in vitro and in vivo lipid peroxidation and protein oxidative damage. Furthermore, the lipid oxidative damage was attenuated or prevented, pending on the doses utilized, by the free radical scavengers α-tocopherol, melatonin and by the NMDA glutamate receptor antagonist MK-801, implying the involvement of reactive species and glutamate receptor activation in these effects. In addition, 2′,7′-dichlorofluorescein diacetate oxidation was significantly increased in synaptosomes by MMA, reinforcing that reactive species generation is elicited by this organic acid. We also verified that glutathione peroxidase activity was inhibited by intrastriatal MMA injection. In contrast, PA did not induce any significant effect on all parameters examined in vitro and in vivo, implying a selective action for MMA. The present data demonstrate that oxidative stress is induced by MMA in vitro in nerve terminals and in vivo in striatum, suggesting the participation of neuronal cells in MMA-elicited oxidative damage.

Keywords Methylmalonic acidemia · Propionic acidemia · Methylmalonic acid · Propionic acid · Oxidative stress · Brain synaptosomes · Striatum

Introduction

Methylmalonic acidemia and propionic acidemia are frequent organic acidurias caused by a severe deficiency of methylmalonyl-CoA mutase (EC 5.4.99.2) and propionyl-CoA carboxylase activities (EC 6.4.1.3), respectively. They are biochemically characterized by predominant blood accumulation of methylmalonic acid (MMA) (1–2.5 mM) and propionic acid (PA) (5 mM), respectively, although the concentrations of 3-hydroxypropionic acid, methylcitric acid, propionylglycine, and tiglylglycine are also increased. Other laboratory findings include ketoacidosis, hypoglycemia, hyperglycinemia, hyperammonemia, neutropenia, and trombocitopenia (Fenton et al. 2001; Hörster et al. 2009). Affected patients usually present early in life lethargy, vomiting, dehydration, hepatomegaly, hypotonia, and

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encephalopathy that may lead to coma and death (Deodato et al. 2006; Manoli and Venditti 2010). Other neurological abnormalities are psychomotor delay/mental retardation, focal and generalized convulsions, EEG alterations, delayed myelination (progressive cortical atrophy), and hypodensity of the basal ganglia (Brismar and Ozand 1994; Chemelli et al. 2000; Harting et al. 2008).

Clinical presentation of propionic acidemia usually occurs in the newborn period with severe metabolic acidosis, vomiting, hypotonia, lethargy, seizures, and hepatomegaly, although in some patients symptoms may occur later, with acute encephalopathy, episodic ketoacidosis, and developmental retardation (Wolf et al. 1981; Surtees et al. 1992; Manoli and Venditti 2010). Treatment of these patients is difficult usually leading to neurological sequelae, including dystonia, chorea, pyramidal signs, developmental delay, focal and general convulsions, cerebral atrophy, and EEG abnormalities (Surtees et al. 1992).

Regarding methylmalonic acidemia, clinical presentation usually occurs in the first week of life and the most common signs and symptoms are lethargy, failure to thrive, recurrent vomiting, respiratory distress and hypotonia, although hepatomegaly and coma is relatively frequent (Shevell et al. 1993; Fenton et al. 2001). Neurological sequelae are similar to that of propionic acidemic patients.

On the other hand, although patients present severe neurological symptoms and cerebral abnormalities, the pathophysiology of brain damage in these disorders is only partly understood. In this context, it has been suggested that brain damage in methylmalonic and propionic acidemias is mainly driven by the accumulating metabolites since it appears that these endogenous compounds are produced and trapped in neural cells (Kölker et al. 2006; Stellmer et al. 2007; Sauer et al. 2006, 2010).

MMA has been demonstrated to provoke behavioral alterations, seizures and striatal lesions in rats after intrastriatal administration through activation of glutamate receptors, energy depletion and oxidative damage (de Mello et al. 1996; Wyse et al. 2000; Fighera et al. 2003; Malfatti et al. 2003; Ribeiro et al. 2005; Royes et al. 2003, 2005, 2006; Furian et al. 2007). Other experimental studies confirmed that impairment of brain mitochondrial energy metabolism, alterations of the redox status and glutamatergic neurotransmission may represent important pathomechanisms of MMA neurotoxicity (Wajner and Coelho 1997; McLaughlin et al. 1998; Fontella et al. 2000; Kölker et al. 2000; Brusque et al. 2001, 2002; Okun et al. 2002; Malfatti et al. 2003; Pettenuzzo et al. 2006).

Furthermore, there is solid evidence showing that oxidative stress and mitochondrial dysfunction play important roles as pathogenic mechanisms in patients and mice models with methylmalonic acidemia (Treacy et al. 1996; Richard et al. 2005, 2007, 2009; Chandler et al. 2009; de Keyzer et al. 2009; Ribas et al. 2010a, b).

Neurotoxic effects of PA have been also reported but to a lesser extent (Wyse et al. 1998; Brusque et al. 1999; de Mattos-Dutra et al. 2000; Fontella et al. 2000; Pettenuzzo et al. 2002; Trindade et al. 2002; Rigo et al. 2006, Ribas et al. 2010a, b). Thus, it has been observed that PA induces lipid peroxidation and decreases non-enzymatic antioxidant defenses in vitro (Fontella et al. 2000) and reduces tissue antioxidant defenses in the hippocampus in vivo (Pettenuzzo et al. 2002). In addition, Rigo et al. (2006) demonstrated that PA causes seizures and induces carbonyl formation in rat striatum after intrastriatal administration and that these effects are prevented by MK-801, a glutamatergic receptor antagonist, suggesting the involvement of glutamate receptors in these effects. Leukocyte DNA damage and induction of lipid and protein oxidative damage were also observed in plasma of patients affected by propionic acidemia and methylmalonic acidemia (Ribas et al. 2010a, b).

However, although oxidative stress was shown to occur in animal models and in humans with methylmalonic and propionic acidemia (Fontella et al. 2000; Furian et al. 2007; Richard et al. 2007, 2009; Mc Guire et al. 2009; Ribas et al. 2010a, b), the reported studies did not distinguish whether neurons or other neural cells are vulnerable to MMA and PA effects. Since synaptosomes are nerve terminals that have been used to investigate the functional consequences of neurotoxins on neurons (Nicholls 2003), our main goal here was to determine whether these neural cells are involved in MMA- and PA-elicited oxidative stress. Therefore, we employed synaptosomal preparations from cerebrum of young rats to study the in vitro effects of MMA and PA on important parameters of lipid and protein oxidative damage and on the production of reactive species. We also investigated the in vivo effects of intrastriatal administration of MMA and PA to young rats on the same parameters and also on the enzymatic antioxidant defenses in the hope to better characterize the deleterious influence of these organic acids on the striatum that is particularly affected in methylmalonic and propionic acidemias.

Methods

Animals and Reagents

Thirty-day-old Wistar rats obtained from the Central Animal House of the Department of Biochemistry, ICBS, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil, were used. The animals were housed six per cage with food (protein commercial chow; SUPRA, Porto Alegre, RS, Brazil) and water available ad libitum and

were maintained on a normal 12 h light/dark cycle (lights on 7:00–19:00 h) in air conditioned constant temperature (22 ± 1°C) colony room. This study was performed in accordance with the "Principles of Laboratory Animal Care" (NIH publication no. 80-23, revised 1996) and with the approval of Ethics Committee for Animal Research of the Universidade Federal do Rio Grande do Sul. All efforts were made to minimize the number of animals used and their suffering.

All chemicals were purchased from Sigma (St. Louis, MO, USA). MMA, PA and NaCl solutions were prepared on the day of the experiments in the incubation medium used for each technique and the pH was adjusted to 7.4.

Synaptosomal Preparation from Brain

For the in vitro studies, synaptosomal preparations were used. The animals were killed by decapitation without anesthesia and the brain without the medulla, pons, olfactory bulb, and cerebellum was cut into small pieces using surgical scissors, extensively washed and then manually homogenized in 15 ml of buffer containing 0.32 M sucrose, 2 mM EDTA, 2 mM EGTA, and 20 mM HEPES, pH 7.2. The homogenate was centrifuged at $450 \times g$ for 10 min at 4°C and the supernatant transferred to a new tube. Then, the supernatant was centrifuged at $20{,}000 \times g$ for 10 min at 4°C, and the resulting crude synaptosomal pellet was resuspended in 2 ml of Locke's buffer (154 mM NaCl, 5.6 mM KCl, 2.3 mM $CaCl_2$, 1 mM $MgCl_2$, 3.6 mM $NaHCO_3$, 5 mM glucose, and 5 mM HEPES, at pH 7.2) (Springer et al. 1998). Synaptosomal preparations were incubated for 60 min at 37°C with MMA or PA at concentrations ranging from 0.2 to 10 mM. Controls did not contain these organic acids in the incubation medium. In some experiments, MK-801 (glutamatergic receptor antagonist) and antioxidants were added to the medium at the beginning of incubation together with MMA, at the following final concentrations: 500 or 1000 μM MK-801, 10 or 20 μM trolox (soluble α-tocopherol; TRO), 1000 or 2000 μM melatonin (MEL), combination of superoxide dismutase (SOD) plus catalase (CAT) (100 or 250 mU/ml each), 100 μM L-carnitine (CAR) or 750 μM N^{ω}-nitro-L-arginine methyl ester (L-NAME). Thereafter, aliquots were taken for the measurement of thiobarbituric acid-reactive substances (TBA-RS) (lipid peroxidation), sulfhydryl oxidation and carbonyl formation (protein oxidation) and 2′,7′-dichlorofluorescein diacetate (DCF-DA) (reactive species production). The doses of the antioxidants employed in this investigation was those reported by other investigators as efficient for their scavenging properties (Guajardo et al. 2006; Halliwell and Gutteridge 2007; Gavazza and Catalá 2009; Stasiak et al. 2010; Stuss et al. 2010; Sadowska-Woda et al. 2010).

Administration of Methylmalonic Acid or Propionic Acid and Striatum Preparation

For these studies MMA or PA was injected bilaterally into the striatum. Male Wistar rats of 30 days of life were anesthetized with ketamine and xylasine (75 and 10 mg/kg ip, respectively). Two small holes were drilled in the skull for microinjection and 1 μl of 8 M MMA, 8 M PA or 8 M NaCl (each solution prepared in water and pH was adjusted to 7.4 with NaOH) was slowly injected over 3 min into each striatum via needles connected by a polyethylene tube to a 10 μl Hamilton syringe. The needle was left in place for another 1 min before being softly removed, so that the total procedure lasted 4 min. The coordinates for injection were as follows: 0.6 mm posterior to the bregma, 2.6 mm lateral to the midline and 4.5 mm ventral from dura (Paxinos and Watson 1986). The correct position of the needle was tested by previous injection of 0.5 μl of a methylene blue solution (4% in saline) and further histological analysis.

Thirty minutes after injection the rats were killed by decapitation, the brain was removed and the striatum isolated. The striatum was homogenized in 1:10 (w/v) in 20 mM sodium phosphate buffer with 140 mM KCl, pH 7.4. The homogenate was then centrifuged at $750 \times g$ for 10 min at 4°C and the supernatant collected. Thereafter, aliquots were separated and used to measure TBA-RS levels (lipid peroxidation), sulfhydryl oxidation (protein oxidation), and the activities of the antioxidant enzymes glutathione peroxidase (GPx), CAT, and SOD.

Thiobarbituric Acid-Reactive Substances

TBA-RS levels were measured according to the method described by Yagi (1998) with slight modifications. Briefly, 200 μl of 10% trichloroacetic acid and 300 μl of 0.67% TBA in 7.1% sodium sulfate were added to 100 μl of tissue supernatant (0.3 mg of protein) and incubated for 2 h in a boiling water bath. The mixture was allowed to cool on running tap water for 5 min. The resulting pink-stained complex was extracted with 400 μl of butanol. Fluorescence of the organic phase was read at 515 and 553 nm as excitation and emission wavelengths, respectively. Calibration curve was performed using 1,1,3,3-tetramethoxypropane and subjected to the same treatment as supernatants. TBA-RS levels were calculated as nmol TBA-RS/mg protein.

Sulfhydryl Content

Total sulfhydryl group content was assessed according to the method of LoPachin et al. (2004), which is based on the reduction of 5,5′-ditio-bis-2-nitrobenzoic acid (DTNB) by

thiol groups present in the sample, forming a yellow derivate (TNB). Sixty hundred and fifty microliters of PBS containing 1 mM EDTA, pH 7.4, and 100 µl of sodium dodecyl sulfate 1% were added to 250 µl of sample (0.3 mg of protein) and rested for 5 min. Soon after, 30 µl of DTNB were added and after an incubation of 5 min the absorption was read at 412 nm. The absorbance due to the amount of TNB is proportional to the amount of reduced thiol groups present in the sample. The results were expressed as nmol TNB/mg protein.

Protein Carbonyl Content

Protein carbonyl formation was measured spectrophotometrically according to Levine et al. (1994). Two hundred microliters of sample (0.3 mg of protein) were treated with 100 µl of 50 mM TRIS buffer and 200 µl of a solution of 10 mM 2,4-dinitrophenylhydrazine (DNPH) prepared in 2.5 N HCl or 2.5 N HCl (blank) and left in the dark at 37°C for 1 h. Samples were then precipitated with 325 µl of 20% trichloroacetic acid and centrifuged for 10 min at 3,000×*g*. The pellet was then washed with a mixture of ethanol:ethyl acetate (1:1, V/V) and suspended in 700 µl of 6 M guanidine prepared in 2.5 N HCl at 37°C for 5 min. The difference between the DNPH- and HCl-treated samples (blank) was used to calculate the carbonyl content determined at 365 nm. The results were calculated as nmol of carbonyl groups/mg of protein.

2′,7′-Dichlorofluorescein Diacetate Oxidation

The production of reactive species was determined according to the method of LeBel et al. (1992) using DCF-DA. DCF-DA prepared in 20 mM sodium phosphate buffer, pH 7.4, containing 140 mM KCl, was incubated with 80 µl of synaptosomal preparation (1 mg of protein) in the presence of MMA or PA during 1 h at 37°C. Intracellular esterases cleave the acetate group of DCF-DA, generating the reduced form DCFH, which is then rapidly oxidized to form the highly fluorescent product DCF in the presence of reactive species. Fluorescence was measured using wavelengths of 480 nm (excitation) and 535 nm (emission). The calibration curve was performed with standard DCF (0-10 mM) and the concentration of reactive species was expressed as pmol DCF/mg protein.

Glutathione Peroxidase Activity

GPx activity was measured according to Wendel (1981) using *tert*-butylhydroperoxide as substrate. The enzyme activity was determined by monitoring the NADPH disappearance at 340 nm in a medium containing 600 µl of buffer (100 mM potassium phosphate containing 1 mM EDTA, pH 7.0), 10 µl of 40 mM sodium azide, 15 µl of 100 mM glutathione, 15 µl of 10 U/ml glutathione reductase, 10 µl of 10 mM NADPH, and 10 µl of sample (3 µg of protein). One GPx unit (U) is defined as 1 µmol of NADPH consumed per minute. The specific activity was calculated as U/mg protein.

Catalase Activity

CAT activity was assayed according to Aebi (1984) by measuring the absorbance decrease at 240 nm at room temperature (22 ± 2°C) in a reaction medium containing 20 mM H_2O_2, 0.1% Triton X-100, 10 mM potassium phosphate buffer, pH 7.0, and the supernatants containing approximately 1 µg of protein. CAT activity was calculated as U/mg protein, using the extinction coefficient of 43.6 M/cm for H_2O_2. One unit (U) of the enzyme is defined as 1 µmol of H_2O_2 consumed per minute.

Superoxide Dismutase Activity

SOD activity was assayed according to Marklund (1985) and is based on the capacity of pyrogallol to autoxidize, a process highly dependent on superoxide anion, which is the substrate for SOD. The inhibition of the autoxidation of pyrogallol occurs in the presence of SOD and, therefore, is proportional to the activity of the SOD present in homogenates. The reaction medium contained 50 mM Tris buffer/1 mM ethylenediaminetetraacetic acid, pH 8.2, 80 U/ml CAT, 0.38 mM pyrogallol and approximately 1 µg of protein and the absorbance was read at 420 nm. A calibration curve was performed with purified SOD as standard, in order to calculate the activity of SOD present in the samples. The results were reported as U/mg protein.

Protein Content

The protein content was determined by the method of Lowry et al. (1951) or Bradford (1976) using bovine albumin as standard.

Statistical Analysis

Data were expressed as means ± SD for absolute values. Assays were performed in duplicate or triplicate and the mean was used for statistical analysis. Data were analyzed using one-way analysis of variance (ANOVA) followed by the post-hoc Duncan multiple range test when F was significant (in vitro) or Student t test for unpaired samples (in vivo). Only significant F and t values are displayed in the text. Differences between groups were rated significant at $P < 0.05$. All analyses were carried out in an

IBM-compatible PC computer using the Statistical Package for the Social Sciences (SPSS) software.

Results

MMA Induces Lipid Peroxidation in Brain Synaptosomes and Striatum of Young Rats

We first observed that TBA-RS levels were significantly increased by MMA in synaptosomal preparations (up to 60%) [$F_{(4,25)} = 59.2$; $P < 0.001$] in a concentration-dependent manner ($\beta = 0.921$; $P < 0.001$) (Fig. 1a), whereas PA did not alter this parameter (results not shown). We also evaluated the effect of 3-nitropropionic acid (3NPA), a known irreversible inhibitor of complex II activity of the respiratory chain on TBA-RS levels and found that, similarly to PA, this acid had no effect on this parameter (results not shown). We also found that the NMDA receptor antagonist MK-801 (500 μM) [$F_{(5,30)} = 25.6$; $P < 0.001$] and the free radical scavengers trolox (soluble α-tocopherol, 10 μM TRO) [$F_{(3,20)} = 44.8$; $P < 0.001$] and melatonin (MEL, 1000 μM) [$F_{(3,20)} = 93.7$; $P < 0.001$] attenuated the MMA-induced lipid peroxidation (Fig. 1b–d), whereas the combination of SOD plus CAT (100 mU/ml each), L-carnitine (100 μM) and L-NAME (750 μM) did not change this effect (data not shown). However, when increasing the amounts of these antioxidants in the medium, we observed that 2000 μM MEL and 1000 μM MK-801 totally prevented and 20 μM TRO only attenuated MMA-induced increase of TBA-RS levels [$F_{(5,18)} = 10.2$; $P < 0.001$], suggesting that the hydroxyl radical was mainly responsible for this effect. In addition, the combination of SOD plus CAT at 250 mU/ml each had no effect on the increased TBA-RS values (Fig. 1e). Similarly, MMA intrastriatal administration provoked a significant increase in TBA-RS levels in striatum of young rats (43%) [$t_{(8)} = 3.88$; $P < 0.01$] (Fig. 2). Taken together, these data indicate that lipid oxidative damage is markedly induced by MMA in vitro in rat brain synaptosomes and in vivo in striatum probably via reactive species generation.

MMA Induces Protein Oxidative Damage in Brain Synaptosomes and Striatum of Young Rats

The next step of the present study was to evaluate the effect of MMA and PA on protein oxidative damage by measuring sulfhydryl oxidation and carbonyl formation in the brain. Our results show that MMA caused an increase of

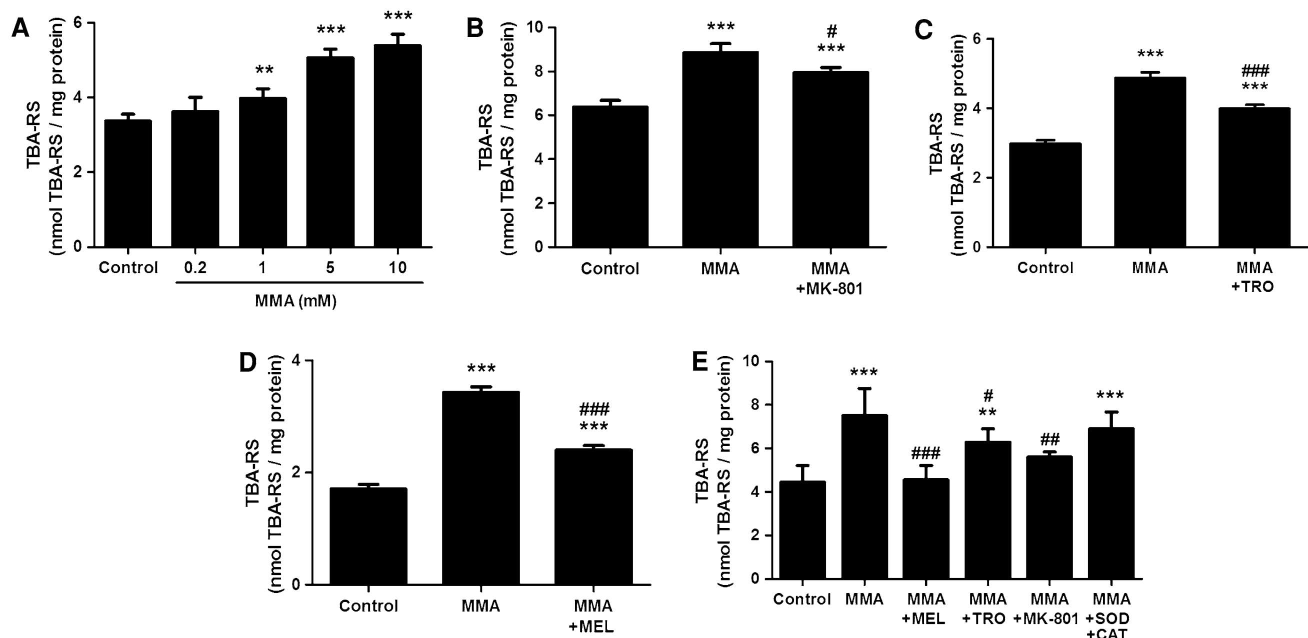

Fig. 1 In vitro effect of methylmalonic acid (*MMA*) on thiobarbituric acid-reactive substances (*TBA-RS*) in brain synaptosomes. Synaptosomes were incubated during 1 h in the presence of MMA (**a**). In some experiments synaptosomes were co-incubated for 1 h with 10 mM MMA and either MK-801 (500 μM, **b**), ∝-tocopherol (TRO, 10 μM, **c**), or melatonin (MEL, 1000 μM, **d**). Higher concentrations of MK-801 (1000 μM), MEL (2000 μM), TRO (20 μM), superoxide dismutase plus catalase (SOD + CAT, 250 mU/ml each) were also tested (**e**). Controls did not contain MMA in the incubation medium, but rather the buffer used in the technique (20 mM sodium phosphate buffer with 140 mM KCl, pH 7.4). Values are means ± standard deviation of six independent experiments (animals) performed in triplicate. $^{**}P < 0.01$, $^{***}P < 0.001$, compared to control; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared to MMA (Duncan multiple range test)

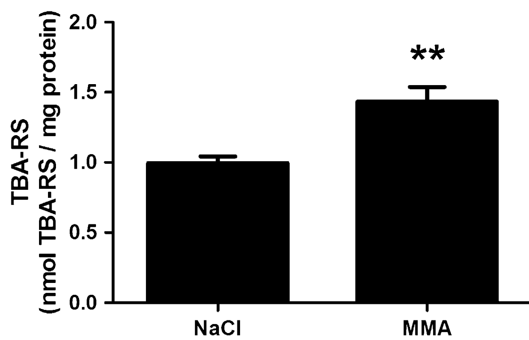

Fig. 2 In vivo effect of intrastriatal administration of methylmalonic acid (*MMA*) (8 μmol) on thiobarbituric acid-reactive substances (*TBA-RS*) in rat striatum. Controls received intrastriatal administration of NaCl (8 μmol). Values are means ± standard deviation of five independent experiments (animals) performed in triplicate. $^{**}P < 0.01$, compared to control (Student *t* test)

carbonyl formation (up to 99%) in synaptosomal preparations when supplemented to the incubation medium [$F_{(4,15)} = 4.58$; $P < 0.05$] (Fig. 3a). Furthermore, MMA moderately but significantly induced sulfhydryl oxidation (10%) in striatum after intrastriatal administration [$t_{(8)} = 3.82$; $P < 0.01$] (Fig. 3b). In contrast, sulfhydryl oxidation was not altered by the exposition of synaptosomal preparations to MMA, whereas PA did not alter these parameters in vivo or in vitro (data not shown). These results indicate that MMA provokes protein oxidative damage in brain synaptosomes and striatum from young rats.

MMA Induces Reactive Species in Brain Synaptosomes from Young Rats

The in vitro effect of MMA and PA on DCF-DA oxidation was also investigated in synaptosomal preparations from brain of young rats. We verified that MMA strongly induced DCF-DA oxidation (up to 177%) [$F_{(4,25)} = 65.3$; $P < 0.001$] (Fig. 4), indicating that this organic acid induces an increase of reactive species formation. In contrast, PA had no effect on this parameter (results not shown).

MMA Intrastriatal Administration Decreases GPx Activity in Striatum of Young Rats

Finally, we investigated the effect of MMA and PA intrastriatal administration on the activities of the antioxidant enzymes GPx, CAT, and SOD (Table 1). MMA significantly decreased the activity of GPx [$t_{(7)} = 9.00$; $P < 0.001$] (37%), but did not affect SOD and CAT activities. In addition, PA did not alter any of these activities (data not shown).

Discussion

In the present work we evaluated the effects of MMA and PA on lipid and protein oxidative damage and on the production of reactive species using synaptosomal preparations from brain of young rats to assess whether oxidative damage is elicited by these metabolites in neuronal cells. We also tested the effect of in vivo intrastriatal administration of MMA and PA on lipid and protein oxidative damage and on the activities of antioxidant enzymes because the striatum is mainly affected in methylmalonic and propionic acidemias, especially during metabolic crises when the concentrations of the accumulating organic acids dramatically increase. We killed the animals 30 min after NaCl, MMA, or PA injection to investigate short-lived effects. It is emphasized that, to our mind, this is the first work that employed synaptosomes to investigate

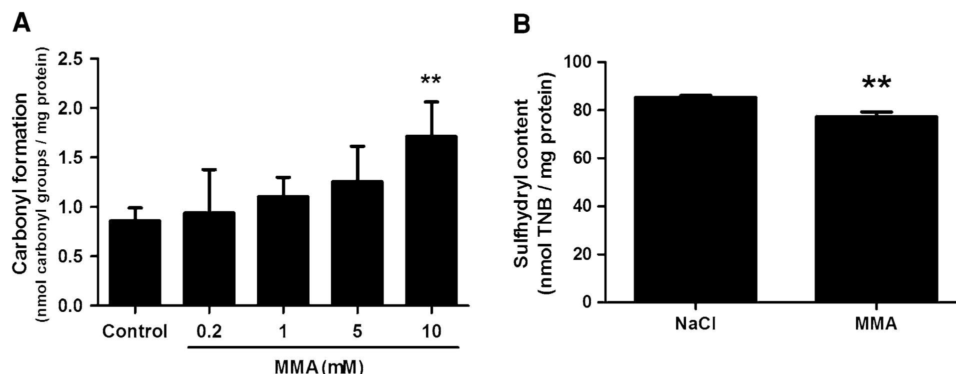

Fig. 3 Effects of methylmalonic acid (MMA) on carbonyl formation (**a**) and sulfhydryl content (**b**). Carbonyl formation was measured after exposing synaptosomal preparations to various concentrations of MMA (**a**), whereas sulfhydryl content was measured after intrastriatal administration of MMA (8 μmol) (**b**). In the in vitro experiments controls did not contain MMA in the incubation medium, but rather the buffer used in the technique (20 mM sodium phosphate buffer with 140 mM KCl, pH 7.4), whereas the controls in vivo received intrastriatal administration of NaCl (8 μmol). Values are means ± standard deviation of five to six independent experiments (animals) performed in triplicate. $^{**}P < 0.01$, compared to control (Duncan multiple range test and Student *t* test)

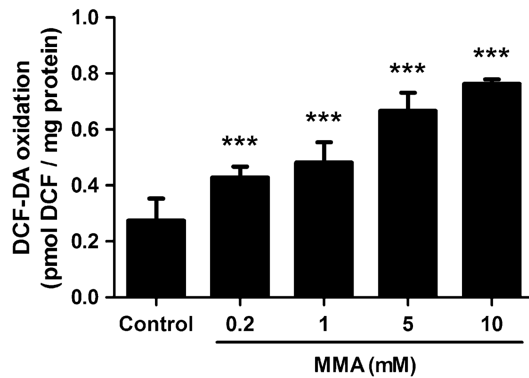

Fig. 4 In vitro effect of methylmalonic acid (MMA) on 2′,7′-dichlorofluorescein diacetate (DCF-DA) oxidation in brain synaptosomes. Controls did not contain MMA in the incubation medium, but rather the buffer used in the technique (20 mM sodium phosphate buffer with 140 mM KCl, pH 7.4). Values are means ± standard deviation of six independent experiments (animals) performed in triplicate. ***$P<0.001$, compared to control (Duncan multiple range test)

Table 1 In vivo effect of intrastriatal administration of methylmalonic acid (MMA, 8 µmol) on the activities of the antioxidant enzymes superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase (CAT) in rat striatum

Activities	NaCl	MMA
SOD	7.30 ± 0.51	8.25 ± 1.26
CAT	4.47 ± 0.72	3.96 ± 0.90
GPx	10.7 ± 0.54	6.68 ± 0.80***

Data are expressed as mean ± SD for five to six independent experiments (animals) performed in triplicate. One SOD unit is defined as 50% inhibition of pyrogallol autoxidation; one CAT unit is defined as one µmol of H_2O_2 consumed per minute; one GPx unit is defined as one µmol of NADPH consumed per minute. Values are expressed as U/mg protein

*** $P < 0.001$, compared to NaCl (Student *t* test)

neurotoxic properties of MMA and PA and, besides, determine whether these organic acids are able to alter the enzymatic antioxidant defenses in the striatum.

We first verified that MMA, but not PA and 3NPA, another inhibitor of complex II of the respiratory chain, significantly increased TBA-RS levels in vitro and in vivo in rat brain. These results indicate a selective oxidative effect of MMA, rather than a nonspecific action of acidic compounds. Considering that TBA-RS measurement reflects the amount of malondialdehyde formation, an end product of membrane fatty acid peroxidation (Halliwell and Gutteridge 2007), the increased values of this parameter elicited by MMA strongly indicate that this metabolite causes lipid peroxidation. We also observed that the lipid oxidative damage induced by MMA in brain synaptosomal preparations (in vitro) was partially prevented by MEL (1000 µM), TRO (10 µM), and MK-801 (500 µM), but not by L-carnitine (100 µM), L-NAME (750 µM) or the combination of SOD and CAT (100 mU/ml each). Furthermore, higher doses of MEL (2000 µM) and MK-801 (1000 µM) fully prevented, whereas TRO (20 µM) attenuated and the combination of SOD plus CAT (250 mU/ml each) did not change MMA-elicited effects. Since TRO and MEL scavenge preferentially peroxyl and hydroxyl radicals, it may be suggested that these reactive oxygen species and especially the hydroxyl radical that is efficiently scavenged by MEL, were at least partly involved in MMA-induced lipid oxidation. It should be emphasized that the antioxidants were added simultaneously with MMA to the incubation medium so that they were able to scavenge reactive species before they could react with cell constituents. On the other hand, it is unlikely that reactive nitrogen species were responsible for the induction of lipid peroxidation in synaptosomes used in the present investigation since the classical inhibitor of nitric oxide synthase L-NAME did not reduce the increase of TBA-RS levels provoked by MMA. Furthermore, the protective effects of the NMDA antagonist MK-801 support the involvement of these glutamate receptors in MMA effects, as previously observed (de Mello et al. 1996; Kölker et al. 2000; Brusque et al. 2001; Malfatti et al. 2007). MMA also provoked a moderate protein oxidative damage, as observed by the increase of carbonyl formation in synaptosomes elicited at a high concentration and by the mild enhancement of sulfhydryl oxidation after intrastriatal administration. In contrast, PA did not affect these parameters. Carbonyl group generation is currently used as a marker of free radical-mediated protein oxidation (Levine et al. 1994), being the amino acid residues Pro, Arg, Lys, and Thr of the side chain of proteins the most vulnerable to oxidative attack (Dalle-Donne et al. 2003). On the other hand, since approximately two-thirds of sulfhydryl groups are bound to proteins, whereas one-third is a component of small molecules such as glutathione (Requejo et al. 2010), oxidation of cellular protein-bound sulfhydryl groups from specific cysteine residues also reflects oxidative damage that may potentially lead to protein inactivation (Aksenov and Markesbery 2001; Davies 2003).

We also found that MMA markedly increased DCF-DA oxidation, which is converted to DCF, mainly by hydroxyl radicals, hydrogen peroxide, and peroxynitrite (LeBel et al. 1992; Ischiropoulos et al. 1999; Ohashi et al. 2002; Myhre et al. 2003; Bonini et al. 2006). These data, allied to the findings showing that peroxyl and hydroxyl scavengers attenuated MMA-elicited lipid peroxidation, reinforce the presumption that these reactive species were involved in MMA-induced oxidative effects. Interestingly, it was recently demonstrated increased levels of reactive species in fibroblasts from patients with methylmalonic acidemia (Richard et al. 2005, 2007, 2009), supporting our present in vitro and in vivo findings.

With regard to the antioxidant defense system, MMA in vivo administration provoked a selective decrease of GPx activity, without altering the activities of CAT and SOD. The reduced activity of GPx caused by MMA in striatum may result from decreased de novo synthesis or from inactivation of the enzyme protein due to a direct binding of MMA to vulnerable groups of the enzyme or indirectly through increased reactive species that attack essential sulfhydryl or other vulnerable groups of the enzyme (Singh et al. 2004; Jafari 2007). On the other hand, reduction of GPx activity may lead to a diminished capacity of the striatum to scavenge hydrogen peroxide and fatty acid hydroperoxides that can generate other forms of carbon-, nitrogen-, and oxygen-centered radicals, such as hydroxyl radicals via the Fenton reaction (Halliwell and Gutteridge 2007). In this context, it should be emphasized that hydroxyl radicals readily initiate the process of lipid peroxidation, which may be related to the lipid oxidative damage induced by MMA.

Since oxidative stress results from an imbalance between the total antioxidant defenses and the reactive species generated in a tissue (Halliwell and Gutteridge 2007), our present data strongly indicate that MMA induces oxidative stress in brain of young rats. At this point, it should be emphasized that the brain has low cerebral antioxidant defenses compared with other tissues. Besides, the high oxygen consumption and high iron and lipid contents, especially polyunsaturated fatty acids (Halliwell and Gutteridge 2007), contribute to make the brain more vulnerable to increased reactive species generation.

Taken together the various observations that oxidative damage occurs in patients affected by methylmalonic acidemia (Treacy et al. 1996; Richard et al. 2005, 2007, 2009; Ribas et al. 2010a, b) and the animal experimental studies demonstrating that MMA induces oxidative stress in the brain (McLaughlin et al. 1998; Brusque et al. 2001; Okun et al. 2002; Fighera et al. 2003; Royes et al. 2005, 2006, 2007; Furian et al. 2007; Richard et al. 2005, 2007, 2009; Ribeiro et al. 2005, 2009), it is presumed that alterations of the biological oxidations in the brain may possibly represent one of the mechanisms by which MMA is neurotoxic. However, to our knowledge, no previous study investigated which neural cells were involved in MMA oxidative effects. Our present work was the first to demonstrate that MMA action inducing oxidative damage occurs in nerve terminals (synaptosomal preparations) used as a model to study the effects of neurotoxins in neuronal cells (Nicholls 2003). A great advantage of the utilization of synaptosomes, that correspond to mini-cell in which mitochondria exist in a physiological milieu and supply ATP to the cytoplasm and plasma membrane, is that these preparations can be made from animals of any age, in contrast to the neonatal requirement for virtually all primary neuronal cell cultures. Therefore, we assume that MMA affects biological oxidations in the central nervous system by acting on neuronal cells.

In contrast, PA had no effect on all parameters examined in vitro and in vivo, strongly indicating that this organic acid does not elicit oxidative stress in neuronal cells and in the striatum. However, a previous report showing that PA provokes lipid peroxidation and reduces the nonenzymatic antioxidant defenses in rat cerebral cortex homogenates, implying that oxidative damage may occur in this cerebral region (Fontella et al. 2000). Furthermore, oxidative stress markers were found to be increased in plasma of patients affected by propionic acidemia, although it was not investigated which accumulating organic acids were related to these findings (Ribas et al. 2010a, b). Therefore, it is possible that different approaches and tissue specific effects may possibly explain these apparently conflicting results.

It is difficult to determine the pathophysiological relevance of our present data since to our knowledge MMA brain concentrations are not yet established in methylmalonic acidemia. However, it should be noted significant effects on some parameters of oxidative stress occurred at concentrations as low as 0.2 and 1 mM, concentrations that are within the levels found in plasma and cerebrospinal fluid (CSF) (3.0 mM) (Fenton et al. 2001) of patients affected by this disorder. Furthermore, it is feasible that even higher brain MMA concentrations may take place in stress situations, such as occurs during episodes of metabolic decompensation characterized by intense catabolism and proteolysis. Under these circumstances, the levels of the accumulating metabolites within neural cells may predominate over those found in plasma and CSF (Hoffmann et al. 1993). We should also consider that MMA may be directly produced in the brain, being thereafter trapped in neural cells (Kölker et al. 2006; Sauer et al. 2006, 2010). This is in line with the data showing that patients suffering liver transplantation do not present a decrease in CSF MMA levels and remain with neurological manifestations after this procedure, which is in accordance with the trapping hypothesis (van't Hoff et al. 1999; Chakrapani et al. 2002; Nyhan et al. 2002; Kölker and Okunm 2005; Nagarajan et al. 2005; Kaplan et al. 2006; Kölker et al. 2006; Sauer et al. 2006, 2010). However, we should also emphasize that the protein oxidative damage induced by MMA was only moderate and occurred at high concentrations of MMA, which may not be physiologically important.

In conclusion, we report for the first time that MMA induces lipid and protein oxidative damage by indirectly increasing the generation of free radicals and other reactive species in brain synaptosomes. Similar findings and a reduction of the enzymatic antioxidant defenses were

found in rat striatum after MMA intrastriatal injection, a brain structure that is severely compromised in methylmalonic acidemia (Brismar and Ozand 1994; Chemelli et al. 2000; Harting et al. 2008). In case the present findings are confirmed in human *postmortem* brain from methylmalonic acidemic patients, the stimulation of highly reactive radical production by MMA in the CNS will potentially lead to deleterious consequences to the brain. Based on the present findings and on previous evidence suggesting that oxidative stress plays a role in this disease and that antioxidants prevent the oxidative damage induced by MMA (Fontella et al. 2000; Malfatti et al. 2003; Royes et al. 2006, 2007; Furian et al. 2007), it is presumed that this pathomechanism may be relevant to explain at least in part the brain dysfunction and abnormalities observed in this disorder, particularly during crises of metabolic decompensation in which the concentrations of MMA dramatically increase. Finally, it is conceivable that administration of antioxidants as adjuvant agents to the usual therapy of methylmalonic acidemia may be useful, especially during these episodes.

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