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DNA damage in an animal model of maple syrup urine disease

Giselli Scaini [a,b], Isabela C. Jeremias [a,b], Meline O.S. Morais [a,b], Gabriela D. Borges [c], Bruna P. Munhoz [c], Daniela D. Leffa [c], Vanessa M. Andrade [c], Patrícia F. Schuck [d], Gustavo C. Ferreira [d], Emilio L. Streck [a,b,*]

[a] Laboratório de Bioenergética, Programa de Pós-Graduação em Ciências da Saúde, Universidade do Extremo Sul Catarinense, Criciúma, SC, Brazil
[b] Instituto Nacional de Ciência e Tecnologia Translacional em Medicina (INCT-TM), Porto Alegre, RS, Brazil
[c] Laboratório de Biologia Celular e Molecular, Programa de Pós-Graduação em Ciências da Saúde, Universidade do Extremo Sul Catarinense, Criciúma, SC, Brazil
[d] Laboratório de Erros Inatos do Metabolismo, Programa de Pós-Graduação em Ciências da Saúde, Universidade do Extremo Sul Catarinense, Criciúma, SC, Brazil

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ABSTRACT

Maple syrup urine disease is an inborn error of metabolism caused by a severe deficiency of the branched chain alpha-ketoacid dehydrogenase complex. Neurological dysfunction is a common finding in patients with maple syrup urine disease. However, the mechanisms underlying the neuropathology of brain damage in this disorder are poorly understood. In this study, we investigated whether acute or chronic administration of a branched chain amino acid pool (leucine, isoleucine and valine) causes transient DNA damage, as determined by the alkaline comet assay, in the brain and blood of rats during development and whether antioxidant treatment prevented the alterations induced by branched chain amino acids. Our results showed that the acute administration of branched chain amino acids increased the DNA damage frequency and damage index in the hippocampus. However, the chronic administration of branched chain amino acids increased the DNA damage frequency and damage index in both the hippocampus and the striatum, and the antioxidant treatment was able to prevent DNA damage in the hippocampus and striatum. The present study demonstrated that metabolite accumulation in MSUD induces DNA damage in the hippocampus and striatum and that it may be implicated in the neuropathology observed in the affected patients. We demonstrated that the effect of antioxidant treatment (N-acetylcysteine plus deferoxamine) prevented DNA damage, suggesting the involvement of oxidative stress in DNA damage.

1. Introduction

Maple syrup urine disease (MSUD) is an autosomal recessive metabolic disorder caused by a severe deficiency in the branched-chain α-keto acid dehydrogenase complex (BCKAD) activity, affecting approximately 1 in 185,000 newborns worldwide [1]. The inability of this enzyme complex to oxidize α-ketoisocaproic acid (KIC), α-keto-β-methylvaleric acid (KMV) and α-ketoisovaleric acid (KIV) leads to the tissue accumulation of these metabolites and their precursor amino acids leucine (Leu), isoleucine (Ile) and valine (Val), respectively, in the affected individuals [1,2]. The major clinical features presented by MSUD patients include convulsions, ketoacidosis, apnea, hypoglycemia, coma, ataxia, psychomotor delay and mental retardation, as well as generalized edema and hypomyelination/demyelination, as evidenced by magnetic resonance imaging studies of the central nervous system (CNS) [1,3]. The mechanisms of the neurological symptoms presented by MSUD patients are still poorly understood. However, considering that increased concentrations of leucine and/or α-ketoisocaproate are associated with the appearance of neurological symptoms, these compounds appear to be the primary neurotoxic metabolites in MSUD [1,4]. It has been postulated that brain injury associated with MSUD could be related to excitotoxicity [5,6], energy deficit [7–11] and oxidative stress in brain subcellular fractions [12–16].

Cellular DNA damage represents a great threat to genome stability, leading to a loss or amplification of chromosomal activity, which can result in carcinogenesis or tissue aging [17]. There is a body of evidence correlating medical conditions with DNA damage [18]. Cellular DNA is a sensitive target of damage following oxidative stress. This damage can include chemical and structural modifications to purine and pyrimidine bases and 2′-deoxyribose and can lead to the formation of single- and double-strand breaks. Strand breaks within DNA can occur either directly, as a result of damage from free radical exposure, or indirectly, as a result of cleavage of the DNA backbone during DNA base excision repair [19,20]. Continued oxidative damage to DNA can alter signaling cascades and gene expression and cause replication errors and genomic instability [21]. Benes and colleagues [22] suggested that changes in the intracellular signaling pathways within the mitochondria can be associated with apoptotic cell death in response to oxidative stress.

Here, we decided to investigate whether acute or chronic administration of a branched chain amino acid (BCAA) pool (Leu, Ile, Val) causes transient DNA damage, as determined by the alkaline comet assay, in

* Corresponding author at: Laboratório de Bioenergética, Universidade do Extremo Sul Catarinense, Av. Universitária, 1105, Criciúma, 88806-000, SC, Brazil. Fax: +55 48 3431 2644.
E-mail address: emiliostreck@gmail.com (E.L. Streck).

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the brain and blood of rats during their development. For this purpose, we used a chemically induced model of MSUD, which produces high sustained levels of BCAA that are similar to those found in the plasma of MSUD patients. Considering that previous studies suggest that oxidative stress may be involved in the pathophysiology of the neurological dysfunction of MSUD [12–16], we also investigated the influence of the concomitant administration of N-acetylcysteine (NAC) and deferoxamine (DFX) in this parameter of DNA damage.

2. Materials and methods

2.1. Animals

Male Wistar rats were obtained from the Central Animal House of the Universidade do Extremo Sul Catarinense. Ten-day-old rats were kept with dams until the day of the experiment, and seven-day-old rats were weaned at 21 days of life. All rats were caged in groups of five, with free access to food and water, and were maintained on a 12-h light–dark cycle (lights on 7:00 am) at a temperature of 23 ± 1 °C. All experimental procedures were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the Brazilian Society for Neuroscience and Behavior recommendations for animal care, with the approval of the Ethics Committee of the Universidade do Extremo Sul Catarinense (protocol number 60/2010).

2.2. Acute administration of the BCAA pool

The animals received three subcutaneous administrations of the BCAA pool (15.8 μL/g body weight at 1-h intervals) containing 190 mmol/L Leu, 59 mmol/L Ile, and 69 mmol/L Val in saline solution (0.85% NaCl) or saline alone (control group). The BCAA pool and saline solution were given to rats on postnatal day (PD) 10 (n = 6). One hour after the last injection, the animals were sacrificed by decapitation, and the blood was collected. The brain was rapidly removed, and the hippocampus, striatum and cerebral cortex were collected for DNA damage assays. The choice of the doses and ages to test was based on a previous study [23], showing that the administration of the BCAA pool to rats (doses and ages similar to those used in our present study) resulted in increased levels of Leu, Ile and Val in the blood and brain, mimicking the main biochemical finding observed in MSUD patients during crises.

2.3. Chronic administration of the BCAA pool and treatment with antioxidants

The animals were divided in to three groups: 1) control (saline), 2) MSUD (induced by the BCAA pool), and 3) MSUD treated with the combination of NAC (20 mg/kg) and DFX (20 mg/kg). The animals received two subcutaneous administrations of the BCAA pool (15.8 μL/g body weight at 12-h intervals) containing 190 mmol/L Leu, 59 mmol/L Ile, and 69 mmol/L Val in saline solution and were administered for 21 days starting at PD 7 (last injection at PD 27) [23] (n = 6). NAC was administered subcutaneously twice a day (at 12-h intervals), and DFX was administered once every 2 days for a total of 21 days [24]. Twelve hours after the last injection, the animals were sacrificed by decapitation, and the blood was collected. The brain was rapidly removed, and the hippocampus, striatum and cerebral cortex were collected for DNA damage assays.

2.4. Comet assay

The alkaline comet assay was performed as described by Singh and colleagues [25] with the modifications suggested by Tice and colleagues [26]. Samples of 5 μL of whole peripheral blood or homogenate hippocampus, striatum or cerebral cortex (cold PBS) were embedded in 95 μL of 0.75% low-melting point agarose and added to a microscope slide (two slides per donor) that was pre-coated with normal agarose (1.5% buffer solution). When the agarose had solidified, the slides were placed in lysis buffer (2.5 M NaCl, 100 mM EDTA and 10 mM Tris; pH 10.0–10.5) containing freshly added 1% (v/v) Triton X-100 and 10% (v/v) dimethylsulfoxide (DMSO) for a minimum of 1 h and a maximum of 2 weeks. After treatment with lysis buffer to allow DNA unwinding, the slides were incubated in a freshly made alkaline electrophoresis buffer (0.3 M NaOH and 1 mM EDTA; pH > 13) for 20 min in a horizontal electrophoresis tank. The DNA was then submitted to electrophoresis for 20 min at 25 V (0.90 V/cm) and 300 mA. Every step was carried out under indirect yellow light. After electrophoresis, the slides were washed three times in a neutralization buffer (0.4 M Tris; pH 7.5) for 5 min, rinsed three times in distilled water, and left to dry overnight at room temperature. Slides were stained with 50 μL of ethidium bromide (20 μg/mL), and images of 100 randomly selected cells (50 cells from each of two replicate slides) were blindly analyzed from each animal using a fluorescence microscope, equipped with an excitation filter of BP546/12 nm and a 590 nm barrier filter. The extent of the DNA damage was assessed using Collins' visual classification method [27]. Images of 100 randomly selected cells were analyzed for each individual. The cells were scored visually into five classes, according to tail size and shape (from undamaged − 0, to maximally damaged − 4), and a value (damage index) was assigned to each comet according to its class (see Fig. 1). Thus, the damage index ranged from 0 (completely undamaged: 100 cells × 0) to 400 (with maximum damage: 100 cells × 4). The calculation of the damage frequency (%) was based on the percentage of damaged cells (0–100%). International guidelines and recommendations for the comet assay consider visual scoring of comets to be a well-validated evaluation method. Visual scoring has a high correlation with computer-based image analysis [27]. According to Collins [27] and Tice and colleagues [26], the apoptosis/necrosis damage cell cannot be assessed by the comet assay technique; thus, the cells that do not present the format required for classification by the Comet Assay were excluded from the slides analyzed.

2.5. Statistical analysis

The results are presented as mean ± standard deviation of the mean. Assays were performed in duplicate, and the mean was used for statistical analysis. Student's *t*-test was used for comparisons between two means. A one-way analysis of variance (ANOVA) followed by Tukey HSD Post-Hoc Tests was used for comparisons among three means. Differences between the groups were considered significant at $p < 0.05$. All analyses were carried out on an IBM-compatible PC computer using the Statistical Package for the Social Sciences (SPSS) software.

3. Results

We investigated the genotoxic effect of acute and chronic administrations of the BCAA pool in the hippocampus, striatum, cerebral cortex and blood of rats by using the comet assay. Tables 1 and 2 show individual values of the number of cells found in each damage class in the hippocampus, striatum, cerebral cortex and blood of animals treated with saline (control group) and BCAA (both acute and chronic exposure) as well as BCAA with chronic antioxidant exposure.

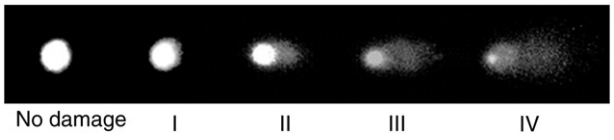

Fig. 1. Comet assay. Evaluation of DNA damage using ethidium bromide (400×). The cells are assessed visually and received scores from 0 (undamaged) to 4 (maximally damaged), according to the size and shape of the tail.