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Lactate Dehydrogenase Activity is Inhibited by Methylmalonate *in vitro*

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Abstract Methylmalonic acidemia (MMAemia) is an inherited metabolic disorder of branched amino acid and odd-chain fatty acid metabolism, involving a defect in the conversion of methylmalonyl-coenzyme A to succinyl-coenzyme A. Systemic and neurological manifestations in this disease are thought to be associated with the accumulation of methylmalonate (MMA) in tissues and biological fluids with consequent impairment of energy metabolism and oxidative stress. In the present work we studied the effect of MMA and two other inhibitors of mitochondrial respiratory chain complex II (malonate and 3-nitropropionate) on the activity of lactate dehydrogenase (LDH) in tissue homogenates from adult rats. MMA potently inhibited LDH-catalyzed conversion of lactate to pyruvate in liver and brain homogenates as well as in a purified bovine heart LDH preparation. LDH was about one order of magnitude less sensitive to inhibition by MMA when catalyzing the conversion of pyruvate to lactate. Kinetic studies on the inhibition of brain LDH indicated that MMA inhibits this enzyme competitively with lactate as a substrate (K_i=3.02 ± 0.59 mM). Malonate and 3-nitropropionate also strongly inhibited LDH-catalyzed conversion of lactate to pyruvate in brain homogenates, while no inhibition was observed by succinate or propionate, when present in concentrations of up to 25 mM. We propose that inhibition of the lactate/pyruvate conversion by MMA contributes to lactate accumulation in blood, metabolic acidemia and inhibition of gluconeogenesis observed in patients with MMAemia. Moreover, the inhibition of LDH in the central nervous system may also impair the lactate shuttle between astrocytes and neurons, compromising neuronal energy metabolism.

Keywords Inborn error of metabolism · Lactic acid · Lactate dehydrogenase · Malonate · Methylmalonic acidemia · 3-nitropropionate · Neurodegeneration

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Introduction

Methylmalonic acidemia (MMAemia) is an inherited autosomic recessive metabolic disorder affecting 1 in every 48,000 newborns [1, 2]. MMAemia is frequently caused by deficiency of methylmalonyl-coenzyme A (CoA) mutase or by abnormalities of its cofactor adenosylcobalamin [2]. Methylmalonyl-CoA mutase catalyses the conversion of methylmalonyl-CoA to succinyl-CoA, a key step in the metabolism of branched chain aminoacids isoleucine, valine, threonine and methionine, as well as odd-chain fatty acid and cholesterol degradation [2]. As a result, methylmalonyl-CoA and methylmalonate (MMA), among other metabolites, accumulate in tissues and body fluids. MMA is produced predominantly in tissues before reaching plasma and cerebrospinal fluid, where 0.2–3 mM levels have been reported during crises of metabolic decompensation [2–4]. MMAemia is characterized by generalized tissue damage with prominent effects in the brain [3–6], possibly because MMA accumulates at higher levels in this tissue, or due to the large dependence of this tissue on aerobic metabolism. In addition, our group has recently demonstrated that

MMA added to Ca^{2+}-loaded isolated brain mitochondria [7] acts as a potent inducer of membrane permeability transition, a process involved in necrotic and apoptotic cell death [8, 9].

MMAemia patients are at constant risk of metabolic decompensation with metabolic acidosis, ketonemia, hyperammonemia, hyperglycinemia, hypoglycemia, metabolic stroke, damage to basal ganglia and further neurological deterioration [2, 6, 10, 11]. The concentration of MMA does not explain the acidemia observed in MMAemia, suggesting the involvement of other processes and metabolites in this alteration. Hypoglycemia, which occurs mainly during crises [2, 12, 13], is attributed to inhibition of pyruvate carboxylase [14] and malate transport through the inner mitochondrial membrane [15], important steps in gluconeogenesis. Other enzymes involved in energy metabolism that are inhibited by MMA include succinate dehydrogenase ([16, 17]; but see [18, 19]), β-hydroxybutyrate dehydrogenase [16] and mitochondrial creatine kinase [20].

In the present study, we investigated the effect of MMA on lactate dehydrogenase (LDH) (EC 1.1.1.27) activity. LDH catalyses the interconversion of pyruvate, a product of glycolysis, and lactate. A first evidence of LDH inhibition by MMA was obtained by our group when evaluating the activity of this enzyme as a potential marker of cellular damage in brain slices exposed to MMA [7]. Based on the importance of LDH in cellular, tissue and whole body metabolism (for a recent review, see [21]), we conducted the present study to characterize the inhibitory effect of MMA and other related compounds on the activity of this enzyme.

Experimental Procedures

Reagents

Most chemicals, including L-lactic dehydrogenase from bovine heart, lactic acid, malonic acid, methylmalonic acid, β-nicotinamide adenine dinucleotide sodium salt (NAD$^+$), β-nicotinamide adenine dinucleotide reduced disodium salt (NADH), 3-nitropropionic acid, propionic acid and pyruvic acid were obtained from Sigma-Aldrich (Sigma-Aldrich Inc., St. Louis, MO, USA). MMA, 3-nitropropionate (3-NP) and malonate (MA) were prepared by dissolving the respective acids in water and adjusting the pH to 7.2 with NaOH.

Preparation of tissue homogenates

Female Wistar rats weighing 200–250 g were used. Rats were sacrificed by decapitation. Brain, liver, heart or gastrocnemial muscles were rapidly removed (within 2 min) and put into 10 ml of ice-cold "isolation buffer" containing 225 mM mannitol, 75 mM sucrose, 1 mM K$^+$-EGTA, 0.1 % bovine serum albumin (BSA) and 10 mM K$^+$-HEPES pH 7.2. Cerebellum and underlying structures were removed from the brain and the remaining material (forebrain) was used. The tissues were cut into small pieces using surgical scissors and extensively washed with "isolation buffer". The tissue was then manually homogenized in a Dounce homogenizer using both a loose-fitting and a tight-fitting pestle. The homogenate was centrifuged for 3 min at 2,000 g (forebrain), 10 min at 2,500 g (liver), 10 min at 800 g (heart) or 10 min at 700 g (muscle) in a Beckman JA 25.5 rotor (Beckman, Palo Alto, CA, USA). After the first centrifugation, the supernatant was recentrifuged for 10 min at 8,000 g (brain, liver and muscle) or 10 min at 6,000 g (heart). The supernatant from this second centrifugation, at an approximate protein concentration of 5–8 mg/ml, was used as "tissue homogenate" for the experiments. The purpose of these two centrifugations was to obtain a fraction rich in cytosolic content, but poor in heavy cellular components, nuclei and mitochondria. Control experiments showed no evidence of a considerable fraction of functioning mitochondria in the "tissue homogenates", as verified by the lack of pyruvate-supported oxygen consumption (results not shown).

Protein determination

The protein content of brain, liver, heart and muscle homogenates was determined by the Biuret assay [22] in the presence of 0.2% deoxycholate, using BSA as a standard.

LDH activity assay

The experiments to evaluate LDH-catalyzed conversion of pyruvate to lactate were carried out at 37°C in a fluorimeter cuvette, with continuous magnetic stirring, in reaction medium containing 50 mM Tris–HCl buffer, pH 7.4, and different concentrations of pyruvate-Na$^+$, as indicated in the figures. Experiments were conducted under these pH conditions to simulate enzymatic reactions in the cytosol. To evaluate LDH-catalyzed conversion of lactate to pyruvate the experiments were carried out in reaction medium containing different concentrations of lactate-Na$^+$, as indicated in the figures. After 30 sec of pre-incubation of "tissue homogenates" (20–40 μg of protein) or bovine heart LDH (1.47 μg) in 2 ml of reaction medium, the reaction was started by the addition of 0.1 mM NADH or 0.8 mM NAD$^+$, for measurement of conversion of pyruvate to lactate or lactate to pyruvate, respectively. The reaction was

stopped after 3 min. Different concentrations of MMA, malonate, 3-nitropropionate, succinate and propionate were present since the beginning of the pre-incubation period. The reagent concentrations and the amount of protein added were chosen to assure linearity of the enzymatic reaction at least during the first 30–40 sec. Rates of produced or consumed NADH were estimated considering the reaction rates between 10 and 40 sec (5–15 sec, for heart and muscle) after NAD^+ or NADH addition, respectively. LDH activity was followed by measuring NADH fluorescence levels recorded on a model F-4010 Hitachi spectrofluorometer (Hitachi Ltd., Tokyo, Japan) operating at excitation and emission wavelengths of 366 and 450 nm, respectively. Both slit widths used were at 2.5 nm or 5.0 nm, for reactions following NADH consumption or production, respectively.

LDH-catalyzed conversion of pyruvate to lactate was 0.97 ± 0.12, 1.87 ± 0.39, 7.72 ± 1.31, and 14.48 ± 6.10 mM NADH min^{-1} mg^{-1}, for brain, liver, heart and muscle tissue homogenates, respectively, using 0.1 mM pyruvate. LDH-catalyzed conversion of lactate to pyruvate was 0.18 ± 0.03 and 0.26 ± 0.03 mM of NAD^+ min^{-1} mg^{-1}, for brain and liver tissue homogenates, respectively, using 2 mM lactate. Using a purified bovine heart LDH, enzymatic activity was 43.53 ± 4.71 mM of NAD^+ min^{-1} mg^{-1}, for lactate/pyruvate conversion. It is important to note that to follow lactate/pyruvate conversion, a higher concentration of the substrate was used. In addition, some experiments were conducted in the presence of 50 mM hydroxylamine, a trapping agent for the pyruvate formed.

Statistical analysis

Data from the experiments were analyzed by one-way ANOVA followed by Tukey's post-hoc test performed by OriginPro 7.5 SRO (OriginLab Corporation, Northampton, MA, USA). When one parameter was compared between two groups, Student's t-test was used. Data are presented as averages ± standard deviation (± SD) of at least three experiments conducted with different preparations.

Results

The *in vitro* effect of MMA, at concentrations varying from 5 to 50 mM, on LDH-catalyzed conversion of pyruvate to lactate was studied in tissue homogenates from adult rat brain (Fig. 1a, b), liver (Fig. 1c, d), heart (Fig. 1e) and muscle (Fig. 1f). Panels a and c depict representat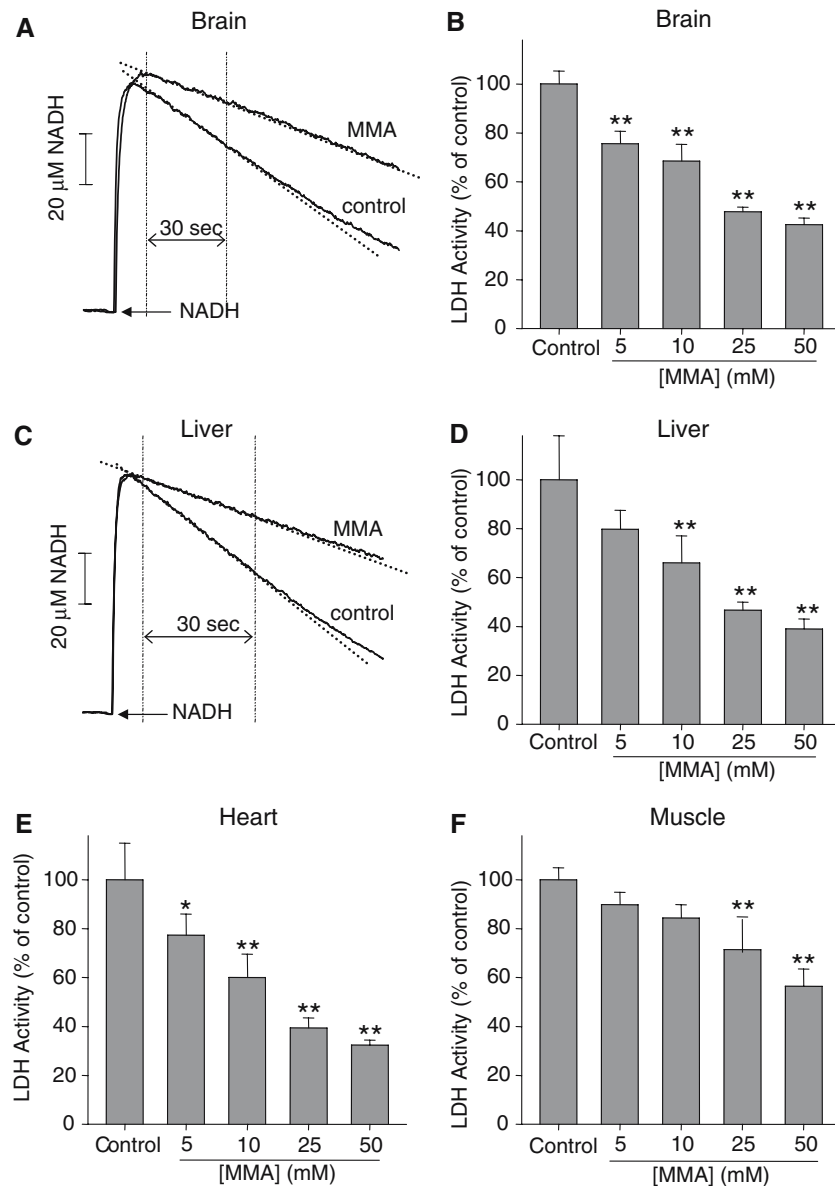ive traces of LDH-catalyzed pyruvate/lactate conversion in brain and liver homogenates, respectively. Similar patterns were obtained in heart and muscle homogenates (results not shown). MMA significantly inhibited LDH activity in a dose-dependent manner, with IC_{50} of 23.3 ± 0.6 mM, 20.8 ± 2.9 mM, 16.5 ± 5.1 mM and 56.2 ± 13.5 mM, for brain, liver, heart and muscle homogenates, respectively. The IC_{50} of MMA for pyruvate/lactate conversion in muscle homogenate was significantly higher than that observed in brain, liver and heart homogenates ($P < 0.05$, Tukey's test). The concentration of pyruvate used in these experiments was in the range of those in tissue and whole blood under physiological conditions [23–26]. Moreover, pyruvate concentrations above 250 μM resulted in a progressive inhibition of the reaction rate in brain homogenates (results not shown), in accordance with an early report [27]. Under our experimental conditions, no significant NADH oxidation was observed in the presence of brain homogenate but in the absence of added pyruvate, indicating that the presence endogenous NADH oxidases is not significantly affecting the measurements (results not shown).

Interestingly, LDH-catalyzed conversion of lactate to pyruvate in homogenates from rat brain (Fig. 2a, b) and liver (Fig. 2c, d) was inhibited by lower concentrations of MMA, with an IC_{50} of 4.6 ± 0.39 mM and 5.6 ± 1.3 mM, respectively. When lactate/pyruvate conversion in brain homogenate was determined in the presence of hydroxylamine, a trapping agent for the pyruvate formed [27], the MMA IC_{50} was 5.50 ± 0.86 mM. The concentration of lactate used in these experiments was in the range of physiological concentrations of lactate (venous blood: 0.6–2.2 mmol/l, arterial blood: 0.3–0.8 mmol/l) [25].

Since tissue homogenates contain many enzymes in addition to LDH that could convert MMA to other metabolites [18, 19], the effect of MMA was tested on purified LDH from bovine heart (Fig. 3). This enzyme preparation was also sensitive to MMA in a dose-dependent manner, when converting lactate to pyruvate, with an IC_{50} of 5.2 ± 0.57 mM.

In order to better characterize the *in vitro* inhibitory effect of MMA on brain LDH activity, we carried out competition experiments between MMA and lactate. The K_m for lactate as substrate was 1.79 ± 0.32 mM ($n=4$). The Lineweaver-Burk plot indicated that MMA inhibited LDH activity by competition with lactate (Fig. 4). The K_i value was 3.02 ± 0.59 mM ($n=4$) for MMA, calculated using the Dixon plot [28] (result not shown).

Since MMA [16, 17] or its metabolites [18, 19] inhibit succinate dehydrogenase, experiments were done to compare the effects of two other succinate dehydrogenase inhibitors: 3-nitropropionate (Fig. 5a) and malonate

Fig. 1 Effect of methylmalonate (MMA) on lactate dehydrogenase (LDH)-catalyzed conversion of pyruvate to lactate in brain (**a**, **b**), liver (**c**, **d**), heart (**e**) and muscle (**f**) homogenates from adult rats. The reactions were started by the addition of 0.1 mM NADH to the reaction medium containing 0.1 mM pyruvate and tissue homogenate. Panels **a** and **c** depicted representative traces of LDH-catalyzed pyruvate/lactate conversion measured by NADH oxidation in brain and liver homogenates, respectively, in the absence (control) and presence of 25 mM MMA. Data in Panels **b**, **d**, **e** and **f** are mean ± S.D. of 3–5 experiments performed in duplicate. *Significantly different from control, $P < 0.05$. **Significantly different from control, $P < 0.01$

(Fig. 5b), on brain LDH-catalyzed conversion of lactate to pyruvate. 3-Nitropropionate and malonate significantly inhibited LDH activity in a dose-dependent manner, with an IC_{50} of 3.58 ± 1.28 mM and 1.41 ± 0.31 mM, respectively. In addition, the effects of propionate (Fig. 5c) and the physiological substrate succinate were also evaluated. Lactate/pyruvate conversion was not inhibited by succinate (results not shown) and was slightly (non-significantly) sensitive to propionate when present in concentrations up to 25 mM (Fig. 5c).

Discussion

In the present work we studied the inhibitory effect of MMA on LDH activity in homogenates obtained from adult rat brain, heart, liver and muscle. LDH-catalyzed conversion of either pyruvate to lactate or lactate to pyruvate was evaluated. Interestingly, MMA levels observed during crises of metabolic decompensation in MMAemia, i.e. up to 3 mM [2–4], extensively inhibit LDH-catalyzed conversion of lactate to pyruvate (Figs. 2–4), but have a
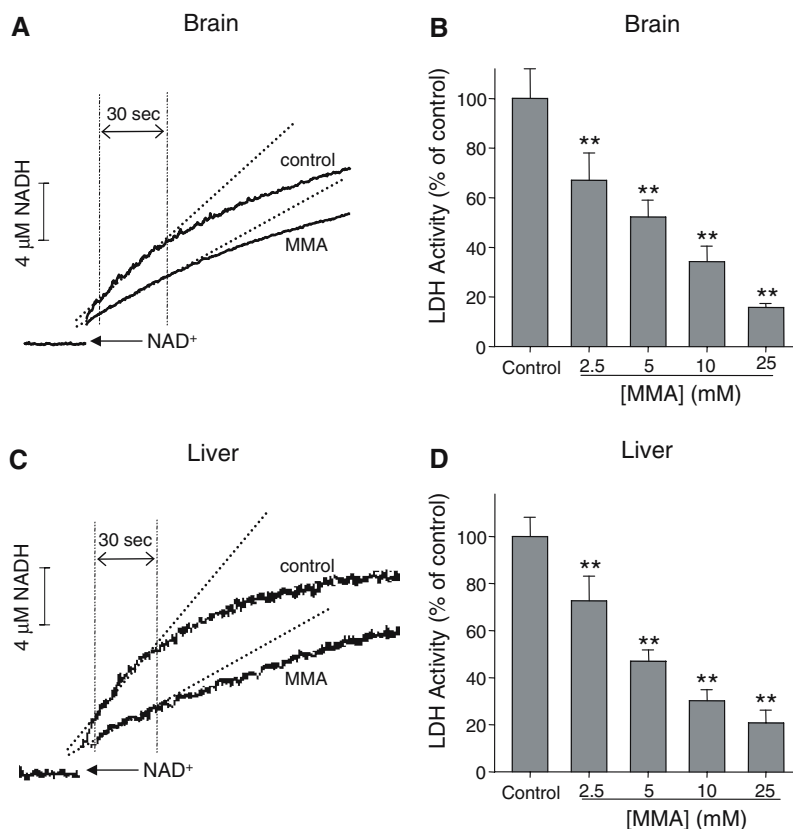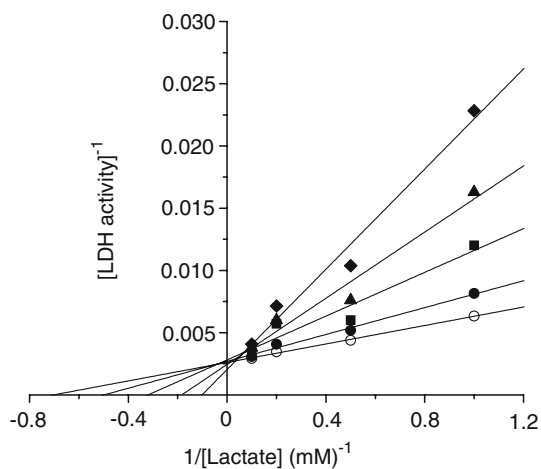
Fig. 2 Effect of MMA on LDH-catalyzed conversion of lactate to pyruvate in brain (**a**, **b**) and liver (**c**, **d**) homogenates from adult rats. The reactions were started by the addition of 0.8 mM NAD$^+$ to the reaction medium containing 2 mM lactate and tissue homogenate. Panels **a** and **c** depicted representative traces of LDH-catalyzed lactate/pyruvate conversion measured by NAD$^+$ reduction in brain and liver homogenates, respectively, in the absence (control) and presence of 5 mM MMA. Data in Panels **b** and **d** are mean ± S.D. of 3–5 experiments performed in duplicate. **Significantly different from control, $P < 0.01$
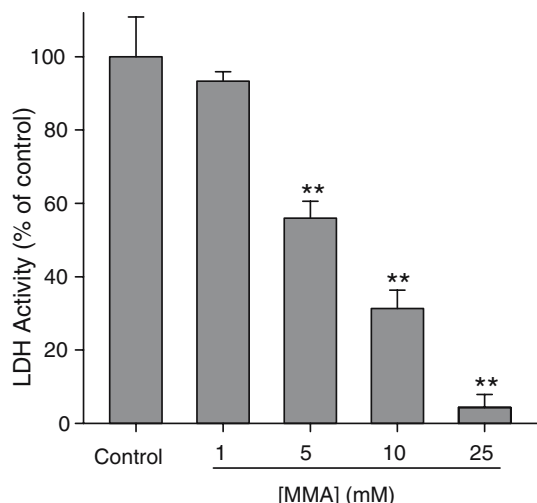
Fig. 3 Effect of MMA on purified bovine heart LDH-catalyzed conversion of lactate to pyruvate. The reactions were started by the addition of 0.8 mM NAD$^+$ to the reaction medium containing 2 mM lactate and tissue homogenate. Data are mean ± S.D. of 3 experiments performed in duplicate. **Significantly different from control, $P < 0.01$

Fig. 4 Lineweaver-Burk competition plots between lactate and MMA on LDH activity (μM NAD$^+$ min^{-1} mg^{-1}) in rat brain homogenates. Data are representative of 4–5 experiments performed in duplicate. (○) control (without inhibitor); (●) 1 mM; (■) 5 mM; (▲) 10 mM; (◆) 25 mM MMA
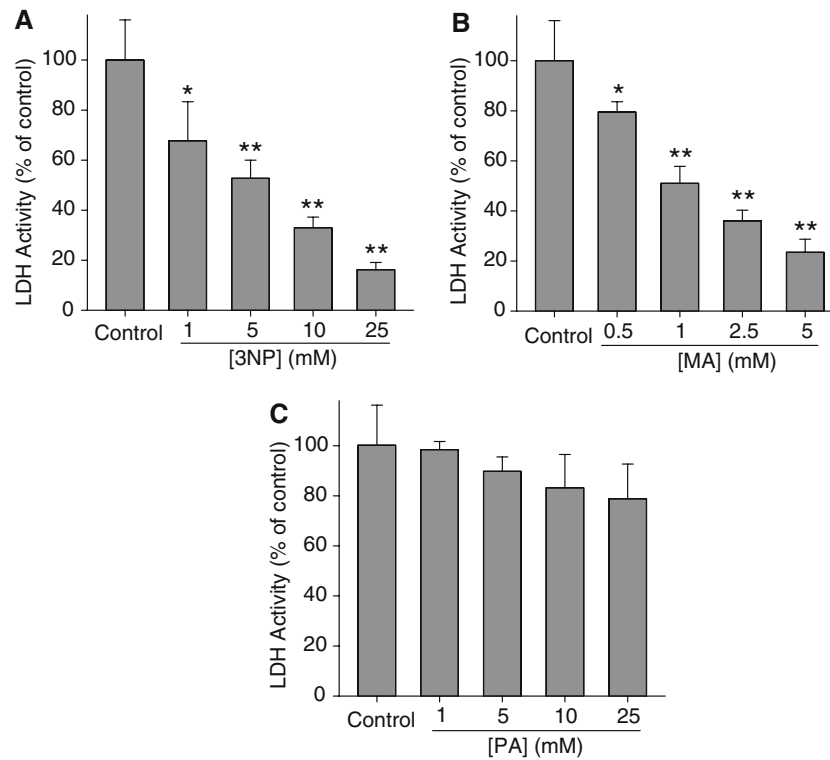
Fig. 5 Effect of 3-nitropropionate (3-NP) (**a**), malonate (MA) (**b**) or propionate (PA) (**c**) on LDH-catalyzed conversion of lactate to pyruvate in rat brain homogenates. The reactions were started by the addition of 0.8 mM NAD^+ to the reaction medium containing 2 mM lactate and tissue homogenate. Data are mean ± S.D. of 3 experiments performed in duplicate. *Significantly different from control, $P < 0.05$. **Significantly different from control, $P < 0.01$

minor inhibitory effect on the conversion of pyruvate to lactate (Fig. 1) when the experiments were conducted in the presence of pyruvate and lactate in the range of physiological concentrations. Since MMA [16, 17] or its metabolites [19] inhibit the respiratory chain enzyme succinate dehydrogenase, we also tested the effects of a competitive (malonate) and of a non-competitive and irreversible (3-nitropropionate) succinate dehydrogenase inhibitors. Both malonate and 3-nitropropionate potently inhibited lactate/pyruvate conversion (Fig. 5). However, LDH activity was not inhibited by the physiological substrate succinate. Recently, most of the inhibitory effects of MMA on respiratory chain have been attributed to intramitochondrial production of metabolites such as malonate and 2-methylcitrate [18, 19]. Under our conditions, we observed a potent inhibition of LDH-catalyzed conversion of lactate to pyruvate in a purified preparation of this enzyme (Fig. 3), in which the production of MMA metabolites is not expected. This indicates that MMA is the primary inhibitor of this reaction.

In mammalian cells, LDH isoenzymes are composed of four polypeptide chains, containing different ratios of two kinds of polypeptide: the A chain (also designated M for muscle) and the B chain (also designated H for heart) [29].

The A chain predominates in tissues subject to anaerobic conditions such as skeletal muscle (A_4 isoenzyme) and the B chain predominates in aerobic tissues such as heart muscle (B_4 isoenzyme). The A chain appears to mainly function in the reduction of pyruvate by NADH into lactate, whereas the B chain appears to be better adapted to catalyze the reverse reaction. In brain tissue, a predominance of B_4 isoenzyme is observed in neurons, while astrocytes present both A_4 and B_4 isoenzymes [30], yielding a mixture of LDH isoenzymes in brain homogenates. Liver presents predominantly the A_4 isoenzyme [31]. Considering that our results show that both liver (A_4) and heart (B_4), but not muscle (A_4), have similar sensitivity to MMA-inhibited pyruvate/lactate conversion (Fig. 1), there is no apparent indication for different susceptibility to the inhibitory effect of MMA regarding the LDH isoenzyme.

Kinetics studies showed that MMA inhibits brain LDH by competition with the substrate lactate (Fig. 4). The K_i value (3.02 ± 0.59 mM) was in the range of the MMA plasma concentration observed in crises of metabolic decompensation in MMAemia (2–4). The K_m for lactate under our experimental conditions (1.79 ± 0.32 mM, brain homogenate) is in the range of physiological

concentrations of lactate (venous blood: 0.6–2.2 mmol/l, arterial blood: 0.3–0.8 mmol/l) [25], indicating that LDH is not saturated in respect to lactate. These observations indicate that MMA could actually be a relevant competitive inhibitor of the lactate/pyruvate conversion under pathological situations.

In conclusion, our findings indicate that MMA is a potent inhibitor of LDH-catalyzed conversion of lactate to pyruvate. This inhibition may contribute to lactate accumulation and inhibition of gluconeogenesis observed in MMAemia [2, 13, 14, 32], since lactate is an important source of carbon atoms for glucose synthesis by gluconeogenesis. Impairment of mitochondrial function due to membrane permeability transition promoted by MMA and Ca^{2+} [7] would enhance glycolytic metabolism and also contribute to lactate accumulation in MMAemia. Moreover, LDH inhibition may impair the lactate shuttle between astrocytes and neurons [21, 33], compromising neuronal energy metabolism and glutamatergic neurotransmission when MMA reaches high concentrations in the CNS.

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