Free Radical Research, January 2005; 39(1): 71–79

Taylor & Francis
Taylor & Francis Group

Evaluation of the mechanisms involved in leucine-induced oxidative damage in cerebral cortex of young rats

RAQUEL BRIDI[1], ALEXANDRA LATINI[1,2], CÉSAR A. BRAUM[1], GIOVANNI K. ZORZI[1], MOACIR WAJNER[1,2], EDUARDO LISSI[3], & CARLOS SEVERO DUTRA-FILHO[1]

[1]*Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2600 Anexo, CEP 90035-003, Porto, Alegre RS, Brazil,* [2]*Serviço de Genética Médica, Hospital de Clínicas de Porto Alegre, Porto Alegre RS, Brazil, and* [3]*Departamento de Ciencias Químicas, Faculdad de Química y Biologia, Universidad de Santiago de, Chile, Santiago, Chile*

Accepted by Professor J. Vina

(Received 10 September 2004; in revised form 21 October 2004)

Abstract

Maple syrup urine disease (MSUD) is a metabolic disorder caused by the deficiency of the activity of the mitochondrial enzyme complex branched-chain L-2-keto acid dehydrogenase. The metabolic block results in tissue and body fluid accumulation of the branched-chain amino acids leucine (Leu), isoleucine and valine, as well as of their respective α-keto acids. Neurological sequelae are usually present in MSUD, but the pathophysiologic mechanisms of neurotoxicity are still poorly known. It was previously demonstrated that Leu elicits oxidative stress in rat brain. In the present study we investigated the possible mechanisms involved in Leu-induced oxidative damage. We observed a significant attenuation of Leu-elicited increase of thiobarbituric acid-reactive substances (TBA-RS) measurement when cortical homogenates were incubated in the presence of the free radical scavengers ascorbic acid plus trolox, dithiothreitol, glutathione, and superoxide dismutase, suggesting a probable involvement of superoxide and hydroxyl radicals in this effect. In contrast, the use of N^{ω}-nitro-L-arginine methyl ester or catalase (CAT) did not affect TBA-RS values. We also demonstrated an inhibitory effect of Leu on the activities of the antioxidant enzymes CAT and gluthathione peroxidase, as well as a significant reduction in the membrane-protein thiol content from mitochondrial enriched preparations. Furthermore, dichlorofluorescein levels were increased although not significantly by Leu. Taken together, our present data indicate that an unbalance between free radical formation and inhibition of critical enzyme activities may explain the mechanisms involved in the Leu-induced oxidative damage.

Keywords: *L-Leucine, gluthathione peroxidase, superoxide dismutase, branched-chain keto aciduria, maple syrup urine disease*

Introduction

Maple syrup urine disease (MSUD; branched-chain keto aciduria) is an autosomal recessive inborn error of metabolism caused by the deficiency of the activity of the mitochondrial enzyme complex branched-chain L-2-ketoacid dehydrogenase (BCKD). This metabolic block results in leucine (Leu) accumulation which undergoes reversible transamination to produce the branched-chain α-keto acid α-ketoisocaproic acid (KIC), as well as of isoleucine and valine and their respective α-keto acids α-keto-β-methylvaleric acid (KMV) and α-ketoisovaleric acid (KIV).[1] The hydroxy derivatives produced by the reduction of their respective α-keto acids also accumulate in this disorder.[2]

MSUD is manifested as heterogeneous clinical phenotypes, ranging from a severe classical form to

Correspondence: C. S. Dutra-Filho, Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2600 Anexo, CEP 90035-003, Porto, Alegre RS, Brazil. Tel: 55-51-3316-5573. Fax: 55-51-3316-5535. E-mail: dutra@ufrgs.br

ISSN 1071-5762 print/ISSN 1029-2470 online © 2005 Taylor & Francis Ltd
DOI: 10.1080/10715760400022350

mild variants, possibly due to the distinct residual enzyme activity.[3] Based on the clinical presentation and biochemical responses to thiamine administration, MSUD patients can be divided into five clinical and biochemical phenotypes[1] If untreated by dietary restriction of branched-chain amino acids (BCCAs), these patients suffer from seizures, psychomotor delay and mental retardation.[1,4] Characteristic neuropathologic findings of the disease are cerebral edema, atrophy of the cerebral hemispheres, spongy degeneration of the white matter and delayed myelination.[1,2] Neurological sequelae are present in most patients, but the fundamental mechanisms of neurotoxicity of this disorder are virtually unknown. However, Leu and/or its keto acid KIC are thought to be main neurotoxins in MSUD[1,5] since they are the metabolites that most accumulate in untreated patients.[6] It has been postulated that Leu alters brain energy metabolism,[7–10] neurotransmitter systems,[6,11,12,13] brain uptake of essential amino acids[14] and induces neuronal apoptosis.[15] Furthermore, our group has demonstrated that Leu provokes oxidative stress in rat brain homogenates by increasing the oxidation of lipids and by reducing the brain non-enzymatic antioxidant defenses.[16]

Therefore, the objective of the present study was to evaluate the role of antioxidants on the effect provoked by Leu on thiobarbituric acid-reactive substances (TBA-RS) levels, a measure of lipid oxidative damage in rat brain. We also evaluated whether Leu alters the protein thiol groups in membrane from mitochondrial enriched fractions, the oxidation of 2,7-dichlorofluorescin (DCFH), cyclic voltammetry, and the activities of the main antioxidant enzymes catalase (CAT), superoxide dismutase (SOD) and gluthathione peroxidase (GPx), in order to better understand the mechanisms of the Leu-induced oxidative stress.

Experimental procedures

Animals and reagents

Male Wistar rats of 30 days of life obtained from the Central Animal House of the Department of Biochemistry, ICBS, Universidade Federal do Rio Grande do Sul, Porto Alegre, (Brazil), were used. The animals were maintained on a 12:12 h light/dark cycle (lights were on from 07:00 to 19:00 h) in an air conditioned controlled temperature (22° ± 1°C) colony room, with free access to water and 20% (*w*/*w*) protein commercial chow (Germani, Porto Alegre, RS, Brazil). The "Principles of laboratory animal care" (NIH publication #80-23, revised 1996) were followed in all the experiments. All chemicals were purchased from Sigma (St. Louis, MO, USA), except for Ransod kit from Randox (Antrim, United Kingdom). L-Leucine solutions were prepared on the day of the experiment in the buffer used for each technique. The final concentrations of the amino acid in the medium were 1.0, 2.5, 5.0 or 10.0 mM.

Tissue preparation

Rats were sacrificed by decapitation without anesthesia, and the brain was rapidly excised and kept on an ice-plate. The olfactory bulbs, pons and medulla were discarded and the cerebral cortex was dissected, extensively rinsed with saline for complete blood removal, weighed and kept chilled until homogenization.

Cerebral cortex homogenates. The isolated cerebral cortex was homogenized in 10 volumes (1:10 *w*/*v*) of the specific medium for measuring TBA-RS levels, DCFH oxidation and CAT, SOD and GPx activities. Homogenates were then centrifuged at 750*g* for 10 min at 4°C to discard nuclei and cell debris. The supernatant, a suspension of mixed and preserved organelles, including mitochondria, was separated and immediately used for analyses and the pellet was discarded.

Mitochondrial preparations from cerebral cortex. Cerebral cortex was homogenized (1:4 *w*/*v*) in 0.25 M sucrose and 10 mM Tris, pH 7.4 on ice. The homogenates were centrifuged at 650*g* for 10 min at 4°C to remove the nuclei and membrane debris. The supernatants were further centrifuged at 25,000*g* at 4°C for 15 min. The mitochondrial pellet was resuspended in 0.1 M Tris, pH 8.0 and stored at − 70°C.[17,18]

In vitro experiments

Cerebral cortex supernatants and mitochondrial preparations were incubated for 1 h at 37°C in the presence of Leu, prepared in the same buffer used for homogenizing the brain tissue, at concentrations ranging from 1.0 to 5.0 mM. Immediately after incubation, aliquots were taken to measure TBA-RS, dichlorofluorescin (DCF), membrane proteic thiol content and the activity of antioxidant enzymes CAT, SOD, and GPx. Controls did not contain Leu in the incubation medium. Measurements of antioxidant enzyme activities were also carried out without incubation by adding Leu to homogenates at the time of assay. In addition, the activity of commercial preparations of CAT (purified from bovine liver—EC 1.11.1.6) and GPx (purified from bovine erythrocytes—EC 1.11.1.9) were measured in the presence of 2.5, 5.0 or 10.0 mM Leu.

The effect of some antioxidants, prepared in 20 mM sodium phosphate buffer, pH 7.4, containing 140 mM KCl, were also investigated. The final concentrations were similar to those described previously,[19,20] as follows: 200 μM ascorbic acid, 10 μM Trolox (a water soluble α-tocopherol mimic), 400 μM dithiothreitol

(DTT); 400 μM gluthatione (GSH), 200 μM N^{ω}-nitro-L-arginine methyl ester (L-NAME), 50 mU/ml SOD (purified from bovine erythrocytes—EC 1.15.1.1) and 50 mU/ml catalase.

Thiobarbituric acid-reactive substances (TBA-RS)

TBA-RS levels were determined according to the method described by Esterbauer and Cheeseman.[21] Briefly, 300 μl of cold 10% (*w*/*v*) trichloroacetic acid were added to 150 μl of cerebral cortex homogenates prepared with 20 mM sodium phosphate buffer, pH 7.4, containing 140 mM KCl (1:10 *w*/*v*) and centrifuged at 300*g* for 10 min. A measure of 300 μl of the supernatant were transferred to a Pyrex tube and incubated with 300 μl of 0.67% (*w*/*v*) thiobarbituric acid in 7.1% (*w*/*v*) sodium sulphate in a boiling water bath for 25 min. The mixture was allowed to cool on water for 5 min. The resulting pink stained TBA-RS were determined in a spectrophotometer at 535 nm. Leu and the free radical scavengers used, ascorbic acid, Trolox, DTT, GSH, CAT, SOD and L-NAME, did not produce pink color along time when tested without the addition of the homogenates, demonstrating an absence of a direct reaction of these compounds with thiobarbituric acid. A calibration curve using 1,1,3,3-tetramethoxypropane, was performed being subjected to the same treatment as that of the samples. TBA-RS levels were calculated as nmol TBA-RS/mg protein.

Determination of membrane protein thiol content

The mitochondrial suspension was submitted to three subsequent freeze-thawing procedures to release matrix proteins and was afterward centrifuged for 2 min at 15,000*g*. The pellet was treated with 200 μl of 6.5% trichloroacetic acid in order to precipitate proteins and two centrifugations were carried out at 15,000*g* for 2 min. The final pellet was suspended in 200 μl of a medium containing 0.5 mM EDTA and 0.5 M Tris (pH 8.3).[22] After incubations, 1.0 ml of 100 μM 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB)[23] was added to the medium and left to stand for 30 min in the dark. Membrane protein oxidized thiol groups from mitochondrial pellet were measured at 412 nm. The thiol content was calculated by using a molar extinction coefficient of 13.6 $mM^{-1}\,cm^{-1}$ and expressed in nmol oxidized DTNB/mg protein.

Catalase (CAT)

CAT activity was assayed by the method of Aebi[24] using a spectrophotometer with temperature control (Hitachi U-2001). Cerebral tissue was homogenized 1:10 (*w*/*v*) in 10 mM potassium phosphate buffer, pH 7.0. The method is based on the disappearance of H_2O_2 at 240 nm in a reaction medium containing 20 mM H_2O_2, 0.1% Triton X-100, 10 mM potassium phosphate buffer, pH 7.0, and 0.1–0.3 mg protein/ml. One unit is defined as one μmol of hydrogen peroxide consumed per minute, and the specific activity is reported as units/mg protein.

Superoxide dismutase (SOD)

SOD activity was determined using the RANSOD kit from Randox using a spectrophotometer with temperature control (Hitachi U-2001). Cerebral tissue was homogenized in 50 mM potassium phosphate buffer, pH 7.0. The method is based on the formation of red formazan from the reaction of 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride and superoxide radical (produced in the incubation medium from the xanthine–xanthine oxidase reaction system), which is assayed spectrophotometrically at 505 nm. The inhibition of the produced chromogen is proportional to the activity of the SOD present in the sample. A 50% inhibition is defined as one unit of SOD and the specific activity is represented as units/mg protein.

Glutathione peroxidase (GPx)

GPx activity was measured by the method of Wendel[25] using *tert*-butyl-hydroperoxide as substrate. Cerebral tissue was homogenized in 100 mM potassium phosphate buffer containing ethylenediaminetetraacetic acid 1.0 mM, pH 7.7. The activity was determined by monitoring the NADPH disappearance at 340 nm using a spectrophotometer with temperature control (Hitachi U-2001). The medium contained 2.0 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 one μmol of NADPH consumed per minute, and the specific activity is given as units/mg protein.

2,7-Dichlorofluorescin oxidation by reactive species

DCF was measured according to LeBel.[26] The membrane-permeable diacetate form of the DCFH prepared in 20 mM sodium phosphate buffer pH 7.4 containing 140 mM KCl was incubated with the homogenates at a final concentration of 100 μM for 30 min at 37°C. Fluorescence was measured using excitation and emission wavelengths of 480 and 535 nm, respectively. Calibration curve was performed with standard DCF (0–10 μM) and the concentration of the reactive species, given by the rate of DCFH oxidation, was calculated as nmol DCF formed/mg protein and expressed as percentage of controls.

Cyclic voltammetry measurements

Stock solutions (0.1 M) of Fe^{2+} or Cu^{+} were prepared in distilled water and diluted to 1.0 mM to carry out the cyclic voltammetry experiments. KCl (0.1 M) was employed as supporting electrolyte. The electrode

potential was set by a Wenking (POS 73) potentioscan. A glassy carbon stationary electrode was used as working electrode, with a platinum wire employed as counter electrode. All potentials were measured against an Ag/AgCl/KClsat electrode. Oxidation cycles were carried out at 100 mV/s, starting from the lowest potential. Cycles were carried out in absence and in presence of 2.0 mM Leu under identical experimental conditions.

Protein determination

Protein concentrations were determined in cerebral cortex preparations by the method of Lowry[27] using bovine serum albumin as standard.

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) followed by the Duncan multiple range test when the F value was significant. Linear regression analysis was also used to test dose-dependent effects. All analyses were carried out 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

Our previous report[16] demonstrated that Leu increased chemiluminescence and TBA-RS levels (lipid peroxidation) and decreased non-enzymatic antioxidant defenses in rat brain. In the present investigation we studied the role of various antioxidants on the effect produced by Leu on TBA-RS levels as well as on the antioxidant enzymatic defenses, the protein thiol content from mitochondrial enriched fractions, the oxidation of DCFH, and cyclic voltammetry in cerebral cortex homogenates from young rats.

Cerebral cortex homogenates were incubated for 1 h with 5.0 mM Leu alone or combined with the free radical scavengers ascorbic acid, Trolox, DTT, GSH, CAT, SOD or L-NAME and the TBA-RS levels were measured afterwards. Figure 1A and B shows that the Leu-induced increase of TBA-RS measurement was abolished by 200 μM ascorbic acid plus 2.5 μM Trolox, by 400 μM DTT and by 400 μM GSH, rendering the measured values very close to the controls. Similar results were obtained with 50 mU/ml SOD (Figure 1C).

In contrast, the use of 50 mU/ml catalase (Figure 1D) or 200 μM of the nitric oxide synthase inhibitor L-NAME (Figure 1E) did not reduced the increased TBA-RS values observed in Leu-treated homogenates. It is important to note that the addition of any of these antioxidants alone did not significantly alter TBA-RS values.

Next, we investigated the effect of Leu at concentrations varying from 1.0 to 10.0 mM on the activity of the antioxidant enzymes CAT, SOD and GPx. These activities were evaluated without incubation and also after exposing the homogenates to the amino acid during 1 h at 37°C. In addition, Leu effect on CAT and GPx activities were also assessed in commercial preparations. It can be observed in Figure 2 that CAT activity was significantly reduced (30%) by Leu in a dose-response manner in both conditions, without incubation [$F(3, 20) = 3.99$; $p < 0.05$; $\beta = -0.70$, $p < 0.05$] (Figure 2A) and after 1 h incubation [$F(3, 20) = 3.75$; $p < 0.05$; $\beta = -0.58$, $p < 0.01$] (Figure 2B). Figure 2C shows that CAT activity

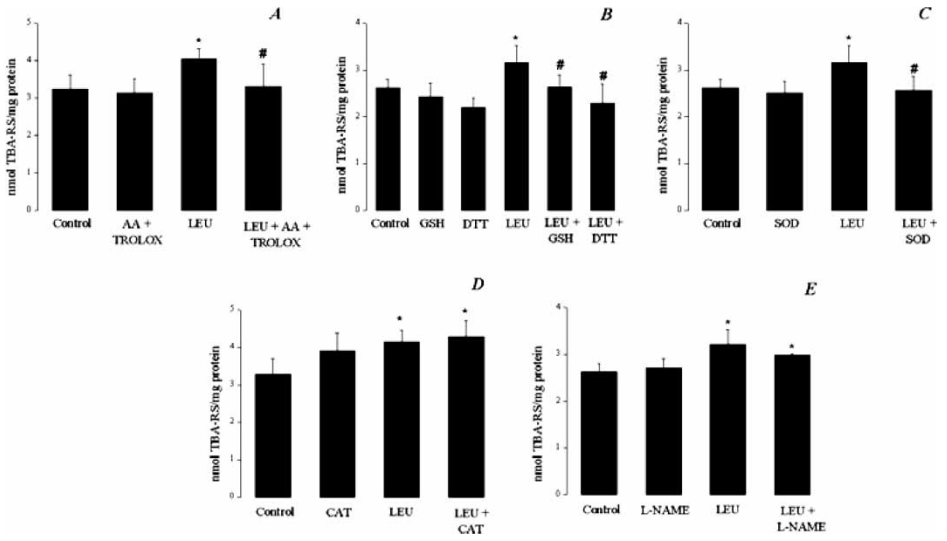

Figure 1. Effect of 5 mM leucine (Leu) on thiobarbituric-acid reactive species (TBA-RS) in cerebral cortex from young rats in the presence of various antioxidants; **A**: ascorbic acid (AA) plus Trolox; **B**: gluthatione (GSH) and dithiothreitol (DTT); **C**: superoxide dismutase (SOD); **D**: catalase (CAT); **E**: N^{ω}-nitro-L-arginine methyl ester (L-NAME). Data represent the mean ± SD for four to five independent experiments (animals) performed in triplicate and are expressed as nmol/mg protein. Difference from control, $\star p < 0.05$. Difference from Leu, $p < 0.05$ (Duncan multiple range test).

from purified preparations was also inhibited by Leu at all concentrations used [$F(3,8) = 5.80$; $p < 0.05$] at a similar degree to that observed in the brain homogenates. These findings suggest a direct effect of Leu on the enzyme activity.

A similar approach was used to evaluate the effect of Leu on SOD activity. It can be observed in Figure 3A and B that the activity of SOD was not affected by Leu treatment without [$F(3,16) = 0.11$; $p > 0.05$] or after1 h incubation with the amino acid [$F(3,12) = 0.03$; $p > 0.05$]. We also tested the effect of Leu on GPx activity (Figure 4). Leu significantly reduced the activity of the enzyme without previous incubation (Figure 4A) [$F(3,16) = 4.23$; $p < 0.01$]. Although Leu also inhibited (by 20%) GPx activity after 1-hour incubation (Figure 4B), this effect was not statistically significant [$F(3,12) = 1,16$; $p > 0.05$]. GPx activity was also evaluated using a commercial purified GPx preparation. Figure 4C shows that the activity of the purified GPx was significantly inhibited by 10 mM Leu [$F(2,6) = 9.20$; $p < 0.05$]. These data suggest that Leu probably exerts a direct effect on GPx.

Our next step was to test whether Leu was able to oxidize membrane protein thiol groups present in mitochondrial enriched fractions. Membranes were incubated for 1 h at 37°C with 1.0 to 5.0 mM Leu. Figure 5 shows that Leu significantly reduced the thiol content of mitochondrial enriched fractions [$F(3,24) = 3.06$; $p < 0.05$].

Finally, reactive species production in cerebral cortex homogenates was measured by the extent of DCFH oxidation. Figure 6 shows that Leu did not provoke a significant increase of DCFH oxidation [$F(3,20) = 1.75$, $p > 0.05$], although there was a tendency for Leu-induced increase this parameter (15% increase; $\beta = 0.45$; $p < 0.02$). We also tested the effect of Leu on the cyclic voltammetry. For Fe^{2+} and Cu^{+}, a reversible oxidation peak with an anodic peak potential at 74 and 170 mV, respectively, was observed. The respective cathodic peaks appear at 0 and 85 mV, generating a well-defined redox couple. In the presence of 2 mM Leu, no changes in the cyclic voltammograms were observed, indicating that Leu did not form complexes with Fe or Cu.

Discussion

Although the mechanisms of brain damage in MSUD are still unclear, it is presumed that Leu and its respective keto acid KIC are the main neurotoxic metabolites.[1,5] We previously reported that Leu induces oxidative stress in rat brain.[16] In order to get a better insight into the mechanisms of Leu-induced oxidative stress we evaluated the effect of Leu on the following parameters TBA-RS levels, antioxidant enzyme activities, protein-SH content, rate of DCFH oxidation and cyclic voltammetry. Considering that the overproduction of reactive

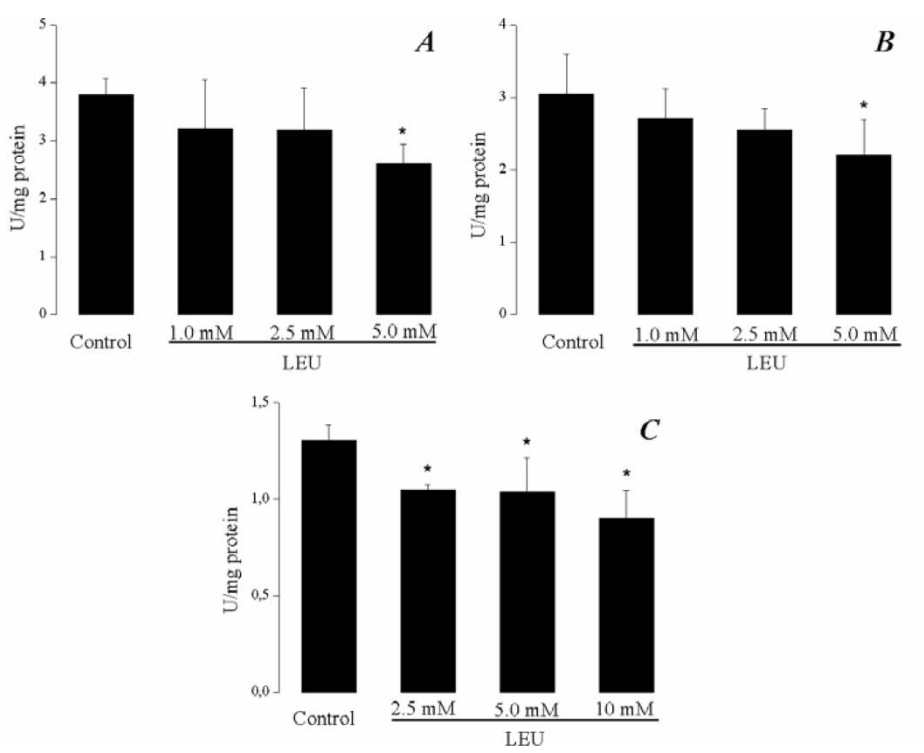

Figure 2. Effect of leucine on catalase (CAT) activity from cerebral cortex homogenates and from commercial purified preparation; **A**: cortical homogenates without incubation; **B**: cortical homogenates after 1 h incubation; **C**: purified CAT activity without incubation. The data represent mean ± SD for five independent experiments (animals) performed in triplicate. The results are expressed as U/mg protein and one CAT unit is defined as one μmol of hydrogen peroxide consumed per minute. Difference from control, $\star p < 0.05$ (Duncan multiple range test).

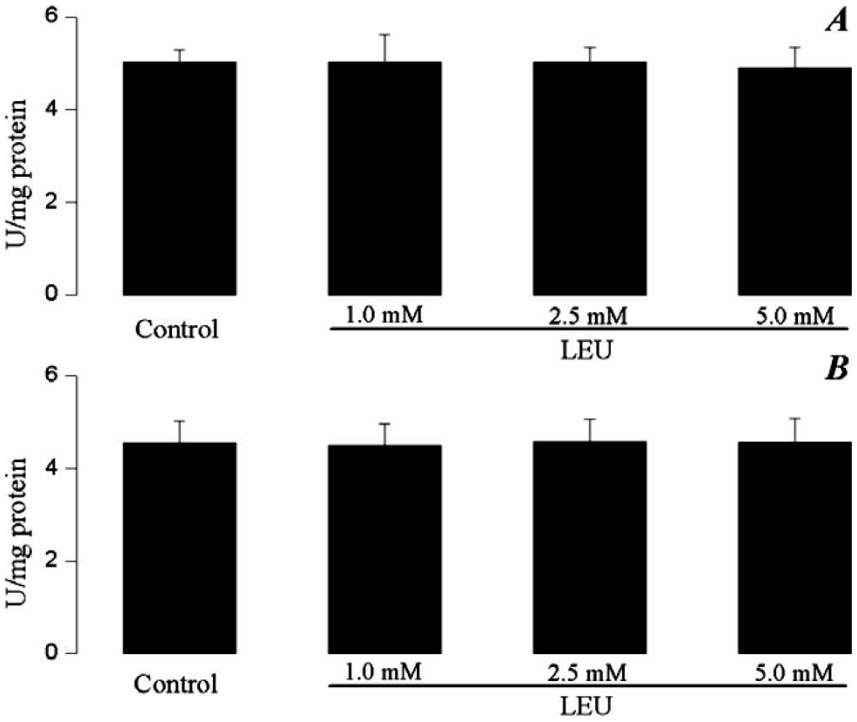

Figure 3. Effect of leucine on superoxide dismutase (SOD) activity in cerebral cortex homogenates from young rats without incubation; **A**: Effect after 1 h incubation; **B**: Data represent the mean ± SD for four independent experiments (animals) performed in duplicate and are expressed as U/mg protein. One SOD unit is defined as 50% inhibition of the produced chromogen. No significant differences were observed (one-way ANOVA).

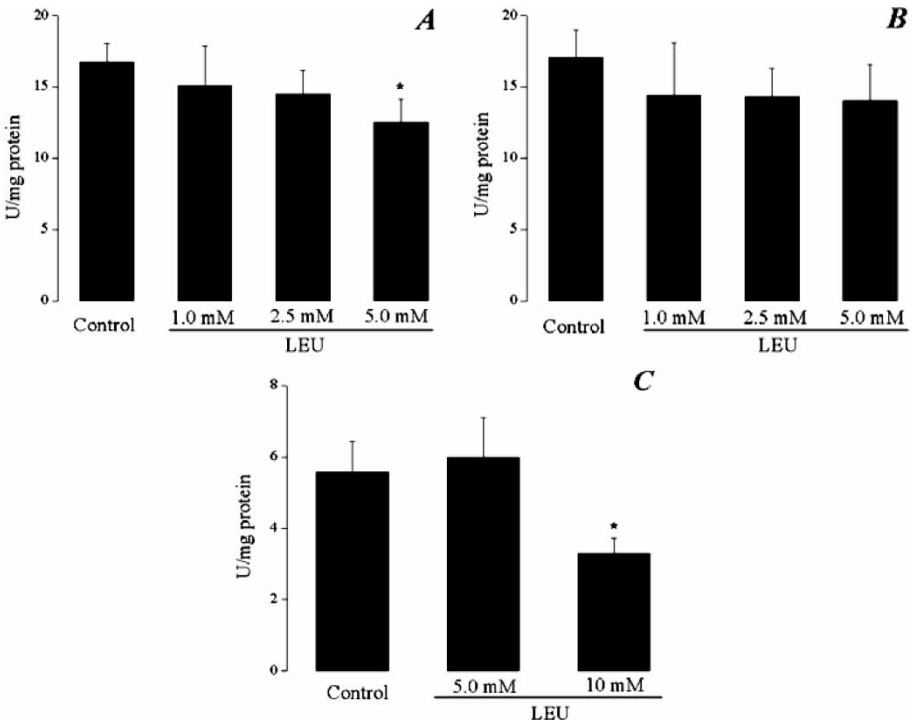

Figure 4. Effect of leucine on glutathione peroxidase (GPx) activity in cerebral cortex homogenates and from commercial purified preparation; **A**: cortical homogenates without incubation; **B**: cortical homogenates after 1 h incubation; **C**: purified GPx activity without incubation. The data represent the mean ± SD for five independent experiments (animals) performed in triplicate and are expressed as U/mg protein. One GPx unit is defined as one μmol of NADPH consumed per minute. Difference from control, $*p < 0.05$ (Duncan multiple range test).

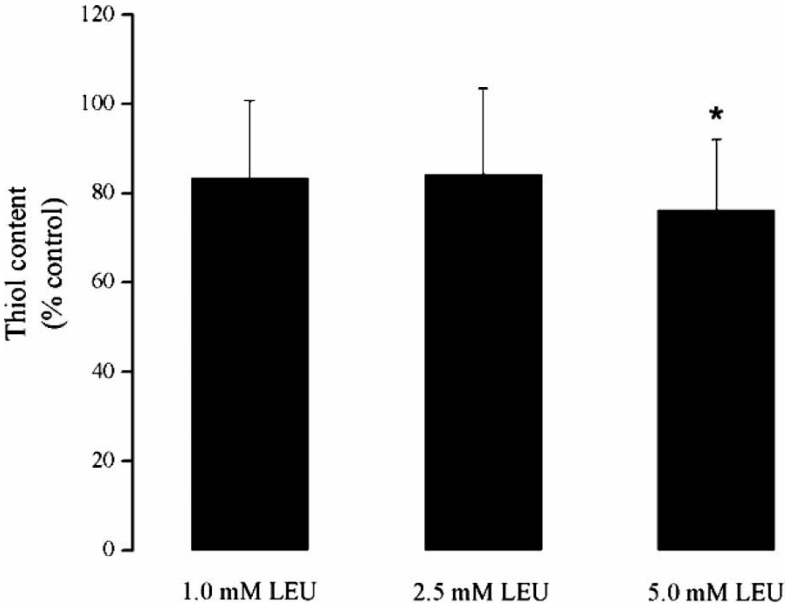

Figure 5. Effect of leucine on membrane protein thiol groups from mitochondrial enriched fractions in cerebral cortex homogenates from young rats. Data represent the mean ± SD for six independent experiments (animals) performed in triplicate and are expressed as percentage of control (Control values: 134.67 ± 14.46 nmol oxidized DTNB/mg protein). Difference from control, $*p < 0.05$ (Duncan multiple range test).

species gives rise to high TBA-RS levels,[28] we studied the influence of various antioxidants on the increased of TBA-RS levels induced by Leu. We observed that ascorbic acid plus trolox, DTT, GSH, and SOD, but no CAT, abolished Leu elicited increase in TBA-RS measurements in cerebral cortex homogenates. These findings might indicate the participation of superoxide and hydroxyl radicals on this effect, since these reactive species are scavenged by the antioxidants used.[29,30] In contrast, nitric oxide (NO) was not probably involved in the increased TBA-RS caused by Leu since when L-NAME was supplemented to Leu-treated samples, the additive was not able to reduce the increase of TBA-RS levels.

It has been previously postulated that the damage associated to the MSUD neuropathology is, at least

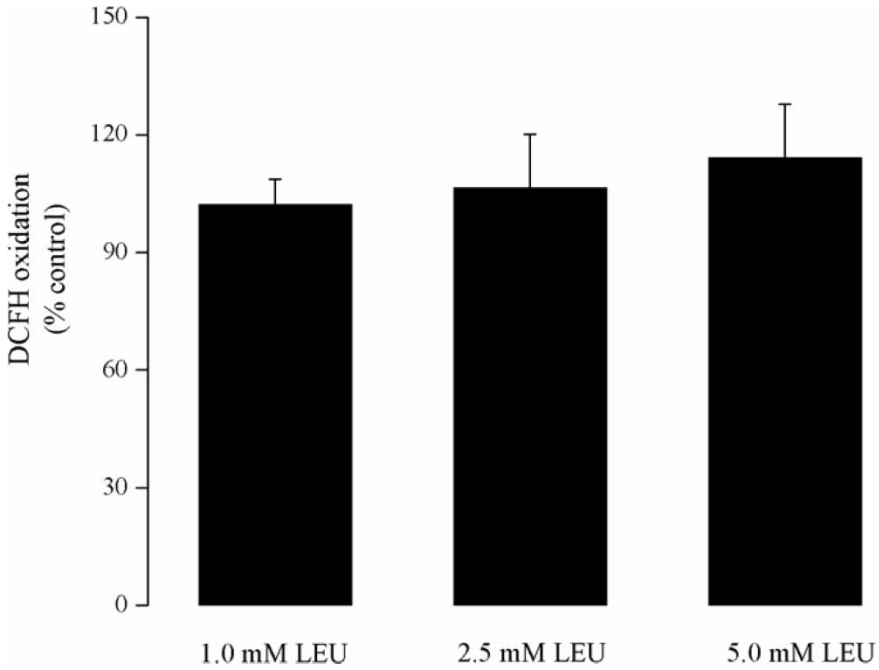

Figure 6. Effect of leucine on DCFH oxidation in cerebral cortex homogenates from young rats. Data represent the mean ± SD for six independent experiments (animals) performed in triplicate and are expressed as percentage of control (Control values: 1.18 ± 0.14 nmol DCF formed/ mg protein). No significant differences were observed (one-way ANOVA).

partially, mediated by an unbalance of the prooxidant/antioxidant ratio.[16,31] The parameters of oxidative stress evaluated in both, our previous (total radical-trapping antioxidant potential, total antioxidant reactivity, TBA-RS, chemiluminescence) and the present investigation support a pro-oxidant behavior of Leu in cerebral cortex homogenates. This pro-oxidant action could be due to several factors, such as changes in the redox cycling of active metals (such as Fe or Cu); decrease in the enzymatic and/or non-enzymatic defenses; and increase in the rate of ROS production. Reactions of autoxidation in cerebral homogenates are strongly dependent of the presence of trace amounts of redox active metals and can be increased by compounds that promote their reduction to Fenton active oxidation states.[29,32,33] However, the addition of Leu does not modify the cyclic voltammogram of Fe or Cu ions, indicating a lack of effect of Leu in the redox cycling of these ions. This lack of activation is further supported by results obtained in the LDL oxidation promoted by Cu(II) or Fe(III)/ascorbate. In these systems, no evidence of pro-oxidant effect of Leu was found (unpublished data).

CAT and GPx activities from cortical homogenates and also from purified preparations were significantly inhibited by Leu, in contrast to the SOD activity, which was not modified by the amino acid. The results presented here on CAT and GPx activities from cortical brain homogenates and purified preparations indicate a direct Leu inhibitory action, since Leu inhibited CAT and GPx activities when the commercial purified enzymes were used. These results indicate that reduction of these activities could also contribute to the increased lipid peroxidation observed in the brain homogenates, as well as on the indirect depletion of the non-enzymatic defenses due to increased free radical production.[16]

Finally, Leu was able to significantly oxidize protein thiol groups (up to 24%), as measured by determining the thiol content in mitochondrial enriched fractions. These data suggest an oxidative role for Leu towards the protein thiol groups. In contrast, Leu was not able to significantly oxidize DCFH, although a 15% increase on DCF values was observed.

We cannot exclude that brain energy metabolism impairment could secondarily lead to an increase in the rate of ROS production. In this context, there is evidence that the BCCAs accumulating in MSUD affect mitochondrial enzymes, resulting in impaired energy metabolism.[7–10,34,35] It has also been reported that Leu is an inhibitor of creatine kinase (CK) activity, a key enzyme for energy homeostasis in brain.[9,10] In this scenario, we cannot determine at the present time whether impairment of energy metabolism, oxidative stress or alterations of GABAergic/glutamatergic systems is the main pathogenetic mechanism leading to brain injury in MSUD. However, it is feasible that all these mechanisms might act together in a complex intricate relationship and might contribute to the neuropathology of MSUD. Based on the results presented here, it is proposed that the administration of antioxidants especially vitamins E and C, should be considered as a potential adjuvant therapy for MSUD affected patients specially during crises when the levels of the BCAAs increase dramatically.

Acknowledgements

We thank Dr Maria Soledad Ureta-Zañartu and her lab members for assistance with cyclic voltammetry measurements. This research was supported by grants from the Excellence Nuclei Program (PRONEX), PROPESQ/UFRGS, Brazilian National Research Council (CNPq) and the International Brain Research Organization (IBRO).

References

[1] Chuang DT, Shih VE. Maple syrup urine disease (branched-chain ketoaciduria). In: Scriver CR, Beaudet AL, Sly WL, Valle D, editors. The Metabolic and Molecular Bases of Inherited Disease. New York: McGraw-Hill; 2001. p 1971–2005.

[2] Treacy E, Clow CL, Reade TR, Chitayat D, Mamer OA, Scriver CR. Maple syrup urine disease: interrelationship between branched chain amino-, oxo-, and hydroxyacids; implications for treatment; association with CNS dysmelination. J Inherit Metab Dis 1992;15:121–135.

[3] Schadewaldt P, Wendel U. Metabolism of branched-chain amino acids in maple syrup urine disease. Eur J Pediatr 1997;156:S62–S66.

[4] Nyhan WL. Maple syrup urine disease: branched-chain ketoaciduria. In: Nyhan WL, editor. Abnormalities in Amino Acid Metabolism in Clinical Medicine. Norwalk: Appleton-Century-Crofts; 1984. p 21–35.

[5] Snyderman SE, Norton PM, Roitman E. Maple syrup urine disease with particular reference to diet therapy. Pediatrics 1964;34:454–472.

[6] Zielke HR, Huang Y, Tildon JT, Zielke CL, Baab PJ. Elevation of amino acids in the interstitial space of the rat brain following infusion of large neutral amino and keto acids by microdialysis: leucine infusion. Dev Neurosci 1996;18:420–425.

[7] Land JM, Mowbray J, Clark JB. Control of pyruvate and β-hydroxybutyrate utilization in rat brain mitochondria and its relevance to phenylketonuria and maple syrup urine disease. J Neurochem 1976;26:823–830.

[8] Danner DJ, Elsas II, LJ. Disorders of branched chain amino acid and keto acid metabolism. In: Scriver CR, Beaudet AL, Sly WL, Valle D, editors. The Metabolic Basis of Inherited Disease. New York: McGraw-Hill; 1989. p 671–692.

[9] Pilla C, Cardozo RF, Dutra-Filho CS, Wyse AT, Wajner M, Wannmacher CM. Creatine kinase activity from rat brain is inhibited by branched-chain amino acids *in vitro*. Neurochem Res 2003a;28:675–679.

[10] Pilla C, Cardozo RF, Dornelles PK, Dutra-Filho CS, Wyse AT, Wajner M, Wannmacher CM. Kinetic studies on the inhibition of creatine kinase activity by branched-chain alpha-amino acids in the brain cortex of rats. Int J Dev Neurosci 2003;21:145–151.

[11] Tashian RE. Inhibition of brain glutamic acid decarboxylase by phenylalanine, valine, and leucine derivatives: A suggestion concerning the etiology of the neurological defect in phenylketonuria and branched-chain ketonuria. Metabolism 1961;10:393–402.

[12] Yuwiler A, Geller E. Serotonin depletion by dietary leucine. Nature 1965;208:83–84.
[13] Tavares RG, Santos CES, Tasca C, Wajner M, Souza DO, Dutra-Filho CS. Inhibition of glutamate uptake into synaptic vesicles of rat brain by the metabolites accumulating in maple syrup urine disease. J Neurol Sci 2000;181:44–49.
[14] Araújo P, Wassermann GF, Tallini K, Furlanetto V, Vargas CR, Wannmacher CMD, Dutra-Filho CS, Wajner M. Reduction of large neutral amino acid levels in plasma and brain of hyperleucinemic rats. Neurochem Int 2001;38:529–537.
[15] Jouvet P, Rustin P, Taylor DL, Pocock JM, Felderhoff-Mueser U, Mazarakis ND, Sarraf C, Joashi U, Koszma M, Greewood K, Edwards AD, Mehmet H. Branched chain amino acids induce apoptosis in neural cells without mitochondrial membrane despolarization or cytochrome c release: Implications for neurological impairment associated with maple syrup urine disease. Mol Biol Cell 2000;11:1919–1932.
[16] Bridi R, Araldi J, Sgarbi MB, Testa CG, Durigon K, Wajner M, Dutra-Filho CS. Induction of oxidative stress in rat brain by the metabolite accumulating in maple syrup urine disease. Int J Dev Neurosci 2003;21:327–332.
[17] Clark JB, Nicklas WJ. The metabolism of the rat brain mithocondria. Preparation and characterization. J Biol Chem 1970;245:4724–4731.
[18] Zheng W, Rean S, Graziano JH. Manganese inhibits mitochondrial aconitase: A mechanism of mangase neurotoxicity. Brain Res 1998;799:334–342.
[19] Latini A, Scussiato K, Rosa RB, Leipnitz G, Llesuy S, Bello-Klein A, Dutra-Filho CS, Wajner M. Induction of oxidative stress by L-2-hydroxyglutaric acid in rat brain. J Neurosci Res 2003;74:103–110.
[20] Latini A, Scussiato K, Rosa RB, Llesuy S, Bello-Klein A, Dutra-Filho CS, Wajner M. D-2-hydroxyglutaric acid induces oxidative stress in cerebral cortex of young rats. Eur J Neurosci 2003;17:2017–2022.
[21] Esterbauer H, Cheeseman KH. Determination of aldehydic lipid peroxidation products: Malonaldehyde and 4-hydroxy-nonenal. Methods Enzymol 1990;186:407–421.
[22] Kowaltowsky AJ, Castilho RF. Ca2 + acting at the external side of the inner mitochondrial membrane can stimulate mitochondrial permeability transition induced by phenylarsine oxide. Biochim Biophys Acta 1997;1322:221–229.
[23] Ellman GL. Tissue sulfhydryl groups. Arch Biochem Biophys 1959;82(1):70–77.
[24] Aebi H. Catalase *in vitro*. Methods Enzymol 1984;105:121–126.
[25] Wendel A. Glutathione peroxidase. Methods Enzymol 1981;77:325–332.
[26] LeBel CP, Ischiropoulos H, Bondy SC. Evaluation of the probe 2′,7′-dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress. Chem Res Toxicol 1992;5:227–231.
[27] Lowry OH, Rosebrough NJ, Lewis-Farr A, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265–275.
[28] Halliwell B, Gutteridge JMC. Detection of free radicals and others reactive species: Trapping and fingerprinting. In: Halliwell B, Gutteridge JMC, editors. Free Radicals in Biology and Medicine. Oxford: Oxford University Press; 1999. p 351–425.
[29] Halliwell B, Gutteridge JMC. The chemistry of free radicals and related 'reactive species'. In: Halliwell B, Gutteridge JMC, editors. Free Radicals in Biology and Medicine. Oxford: Oxford University Press; 1999. p 37–104.
[30] Bains JS, Shaw CA. Neurodegenerative disorders in humans: The role of glutathione in oxidative stress-mediated neuronal death. Brain Res Rev 1997;25:335–358.
[31] Fontella FU, Gassen E, Pulrolnik V, Wannmacher CMD, Klein AB, Wajner M, Dutra-Filho CS. Stimulation of lipid peroxidation in vitro in rat brain by metabolites accumulating in maple syrup urine disease. Metab Brain Dis 2002;17:47–54.
[32] Annopkumar-Dukie S, Lack B, McPhail TN, Lambat Z, Maharaj D, Daya S. Indomethacin reduces lipid peroxidation in rat brain homogenate by binding Fe^{2+}. Metab Brain Dis 2003;18:1–9.
[33] Kohen R, Vellaichamy E, Hrbac J, Gati I, Tirosh O. Quantification of the overall reactive oxygen species scavenging capacity of biological fluids and tissues. Free Radic Biol Med 2000;28:871–879.
[34] Howell RK, Lee M. Influence of alpha-ketoacids on the respiration of brain *in vitro*. Proc Soc Exp Biol Med 1963;113:660–663.
[35] Halestrap AP, Brand MD, Denton RM. Inhibition of mitochondrial pyruvate transport by phenylpyruvate and α-ketoisocaproate. Biochim Biophys Acta 1974;367:102–108.