In vivo neuroprotective effect of L-carnitine against oxidative stress in maple syrup urine disease

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Abstract Maple syrup urine disease (MSUD) is an autosomal recessive inborn error of metabolism caused by deficiency of the activity of the mitochondrial enzyme complex branched-chain α-keto acid dehydrogenase (BCKAD) leading to accumulation of the branched-chain amino acids (BCAA) leucine, isoleucine and valine and their corresponding branched-chain α-keto acids. Affected patients present severe brain dysfunction manifested such as ataxia, seizures, coma, psychomotor delay and mental retardation. The mechanisms of brain damage in this disease remain poorly understood. Recent studies have shown that oxidative stress may be involved in neuropathology of MSUD. L-Carnitine (L-Car) is considered a potential antioxidant through its action against peroxidation as a scavenger of reactive oxygen species and by its stabilizing effect of damage to cell membranes. In this study we evaluate the possible neuroprotective in vivo effects of L-Car against pro-oxidative effects of BCAA in cerebral cortex of rats. L-Car prevented lipoperoxidation, measured by thiobarbituric acid-reactive substances, protein damage, measured by sulfhydryl and protein carbonyl content and alteration on catalase and glutathione peroxidase activity in rat cortex from a chemically-induced model of MSUD. Our data clearly show that L-Car may be an efficient antioxidant, protecting against the oxidative stress promoted by BCAA. If the present results are confirmed in MSUD patients, this could represent an additional therapeutic approach to the patients affected by this disease.

Keywords Maple syrup urine disease · L-carnitine · Branched-chain amino acids · Oxidative stress · Neuroprotection · Antioxidant

Introduction

Maple Syrup Urine Disease (MSUD; branched-chain ketoaciduria) is an autosomal recessive inborn error of metabolism caused by deficiency of mitochondrial enzyme complex branched-chain α-keto acid dehydrogenase (BCKAD) activity. The metabolic defect leads to accumulation of the branched chain amino acids (BCAA) leucine (Leu), isoleucine (Ile) and valine (Val) and the corresponding branched-chain α-keto acids (BCKA), α-ketoisocaproic acid (KIC), α-keto-β-methylvaleric acid and α-ketoisovaleric acid, as well as the corresponding α-hydroxy acids in tissue and body fluids (Chuang and Shih 2001; Treacy et al. 1992).

Based on distinct residual enzyme activity, clinical presentation and biochemical responses to thiamine administration, MSUD can be divided in five heterogeneous phenotypes: classic, intermittent, intermediate, thiamine-responsive and E3-deficient forms. Patients with classical form of this disease present poor feeding, apnea, ketoacidosis, convulsion, coma,
psychomotor delay and variable degree of mental retardation (Chuang and Shih 2001). MSUD treatment consists of a low protein diet and a semi-synthetic formula poor in Leu, Ile and Val and supplemented by essential amino acids and vitamins. (Chuang and Shih 2001; Snyderman et al. 1964).

The mechanisms by which high concentrations of Leu and its keto acid KIC, the main metabolites accumulated in untreated patients, are toxic to the central nervous system are still poorly understood. In this context, it has been established that the metabolites accumulating in MSUD leads to neuronal apoptosis (Jouvet et al. 2000), and reduce the uptake of essential amino acids by brain tissue (Araújo et al. 2001). Furthermore, the BCAA and BCKA may alter energy metabolism in rat brain (Sgaravatti et al. 2003; Ribeiro et al. 2008; Amaral et al. 2010), and can also decrease neurotransmitter metabolism (Zielke et al. 1996; Tavares et al. 2000).

Free radicals participate in both pathological and physiological processes in the organism. An imbalance between production and removal systems can result in pathological consequences such as oxidative damage to proteins, lipids and DNA (Halliwell and Gutteridge 2007). Recent studies have shown the involvement of oxidative stress in the pathophysiology of various inborn errors of metabolism including MSUD (Pederzoli et al. 2010; Sitta et al. 2009). Studies in animals have demonstrated that lipid peroxidation is stimulated by BCAA and BCKA in brain of rats and these metabolites reduce in vitro and in vivo the cerebral capacity to modulate the damage associated to increased free radical production (Bridi et al. 2005a, b; Bridi et al. 2003; Fontella et al. 2002). Also, there are evidences that oxidative stress occurs in MSUD patients at diagnosis and during treatment (Barschak et al. 2006; Barschak et al. 2008). Besides, selenium deficiency and decrease of erythrocyte glutathione activity could be associated to the development of the oxidative stress (Barschak et al. 2007).

L-Carnitine (L-Car) [L-3-hydroxy-4-N,N,N-trimethylamino- butyrate] is a highly polar, water soluble, small quaternary amine, which is present in the organism mainly from exogenous origin (Agarwal and Said 2004). Classically, the function of L-Car is the transport of long chain fatty acids across the inner mitochondrial membrane for their utilization in metabolism through β-oxidation (Hoppel 2003). However, some researchers have established that L-Car also develops a protective role as antioxidant and antiperoxidative against reactive oxygen species by scavenging hydroxyl radical, superoxide anion, hydrogen peroxide and by inhibiting hydroxyl radical formation in the Fenton reaction system (Reznick et al. 1992; Derin et al. 2004). The ability of L-Car to scavenge free radicals is explained by the action of the carbonyl group (included in the carboxylate group) which stabilizes a radical formed after hydrogen atom abstraction from L-carnitine α-carbon (Derin et al. 2004; Gulcin 2006). Studies have been shown that treatment with L-Car may reduce lipid peroxidation in patients with propionic acidemia and methylmalonic acidemia and is also able to prevent DNA damage caused by propionic and methylmalonic acids in human peripheral leukocytes in vitro (Ribas et al. 2010a, b). L-Car was also associated with the reduction of lipid peroxidation in patients with phenylketonuria, an inborn error of phenylalanine metabolism (Sitta et al. 2009).

So, the objective of this study was to evaluate the possible antioxidant and neuroprotective effects of L-Car treatment in a chemically-induced acute model of MSUD. The preventive effect of L-Car against oxidative stress caused by BCAA was verified assessing enzymatic antioxidant defenses, lipid peroxidation and protein oxidative damage.

Materials and methods

Materials

All chemicals were purchased from Sigma/Aldrich (St. Louis, MO, USA). BCAA and L-Car solutions were freshly prepared in saline solution, and pH was adjusted to 7.4.

Experimental design

Fourteen-day-old Wistar rats bred in the Department of Biochemistry, ICBS, UFRGS, were used. Rats were kept with dams until they were sacrificed. The dams had free access to water and 20% (w/w) protein commercial chow (Supra, Porto Alegre, RS, Brazil). They were kept in a room with a 12:12 h light/dark cycle (lights on 07:00–19:00 h), and with air conditioned controlled temperature (22±1°C). The “Principles of Laboratory Animal Care” (NIH publication # 80–23, revised 1996) were followed in all the experiments. A chemically-induced acute model of MSUD was conducted in according to Bridi et al. (2006). BCAA solution was prepared at the day of experiment in saline solution (0.85% NaCl). The animals received three subcutaneously administrations (at 1 h interval from each other) of BCAA pool (15.8 μL/g body weight) containing 190 mmol/L Leu, 59 mmol/L Ile, and 69 mmol/L of Val. The administration of L-Car was performed intraperitoneally (100 mg/kg body weight), starting 1 day before the administration of BCAA pool and continued concomitantly until the last injection of the BCAA pool. Animals were divided into four groups: Control group (saline solution); MSUD group (BCAA pool); L-Car group (L-Car) and...
MSUD+L-Car group (L-Car and BCAA pool) and killed 1 h after the last injection.

Tissue preparation

Rats were killed by decapitation without anesthesia; the brain was immediately removed and kept on an ice-plate. The olfactory bulbs, pons, cerebellum and medulla were discarded and cerebral cortex was dissected and kept chilled until homogenization. Cerebral cortex was homogenized 1:10 w/v in 20 mM sodium phosphate and 140 mM KCl (pH 7.4) buffer. Homogenates were centrifuged at 750 g for 10 min at 4°C and the supernatant was immediately used for measurements.

Catalase assay (CAT)

CAT activity was assayed by the method of Aebi (1984) using SpectraMax M5/M5 Microplate Reader (Molecular Devices, MDS Analytical Technologies, Sunnyvale, CA, USA). This method is based on the disappearance of H₂O₂ at 240 nm in a reaction medium containing 20 mM H₂O₂, 0.1% Triton X-100, 10 mM potassium phosphate buffer, pH 7.0, and 0.1–0.3 mg protein/mL. One CAT unit is defined as 1 μmol of hydrogen peroxide consumed per minute and the specific activity is calculated as units/mg protein.

Superoxide dismutase assay (SOD)

The assay of SOD activity was carried out as described by Marklund (1985). Cerebral tissue was homogenized 1:10 (w/v) in 50 mM Tris-HCl buffer containing 1 mM ethylenediaminetetraacetic acid (EDTA), pH 8.2. This method is based on the capacity of pyrogallol to autoxidize, a process highly dependent on superoxide radical. The inhibition of autoxidation of this compound occurs in the presence of SOD, whose activity can be then indirectly assayed spectrophotometrically at 420 nm, using a SpectraMax M5/M5 Microplate Reader (Molecular Devices, MDS Analytical Technologies, Sunnyvale, CA, USA). A calibration curve was generated using 1,1,3,3-tetramethoxypropane as a standard. A 50% inhibition of pyrogallol autoxidation is defined as one unit of SOD and the specific activity is represented as units/mg protein.

Glutathione peroxidase assay (GPx)

GPx activity was measured using tert-butyl-hydroperoxide as substrate according to the method described by Wendel (1981). NADPH disappearance was monitored at 340 nm using SpectraMax M5/M5 Microplate Reader (Molecular Devices, MDS Analytical Technologies, Sunnyvale, CA, USA). The medium contained 2 mM glutathione, 0.15 U/mL glutathione reductase, 0.4 mM azide, 0.5 mM tert-butyl-hydroperoxide and 0.1 mM NADPH. One GPx unit is defined as 1 μmol of NADPH consumed per minute and the specific activity is represented as GPx units/mg protein.

Protein carbonyl content

Oxidatively modified proteins present a reinforcement of carbonyl content (Stadtman and Levine 2003). In this paper, protein carbonyl content was assayed by a method based on the reaction of protein carbonyls with dinitrophenylhydrazine forming dinitrophenylhydrazone, a yellow compound, measured spectrophotometrically at 370 nm (Reznick and Packer 1994). Briefly, 200 μL of sample were added to 400 μL of 10 mM dinitrophenylhydrazine (prepared in 2 M HCl). This mixture was kept in the dark for 1 h, and vortexed every 15 min. Behind this, 500 μL of 20% trichloroacetic acid was added to each tube. The mixture was vortexed and centrifuged at 20,000 g for 3 min. The supernatant obtained was discarded. The pellet was washed with 1 ml ethanol: ethyl acetate (1:1, v/v), vortexed, and centrifuged at 20,000 g for 3 min. The supernatant was discarded, and the pellet re-suspended in 600 μL of 6 M guanidine (prepared in a 20 mM potassium phosphate solution pH 2.3). The sample was vortexed and incubated at 60°C for 15 min. Subsequently, it was centrifuged at 20,000 g for 3 min, and the absorbance was measured at 370 nm (UV) in a quartz cuvette with a Hitachi U-2001 double-beam spectrophotometer with temperature control (Hitachi High Technologies America, Inc., Life Sciences Division, Pleasanton, CA, USA). The results were represented as protein carbonyl content (nmol/mg protein).
Sulfhydryl content

This parameter was performed according to Aksenov and Markesbery (2001). The oxidation of free thiols in the sample leads to the formation of disulfide bonds. The 5,5′ dithio-bis(2-nitrobenzoic acid) (DTNB), color reagent is not reduced by the thiols oxidized, generating a yellow derivative (TNB), read spectrophotometrically at 412 nm. The sulfhydryl content is inversely correlated to oxidative damage to proteins. The results were expressed as nmol TNB/mg protein.

Protein determination

Protein concentration was determined in cerebral cortex supernatants by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Statistical analysis

Statistical analysis was performed by the one-way analysis of variance (ANOVA), followed by the Tukey test for multiple comparisons when the F value was significant. All the analyses were performed using the Statistical Package for the Social Sciences (SPSS) software in a PC-compatible computer. A value of \( P<0.05 \) was considered to be significant.

Results

In this study, we investigated the possible protective role of L-Car against acute toxicity of BCAA by analyzing a series of oxidative stress parameters in cerebral cortex of 14-day-old rats.

Figure 1 shows that acute administration of BCAA pool markedly reduced CAT and GPx activities in cerebral cortex homogenates \([F(3,20)=26.45; p<0.05 \text{ and } F(3,18)=8.290; p<0.05, \text{ respectively}] \) and these effects were prevented by L-Car treatment. BCAA pool did not alter the activity of SOD \([F(3,22)=0.889; p>0.05]\) (results not shown).

Lipid peroxidation was investigated by measuring TBA-RS levels in rat cerebral cortex (Fig. 2). TBA-RS were increased by BCAA administration in the MSUD group and L-Car treatment inhibited this increase \([F(3,20)=19.872, P<0.05]\).

Finally, we tested whether tissue proteins were affected by BCAA pool. To accomplish this, two different parameters of oxidative protein damage were measured– carbonyl and sulfhydryl contents (Fig. 2). Carbonyl content was significantly enhanced in MSUD group in cerebral cortex \([F(3,24)=10.779, P<0.05]\) while sulfhydryl content was significantly reduced \([F(3,21)=21.079, P<0.05]\), indicating the occurrence of oxidized proteins. These effects were prevented by L-Car treatment since MSUD+L-Car group was not different from controls.

Discussion

Patients affected by severe form of MSUD present BCAA and BCKA accumulation as a biochemical hallmark and are clinically characterized by ketoacidosis, convulsions, coma, failure to thrive, poor feeding, apnea, ataxia, seizures, psychomotor delay and mental retardation (Chuang and Shih 2001). The exact mechanisms underlying brain damage in this disorder remain far poorly known, but it is well established that high concentrations of Leu and/or KIC were associated with the appearance of the neurological symptoms and these compounds seem to be the main neurotoxic metabolites in the illness (Chuang and Shih 2001).

It is considered that oxidative stress can result from different conditions such as generation of reactive species at an abnormally high rate, insufficient antioxidant defenses, increased concentrations of transition metal ions, or due to...
a combination of above status which can potentially induce biomolecular oxidative damage (Halliwell and Gutteridge 2007; Halliwell 2001). In this context it is necessary to emphasize that the central nervous system is highly sensitive to oxidative stress owing to its high oxygen consumption, low activity of antioxidant enzymatic defenses, its high lipid content, specially unsaturated fatty acids, and iron content that stimulates the Fenton reaction being therefore highly susceptible to reactive species (Halliwell 2001).

Moreover, the adverse consequences of oxidative stress have been implicated in a variety of central nervous system diseases, including inherited metabolic disorders (Wajner et al. 2004; Halliwell 2006). Our group demonstrated that the metabolites accumulated in MSUD stimulate lipid peroxidation and decrease of brain antioxidant defenses (Bridi et al. 2005a, b; Bridi et al. 2003; Fontella et al. 2002). It is important to emphasize that several studies in the literature correlating oxidative stress and maple syrup urine disease in animal model were performed in rat cerebral cortex with 14 and/or 30 days of life (Fontella et al. 2002; Bridi et al. 2003; Bridi et al. 2005a, b; Bridi et al. 2006). For this reason, in this study we prioritized the cerebral cortex to evaluate a possible neuroprotective role of L-Car against a pathological increase of free radicals caused by acute administration of branched chain amino acids accumulating in this disease. More recently, we have also reported that oxidative stress is induced in MSUD patients at diagnosis and during treatment (Barschak et al. 2006; Barschak et al. 2008). Altogether, these results strongly suggest that oxidative stress may be involved in the pathophysiology of MSUD.

According to Gutteridge and Halliwell (2010) antioxidants are substances that, at low concentrations, delays, prevents, or removes oxidative damage to a target molecule, competing with oxidizable substrates inhibiting their oxidation. L-Car plays a central role in the cellular energy metabolism because it transports long-chain fatty acids for oxidation and ATP generation. In recent years, many studies have demonstrated the antioxidant role of this compound, through its action against peroxidation in different tissues by various mechanisms: a “scavenger” of reactive oxygen species, stabilizing effect of damage to cell membranes and increasing enzymatic and non-enzymatic antioxidant levels (Derin et al. 2004; Gulcin 2006; Agarwal and Said 2004). Thus, L-Car has been increasingly used therapeutically due to its antioxidant role, including diseases such those affecting the central nervous system (Augustyniak and Skrzydlewska 2010; Mancuso et al. 2007) since it is capable of crossing the blood brain barrier (Kido et al. 2001), and also in metabolic disorders, including inborn errors of metabolism (Sitta et al. 2009; Ribas et al. 2010a; b). So, in the present study, we evaluated the possible neuroprotective effect of L-Car in cerebral cortex of rats subjected to a chemically-induced acute model of MSUD.

Primarily, we analyze the activity of the antioxidant enzymes CAT, GPx and SOD. We started showing that CAT and GPx activities were significantly reduced in cerebral cortex of rats subjected to an acute administration of BCAA pool and L-Car prevented this inhibitory effect.
Interestingly, previous findings showed that L-Car are able to scavenger hydrogen peroxide (Derin et al. 2004; Gulcin 2006). CAT is an enzyme that directly catalyses the decomposition of hydrogen peroxide into water and molecular oxygen. GPx removes hydrogen peroxide and other peroxides by coupling its reduction to H₂O with oxidation of reduced glutathione (GSH), using selenium (Se) as cofactor. Acting together, both enzymes are responsible for hydrogen peroxide detoxification and the inhibition of CAT and GPx activities may lead to an impairment of detoxification of this substrate. An increase in CAT activity after administration of L-Car may also be related to the improvement of activity of glucose-6-phosphate dehydrogenase generating NADPH which is used for the regeneration of CAT activity (Muthuswamy et al. 2006).

Previous results in vitro have already shown that Leu inhibits CAT and GPx activity (Bridi et al. 2005b). In addition, Barschak et al. (2007) demonstrated that MSUD patients have selenium deficiency and decrease of erythrocyte glutathione activity, so the action of L-Car to restore the activity of GPx may be very important to treatment of these patients.

In contrast, the activity of SOD, enzyme responsible for removing anion superoxide by accelerating the rate of its dismutation to hydrogen peroxide and water was not altered by acute administration of BCAA pool. This result is in agreement with previous studies showing that SOD activity is not modified by the increase in the concentration of MSUD metabolites accumulated both in vitro (Bridi et al. 2005a, b) and in erythrocytes of patients (Barschak et al. 2007).

Finally, the influence of acute administration of pool BCAA and possible prevention by L-Car treatment on lipid and protein oxidation in cerebral cortex of rats were investigated. TBA-RS reflects the content of malondialdehyde, the most abundant individual aldehyde resulting from lipid breakdown due to lipid peroxidation process. On the other hand, protein carbonylation and sulphydryl content are the most general and a useful index of protein oxidation (Reznick and Packer 1994; Stadtman and Levine 2003). We have showed that BCAA pool was able to cause lipoperoxidation, as verified by increased TBA-RS levels and protein oxidative damage, as analyzed by significant alterations of sulphydryl and carbonyl contents compared to control group, suggesting that oxidative damage to lipids and proteins may be involved in the neuropathology of MSUD. This is in agreement with previous results from our laboratory (Fontella et al. 2002; Bridi et al. 2003, 2005b) and interestingly, we have also found increased serum TBA-RS levels in MSUD patients (Barschak et al. 2006, 2008). The present results showed that the treatment with L-Car was able to prevent lipoperoxidation and protein damage. This is probably associated with the antioxidant activity of L-Car, which is based on the capacity to scavenge free radicals as well as by chelating metal ions that participate in free radical reaction (Muthuswamy et al. 2006; Gulcin 2006). These antioxidant action of L-Car need to be clarified and further studied, but possible underlying mechanisms may involve scavenging of free radicals and/or the chelation of transition metals, prevention of BCAA from crossing the blood-brain barrier or their uptake by brain mitochondria. Moreover, L-carnitine may be stabilizing mitochondria resulting in lower production of superoxide radicals.

Plasma BCAAs are transported into the brain (and other portions of the central nervous system (CNS)) by a transporter, located at the blood–brain barrier (BBB) on CNS capillary endothelial cells, called of large neutral amino acids (LNAAs) transporter. The BCAA influence brain function by modifying LNAAs transport at the blood–brain barrier. Transport is shared by several LNAAs, notably the BCAA and the aromatic amino acids, and is competitive. Consequently, when plasma BCAA concentrations rise, such reductions in brain aromatic amino acid concentrations have functional consequences: biochemically, they reduce the synthesis and the release of neurotransmitters derived from aromatic amino acids, notably serotonin (from tryptophan) and catecholamines (from tyrosine and phenylalanine) (Bridi et al. 2006). Moreover, several in vitro studies have shown that increasing the concentration of MSUD metabolites in cerebral cortex of rats induces an increased production of oxidative stress (Fontella et al. 2002; Bridi et al. 2003; Bridi et al. 2005a, b). Still, the present acute chemically-induced MSUD model have a similar amino acid profile to that of MSUD untreated patients reaching concentrations of the BCAA comparable to those found in these patients in plasma, which may contribute to investigate the mechanisms of brain damage characteristic of this disorder (Bridi et al. 2006).

Through the chemically-induced acute model of MSUD conducted in this work, it can be presumed that oxidative stress may contribute, along with other mechanisms, to the pathophysiology of the brain dysfunction characteristic of MSUD. Efficiently, L-Car was able to prevent all effects on oxidative stress caused by BCAA pool injection reported now by improving activity of antioxidant enzymes and reducing lipid and protein oxidative damage. These results indicate a possible neuroprotective role for L-Car as a potential adjuvant therapy to the MSUD affected patients, especially during crises when the levels of the BCAAs increase dramatically. Thereby, these data may contribute to the understanding of the mechanism of action of the cytotoxic effect of metabolites accumulated in MSUD and the effect of L-Car upon this process. The study of antioxidants like L-Car, can open an additional therapeutic approach to the currently employed for MSUD patients, which is primarily dietary and therefore difficult to handle.
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Conflict of interest The authors declare that they have no conflict of interest.

References


