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Disruption of redox homeostasis in cerebral cortex of developing rats by acylcarnitines accumulating in medium-chain acyl-CoA dehydrogenase deficiency

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ABSTRACT

Medium-chain fatty acids and acylcarnitines accumulate in medium-chain acyl-CoA dehydrogenase deficiency (MCADD), the most frequent fatty acid oxidation defect clinically characterized by episodic crises with vomiting, seizures and coma. Considering that the pathophysiology of the neurological symptoms observed in MCADD is poorly known and, to our knowledge, there is no report on the involvement of acylcarnitines in the brain damage presented by the affected patients, the objective of the present study was to investigate the *in vitro* effects of hexanoylcarnitine (HC), octanoylcarnitine, decanoylcarnitine (DC) and cis-4-decenoylcarnitine (cDC) at concentrations varying from 0.01 to 1.0 mM on important oxidative stress parameters in cerebral cortex of young rats. HC, DC and cDC significantly induced lipid peroxidation, as determined by increased thiobarbituric acid-reactive substances (TBA-RS) values. In addition, carbonyl formation was significantly augmented and sulfhydryl content diminished by DC, reflecting induction of protein oxidative damage. HC, DC and cDC also decreased glutathione (GSH) levels, the most important brain antioxidant defense. Furthermore, DC-induced elevation of TBA-RS values and decrease of GSH levels were prevented by the free radical scavengers melatonin and α-tocopherol, indicating the involvement of reactive oxygen species in these effects. We also found that L-carnitine itself did not induce lipid and protein oxidative damage, neither reduced the antioxidant defenses. Our present data show that the major medium-chain acylcarnitines accumulating in MCADD elicit oxidative stress in rat brain. It is therefore presumed that these compounds may be involved to a certain extent in the pathogenesis of the neurologic dysfunction of MCADD.

1. Introduction

Medium-chain acyl-CoA dehydrogenase deficiency (MCADD), the most frequent inherited disorder of fatty acid oxidation (FAO), is biochemically characterized by accumulation of medium-chain fatty acids (MCFA) and their L-carnitine esters (Rinaldo et al., 2002) in tissues and body fluids. MCAD-deficient patients present characteristic episodes of acute decompensation under metabolic stress usually due to prolonged fasting or following infections. During these crises of metabolic decompensation, when energy from FAO is most needed, the affected individuals manifest severe symptoms, such as vomiting, hypotonia, seizures and coma that may be associated with hypoglycemia and hyperammonemia, and marked increases of the free MCFA hexanoic, octanoic, decanoic and cis-4-decenoic acids, as well as their L-carnitine conjugates hexanoylcarnitine (HC), octanoylcarnitine (OC), decanoylcarnitine (DC) and cis-4-decenoylcarnitine (cDC) (Roe and Ding, 2001). The most dramatic outcome is sudden unexplained death that occurs in approximately 15–20% of children with MCADD. Furthermore, approximately 20% of unselected patients diagnosed during crises before introduction of expanded newborn screening in the general population present sustained neurological damage (Pollitt and Leonard, 1998; Wilcken et al., 1994, 2007). However, recent data from affected children diagnosed in the neonatal period indicate that the risk of intellectual deficit or other morbidities is extremely small, probably because of better emergency treatment, which usually prevents crises of metabolic decompensation (Wilcken, 2010).

Abbreviations: HA, hexanoic acid; HC, hexanoylcarnitine; HG, hexanoylglycine; OC, octanoylcarnitine; DC, decanoylcarnitine; cDC, cis-4-decenoylcarnitine; MCAC, medium-chain acylcarnitines.

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Despite the high prevalence of MCADD in the population, which is similar to phenylketonuria (1:10,000 newborns), the exact underlying mechanisms responsible for the neuropathology of MCADD are still poorly established. In this scenario, the rapid deterioration of the clinical features in affected patients generally precedes the development of hypoglycemia, suggesting a potential toxic role for the accumulating MCFA and their derivatives in the pathogenesis of MCADD, in addition to hypoketotic hypoglycemia (Gregersen et al., 2008). This is consistent with the observations that the MCFA accumulating in this disease disturb mitochondrial homeostasis by inhibiting the citric acid cycle, the respiratory chain flow, Na^+ K^+ ATPase and mitochondrial creatine kinase activities, besides inducing oxidative stress in rat brain (de Assis et al., 2006; Reis de Assis et al., 2004; Schuck et al., 2007, 2009). However, regarding to the medium-chain acylcarnitines (MCAC), the only study reporting their effects on metabolism revealed that these compounds do not alter the respiratory chain complex activities measured in submitochondrial particles, neither commercially purified α-ketoglutarate dehydrogenase or pyruvate dehydrogenase activities prepared from bovine and porcine heart (Sauer et al., 2008).

Since oxidative damage has been associated with common neurodegenerative diseases and considered an important pathogenic mechanism of these disorders (Behl and Moosmann, 2002; Bogdanov et al., 2001; Perez-Severiano et al., 2000), the aim of the present study was to investigate the role of the MCAC that most accumulate in MCADD on important parameters of redox homeostasis in rat brain. We tested the *in vitro* effects of HC, OC, DC and cDC at concentrations varying from 0.01 to 1.0 mM on thiobarbituric acid-reactive substances (TBA-RS) values (lipid oxidation), carbonyl formation and sulfhydryl oxidation (protein oxidation) and reduced glutathione (GSH) levels (antioxidant defenses) in cerebral cortex of developing rats (Halliwell and Gutteridge, 2007). We also tested the effects of L-carnitine on some parameters of oxidative stress and the role of antioxidants on the alterations elicited by the MCAC on TBA-RS and GSH levels.

2. Material and methods

2.1. Animals and reagents

Seventy two thirty-day-old male Wistar rats obtained from the Central Animal House of the Department of Biochemistry, ICBS, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil, were used (6 animals in each experiment). The animals were maintained on a 12:12 h light/dark cycle (lights on 07.00–19.00 h) in air conditioned constant temperature (22 ± 1 °C) colony room, with free access to water and 20% (w/w) protein commercial chow (SUPRA, Porto Alegre, RS, Brazil). The experimental protocol was approved by the Ethics Committee for animal research of the Federal University of Rio Grande do Sul, Porto Alegre, Brazil and followed the Principles of Laboratory Animal Care (NIH publication 85-23, revised 1996). All efforts were made to minimize the number of animals used and their suffering.

All chemicals were purchased from Sigma (St. Louis, MO, USA), except for the acylcarnitines and hexanoylglycine (99% purity) that were prepared by Dr. Ernesto Brunet, Madrid, Spain. The metabolites used were dissolved on the day of the experiments in the incubation medium used for each technique and had their pH adjusted to 7.4. The final concentrations of these metabolites in the medium, as well as L-carnitine that was used in some experiments, ranged from 0.01 to 1 mM.

2.2. Tissue preparation and incubation

On the day of the experiments the animals were sacrificed by decapitation without anesthesia, and the brain was rapidly excised on a Petri dish placed on ice. The olfactory bulbs, pons, medulla, cerebellum and striatum were discarded, and the cerebral cortex was peeled away from the subcortical structures, weighed and homogenized in 10 volumes (1:10, w/v) of 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl. Homogenates were centrifuged at 750 × *g* for 10 min at 4 °C to discard nuclei and cell debris (Evelson et al., 2001). The pellet was discarded and the supernatant, a suspension of mixed and preserved organelles, including mitochondria, was separated and incubated in 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl at 37 °C for 1 h with the acylcarnitines (HC, OC, DC, cDC). We also utilized hexanoic acid (HA), hexanoylglycine (HG) and L-carnitine in some assays. Controls did not contain any of these metabolites in the incubation medium.

In some experiments, antioxidants were co-incubated with supernatants at the following final concentrations: 10 μM Trolox (TRO, soluble α-tocoferol), 1500 μM melatonin (MEL), 500 μM reduced glutathione (GSH), 500 μM N^{ω}-nitro-L-arginine methyl ester (L-NAME) and the combination of SOD plus CAT (100 mU each). The chosen concentrations of the antioxidants were those capable to efficiently scavenge free radicals (Halliwell and Gutteridge, 2007; Leipnitz et al., 2011).

We always carried out parallel experiments with blanks (controls) in the presence or absence of the tested metabolites and without cortical supernatants in order to detect artifacts caused by the MCAC in the assays.

2.3. Determination of thiobarbituric acid-reactive substances (TBA-RS) levels

TBA-RS levels were determined according to the method of Esterbauer and Cheeseman (1990). Briefly, 300 μL of cold 10% trichloroacetic acid were added to 150 μL of pre-incubated cerebral cortex supernatants and centrifuged at 750 × *g* for 10 min. Three hundred microliters of the supernatant (containing approximately 0.3 mg of protein) were transferred to a pyrex tube and incubated with 300 μL of 0.67% TBA in 7.1% sodium sulfate on a boiling water bath for 25 min. The tubes containing the mixture were allowed to cool on running tap water for 5 min. The resulting pink-stained TBA-RS was determined in a spectrophotometer at 532 nm. A calibration curve was performed using 1,1,3,3-tetramethoxypropane, and each curve point was subjected to the same treatment as supernatants. TBA-RS values were calculated as nmol of TBA-RS/mg protein.

2.4. Determination of protein carbonyl formation

Protein carbonyl content, a marker of oxidized proteins, was measured spectrophotometrically according to Reznick and Packer (1994). One hundred microliters of the aliquots from the incubation (containing approximately 0.3 mg of protein) were treated with 400 μL of 10 mM 2,4-dinitrophenylhidrazine (DNPH) dissolved in 2.5 N HCl or with 2.5 N HCl (blank control) and left in the dark for 1 h. Samples were then precipitated with 500 μL 20% TCA and centrifuged for 5 min at 10,000 × *g*. The pellet was then washed with 1 mL ethanol:ethyl acetate (1:1, v/v) and re-dissolved in 550 μL 6 M guanidine prepared in 2.5 N HCl. Then, the tubes were incubated at 37 °C for 5 min to assure the complete dissolution of the pellet and the resulting sample was determined at 365 nm. The difference between the DNPH-treated and HCl-treated samples was used to calculate the carbonyl content. The results were calculated as nmol of carbonyl groups/mg of protein, using the extinction coefficient of 22,000 × 106 nmol/mL for aliphatic hydrazones.

2.5. Determination of sulfhydryl (thiol) group content

This assay is based on the reduction of 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) by thiols, generating a yellow derivative (TNB) whose absorption is measured spectrophotometrically at 412 nm (Aksenov and Markesbery, 2001). Briefly, 0.1 mM DTNB was added to pre-treated cortical supernatants (containing approximately 0.3 mg of protein). This was followed by 30 min incubation at room temperature in a dark room. Absorption was measured at 412 nm. The protein-bound sulfhydryl content is inversely correlated to oxidative damage to proteins. Results were reported as nmol TNB/mg protein.

2.6. Determination of reduced glutathione (GSH) concentrations

Reduced glutathione (GSH) concentrations were measured according to Browne and Armstrong (1998). Metaphosphoric acid solution (1.8%) was added to the pre-treated cerebral cortex supernatants (1:1, v/v) to precipitate proteins and centrifuged for 10 min at 7000 × *g*. Then, the supernatants (containing approximately 0.3 mg of protein) were diluted (1:20, v/v) in 100 mM sodium phosphate buffer pH 8.0, containing 5 mM EDTA. One hundred microliters of this preparation were incubated with an equal volume of *o*-phthaldialdehyde (1 mg/mL methanol) at room temperature during 15 min. Fluorescence was measured using excitation and emission wavelengths of 350 nm and 420 nm, respectively. Calibration curve was prepared with standard GSH (0.01–1 mM) and the concentrations were calculated as nmol/mg protein.

2.7. Protein determination

Protein was measured by the method of Lowry et al. (1951) using bovine serum albumin as standard.

2.8. Statistical analysis

Results are presented as mean ± standard deviation of the mean. Assays were performed in triplicate and the mean was used for statistical analysis. Data were analyzed using one-way analysis of variance (ANOVA) followed by the *post hoc* Duncan multiple range test when F was significant. Pearson's linear coefficient of correlation and linear regression analysis were also used in some experiments. Differences between groups were rated significant at $P < 0.05$. All analyses were carried out in an IBM-compatible PC computer using the Statistical Package for the Social Sciences (SPSS) software.