J Inherit Metab Dis (2008) 31:44–54
DOI 10.1007/s10545-007-0798-1

ORIGINAL ARTICLE

Methylmalonate inhibits succinate-supported oxygen consumption by interfering with mitochondrial succinate uptake

S. R. Mirandola · D. R. Melo · P. F. Schuck · G. C. Ferreira · M. Wajner · R. F. Castilho

Received: 25 October 2007 / Submitted in revised form: 2 December 2007 / Accepted: 4 December 2007 /
Published online: 24 January 2008

Summary The effect of methylmalonate (MMA) on mitochondrial succinate oxidation has received great attention since it could present an important role in energy metabolism impairment in methylmalonic acidaemia. In the present work, we show that while millimolar concentrations of MMA inhibit succinate-supported oxygen consumption by isolated rat brain or muscle mitochondria, there is no effect when either a pool of NADH-linked substrates or *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylendiamine (TMPD)/ascorbate were used as electron donors. Interestingly, the inhibitory effect of MMA, but not of malonate, on succinate-supported brain mitochondrial oxygen consumption was minimized when nonselective permeabilization of mitochondrial membranes was induced by alamethicin. In addition, only a slight inhibitory effect of MMA was observed on succinate-supported oxygen consumption by inside-out submitochondrial particles. In agreement with these observations, brain mitochondrial swelling experiments indicate that MMA is an important inhibitor of succinate transport by the dicarboxylate carrier. Under our experimental conditions, there was no evidence of malonate production in MMA-treated mitochondria. We conclude that MMA inhibits succinate-supported mitochondrial oxygen consumption by interfering with the uptake of this substrate. Although succinate generated outside the mitochondria is probably not a significant contributor to mitochondrial energy generation, the physiopathological implications of MMA-induced inhibition of substrate transport by the mitochondrial dicarboxylate carrier are discussed.

Communicating editor: Garry Brown

Competing interests: None declared

References to electronic databases: Methylmalonyl-CoA mutase: EC 5.4.99.2).

S.R. Mirandola and D.R. Melo contributed equally to this work. Drs. M. Wajner and R.F. Castilho share senior authorship.

S. R. Mirandola · D. R. Melo · R. F. Castilho (✉)
Departamento de Patologia Clínica, Faculdade de Ciências Médicas, Universidade Estadual de Campinas, Campinas, SP 13083-887, Brazil
e-mail: roger@fcm.unicamp.br

P. F. Schuck · G. C. Ferreira · M. Wajner
Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre RS, Brazil

Abbreviations

BSA	bovine serum albumin
BtMA	butylmalonate
DCIP	2,6-dichlorophenolindophenol
MA	malonate
2-MCA	2-methylcitrate
MCM	methylmalonyl-CoA mutase
MMA	methylmalonate
PMS	phenazine methosulfate
SDH	succinate dehydrogenase
TMPD	*N*,*N*,*N*′*N*′-tetramethyl-*p*-phenylenediamine

Introduction

Methylmalonic acidaemias consist of a group of autosomal recessive genetic disorders affecting the catabolic pathway of the amino acids isoleucine, valine, methionine and threonine, as well as of odd-chain fatty acids and the side-chain of cholesterol. Methylmalonic acidaemias are caused by complete (mut^0) or partial (mut^-) deficiency of methylmalonyl-

CoA mutase (MCM; EC 5.4.99.2) or by defects in the genes encoding the synthesis, activation or transport of adenosylcobalamin, which is the cofactor of MCM. MCM deficiency leads to the primary accumulation of methylmalonate (MMA) from L-methylmalonyl-CoA, and to the secondary accumulation of other metabolites such as propionate, 3-hydroxypropionate and 2-methylcitrate (2-MCA) (Fenton et al 2001). Levels of MMA in the blood and cerebrospinal fluid have been determined as being as high as 2.9 mmol/L during crises (Fenton et al 2001), and may be even higher in the brain because dicarboxylic acids tend to be trapped inside neural cells (Hoffmann et al 1993; Sauer et al 2006).

The clinical phenotype of affected individuals includes failure to thrive, developmental delay with subsequent mental retardation, acute metabolic decompensation with ketoacidosis, vomiting, lethargy, coma and seizures, and, in the most severe cases, infant death. Cerebral imaging has revealed a symmetric degeneration of the basal ganglia, particularly the globus pallidus (Brismar and Ozand 1994; Larnaout et al 1998). Although the physiopathological mechanisms underlying the neurological signs in this disorder are not well established, these pathological changes may be caused by the accumulation of toxic organic acids (Heidenreich et al 1988).

Furthermore, during acute encephalopathic crises, patients with methylmalonic acidaemias present enhanced amounts of lactic acid in the globus pallidus, suggesting inhibition of mitochondrial energy metabolism *in vivo* (Trinh et al 2001). There is a great deal of evidence suggesting that MMA compromises mitochondrial function, since it competes with malate transport (Halperin et al 1971), increases lactate production *ex vivo* and *in vitro* (Royes et al 2003; Saad et al 2006; Wajner and Coelho 1997; Wajner et al 1992) and decreases ATP levels (McLaughlin et al 1998), CO_2 production (Wajner et al 1992) and succinate-supported O_2 consumption (Kowaltowski et al 2006; Maciel et al 2004; Toyoshima et al 1995). It has also been proposed that MMA is a weak competitive inhibitor of succinate dehydrogenase (SDH) in rat brain and liver, an effect that has been attributed to the structural similarity between MMA, malonate (MA), the classical SDH inhibitor, and succinate, the substrate of SDH (Brusque et al 2002; Dutra et al 1993; Fleck et al 2004; Marisco et al 2003; Pettenuzzo et al 2006; Toyoshima et al 1995). However, the inhibitory effect of MMA on SDH activity has not been detected in purified submitochondial particles from bovine heart (Kolker et al 2003; Okun et al 2002) and when high concentrations of succinate were used in the medium to measure this enzymatic activity (Brusque et al 2002; Pettenuzzo et al 2006). It has been also argued that MMA is not the major neurotoxin in methylmalonic acidaemias and part of the effects obtained with MMA were considered to be artefactual or a result of intracellular formation of MA and 2-MCA from MMA (Kolker and Okun 2005; Kolker et al 2003; Okun et al 2002). However, cleavage of the methyl group of MMA by esterases is unlikely to explain MA formation (Okun et al 2002), as is the reversibility of the propionyl-CoA pathway to form 2-MCA (Reszko et al 2003). Thus, at this stage there is an intense debate on whether MMA impairs mitochondrial bioenergetics and more particularly SDH/respiratory chain complex II activity.

Therefore, the major purpose of the present study was to investigate the *in vitro* effects of MMA on succinate-supported oxygen consumption by isolated rat brain and muscle mitochondria in order to clarify this issue. The most interesting findings observed were that MMA is an important inhibitor of succinate transport through mitochondrial membranes.

Material and methods

Materials

Most chemicals, including ADP, alamethicin, ascorbic acid, butylmalonic acid, glutaric acid, malonic acid, methylmalonic acid, succinic acid and *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD) were obtained from Sigma-Aldrich (St Louis, MO, USA). ADP, ascorbate, glutamate, α-ketoglutarate, malate, pyruvate, succinate, butylmalonate (BtMA), MA and MMA solutions were prepared by dissolving the respective acids in water and adjusting the pH to 7.2 with KOH.

Animals

Nine-week-old female Wistar rats (*Rattus novergicus albinos*) were obtained from the UNICAMP Central Animal Breeding Center. The animals were kept under standard laboratory conditions (20–22°C and 12 h/12 h light/dark cycle) with free access to a standard diet (Labina/Purina, Campinas, SP, Brazil) and tap water. Animal experiments followed University guidelines for the use of animals in experimental studies and the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH publication no. 85-23, revised in 1996).

Isolation of rat forebrain mitochondria

Forebrain mitochondria were isolated as described by Rosenthal and colleagues (1987), with minor modifications, from female Wistar rats weighing 200–240 g. Rats were killed by decapitation and their brains were rapidly removed (within 1 min) and put into 10 ml of ice-cold 'isolation buffer' containing 225 mmol/L mannitol, 75 mmol/L sucrose, 1 mmol/L K^+-EGTA, 0.1% bovine serum albumin (BSA; fatty acid-free) and 10 mmol/L K^+-Hepes, pH 7.2. The cerebellum and underlying structures were removed and the remaining material was used as the forebrain. The tissue was cut into small pieces using surgical scissors and extensively washed. The tissue was then manually homogenized in a Dounce homogenizer using 6 strokes with a loose-fitting glass pestle followed by 7–8 strokes with a tight-fitting pestle. The homogenate was centrifuged for 3 min at 2000 *g* in a Beckman JA-25.50 rotor (Beckman, Palo Alto, CA, USA). After centrifugation, the supernatant was recentrifuged for 8 min at 12 000*g*. The pellet was resuspended in 20 ml 'isolation buffer' containing 40 µl of 10% digitonin and recentrifuged for 10 min at 12 000g. The supernatant and the light layer of the pellet were discarded and the dark pellet was resuspended in 'isolation buffer' devoid of EGTA. This was then centrifuged for 10 min at 12 000*g*. The supernatant was discarded and the final pellet was gently washed and resuspended in 'isolation buffer' devoid of EGTA, at an approximate protein concentration of 30–40 mg/ml. The respiratory control ratio (state 3/state 4 respiratory rate) was over 5.0, measured using 5 mmol/L glutamate plus 5 mmol/L malate as substrates.

Isolation of rat muscle and liver mitochondria

Rat muscle and liver mitochondria were isolated as described previously (Velho et al 2006). The final pellets from muscle and liver mitochondrial preparations were resuspended in 250 mmol/L sucrose and 10 mmol/L K^+-Hepes pH 7.2 to final protein concentrations of approximately 50 and 80 mg/ml, respectively.

Preparation of inside-out submitochondrial particles from rat liver

A fraction enriched in inside-out submitochondrial particles was obtained from isolated rat liver mitochondria as described by Harmon (1982) with minor modifications. Frozen aliquots (4–5 ml) of rat liver mitochondria were thawed, homogenized, and resuspended in 0.25 mol/L sucrose and 10 mmol/L K^+-Hepes pH 7.4 to a volume of 10 ml. Following centrifugation at 27 000 *g* for 15 min, the mitochondrial pellet was resuspended at protein concentration of approximately 30 mg/ml in 0.25 mol/L sucrose, 10 mmol/L K^+-Hepes and 10 mmol/L sodium phosphate, pH 7.4. This mitochondrial suspension was sonicated three times for 30 s each with intervals of 1 min in an ice bath, using a 3 mm titanium microtip in an ultrasonic processor (50 W, 20 kHz) at 30% of the maximal output. The sonicated mixture was centrifuged at 23 500*g* for 10 min. The pellet was discarded and the supernatant was centrifuged at 44 000*g* for 30 min. The supernatant was discarded, and the pellet was resuspended in 0.25 mol/L sucrose, 10 mmol/L K^+-Hepes pH 7.4 and 0.15 mol/L KCl medium and centrifuged again twice under the same conditions. The resultant pellet was resuspended in 0.25 mol/L sucrose and 10 mmol/L K^+-Hepes pH 7.4 and centrifuged at 15 000*g* for 10 min. The pellet was discarded and the supernatant was centrifuged again at 44 000 *g* for 30 min. The final pellet was resuspended in 0.25 mol/L sucrose and 20 mmol/L K^+-Hepes pH 7.4 and stored in an ice bath until used. This preparation procedure for obtaining submitochondrial particles resulted in more than 85% of inverted (inside-out) vesicles as evaluated by the effect of exogenous cytochrome *c* (5 µmol/L) on NADH-supported oxygen consumption (Harmon 1982).

Protein determination

The protein content of mitochondrial and submitochondrial particle suspensions was determined by the Biuret assay (Gornall et al 1949) in the presence of 0.2% deoxycholate, using bovine serum albumin as a standard.

Oxygen uptake measurements

Oxygen consumption was measured using a Clark-type electrode (Hansatech Instruments Limited, Norfolk, UK) in a 1.0 ml sealed glass cuvette equipped with a magnetic stirrer and kept at 37°C. The experiments measuring oxygen consumption by isolated mitochondria and submitochondrial particles were carried out in a standard reaction medium containing 130 mmol/L KCl, 10 mmol/L K^+-Hepes pH 7.2, 2 mmol/L KH_2PO_4, 1 mmol/L $MgCl_2$, 200 µmol/L EGTA and 800 µmol/L ADP. ADP was present to stimulate respiration by oxidative phosphorylation. Other additions are indi-

cated in the figure legends. Under control conditions, brain mitochondrial oxygen consumption rates (nmol O_2/min per mg protein) were 56.9±4.3 using glutamate, malate, α-ketoglutarate and pyruvate (1.25 mmol/L each), 70.3±4.0 using 5 mmol/L succinate plus 2 μmol/L rotenone, or 51.3±3.2 using 200 μmol/L TMPD–1 mmol/L ascorbate plus 1 μmol/L antimycin A. Using TMPD–ascorbate, we noticed that respiratory rates in our brain mitochondrial preparations were lower that those seen with succinate. This is probably due to limited accessibility of cytochrome *c* to electrons donated by TMPD.

Assay of mitochondrial succinate transport

Succinate transport was estimated in a suspension of isolated brain mitochondria using a model F-4010 Hitachi spectrofluorometer (Hitachi Ltd, Tokyo, Japan) operating at excitation and emission wavelengths of 540 nm, with slit widths of 3.0 nm. This methodology is based on the decrease in mitochondrial light scattering due to swelling that accompanies net salt transport into the organelles (Liu et al 1996). Rat brain mitochondria (0.4 mg/ml) were incubated in medium containing 0.1 mmol/L K^+-EGTA, 0.1 mmol/L K^+-EDTA, 5 mmol/L K^+-MOPS (pH 7.0), 0.2 μmol/L antimycin A, 0.7 μmol/L nigericin and 36.7 mmol/L K^+ salts of succinate, glutarate and/or MMA, at 37°C. All media were prepared to approximately 110 mOsm. Under these experimental conditions, stimulation of mitochondrial swelling by 2 mmol/L P_i (potassium salt, pH 7.0) reflects activity of the dicarboxylate carrier (Liu et al 1996). Mitochondrial swelling rates between 5 and 35 seconds after P_i addition were used to quantify the measurements.

Succinate dehydrogenase (SDH) activity

The assay was based on the reduction of phenazine methosulfate (PMS) by SDH (Singer 1974). Reduced PMS is immediately reoxidized by 2,6-dichlorophenolindophenol (DCIP), and bleaching of the latter dye was followed spectrophotometrically at 600 nm. Mitochondria (0.2 mg/ml) were incubated in a 2 ml glass spectrophotometer cuvette at 37°C in medium containing 50 mmol/L Tris-HCl (pH 7.4), 1 mmol/L succinate, 1 μmol/L antimycin A, 1 mmol/L KCN, 1% Triton X-100, 1 mmol/L PMS and 80 μmol/L DCIP. In Fig. 6, these reagents were added to the reaction media after a 10 min pre-incubation period, resulting in a two-fold dilution of the original mitochondrial suspension. Where indicated, the assays were conducted in the presence of MMA, MA or BtMA. The decrease in absorbance at 600 nm was recorded during 5 min.

Malonate (MA) and 2-methylcitrate (2-MCA) determination

Mitochondrial preparations from rat forebrain (0.5 mg/ml) were incubated for 10 min at 37°C in reaction medium containing 130 mmol/L KCl, 10 mmol/L K^+-Hepes pH 7.2, 2 mmol/L KH_2PO_4, 1 mmol/L $MgCl_2$, 200 μmol/L EGTA and 2 mmol/L succinate in the absence or presence of 10 mmol/L MMA in a total volume of 2 ml. MA and 2-MCA were determined in the mitochondrial suspension by capillary gas chromatography–mass spectrometry, as previously described (Wajner et al 2002). MA and 2-MCA were used for external calibration. Correlation coefficients of standard curves were >0.92.

Statistical analysis

Data from the experiments were analysed by one-way ANOVA followed by Tukey's post-hoc test. When one parameter was compared between two groups, Student's *t*-test was used. Data are presented as averages±standard error of the mean (±SEM) of at least four experiments conducted with different preparations.

Results

MMA inhibits succinate-supported mitochondrial oxygen consumption

The effect of MMA on brain mitochondrial oxygen consumption was studied at concentrations varying from 1 to 10 mmol/L (Fig. 1). MMA significantly inhibited succinate-supported mitochondrial oxygen consumption in a dose-dependent manner. In addition, the results displayed in Fig. 1A show no inhibitory effect of MMA on mitochondrial respiration when either a pool of NADH-linked substrates or TMPD/ascorbate were used as electron donors. A pool of respiratory chain complex I substrates was used to avoid the effect of MMA on the transport of single substrates through the inner mitochondrial membrane, either stimulating or inhibiting their transport (Halperin et al 1971). Representative traces in Fig. 1B show that the degree of inhibition of succinate-supported mitochondrial oxygen consumption by MMA was constant during the time course of the experiments.

Since Kolker and colleagues (2003) did not observe a significant inhibitory effect of MMA (1 mmol/L)

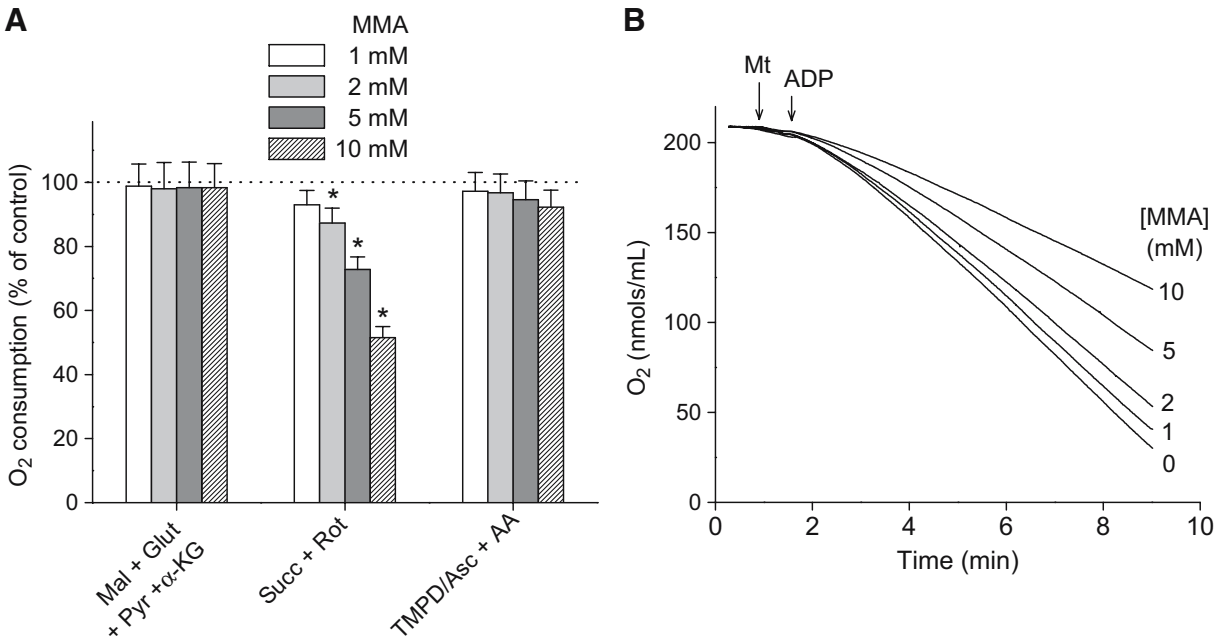

Fig. 1 Effect of MMA on brain mitochondrial oxygen consumption. Isolated rat forebrain mitochondria (Mt; 0.5 mg/ml) were incubated at 37°C in standard reaction medium containing either a pool of NADH-linked substrates (malate, glutamate, pyruvate and α-ketoglutarate (Mal + Glut + Pyr + α-KG); 1.25 mmol/L each), 5 mmol/L succinate plus 2 μmol/L rotenone (Succ + Rot), or 200 μmol/L TMPD–1 mmol/L ascorbate plus 1 μmol/L antimycin A (TMPD/Asc + AA). Rotenone and antimycin A were used to ensure respiratory chain complex I and III inhibition, respectively. (**A**) Mitochondrial oxygen consumption in the presence of different respiratory chain substrates and varying concentrations of MMA (1–10 mmol/L), as indicated in the figure. Data are shown as percentage of the respective control rates. (**B**) Representative traces of succinate-supported mitochondrial oxygen consumption in the presence of varying concentrations of MMA (1–10 mmol/L). Data in (**A**) represent the mean ± SEM of at least 5 independent experiments performed in duplicate. *Significantly different from the respective control at $p < 0.01$

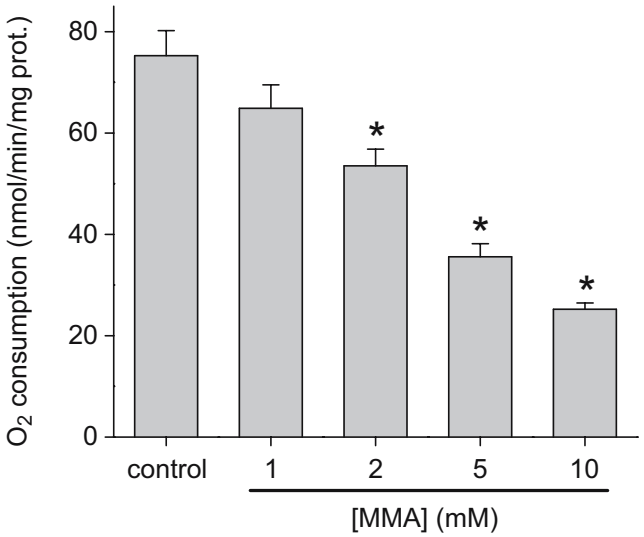

Fig. 2 Effect of MMA on succinate-supported muscle mitochondrial oxygen consumption. Isolated muscle mitochondria (0.5 mg/ml) were incubated at 37°C in standard reaction medium containing 1 mmol/L succinate and 2 μmol/L rotenone. Mitochondrial oxygen consumption measurements were done in the presence of varying concentrations of MMA (1–10 mmol/L), as indicated in the figure. Data represent the mean ± SEM of at least 5 independent experiments performed in duplicate. *Significantly different from control at $p < 0.01$

on succinate-supported oxygen consumption using a crude mouse muscle mitochondrial preparation, we decided to test the effect of MMA (1–10 mmol/L) on isolated rat muscle mitochondria under our experimental conditions. In fact, when tested at 1 mmol/L, MMA promoted only a trend for inhibition of oxygen consumption by muscle mitochondria (Fig. 2). However, when MMA was present at higher concentrations (2–10 mmol/L), a significant and dose-dependent inhibition of mitochondrial respiration was observed. These results indicate that the inhibitory effect of MMA on succinate-supported mitochondrial respiration is not restricted to brain (Fig. 1; Kowaltowski et al 2006; Maciel et al 2004) and liver (Toyoshima et al 1995).

Substrate transport inhibition causes the MMA effect on succinate-supported mitochondrial oxygen consumption

Recent publications from Kolker's group (Kolker et al 2003; Okun et al 2002) show that MMA, at millimolar concentrations, does not inhibit SDH activity in bovine

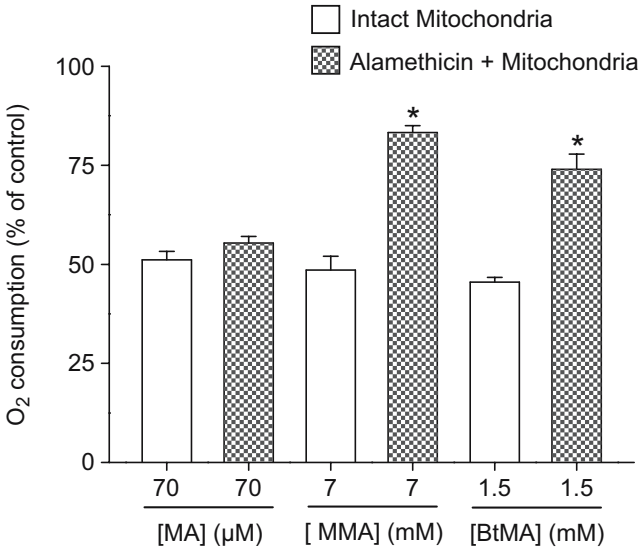

Fig. 3 Effect of MA, MMA and BtMA on oxygen consumption by intact or alamethicin-permeabilized mitochondria. Isolated rat forebrain mitochondria (0.5 mg/ml) were incubated at 37°C in standard reaction medium containing 2 mmol/L succinate, 2 μmol/L rotenone and 5 μmol/L cytochrome *c*. Mitochondrial oxygen consumption measurements were done in the presence of 70 μmol/L MA, 7 mmol/L MMA or 1.5 mmol/L BtMA. Alamethicin (40 μg/mg protein) was present where indicated. Data represent the mean±SEM of at least 5 independent experiments performed in duplicate. *Significantly different from intact mitochondria at $p<0.01$

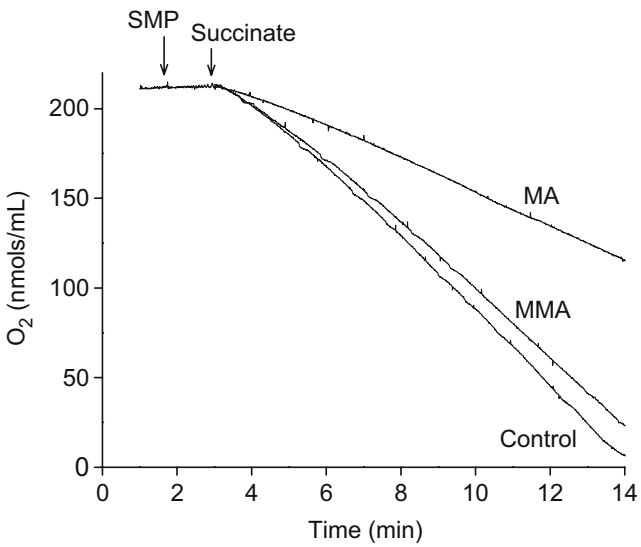

Fig. 4 Effects of MA and MMA on submitochondrial particle oxygen consumption. Rat liver submitochondrial particles (SMP, 0.1 mg/ml) were incubated at 37°C in standard reaction medium containing 1 mmol/L succinate under control conditions or in the presence of either 70 μmol/L MA or 7 mmol/L MMA. Traces are representative of 4 independent experiments performed in duplicate

heart submitochondrial particles. On the other hand, MA potently inhibits this enzyme at micromolar concentrations. One possibility to reconcile these findings with our previous results (Kowaltowski et al 2006; Maciel et al 2004) and the present findings is an inhibitory effect of MMA on mitochondrial succinate transport. In fact, an early report by Halperin and colleagues (1971) reported that MMA is an important inhibitor of malate transport by the mitochondrial dicarboxylate carrier. To assess this hypothesis, we monitored the effect of MMA on succinate-supported mitochondrial oxygen consumption under situations in which the transport of this substrate is not required. We first used brain mitochondria nonselectively permeabilized by the pore-forming compound alamethicin (He et al 1996). MA, MMA or BtMA, an inhibitor of mitochondrial dicarboxylate transport (Robinson and Chappell 1967), were used at concentrations necessary to ensure approximately 50% inhibition of succinate-supported oxygen consumption by intact organelles (Fig. 3, open bars). When the same concentrations of MA, MMA and BtMA were used in the presence of alamethicin, MA still exhibited a similar inhibition of succinate-supported oxygen consumption, but MMA and BtMA lost most of their inhibitory effect (Fig. 3, chequered bars). SDH activity was determined under similar experimental conditions using Triton X-100-permeabilized brain mitochondria. We observed that, while MA inhibits approximately 50% of enzyme acitivity, no significant inhibitory effect was promoted either by MMA or BtMA (Table 1).

A second experimental protocol to study succinate-supported mitochondrial respiration independent of substrate transport was designed, using a preparation enriched in inside-out submitochondrial particles (Harmon 1982) (Fig. 4). Under these conditions, only a slight inhibitory effect was obtained

Table 1 Effects of malonate, methylmalonate and butylmalonate on SDH activity

Conditions	SDH activity (% of control)
Malonate	53.1±3.4*
Methylmalonate	94.5±6.0
Butylmalonate	93.0±6.8

Activity of brain mitochondrial SDH was determined as described in Material and methods under control conditions or in the presence of 70 μmol/L malonate, 7 mmol/L methylmalonate or 1.5 mmol/L butylmalonate. Under control conditions, SDH activity was 81.9±5.5 nmol of reduced DCIP per min per mg protein. Values represent the mean±SEM of 4 independent experiments performed in duplicate.
*Significantly different from control at $p<0.01$.

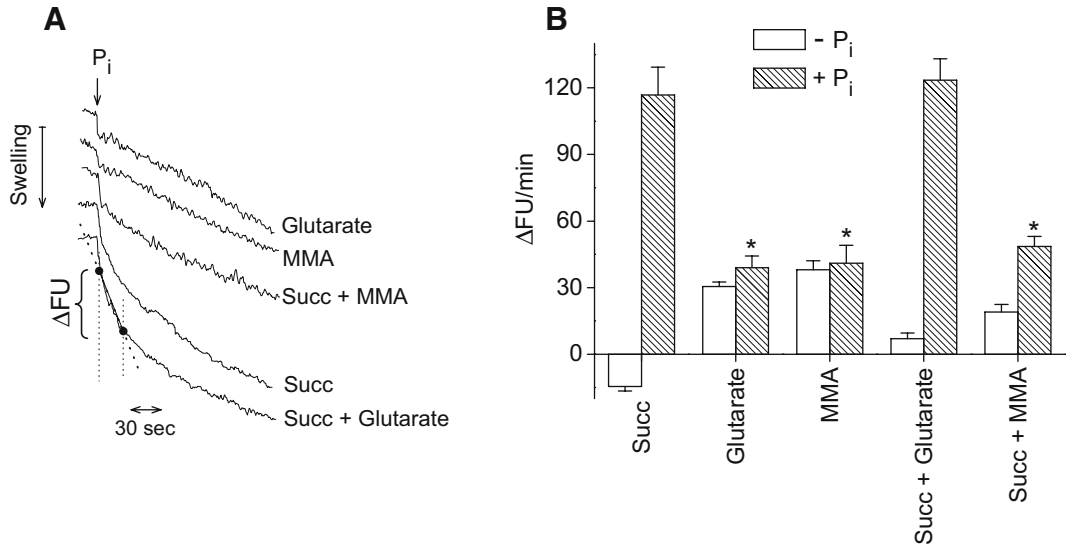

Fig. 5 Inhibitory effect of MMA on mitochondrial succinate transport by the dicarboxylate carrier. Rat forebrain mitochondria (0.4 mg/ml) were incubated at 37°C in reaction medium containing 0.1 mmol/L K^+-EGTA, 0.1 mmol/L K^+-EDTA, 5 mmol/L K^+-MOPS pH 7.0, 0.2 μmol/L antimycin A and 0.7 μmol/L nigericin. The medium contained a total of 36.7 mmol/L of potassium salts of succinate (Succ), glutarate and/or MMA: when two different salts were present, they were added at equal concentrations (18.35 mmol/L). (**A**) Representative figure of mitochondrial swelling in the presence of different dicarboxylates. Where indicated by the arrow, 2 mmol/L P_i (potassium salt) was added to the reaction medium. (**B**) Rates of mitochondrial swelling in the absence or presence of 2 mmol/L P_i (potassium salt). Data represent the mean±SEM of at least 5 independent experiments performed in duplicate. FU, fluorescence units. *Significantly different from 'Succ+P_i' at $p<0.01$

with MMA at millimolar concentrations, while MA, at micromolar concentrations, induced a potent inhibition of succinate-supported submitochondrial particle oxygen consumption.

Mitochondrial dicarboxylate carrier inhibition by MMA

In order to study the inhibitory effect of MMA on succinate transport by the dicarboxylate carrier, mitochondrial swelling experiments were conducted in a hyposmotic medium containing potassium salts, the respiratory chain inhibitor antimycin A and the K^+/H^+ ionophore nigericin. Under these conditions, the addition of P_i promotes transport of dicarboxylates into the mitochondrial matrix and consequent organellar swelling (Liu et al 1996). Figure 5 shows the initial rates of mitochondrial swelling in the absence and presence of P_i in reaction media containing K^+ salts of succinate, glutarate and/or MMA. Glutarate was used as a control anion that is not transported by the dicarboxylate carrier (Liu et al 1996). As expected, extensive mitochondrial swelling was promoted by addition of P_i to brain mitochondria incubated in succinate. This swelling was not inhibited when medium was prepared containing equal quantities of succinate and glutarate as osmotic support. Nevertheless, when succinate and MMA were used, an almost complete inhibition of P_i-induced swelling was observed.

MA production and SDH activity in MMA-treated mitochondria

A potential explanation for the result obtained would be that MMA generates MA in the mitochondrial matrix, which then inhibits SDH. To investigate this possibility, MA and 2-MCA were measured in brain mitochondrial suspensions after incubation with 10 mmol/L MMA during 10 min. Analyses performed by gas chromatography–mass spectrometry revealed no traces of these organic acids (results not shown; sensitivity of the assay ~1 nmol/mg protein).

The experiment reported in Fig. 6 was conducted to further verify whether isolated mitochondria incubated with MMA generated functionally significant quantities of MA. For these experiments, liver mitochondria were chosen because they resulted in more stable and reproducible results of SDH activity than isolated brain organelles under these conditions (results not shown). In intact liver mitochondria, we found that either 70 μmol/L MA or 7 mmol/L MMA lead to a decrease in oxygen consumption of approximately 50% (Fig. 6A). Samples of intact mitochondria incubated under these conditions for 10 min were collected

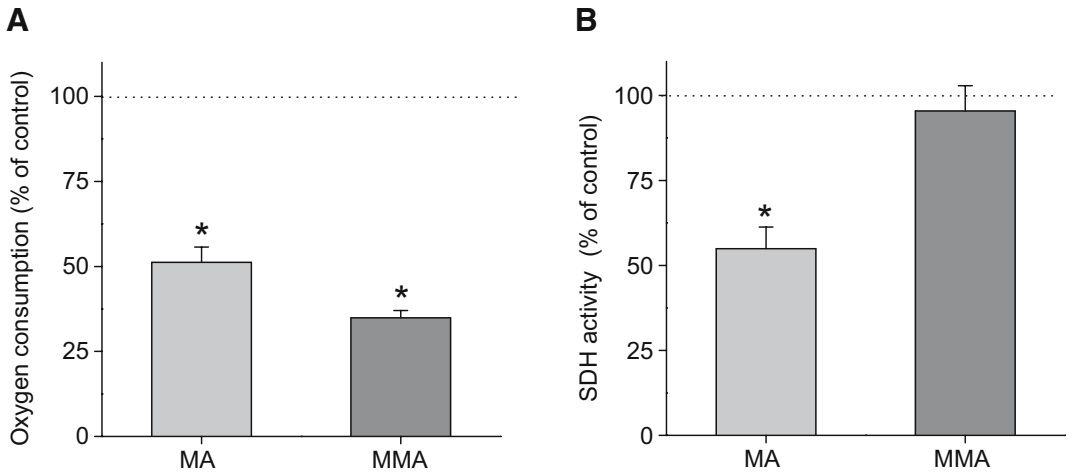

Fig. 6 Effects of pre-incubation with MMA and MA on liver mitochondrial SDH activity. (**A**) Oxygen consumption by rat liver mitochondria (0.4 mg/ml) during a 10 min incubation in standard reaction medium containing 1 mmol/L succinate and 2 μmol/L rotenone in the presence of 70 μmol/L MA or 7 mmol/L MMA. Data are represented as percentages of oxygen consumption in the absence of MA or MMA. (**B**) SDH activity after a 10 min pre-incubation under conditions indicated in the abscissa. After the pre-incubation period, 1% Triton X-100 and reagents for determination of SDH activity were added to the mitochondrial suspension (see Materials and methods). Under control conditions, SDH activity was 90.6±5.9 nmol of reduced DCIP/min per mg protein. Data represent the mean±SEM of 4 independent experiments performed in duplicate. *Significantly different from control at $p<0.01$

and treated with the detergent Triton X-100 in order to permeabilize mitochondria and release any MMA-generated metabolites accumulated in the matrix. SDH activity was then assayed as described in Material and methods. Samples pre-incubated in the presence of MA showed an important inhibition of SDH activity, indicating that MA-induced SDH inhibition could be readily detected under these conditions (Fig. 6B). The minimal concentration of MA present during the pre-incubation period that resulted in a significant inhibitory effect in the subsequent SDH assay was 10 μmol/L (result not shown), supporting the idea that this assay is a sensitive method for evaluating the presence of MA in the suspension. On the other hand, samples pre-incubated with 7 mmol/L MMA did not show any inhibitory effect on SDH after mitochondrial permeabilization with detergent. This result clearly indicates that no functionally significant amounts of MA accumulate in mitochondrial suspensions treated with MMA.

Discussion

In the present work we studied the inhibitory effect of MMA on mitochondrial succinate oxidation. In intact isolated brain and muscle mitochondria, millimolar MMA inhibited oxygen consumption maintained by succinate (Figs. 1 and 2). This observation is in accordance with previous studies reporting an inhibitory effect of MMA on succinate-supported oxygen consumption in isolated liver (Toyoshima et al 1995) and brain (Kowaltowski et al 2006; Maciel et al 2004) mitochondria. In addition, we showed that the inhibitory effect of MMA on mitochondrial oxygen consumption was not observed when electron donors to respiratory chain complexes I or IV were used. Under our experimental conditions, an immediate and uniform inhibition pattern of succinate-supported oxygen consumption was obtained, suggesting a direct effect of MMA on succinate-supported mitochondrial respiration and not a progressive interaction of this compound with mitochondrial components or formation of reactive products. In fact, we did not observe significant MA production in brain and liver mitochondria incubated in the presence of MMA. Okun and colleagues (2002) reported formation of MA in cultured striatal neurons treated with MMA (10 mmol/L), but MA was detected only in nanomolar concentrations and after 2 h of incubation.

In contrast to the results obtained with MMA-treated intact mitochondria oxidizing succinate, only a small effect of MMA was observed on succinate-supported oxygen consumption in permeabilized mitochondria (Fig. 3) or inverted submitochondrial particles (Fig. 4). In addition, no inhibitory effect of MMA was observed on SDH activity from detergent-permeabilized mitochondria (Table 1). In agreement with this last observation, Kolker's group also did not observe any important effects of MMA on SDH activity in submitochondrial particles (Kolker

et al 2003; Okun et al 2002), and recent publications from Wajner's group reported only a marginal inhibition of SDH activity by MMA in brain homogenates (Brusque et al 2002; Pettenuzzo et al 2006). These results suggest that the inhibitory effect of MMA on mitochondrial succinate oxidation is most likely due to inhibition of mitochondrial transport of this substrate. We showed evidence that succinate is transported by the dicarboxylate carrier in brain mitochondria (Fig. 5), as is the case for liver and kidney organelles (Palmieri et al 1971; Rumbach et al 1989; Schoolwerth and LaNoue 1985). In addition, our results identified MMA as a potent inhibitor of mitochondrial succinate transport by the dicarboxylate carrier (Fig. 5). This MMA effect explains the result of MMA on succinate-supported oxygen consumption observed in isolated brain (Kowaltowski et al 2006; Maciel et al 2004; present study) and liver (Toyoshima et al 1995) mitochondria. On the other hand, muscle mitochondria express very low amounts of the dicarboxylate carrier (Fiermonte et al 1999). Succinate transport into muscle mitochondria occurs most likely via the α-ketoglutarate carrier (Palmieri et al 1972; Sluse et al 1971) or other transport systems such as the mitochondrial inner membrane anion channel (IMAC) (Liu et al 1996). The mechanism by which MMA inhibits succinate transport in muscle mitochondria is unknown, but probably also involves competition with succinate for uptake into mitochondria.

Although succinate generated outside mitochondria is probably not a significant contributor to oxidative phosphorylation, mitochondrial dicarboxylate carrier inhibition by MMA probably has important physiopathological implications, such as inhibition of gluconeogenesis in liver and kidney, impairment of neuronal energy metabolism, glutamatergic neurotransmission and induction of oxidative stress, as discussed below. The inhibition of the mitochondrial dicarboxylate carrier by MMA in liver and kidney probably impairs gluconeogenesis. In fact, Halperin and colleagues (1971) proposed that MMA inhibition of malate transport through the mitochondrial dicarboxylate carrier contributes to hypoglycaemia in methylmalonic acidaemia. Malate is the main metabolic substrate transported by the dicarboxylate carrier (Schoolwerth and LaNoue 1985), while the transport of succinate or oxaloacetate by this carrier is either minor or less understood under physiological conditions. In the central nervous system, where gluconeogenesis does not occur, the physiological role of the mitochondrial dicarboxylate carrier is less understood, but is probably associated with supplying substrates to the tricarboxylic acid cycle, since intermediates of this cycle are expended during *de novo* glutamate synthesis (Hertz 2004). Neurons are considered incapable of *de novo* synthesis of tricarboxylic acid cycle intermediates, which requires pyruvate carboxylation present only in astrocytes (Yu et al 1983). In this regard, neuronal energy metabolism and glutamatergic neurotransmission may be compromised when MMA reaches high concentrations in the central nervous system. In addition, as recently proposed by Morath and colleagues (2007), mitochondrial dicarboxylate carrier inhibition by MMA may also inhibits glutathione (GSH) transport into kidney mitochondria (Lash 2006), promoting oxidative stress and chronic renal failure. This may be true for the brain as well, since *in vitro* and *in vivo* studies have demonstrated that MMA elicits oxidative stress in cerebral tissue (Fighera et al 1999; Fontella et al 2000; Malfatti et al 2003), the most affected tissue in methylmalonic acidaemia patients.

We conclude that results showing an inhibitory effect of MMA on either SDH activity (Brusque et al 2002; Dutra et al 1993; Fleck et al 2004; Pettenuzzo et al 2006) or succinate-supported mitochondrial oxygen consumption (Kowaltowski et al 2006; Maciel et al 2004; Toyoshima et al 1995) can be explained by an inhibitory effect of this organic acid on succinate transport through membranes and not by a direct effect on SDH activity (Kolker et al 2003; Okun et al 2002; present study).

Acknowledgements We thank Dr Alicia Kowaltowski for discussions, Edilene S. Santos and Elisangela J. Gomes for technical support and Dr. Luciane C. Alberici for help regarding rat muscle mitochondrial isolation. This study was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and the National Institutes of Health (NIH) (grant number: 1 R03 HD047388–01A1). S.R.M and D.R.M. are graduate students supported by CNPq and FAPESP fellowships, respectively.

References

Brismar J, Ozand PT (1994) CT and MR of the brain in disorders of propionate and methylmalonate metabolism. *Am J Neuroradiol* **15**: 1459–1473.

Brusque AM, Borba Rosa R, Schuck PF, et al (2002) Inhibition of the mitochondrial respiratory chain complex activities in rat cerebral cortex by methylmalonic acid. *Neurochem Int* **40**: 593–601.

Dutra JC, Dutra-Filho CS, Cardozo SE, Wannmacher CM, Sarkis JJ, Wajner M (1993) Inhibition of succinate dehydrogenase and beta-hydroxybutyrate dehydrogenase activities by methylmalonate in brain and liver of developing rats. *J Inherit Metab Dis* **16**: 147–153.

Fenton WA, Gravel RAA, Rosenblatt DS (2001) Disorders of propionate and methylmalonate metabolism. In: Scriver CR, Beaudet AL, Sly WS, Valle D, eds; Childs B, Kinzler KW, Vogelstein B, assoc. eds. *The Metabolic and Molecular*

Bases of Inherited Disease, 8th edn. New York: McGraw-Hill, 2165–2193.

Fiermonte G, Dolce V, Arrigoni R, Runswick MJ, Walker JE, Palmieri F (1999) Organization and sequence of the gene for the human mitochondrial dicarboxylate carrier: evolution of the carrier family. *Biochem J* **344**: 953–960.

Fighera MR, Queiroz CM, Stracke MP, et al (1999) Ascorbic acid and alpha-tocopherol attenuate methylmalonic acid-induced convulsions. *NeuroReport* **10**: 2039–2043.

Fleck J, Ribeiro MC, Schneider CM, Sinhorin VD, Rubin MA, Mello CF (2004) Intrastriatal malonate administration induces convulsive behaviour in rats. *J Inherit Metab Dis* **27**: 211–219.

Fontella FU, Pulrolnik V, Gassen E, et al (2000) Propionic and L-methylmalonic acids induce oxidative stress in brain of young rats. *Neuroreport* **11**: 541–544.

Gornall AG, Bardawill CJ, Donid MM (1949) Determination of serum protein by means of the Biuret reaction. *J Biol Chem* **177**: 751–766.

Halperin ML, Schiller CM, Fritz IB (1971) The inhibition by methylmalonic acid of malate transport by the dicarboxylate carrier in rat liver mitochondria. A possible explantation for hypoglycemia in methylmalonic aciduria. *J Clin Invest* **50**: 2276–2282.

Harmon HJ (1982) Isolation of totally inverted submitochondrial particles by sonication of beef heart mitochondria. *J Bioenerg Biomembr* **14**: 377–386.

He K, Ludtke SJ, Worcester DL, Huang HW (1996) Neutron scattering in the plane of membranes: structure of alamethicin pores. *Biophys J* **70**: 2659–2666.

Heidenreich R, Natowicz M, Hainline BE, et al (1988) Acute extrapyramidal syndrome in methylmalonic acidemia: "metabolic stroke" involving the globus pallidus. *J Pediatr* **113**: 1022–1027.

Hertz L (2004) Intercellular metabolic compartmentation in the brain: past, present and future. *Neurochem Int* **45**: 285–296.

Hoffmann GF, Meier-Augenstein W, Stocker S, Surtees R, Rating D, Nyhan W (1993) Physiology and pathophysiology of organic acids in cerebrospinal fluid. *J Inherit Metab Dis* **16**: 648–669.

Kolker S, Okun JG (2005) Methylmalonic acid—an endogenous toxin? *Cell Mol Life Sci* **62**: 621–624.

Kolker S, Schwab M, Horster F, et al (2003) Methylmalonic acid, a biochemical hallmark of methylmalonic acidurias but no inhibitor of mitochondrial respiratory chain. *J Biol Chem* **278**: 47388–47393.

Kowaltowski AJ, Maciel EN, Fornazari M, Castilho RF (2006) Diazoxide protects against methylmalonate-induced neuronal toxicity. *Exp Neurol* **201**: 165–171.

Larnaout A, Mongalgi MA, Kaabachi N, et al (1998) Methylmalonic acidaemia with bilateral globus pallidus involvement: a neuropathological study. *J Inherit Metab Dis* **21**: 639–644.

Lash LH (2006) Mitochondrial glutathione transport: physiological, pathological and toxicological implications. *Chem Biol Interact* **163**: 54–67.

Liu G, Hinch B, Beavis AD (1996) Mechanisms for the transport of alpha,omega-dicarboxylates through the mitochondrial inner membrane. *J Biol Chem* **271**: 25338–25344.

Maciel EN, Kowaltowski AJ, Schwalm FD, et al (2004) Mitochondrial permeability transition in neuronal damage promoted by Ca^{2+} and respiratory chain complex II inhibition. *J Neurochem* **90**: 1025–1035.

Malfatti CR, Royes LF, Francescato L, et al (2003) Intrastriatal methylmalonic acid administration induces convulsions and TBARS production, and alters Na^{+},K^{+}-ATPase activity in the rat striatum and cerebral cortex. *Epilepsia* **44**: 761–767.

Marisco PC, Ribeiro MCP, Bonini JS, et al (2003) Ammonia potentiates methylmalonic acid-induced convulsions and TBARS production. *Exp Neurol* **182**: 455–460.

McLaughlin BA, Nelson D, Silver IA, Erecinska M, Chesselet MF (1998) Methylmalonate toxicity in primary neuronal cultures. *Neuroscience* **86**: 279–290.

Morath MA, Okun JG, Muller IB, et al (2007) Neurodegeneration and chronic renal failure in methylmalonic aciduria—a pathophysiological approach. *J Inherit Metab Dis*, doi:10.1007/s10545-007-0571-5.

Okun JG, Horster F, Farkas LM, et al (2002) Neurodegeneration in methylmalonic aciduria involves inhibition of complex II and the tricarboxylic acid cycle, and synergistically acting excitotoxicity. *J Biol Chem* **277**: 14674–14680.

Palmieri F, Prezioso G, Quagliariello E, Klingenberg M (1971) Kinetic study of the dicarboxylate carrier in rat liver mitochondria. *Eur J Biochem* **22**: 66–74.

Palmieri F, Quagliariello E, Klingenberger M (1972) Kinetics and specificity of the oxoglutarate carrier in rat-liver mitochondria. *Eur J Biochem* **29**: 408–416.

Pettenuzzo LF, Ferreira Gda C, Schmidt AL, Dutra-Filho CS, Wyse AT, Wajner M (2006) Differential inhibitory effects of methylmalonic acid on respiratory chain complex activities in rat tissues. *Int J Dev Neurosci* **24**: 45–52.

Reszko AE, Kasumov T, Pierce BA, et al (2003) Assessing the reversibility of the anaplerotic reactions of the propionyl-CoA pathway in heart and liver. *J Biol Chem* **278**: 34959–34965.

Robinson BH, Chappell JB (1967) The inhibition of malate, tricarboxylate and oxoglutarate entry into mitochondria by 2-n-butylmalonate. *Biochem Biophys Res Commun* **28**: 249–255.

Rosenthal RE, Hamud F, Fiskum G, Varghese PJ, Sharpe S (1987) Cerebral ischemia and reperfusion: prevention of brain mitochondrial injury by lidoflazine. *J Cereb Blood Flow Metab* **7**: 752–758.

Royes LF, Fighera MR, Furian AF, et al (2003) Creatine protects against the convulsive behavior and lactate production elicited by the intrastriatal injection of methylmalonate. *Neuroscience* **118**: 1079–1090. Erratum in: *Neuroscience* (2004) **125**: 533.

Rumbach L, Cremel G, Marescaux C, Warter JM, Waksman A (1989) Succinate transport inhibition by valproate in rat renal mitochondria. *Eur J Pharmacol* **164**: 577–581.

Saad LO, Mirandola SR, Maciel EN, Castilho RF (2006) Lactate dehydrogenase activity is inhibited by methylmalonate in vitro. *Neurochem Res* **31**: 541–548.

Sauer SW, Okun JG, Fricker G, et al (2006) Intracerebral accumulation of glutaric and 3-hydroxyglutaric acids secondary to limited flux across the blood–brain barrier constitute a biochemical risk factor for neurodegeneration in glutaryl-CoA dehydrogenase deficiency. *J Neurochem* **97**: 899–910.

Schoolwerth AC, LaNoue KF (1985) Transport of metabolic substrates in renal mitochondria. *Annu Rev Physiol* **47**: 143–171.

Singer TP (1974) Determination of the activity of succinate, NADH, choline, and alpha-glycerophosphate dehydrogenases. *Methods Biochem Anal* **22**: 123–175.

Sluse FE, Meijer AJ, Tager JM (1971) Anion translocators in rat-heart mitochondria. *FEBS Lett* **18**: 149–153.

Toyoshima S, Watanabe F, Saido H, Miyatake K, Nakano Y (1995) Methylmalonic acid inhibits respiration in rat liver mitochondria. *J Nutr* **125**: 2846–2850.

Trinh BC, Melhem ER, Barker PB (2001) Multi-slice proton MR spectroscopy and diffusion-weighted imaging in methylmalonic

acidemia: report of two cases and review of the literature. *Am J Neuroradiol* **22**: 831–833.

Velho JA, Okanobo H, Degasperi GR, et al (2006) Statins induce calcium-dependent mitochondrial permeability transition. *Toxicology* **219**: 124–132.

Wajner M, Coelho JC (1997) Neurological dysfunction in methylmalonic acidaemia is probably related to the inhibitory effect of methylmalonate on brain energy production. *J Inherit Metab Dis* **20**: 761–768.

Wajner M, Dutra JC, Cardozo SE, Wannmacher CMD, Motta ER (1992) Effect of methylmalonate on *in vivo* lactate release and carbon dioxide production by brain of suckling rats. *J Inherit Metab Dis* **15**: 92–96.

Wajner M, Raymond K, Barschak A, et al (2002) Detection of organic acidemias in Brazil. *Arch Med Res* **33**: 581–585.

Yu ACH, Drejer J, Hertz L, Schousboe A (1983) Pyruvate carboxylase activity in primary cultures of astrocytes and neurons. *J Neurochem* **41**: 1484–1487.