

Chronic postnatal administration of methylmalonic acid provokes a decrease of myelin content and ganglioside N-acetylneuraminic acid concentration in cerebrum of young rats

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Abstract

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Levels of methylmalonic acid (MMA) comparable to those of human methylmalonic acidemia were achieved in blood (2-2.5 mmol/l) and brain (1.35 $\mu\text{mol/g}$) of rats by administering buffered MMA, pH 7.4, subcutaneously twice a day from the 5th to the 28th day of life. MMA doses ranged from 0.76 to 1.67 $\mu\text{mol/g}$ as a function of animal age. Control rats were treated with saline in the same volumes. The animals were sacrificed by decapitation on the 28th day of age. Blood was taken and the brain was rapidly removed. Medulla, pons, the olfactory lobes and cerebellum were discarded and the rest of the brain ("cerebrum") was isolated. Body and "cerebrum" weight were measured, as well as the cholesterol and triglyceride concentrations in blood and the content of myelin, total lipids, and the concentrations of the lipid fractions (cholesterol, glycerolipids, phospholipids and ganglioside N-acetylneuraminic acid (ganglioside-NANA)) in the "cerebrum". Chronic MMA administration had no effect on body or "cerebrum" weight, suggesting that the metabolites *per se* neither affect the appetite of the rats nor cause malnutrition. In contrast, MMA caused a significant reduction of plasma triglycerides, but not of plasma cholesterol levels. A significant diminution of myelin content and of ganglioside-NANA concentration was also observed in the "cerebrum". We propose that the reduction of myelin content and ganglioside-NANA caused by MMA may be related to the delayed myelination/cerebral atrophy and neurological dysfunction found in methylmalonic acidemic children.

Key words

- Methylmalonic acid
- Glycerides
- Lipids
- Myelin
- Rat brain
- Methylmalonic acidemia

Methylmalonic acidemia is a relatively common organic acidemia due to the deficient activity of L-methylmalonyl-CoA mutase (EC 5.4.99.2), primarily leading to the accumulation of methylmalonyl-CoA and secondarily to the accumulation of propionyl-CoA. Increased amounts of methylmalonic acid (MMA) (1-2.5 mmol/l) are commonly found in blood, and increased MMA and some propionyl-CoA metabolites (methylcitrate and 3-hydroxypropionate) are found in the urine of these patients (1). Encephalopathy is the clinical hallmark of this disease. Among the neurological signs often present, psychomotor delay/mental retardation, focal and generalized convulsions, EEG abnormalities and delayed myelination and hypodensity of globi pallidi are the most frequent. Laboratory findings include metabolic acidosis, ketonemia/ketonuria, hypoglycemia, neutropenia, and thrombocytopenia (1). Methylmalonic acidemia was recently included in the group of disorders called "cerebral" organic acidemias, because the acids can also accumulate in the brain, suggesting that these metabolites may be produced in this organ (2).

Brain lipids comprise approximately one half of neuronal tissue dry weight (3). They are important components of the neuronal membranes, but, apart from their structural function, they also have regulatory roles in controlling cellular metabolism and growth (4). Myelin, an important component of the central nervous system (CNS), is composed of lipids and proteins (5). Various degenerative diseases are caused by inappropriate myelination (hypomyelination) or myelin destruction (demyelination). Among them is methylmalonic acidemia, which presents brain atrophy as a hallmark (1). We have recently reported that 5 mM MMA inhibits the *in vitro* incorporation of [^{14}C]acetate into total lipids in rat cerebral cortex (6), indicating a suppression of brain lipid biosynthesis caused by the acid. We have also demonstrated that rats chronically treated

with MMA present a reduction of ganglioside N-acetylneuraminic acid (ganglioside-NANA) concentration in cerebellum (7), but we did not evaluate the other brain lipids or the amount of myelin in these animals.

Therefore, in the present investigation we studied the effects of chronic postnatal MMA administration to young rats on the content of myelin and on the concentrations of total lipids and the various lipid fractions in the "cerebrum". We also measured the concentration of cholesterol and triglycerides in the blood of the rats.

Wistar rats bred in our laboratory were housed in groups of 9 with their mothers on the day of birth (day 1) and used for the experiments. Rats had free access to a 20% (w/w) protein commercial chow (Germani, Porto Alegre, RS, Brazil) and water and were kept on a 12-h light/dark cycle. Temperature was $24 \pm 1^\circ\text{C}$. The rats were weaned at day 21.

One pup from each litter was randomly assigned to one of the two experimental conditions. One group received subcutaneous injections of buffered MMA, pH 7.4, at increasing concentrations according to age, twice a day with an 8-h interval. This group received $9 \mu\text{l/g}$ ($0.72 \mu\text{mol/g}$ body weight) of a 1 g% solution of MMA during the first 8 days of treatment (5th-12th day). The animals were injected with $8 \mu\text{l/g}$ ($0.89 \mu\text{mol/g}$) of a 1.5 g% solution of MMA from the 13th to the 17th day and with $11 \mu\text{l/g}$ ($1.67 \mu\text{mol/g}$) of a 2.0 g% solution of MMA from the 18th to the 28th day. Doses were calculated from pharmacokinetic parameters determined in our laboratory in order to achieve serum MMA levels of 2.0-2.5 mM. The control group was injected with equivalent volumes of saline (0.9 g% NaCl).

Blood was obtained by cardiac puncture with heparinized syringes from 28-day-old rats 1 h after injecting saline or MMA. Plasma was separated by centrifugation (400 g for 15 min) and used for the determination of cholesterol and triglyceride concentrations

by classical enzymatic methods (8,9). Results are expressed as mg/dl.

Rats were killed by decapitation without anesthesia after blood collection. All animals had their brain rapidly removed. Medulla, pons, olfactory lobes and cerebellum were discarded. The rest of the brain ("cerebrum") was separated, weighed and homogenized (1:29, w/v) in a chloroform-methanol mixture (1:2), and the homogenates were processed for total lipid extraction by the method of Postma and Stroes (10). The cholesterol and glyceride content of the "cerebrum" was determined in the extracts by the above described methods of Kostner and colleagues (8) and Soloni (9), respectively. Since the method of Soloni can also be used to measure glycerol, the amount of the various glycerolipids was quantitated in the brain. Total lipids, ganglioside-NANA and phospholipids were determined by previously reported methods (10-13). Results are expressed as mg/g cerebrum.

Other chronically treated rats were used for myelin preparation. The animals were sacrificed as described and their "cerebrum" separated, weighed and finally homogenized (1:11, w/v) in cold 0.32 M sucrose. The homogenate was submitted to a discontinuous sucrose gradient of 0.32 to 0.85 M to prepare myelin (14). The amount of myelin was determined as dry weight and is expressed as mg/g cerebrum.

The protein concentration in myelin was measured by the method of Lowry et al. (15) using bovine serum albumin as standard.

Concentrations of MMA reached their peaks in blood (2.5 mmol/l) 30 min after drug injection and in "cerebrum" (1.35 μ mol/g) 60 min after drug injection.

Body weight did not differ between MMA (57.8 \pm 4.4 g, N = 12)- and saline (59.9 \pm 3.75 g, N = 12)-treated rats (control group) along treatment (t(22) = 0.15, P = 0.88). These results imply that MMA *per se* does not cause undernutrition or loss of appetite in the animals. Cerebral weight was also the same in MMA-treated rats (1126 \pm 60.8 mg, N = 12) and in controls (1121 \pm 77.3 mg, N = 12; t(22) = 0.18, P = 0.86). In contrast, we observed significantly reduced plasma concentrations of triglycerides in rats treated with MMA (186 \pm 49.49 mg/dl, N = 10), as compared to the saline group (236 \pm 38.3 mg/dl, N = 10; t(18) = 2.53, P = 0.021), whereas plasma cholesterol levels were not affected by MMA treatment (MMA: 123 \pm 22.0 mg/dl, N = 10; saline: 148 \pm 31.6 mg/dl, N = 10; t(18) = 2.01, P = 0.060). We recently showed that this acidic metabolite inhibits the *in vitro* incorporation of labeled acetate into total lipids in rat brain (6) and liver (Coelho JC and Wajner M, unpublished results). Since plasma triglyceride levels are predominantly due to very- low-density lipoproteins produced by the liver, it is feasible that an inhibition of liver lipid synthesis by MMA may explain our present *in vivo* results of plasma triglyceride reduction. We are not aware of any other study on the action of MMA on lipid biosynthesis.

The effect of MMA on the cerebral con-

Table 1 - Effect of chronic administration of methylmalonic acid (MMA) on the concentration of total lipids, cholesterol, glycerolipids, phospholipids and ganglioside N-acetylneuraminic acid in rat cerebrum.

Data are reported as means \pm SD for 9-11 rats and are expressed as mg/g cerebrum. *P<0.05 compared with the saline group (Student t-test).

Treatment	Total lipids	Cholesterol	Glycerolipids	Phospholipids	Gangliosides
Saline	67.20 \pm 1.56	9.72 \pm 0.59	2.10 \pm 0.26	50.95 \pm 1.85	1.41 \pm 0.053
MMA	69.05 \pm 2.36	9.59 \pm 0.22	2.18 \pm 0.17	47.57 \pm 2.58	1.09 \pm 0.142*

Table 2 - Effect of chronic administration of methylmalonic acid (MMA) on dry myelin content and myelin protein concentration in rat cerebrum.

Data are reported as means \pm SD for 6 rats in each group. * $P < 0.05$ compared with the saline group (Student t-test).

Treatment	Myelin dry weight (mg/g cerebrum)	Protein concentration in myelin (mg/mg myelin)
Saline	45.12 \pm 6.37	0.158 \pm 0.097
MMA	36.87 \pm 5.56*	0.150 \pm 0.110

centrations of total lipid and the various lipid fractions is shown in Table 1. Ganglioside-NANA concentration was significantly reduced in MMA-treated rats ($t(16) = 2.63$, $P = 0.018$), whereas the concentrations of the other fractions and of total lipid were not changed (total lipids: $t(20) = 0.65$, $P = 0.52$; cholesterol: $t(16) = 0.54$, $P = 0.596$; glycerolipids: $t(16) = 1.78$, $P = 0.094$; phospholipids: $t(18) = 0.11$, $P = 0.910$; gangliosides: $t(16) = 2.63$, $P = 0.018$). These results confirm our previous findings of a reduced amount of ganglioside-NANA in the brain of rats receiving MMA (7), and may reflect a higher susceptibility of ganglioside synthesis and/or degradation to the effects of the postnatal administration of the metabolite. In our opinion, this may be important in the light of several independent studies showing the highest concentration of gangliosides within the brain among subcellular fractions in nerve ending membranes, supporting the concept that gangliosides are important constituents of the synaptosomal membranes (16-18). Within this context, since a reduction of ganglioside-NANA may be associated with the amount of dendritic surface

and synaptic transmission (16,17), it would be interesting to look at the neurohistological findings of MMA-treated rats. A reduction in the number of synapses would be in agreement with our present findings and may explain the compromised performance of rats on learning/memory tasks previously observed (19,20).

We also observed a significant reduction of cerebral myelin content ($t(14) = 2.39$, $P = 0.038$) in chronically MMA-treated rats and no alteration of protein concentration in myelin ($t(10) = 1.31$, $P = 0.219$) (Table 2). Although we do not have an explanation for these findings, it is feasible that a reduction of brain energy production, which is provoked by MMA through inhibition of succinate dehydrogenase and β -hydroxybutyrate dehydrogenase (21,22), may indirectly affect the biosynthesis of important macromolecules such as the components of myelin. The identification of reduced brain myelin content is interesting in view of the hypomyelination/demyelination characteristic of the CNS of untreated methylmalonic acidemic children.

In conclusion, the present study demonstrates a deficit of important structural lipid components in the brain of young rats chronically treated with MMA. If these findings can be extrapolated to the human condition, it is feasible to propose that the effects of MMA on myelin biosynthesis, i.e., hypomyelination or demyelination may be related to the disturbances of CNS myelination in methylmalonic acidemia, and that the reduction of ganglioside-NANA may be associated with impairment of synaptic transmission, both mechanisms possibly contributing to the neurologic symptoms of patients affected by methylmalonic acidemia.

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