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Prevention by L-carnitine of DNA damage induced by propionic and L-methylmalonic acids in human peripheral leukocytes *in vitro*

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ABSTRACT

Propionic acidemia (PAemia) and methylmalonic acidemia (MMAemia) are inborn errors of propionate metabolism characterized by the accumulation of, respectively, propionic and L-methylmalonic acids (and their metabolites) in the blood and tissues of affected patients. The conditions lead to severe metabolic complications in the neonatal period and to long-term neurological manifestations. Treatment for these disorders consists of a protein-restricted diet, supplemented with synthetic formulas of amino acids, but excluding isoleucine, threonine, valine and methionine; and L-carnitine, to promote detoxication. *In vitro* and *in vivo* studies have demonstrated that lipid and protein oxidative damage may be involved in the pathophysiology of these diseases, but DNA damage has not been fully investigated. In this work, we evaluated *in vitro* the effects of PA and MMA, in the presence or absence of L-carnitine, on DNA damage in peripheral leukocytes, as determined by the alkaline comet assay, using silver staining and visual scoring. PA and MMA induced a DNA damage index (DI) significantly higher than that of the control group. L-Carnitine significantly reduced PA- and MMA-induced DNA damage, in a concentration-dependent manner. Our findings indicate that PA and MMA induce DNA damage and L-carnitine is able to prevent this damage.

1. Introduction

The organic acids propionic acid (PA) and L-methylmalonic acid (MMA) are found at high levels in the blood and urine of patients with propionic acidemia (PAemia) and methylmalonic acidemia (MMAemia), respectively. These inherited disorders are caused by the reduced activity of the enzymes propionyl-CoA carboxylase and L-methylmalonyl-CoA mutase, respectively, which catalyze steps in the conversion of propionyl-CoA to succinyl-CoA, a key process in the metabolism of the amino acids isoleucine, threonine, valine, and methionine; odd-chain fatty acids; the bases thymine and uracil; and cholesterol [1–3].

Individuals affected by these disorders are at constant risk for metabolic decompensation with metabolic acidosis, ketonemia, hyperammonemia, hyperglycinemia, hypoglycemia, and metabolic stroke [4]. During the crises of metabolic decompensation, the levels of MMA and PA can be as high as 2.5–5 mM in the blood and cerebrospinal fluid, and even higher in the neuronal cells [5]. Neurological symptoms, such as encephalopathy, lethargy, convulsions and mental retardation, are common in these disorders, but the mechanisms underlying the brain damage pathophysiology are not yet fully established. Nevertheless, there is compelling evidence that mitochondrial dysfunction, excitotoxicity, and oxidative stress contribute to the neuropathology of these organic acidurias [6–13].

Treatment of PAemia and MMAemia consists of a low-protein high-energy diet and synthetic amino acid-based formulas. L-Carnitine forms conjugates with propionyl and methylmalonyl acids, increasing their excretion in the form of carnitine esters (propionylcarnitine and methylmalonylcarnitine) and leading to secondary carnitine deficiency [3,14]. Therefore, L-carnitine supplementation (approximately 100 mg/kg per day) is essential, to replenish depleted tissue stores of this compound. Oxidative stress may play an important role in the pathophysiology of some inborn errors of metabolism, since accumulation of toxic metabolites may lead to excessive production of free radicals [15–17], which can react with lipids, proteins, DNA, and RNA. The hydroxyl radical, the most harmful reactive oxygen species, can induce a

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variety of lesions in DNA, including single-strand breaks (SSBs), double-strand breaks (DSBs), alkali-labile sites, oxidized purine and pyrimidine bases, and DNA–protein cross-links [15,18–20].

PA and MMA stimulate lipid peroxidation and protein oxidation, as well as reduce antioxidant defenses in rat brain [9,21,22]. We have demonstrated that, at diagnosis, patients with PAemia and MMAemia present higher levels of protein and lipid oxidation compared to healthy subjects, and treatment with L-carnitine may reduce lipid peroxidation in patients with these disorders [23]. L-Carnitine was also associated with the reduction of lipid peroxidation in patients with phenylketonuria, an inborn error of phenylalanine metabolism [16]. Several studies have reported antioxidant and antiperoxidative properties for this compound, which can act as a metal chelator and a scavenger of reactive oxygen species, such as hydrogen peroxide (H_2O_2), superoxide radical, and hydroxyl radical [24–27]. In the present study, using the comet assay, we have tested the effects of PA and MMA on DNA damage *in vitro* and we have tested whether L-carnitine can mitigate such damage.

2. Materials and methods

2.1. In vitro induction of DNA damage by PA and MMA

This study was approved by the Ethics Committee of Hospital de Clínicas de Porto Alegre. Venous blood specimens from healthy volunteers were collected in heparinized vials under sterile conditions. Leukocytes isolated from whole blood were incubated with PA (2, 3, or 5 mM) or MMA (0.5, 2, or 5 mM) at 37 °C for 6 h.

2.2. Effect of L-carnitine on DNA damage in vitro

Leukocytes from healthy volunteers were co-incubated with L-carnitine (30, 60, 90, 120, or 150 μM) and PA (5 mM) or MMA (5 mM) at 37 °C for 6 h. The final concentrations of L-carnitine in the assays were based on previous findings for patients with PAemia and MMAemia, showing that plasma levels of this compound can vary from 30 μmol/L at diagnosis to almost 100 μmol/L under supplementation [23].

2.3. Single cell gel electrophoresis (comet assay)

The alkaline comet assay followed the method described by Singh et al. [28] and was performed in accordance with general guidelines for the assay [29,30]. Isolated human leukocytes were suspended in agarose and spread onto a glass microscope slide pre-coated with agarose. Agarose was allowed to set at 4 °C for 5 min. Slides were incubated in ice-cold lysis solution to remove cell proteins, leaving DNA as "nucleoids". After the lysis procedure, the slides were placed on a horizontal electrophoresis unit and covered with fresh buffer (300 mM NaOH and 1 mM EDTA, pH > 13) at 4 °C for 20 min, to allow DNA unwinding and the expression of alkali-labile sites. Electrophoresis was performed for 20 min (25 V; 300 mA; 0.9 V/cm). Slides were then neutralized, washed in double-distilled water, and stained according to a silver-staining protocol [31]. After drying overnight at room temperature, the gels were analyzed using an optical microscope. One hundred cells (50 cells from each of the two replicate slides) were selected and analyzed. Cells were visually scored according to tail length and received scores from 0 (no migration) to 4 (maximal migration) according to tail intensity. Therefore, the damage index (DI) for cells ranged from 0 (all cells with no migration) to 400 (all cells with maximal migration). The slides were analyzed under blind conditions by at least two different individuals.

2.4. Statistical analysis

Data were analyzed using the nonparametric Kruskal–Wallis test followed by the Mann–Whitney *U*-test. A *p* value lower than 0.05 was considered significant. The values were presented as medians (min; max). All analyses were performed using Statistical Package for the Social Sciences (SPSS) software, v. 15.0, on a PC-compatible computer.

3. Results

Figs. 1 and 2 show the effects of PA and MMA, respectively, on DNA damage in white blood cells *in vitro*. All concentrations of PA and MMA tested resulted in a DNA damage index (DI) significantly higher than that of the control ($p < 0.01$). No significant difference was found in DNA damage between 2 and 5 mM MMA, or among the tested concentrations of PA. The DI values and the number of cells found in each damage class for PA and MMA, obtained from three independent experiments, are presented in Tables 1 and 2, respectively.

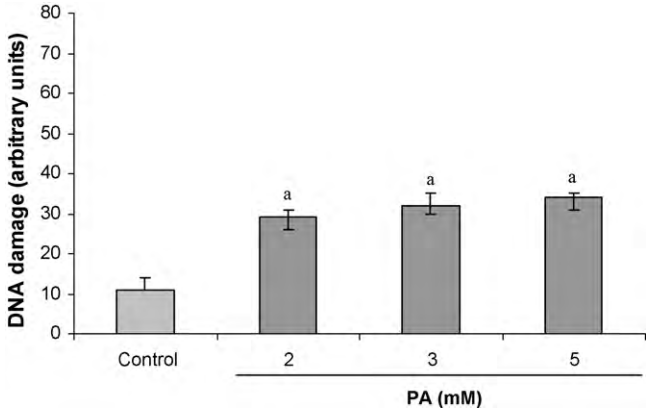

Fig. 1. *In vitro* effect of propionic acid (2, 3 and 5 mM) on DNA damage (comet assay) in leukocytes from whole blood. Data represent median (min; max) of three independent experiments (individuals). a, $p < 0.01$ compared to the control group (Kruskal–Wallis test followed by Mann–Whitney *U*-test).

Next, we evaluated the effect of L-carnitine on DNA damage induced by 5 mM PA (Fig. 3) or 5 mM MMA (Fig. 4) *in vitro*. L-Carnitine reduced the DI induced by PA or MMA, in a concentration-dependent manner, with a particularly large inhibitory effect on MMA. Tables 3 and 4 show DI values and the numbers of cells found in each damage class for PA and MMA, respectively, after the addition of L-carnitine.

4. Discussion

The disorders of propionate metabolism PAemia and MMAemia are genetic diseases characterized by accumulation of, predominantly, PA and MMA, respectively. Patients affected by these disorders present acute episodes of metabolic acidosis with neurological manifestations, including coma and convulsions, and long-term neurological symptoms. Treatment with L-carnitine increases excretion of propionylcarnitine and methylmalonylcarnitine, thereby promoting detoxication [1–3].

Oxidative stress is involved in a large number of human diseases, including neurodegenerative diseases and some inborn errors of metabolism [15,32,33]. Free radicals can attack cellular DNA, causing lesions ranging from base and sugar damage to DNA breaks and DNA–protein cross-links [34]. DNA lesions may hamper pro-

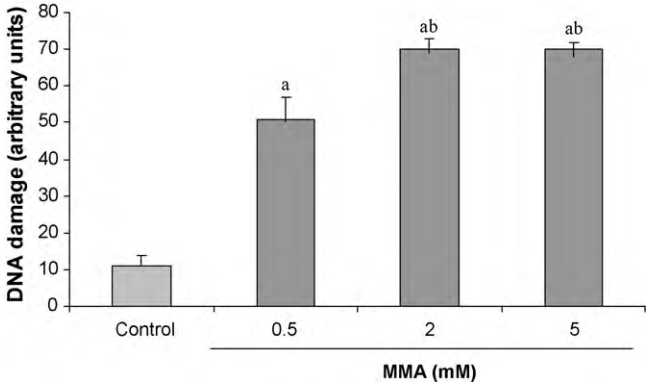

Fig. 2. *In vitro* effect of methylmalonic acid (0.5, 2 and 5 mM) on DNA damage (comet assay) in leukocytes from whole blood. Data represent median (min; max) of three independent experiments (individuals). a, $p < 0.01$ compared to the control group and b, $p < 0.01$ compared to the 0.5 mM MMA group (Kruskal–Wallis test followed by Mann–Whitney *U*-test).