*Metabolic Brain Disease, Vol. 20, No. 2, June 2005 (© 2005)*
DOI: 10.1007/s11011-005-4152-8

# $\alpha$-Keto Acids Accumulating in Maple Syrup Urine Disease Stimulate Lipid Peroxidation and Reduce Antioxidant Defences in Cerebral Cortex From Young Rats

**Raquel Bridi,[1] César A. Braun,[1] Giovanni K. Zorzi,[1] Clóvis M. D. Wannmacher,[1] Moacir Wajner,[1,2] Eduardo G. Lissi,[3] and Carlos Severo Dutra-Filho[1,4]**

*Received November 1, 2004; accepted February 7, 2005*

Maple syrup urine disease (MSUD) is an inherited neurometabolic disorder caused by deficiency of branched-chain $\alpha$-keto acid dehydrogenase complex activity which leads to tissue accumulation of the branched-chain $\alpha$-keto acids (BCKAs) $\alpha$-ketoisocaproic acid (KIC), $\alpha$-ketoisovaleric acid (KIV) and $\alpha$-keto-$\beta$-methylvaleric acid (KMV) and their respective amino acids. Neuropathologic findings characteristic of the disease are cerebral edema and atrophy, whose pathophysiology is poorly known. In the present study, we investigated the in vitro effect of BCKAs on various parameters of oxidative stress, namely chemiluminescence (CL), thiobarbituric acid–reactive substances (TBA-RS), total radical-trapping antioxidant potential (TRAP), total antioxidant reactivity (TAR), and the activities of the antioxidant enzymes catalase (CAT), glutathione peroxidase (GPx), and superoxide dismutase (SOD) in cerebral cortex of 30-day-old rats. The major effects observed were with KIC, which significantly increased CL and TBA-RS measurements, decreased TRAP and TAR values, and markedly inhibited GPx activity. KMV and KIV increased CL and decreased TRAP and TAR values. In contrast, these compounds did not affect CAT and SOD activities. Taken together, it was shown that: the BCKAs studied stimulated lipid peroxidation and reduced the brain antioxidant defences, suggesting an increased production of free radicals. In case the in vitro effects here detected also occur in vivo in MSUD, it can be presumed that oxidative stress might contribute, at least in part, to the brain damage found in the affected patients.

**Key words:** Maple syrup urine disease; $\alpha$-keto acids; oxidative stress; antioxidant brain defences.

## INTRODUCTION

Mammalian[24.6pc] mitochondrial branched-chain $\alpha$-keto acid dehydrogenase (BCKD) catalyzes the oxidative decarboxylation of the branched-chain $\alpha$-keto acids (BCKAs) $\alpha$-ketoisocaproic acid (KIC), $\alpha$-keto-$\beta$-methylvaleric acid (KMV), and $\alpha$-ketoisovaleric acid (KIV), which can originate their corresponding branched-chain amino acids (BCAAs)

[1]Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil.
[2]Serviço de Genética Médica, Hospital de Clínicas de Porto Alegre, Porto Alegre, RS, Brazil.
[3]Departamento de Ciencias Químicas, Facultad de Química y Biología, Universidad de Santiago de Chile, Santiago, Chile.
[4]To whom correspondence should be addressed at Departamento de Bioquímica, ICBS, Universidade Federal do Rio Grande do Sul, Rua Ramiro Barcelos, 2600-Anexo, CEP 90035-003, Porto Alegre, RS, Brazil. E-mail: dutra@ufrgs.br

leucine, isoleucine and valine, respectively. In patients with maple syrup urine disease (MSUD), or branched-chain keto aciduria, the activity of the BCKD is severely deficient. The metabolic blockage at this step results in the accumulation of BCKAs and BCAAs (Chuang and Shih, 2001; Ogier de Baulny and Saudubray, 2002). The hydroxy derivatives $\alpha$-hydroxyisovaleric acid, $\alpha$-hydroxy-$\beta$-methylvaleric acid, and $\alpha$-hydroxyisocaproic acid, produced by the reduction of their respective $\alpha$-keto acids, also accumulate but to a lesser extent (Treacy *et al*., 1992).

The symptomatology of MSUD includes ketoacidosis, failure to thrive, poor feeding, apnea, ataxia, seizures, coma, psychomotor delay and mental retardation (Chuang and Shih, 2001; Nyhan, 1984). Neuropathologic findings characteristic of the disease are cerebral edema, atrophy of the cerebral hemispheres, spongy degeneration of the white matter and delayed myelination (Chuang and Shih, 2001; Treacy *et al*., 1992). MSUD usually presents as a heterogeneous clinical phenotype, ranging from the severe classical form to mild variants, possibly due to distinct residual enzyme activity (Schadewaldt and Wendel, 1997). Although the mechanisms of brain damage in MSUD are still unclear, it appears that leucine and KIC are considered to be the main neurotoxic metabolites since increased plasma concentrations of these metabolites are associated with the appearance of neurological symptoms (Chuang and Shih, 2001; Snyderman *et al*., 1964) and can reach concentrations as high as 5.0 mM in untreated patients (Zielke *et al*., 1996). In this context, it has been demonstrated that BCKAs may affect energy metabolism in rat brain (Danner and Elsas, 1989; Halestrap *et al*., 1974; Land *et al*., 1976; Sgaravatti *et al*., 2003; Yudkoff *et al*., 1994; Zielke *et al*., 2002). Furthermore, the BCAAs and BCKAs accumulating in MSUD have been reported to cause significant alterations of the concentrations of the neurotransmitters glutamate, aspartate and $\gamma$-aminobutyric (GABA) in the brain (Dodd *et al*., 1992; Prensky and Moser, 1967; Tavares *et al*., 2000; Yudkoff *et al*., 1994). On the other hand, it has been proposed that the brain injury of this disorder may also be related to reduce brain uptake of essential amino acids (Araújo *et al*., 2001) and to apoptosis in neural cells (Jouvet *et al*., 2000).

We have previously demonstrated that the BCAAs, as well as their $\alpha$-keto acids and $\alpha$-hydroxy acids derivatives stimulate lipid peroxidation in brain homogenates from 7- and 30-day-old rats (Fontella *et al*., 2002). More recently, we have shown that the amino acids accumulating in MSUD, particularly leucine, promote a reduction of the nonenzymatic antioxidant defences in rat brain homogenates (Bridi *et al*., 2003). In the present work, we extend our previous studies by investigating the in vitro effect of the BCKAs on the nonenzymatic antioxidant defences, evaluated by the total radical-trapping antioxidant potential (TRAP) and the total antioxidant reactivity (TAR) and on the lipid peroxidation assessed by chemiluminescence (CL) and thiobarbituric acid–reactive substances (TBA-RS) in cerebral cortex homogenates. We also tested the effects of these compounds on the activity of the antioxidant enzymes catalase (CAT), glutathione peroxidase (GPx), and superoxide dismutase (SOD).

## MATERIALS AND METHODS

### 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 on 07:00–19:00 h) in 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) except by thiobarbituric acid, which was purchased from Merck (Darmstadt, Germany), 2,2′-azo-bis-(2-amidinopropane) that was purchased from Wako Chemicals (USA) and Ransod kit from RANDOX (Antrim, United Kingdom). Keto acids (KIC, KMV, and KIV) solutions were prepared on the day of the experiment in the incubation medium used for each technique.

## Tissue Preparation

Rats were sacrificed by decapitation without anesthesia, and the brain was rapidly excised and kept on an ice-plate. The olfactory bulb, pons, and medulla were discarded and the cerebral cortex was dissected, rinsed with saline for complete blood removal, weighed and kept chilled until homogenization. Cerebral cortex was homogenized in 10 volumes (1:10, w/v) of the appropriate medium. Homogenates were centrifuged at 750 × *g* for 10 min at 4°C to discard nuclei and cell debris (González-Flecha *et al*., 1991; Llesuy *et al*., 1985). The supernatant, a suspension of mixed and preserved organelles, including mitochondria, was separated and immediately used for analyses and the pellet was discarded.

## In Vitro Experiments

Cerebral cortex supernatants were incubated for 1 h at 37°C in the presence of KIC, KMV or KIV at concentrations ranging from 1.0 to 5.0 mM. Controls did not contain keto acids in the incubation medium. Immediately after incubation, aliquots were taken to measure CL, TBA-RS, TRAP, and TAR. In order to test a direct action of KIC, KIV, and KMV on the antioxidant enzymes CAT, SOD, and GPx, the keto acids were added to the cerebral cortex homogenates at the time of assay of each enzyme activity without previous incubation. In addition, the activity of a commercial preparation of GPx purified from bovine erythrocytes (EC 1.11.1.9) was measured in presence of KIC.

## Chemiluminescence (CL)

Samples were assayed for CL in a dark room by the method of Lissi *et al*. (1988) using a beta liquid scintillation spectrometer Tri-Carb 2100TR. Cerebral tissue was homogenized 1:10 (w/v) in 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl. Incubation flasks contained 3.5 mL of the medium consisting in 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl. The background CL was measured for 5 min. An aliquot of 0.5 mL of supernatant was added and CL was measured for 10 min at room temperature. The background CL was subtracted from the total value. CL was calculated as cpm/mg protein and represented as percentage of controls.

### Thiobarbituric Acid–Reactive Substances (TBA-RS)

TBA-RS were determined according to the method described by Esterbauer and Cheeseman (1990) using a Beckman DU®640 Spectrophotometer. Cerebral tissue was homogenized 1:10 (w/v) in 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl. Briefly, 300 $\mu$L of cold 10% trichloroacetic acid were added to 150 $\mu$L of supernatant and centrifuged at 300 $\times$ $g$ for 10 min. Three hundred microliters of the supernatant were transferred to a Pyrex tube and incubated with 300 $\mu$L of 0.67% thiobarbituric acid in 7.1% 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 was determined in a spectrophotometer at 535 nm. The amino acids did not produce color when tested without the addition of the supernatant, demonstrating the absence of a direct reaction to thiobarbituric acid. Calibration curve was performed using 1,1,3,3-tetramethoxypropane that was subjected to the same treatment as that of the supernatants. TBA-RS was calculated as nanomole TBA-RS per milligram protein and represented as percentage of controls.

### Total Radical-Trapping Antioxidant Potential (TRAP)

TRAP was determined by measuring the chemiluminescence intensity of luminol induced by 2,2′-azo-bis-(2-amidinopropane) (ABAP) (Evelson *et al*., 2001; Lissi *et al*., 1992) using a Wallac 1409 Scintillation Counter. Cerebral tissue was homogenized 1:10 (w/v) in 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl. The background chemiluminescence was measured by adding 3 mL of 10 mM ABAP dissolved in 50 mM sodium phosphate buffer pH 7.4 into a glass scintillation vial. Ten microliters of luminol (4 mM) were added to each vial and the chemiluminescence was measured. This was considered to be the initial value. Ten microliters of 300 $\mu$M Trolox (water-soluble $\alpha$-tocopherol analogue) or tissue supernatant was added and the chemiluminescence was measured. The time necessary for the chemiluminescence intensity show a fast raise is called induction time (IT). IT is directly proportional to the antioxidant capacity of the tissue and the IT of each sample was compared with the IT of Trolox. TRAP values were calculated as nanomole Trolox per milligram protein and represented as percentage of controls.

### Total Antioxidant Reactivity (TAR)

TAR was determined by measuring the luminol chemiluminescence intensity induced by ABAP according to the method of Lissi *et al*. (1995) using a Wallac 1409 Scintillation Counter. Cerebral tissue was homogenized 1:10 (w/v) in 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl. The background chemiluminescence was measured by adding 4 mL 2 mM ABAP (in 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl) into a glass scintillation vial. Fifteen microliters of luminol (4 mM) were added to each vial and the chemiluminescence was measured. This was considered to be the basal value. Five microliters of 10–100 $\mu$M Trolox (curve calibration) or tissue supernatant was then added and the chemiluminescence was measured during 60 s. The rapid reduction in luminol intensity is considered as a measure of its TAR capacity. TAR measurement

was calculated as nanomole Trolox per milligram protein and represented as percentage of controls.

### Catalase Assay

CAT activity was assayed by the method of Aebi (1984) using a double-beam spectrophotometer with temperature control (Hitachi U-2001). Cerebral tissue was homogenized 1:10 (w/v) in 10 mM potassium phosphate buffer, pH 7.0. This 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 1 $\mu$mol of hydrogen peroxide consumed per minute, and the specific activity is reported as units per milligram protein.

### Superoxide Dismutase Assay

The assay of SOD activity was carried out with the RANSOD kit (Randox, USA). Cerebral tissue was homogenized 1:10 (w/v) in 10 mM potassium phosphate buffer, pH 7.0. This method is based on the formation of red formazan from the reaction of 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (I.N.T.) and superoxide radical (produced in the incubation medium from xanthine oxidase reaction), which is assayed spectrophotometrically at 505 nm using a double-beam spectrophotometer with temperature control (Hitachi U-2001). 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 specific activity is represented as units per milligram protein.

### Glutathione Peroxidase Assay

GPx activity was measured by the method of Wendel (1981) using *tert*-butylhydroperoxide as substrate. Cerebral tissue was homogenized in 100 mM potassium phosphate buffer containing ethylenediaminetetraacetic acid 1 mM, pH 7.7. The activity was determined by monitoring the NADPH disappearance at 340 nm using a double-beam spectrophotometer with temperature control (Hitachi U-2001). The medium containing 2 mM glutathione, 0.15 U/mL glutathione reductase, 0.4 mM azide, 0.5 mM *tert*-butylhydroperoxide and 0.1 mM NADPH. One GPx unit is defined as 1 $\mu$mol of NADPH consumed per minute and the specific activity is represented as units per milligram 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

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. The Student's *t*-test was also used for comparison of two means. All 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

The in vitro effect of KIC, KIV and KMV on lipid peroxidation parameters (CL and TBA-RS) was studied. Figure 1 shows that CL was significantly increased, as compared to controls, in a concentration-dependent manner in cerebral cortex homogenates exposed to KIC [$F(3, 16) = 5.65$; $p < 0.01$] [$\beta = 0.71$; $p < 0.001$], KIV [$F(3, 16) = 4.01$; $p < 0.05$] [$\beta = 0.64$; $p < 0.005$] and KMV [$F(3, 16) = 5.06$; $p < 0.05$] [$\beta = 0.69$; $p < 0.001$].

TBA-RS measurement was also enhanced in homogenates exposed to KIC [$F(3, 16) = 4.81$, $p < 0.05$] in a dose-dependent manner [$\beta = 0.69$; $p < 0.001$] (Fig. 2). In contrast, KIV [$F(3, 16) = 1.20$, $p > 0.05$] and KMV [$F(3, 16) = 1.72$, $p > 0.05$] did not alter TBA-RS levels. These results show that lipid peroxidation is predominantly stimulated by KIC and, to a lesser degree, by KIV and KMV in cerebral cortex homogenates.

Next, we observed that TRAP measurement, which reflects the tissue nonenzymatic antioxidant defences, was markedly reduced in a concentration-dependent manner when homogenates were incubated with KIC [$F(3, 16) = 3.33$; $p < 0.05$] [$\beta = -0.58$; $p < 0.01$], KIV [$F(3, 20) = 3.96$; $p < 0.05$] [$\beta = -0.59$; $p < 0.005$] and KMV [$F(3, 16) = 3.19$; $p < 0.05$] [$\beta = -0.56$; $p < 0.01$] (Fig. 3).

TAR values, which is a measure of the tissue capacity to react with free radicals, were also reduced in a concentration-dependent manner when cerebral cortex was exposed to KIC [$F(3, 16) = 3.24$; $p < 0.05$] [$\beta = -0.49$; $p < 0.05$] and KIV [$F(3, 16) = 8.55$; $p < 0.001$] [$\beta = -0.71$; $p < 0.001$] (Fig. 4). It can be also observed in the figure that

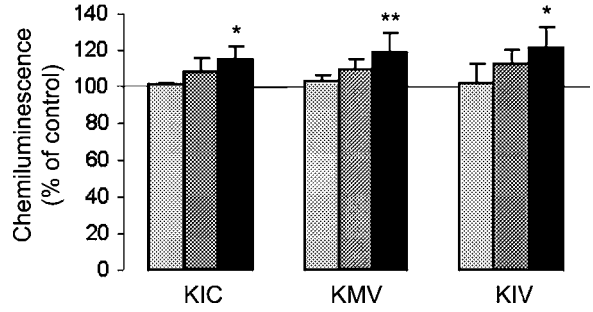


**Figure 1.** Effect of $\alpha$-ketoisocaproic (KIC), $\alpha$-keto-$\beta$-methylvaleric (KMV) and $\alpha$-ketoisovaleric (KIV) on chemiluminescence in cerebral cortex from young rats. Data are expressed as percentage of control and represent the mean ± SD for five independent experiments (animals) performed in duplicate. The straight line represents control values obtained in each experiment are as follows: KIC: 269 ± 19 cpm/mg protein; KMV: 147 ± 17 cpm/mg protein; KIV: 143 ± 17 cpm/mg protein. $^{*}p < 0.05$, $^{**}p < 0.01$, compared to control (Duncan multiple-range test). (▨) 1.0 mM; (▩) 2.5 mM; (■) 5.0 mM.

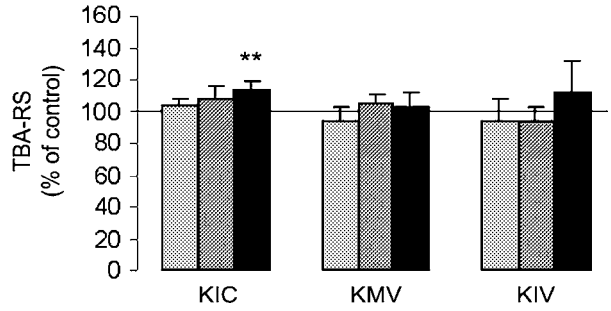


**Figure 2.** Effect of α-ketoisocaproic (KIC), α-keto-β-methylvasleric (KMV) and α-ketoisovaleric (KIV) on thiobarbituric–acid reactive substances (TBA-RS) in cerebral cortex from young rats. Data are expressed as percentage of control and represent the mean ± SD for five to six independent experiments (animals) performed in duplicate. The straight line represents control values obtained in each experiment are as follows: KIC: 3.45 ± 0.21 nmol TBA-RS/mg protein; KMV: 4.85 ± 0.29 nmol TBA–RS/mg protein; KIV: 3.10 ± 0.79 nmol TBA-RS/mg protein. $^{**}p < 0.01$ compared to control (Duncan multiple-range test). (▨) 1.0 mM; (▩) 2.5 mM; (■) 5.0 mM.

KMV reduced these values, although without statistical significance [$F(2, 20) = 2.25$; $p = 0.11$].

These findings suggest that the content of nonenzymatic antioxidants (TRAP) in the cerebral cortex was significantly reduced by the keto acids evaluated. In a similar fashion, the keto acids decreased the antioxidant reactivity (TAR), which indicates a deficient capacity to modulate the associated oxidative damage.

We then examined the effects of KIC, KIV, and KMV on the activities of the antioxidant enzymes CAT, SOD and GPx (Fig. 5). The activity of CAT was not affected by KIC [$F(3, 16) = 0.37$; $p > 0.05$], KIV [$F(3, 16) = 0.58$; $p > 0.05$] and KMV [$F(3, 20) =$

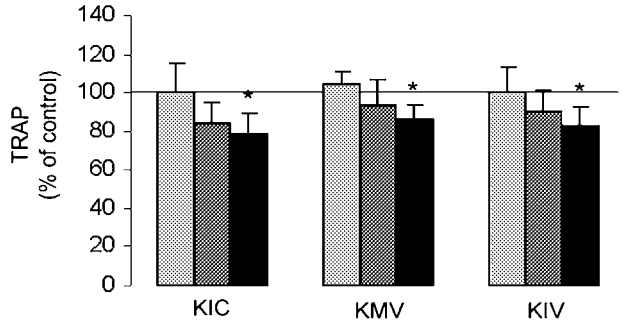


**Figure 3.** Effect of α-ketoisocaproic (KIC), α-keto-β-methylvaleric (KMV) and α-ketoisovaleric (KIV) on total radical-trapping antioxidant potential (TRAP) measurement in cerebral cortex from young rats. Data are expressed as percentage of control and represent the mean ± SD for five or six independent experiments (animals) performed in duplicate. The straight line represents control values obtained in each experiment are as follows: KIC: 29.5 ± 4.72 nmol Trolox/mg protein; KMV: 20.5 ± 2.15 nmol Trolox/mg protein; KIV: 27.1 ± 2.99 nmol Trolox/mg protein. $^{*}p < 0.05$ compared to control (Duncan multiple-range test). (▨) 1.0 mM; (▩) 2.5 mM; (■) 5.0 mM.

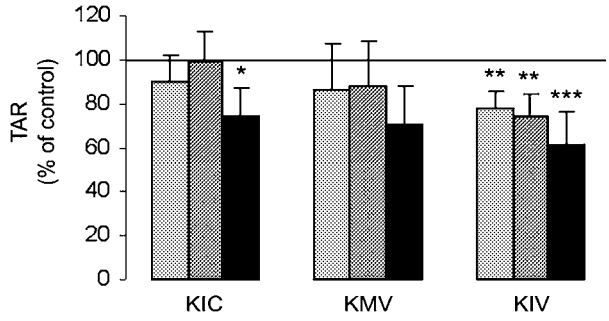


**Figure 4.** Effect of $\alpha$-ketoisocaproic (KIC), $\alpha$-keto-$\beta$-methylvaleric (KMV) and $\alpha$-ketoisovaleric (KIV) on total antioxidant reactivity (TAR) in cerebral cortex from young rats. Data are expressed as percentage of control and represent the mean $\pm$ SD for five independent experiments (animals) performed in duplicate. The straight line represents control values obtained in each experiment are as follows: KIC: $10.8 \pm 2.15$ nmol Trolox/mg protein; KMV: $11.1 \pm 2.72$ nmol Trolox/mg protein; KIV: $18.9 \pm 4.83$ nmol Trolox/mg protein. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$ compared to control (Duncan multiple-range test). (▨) 1.0 mM; (▩) 2.5 mM; (■) 5.0 mM.

0.09; $p > 0.05$] (Fig. 5(B)). Similarly, the activity of SOD was also not affected by KIC [$F(3, 12) = 0.11$; $p > 0.05$], KIV [$F(3, 8) = 0.43$; $p > 0.05$] and KMV [$F(3, 12) = 0.08$; $p > 0.05$] (Fig. 5(C)). In contrast, it can be seen that KIC significantly decreased GPx activity in a concentration-dependent manner in cerebral cortex [$F(3, 16) = 6.07$; $p < 0.01$] [$\beta = -0.66$; $p < 0.01$], whereas KIV [$F(3, 16) = 0.39$; $p > 0.05$] and KMV [$F(3, 16) = 0.13$; $p > 0.05$] caused no effect on this parameter (Fig. 5(A)). In order to test this direct effect of the KIC on GPx activity we evaluated the influence of this keto acid on the activity of a commercial preparation of GPx. It can be observed in Fig. 6 that purified GPx was also inhibited by KIC (10 mM).

## DISCUSSION

Neurological sequelae are frequent in untreated patients affected by MSUD, but the mechanisms underlying the neurotoxicity in this disease are yet not well established. We have previously demonstrated that the BCAAs, at concentrations ranging from 1.0 to 5.0 mM, have a pro-oxidant action manifested by increased lipid peroxidation in brain homogenates (Bridi *et al*., 2003). In the present report, we investigated the effects of the BCKAs, at concentrations similar to those found in MSUD patients (1.0–5.0 mM) on various parameters of lipoperoxidation, as well as on the nonenzymatic antioxidant defences, determined as TRAP and TAR measurements, and on the enzymatic antioxidant defences CAT, SOD, and GSH-Px in cerebral cortex homogenates.

We demonstrated that KIC significantly promotes the oxidation of brain homogenates, as evidenced by the increase in CL and TBA-RS, whereas KIV and KMV caused an increase of CL but no alteration of TBA-RS values. In this context, it should be emphasized that light emitted in the CL assay usually arises from peroxidizing lipids and is interpreted in terms of the formation of singlet oxygen and/or excited carbonyl compounds, although it has to

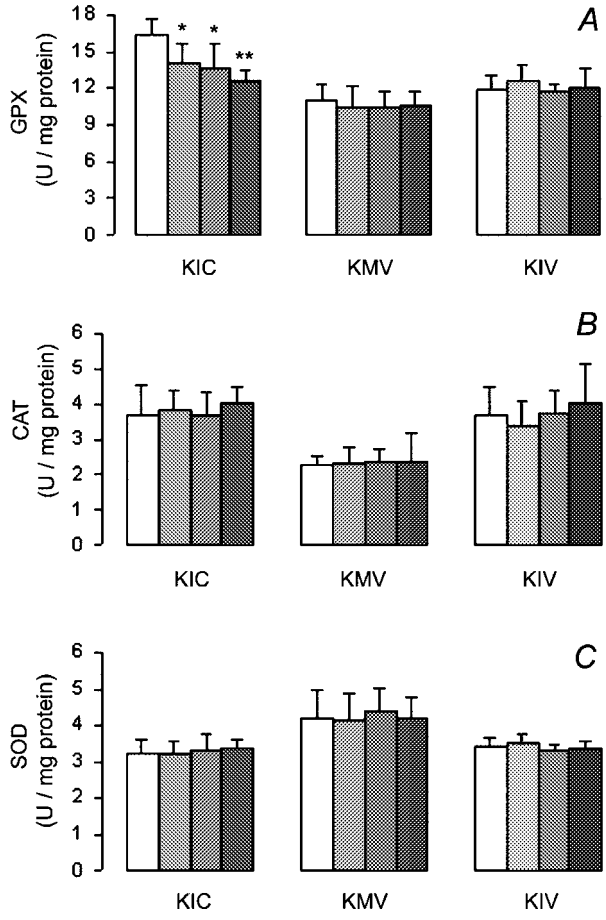


**Figure 5.** Effect of α-ketoisocaproic (KIC), α-keto-β-methylvaleric (KMV) and α-ketoisovaleric (KIV) on gluthatione peroxidase (A), catalase (B) and superoxide dismutase (C) activities in cerebral cortex from young rats. Data represent the mean ± SD for five independent experiments (animals) performed in triplicate and expressed as U/mg protein. One GPx unit is defined as 1 μmol of NADPH consumed per minute and the specific activity. One catalase unit is defined as one μmol of hydrogen peroxide consumed per minute. One SOD unit is defined as 50% inhibition of the produced chromogen. Difference from control, $^{*}p < 0.05$; $^{**}p < 0.01$ (Duncan multiple range test). (□) control; (▨) 1.0 mM; (▩) 2.5 mM; (■) 5.0 mM.

be considered that CL can also reflect protein oxidation (Aspée and Lissi, 2000; Halliwell and Gutteridge, 2001a). On the other hand, TBA-RS reflects the amount of malondialdehyde formation, the last product of lipid breakdown caused by lipid peroxidation process (Esterbauer and Cheeseman, 1990). Considering that the overproduction of oxygen and nitrogen free radicals leads to higher chemiluminescence and TBA-RS levels (González-Flecha *et al.*, 1991; Halliwell and Gutteridge, 2001a), our present results implicate that oxidative stress is probably induced by BCKAs tested, and, particularly, by KIC.

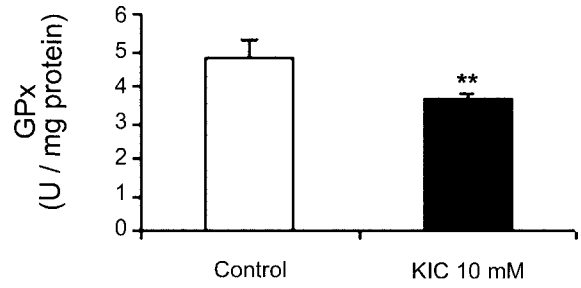


**Figure 6.** Effect of $\alpha$-ketoisocaproic (KIC) on commercial purified gluthatione peroxidase. Data represent the mean $\pm$ SD for three experiments performed in triplicate and expressed as U/mg protein. One GPx unit is defined as one $\mu$mol of NADPH consumed per minute and the specific activity. Difference from control, $^{**}p < 0.01$ (Student' *t*-test). (□) control; (■) 10 mM.

We also observed that all keto acids tested significantly reduce TRAP values, which reflects a decreased in the nonenzymatic antioxidant defences in the brain. Since ascorbic acid and glutathione (GSH) are the most abundant and broad-spectrum antioxidants of low molecular weight in the CNS (Evelson *et al.*, 2001; Rice and Russo-Menna, 1998), that readily react against many ROS, it is feasible that the reduction of TRAP levels provoked by the BCKAs may result from the generation of free radicals with the consequent consumption of these important antioxidants. Moreover, a deficient capacity of the tissue to modulate the steady-state increase of reactive species caused by KIC and KIV was demonstrated by a significant decrease of TAR measurement caused by these organics acids, a fact that strengthen the diminution of nonenzymatic antioxidant defences in the brain (Lissi *et al.*, 1995). Given that increased lipid peroxidation and decreased antioxidant defences was found to be cause by the BCKAs accumulating in MSUD, it can be presumed that oxidative stress plays an important role in the activity of these compounds.

We also demonstrated that GPx activity, which catalyses the reduction of hydroperoxides using GSH, thereby protecting mammalian cells against oxidative damage, was significantly inhibited by KIC at all concentrations used in brain homogenates, but not by KIV and KMV. Moreover, we also observed that KIC decreased GPx activity in a purified enzyme preparation in absence of homogenates a fact that points to a direct inhibition of the protein function by this keto acids. On the other hand, CAT and SOD activities were not affected in vitro by the BCKAs in brain preparations.

Regarding the mechanism, by which free radicals are elicited by these compounds, it should be considered that the increased rate of ROS production may be associated to the impaired brain energy metabolism previously demonstrated for the BCKAs. These compounds were shown to reduce $^{14}CO_2$ production (around 40%), stimulate lactate production (20–30%) and glucose uptake (around 40%), and provoke an inhibition of the electron transport chain (60%) at complex I (Sgaravatti *et al.*, 2003). In this context, inhibition of complex I increases ROS production, and isolated human complex I defects have been identified in a number of neurodegenerative diseases, a fact that suggest that the activity of this respiratory chain complex is important for normal CNS function (Schapira, 1998). Furthermore, administration of 1-methyl-4-phenyl-1,2,3,6 tetrahydropyridine (MPTP), which is used to produce an animal model of Parkinson disease, inhibits complex I activity probably

by oxidative damage since this inhibition is prevented by free-radical scavengers (Cleeter *et al*., 1992; Tipton and Singer, 1993).

Cerebral homogenate autoxidation is strongly dependent on the presence of traces of redox active metals and can be increased by compounds that can promote their reduction to Fenton active oxidation states (Annopkumar-Dukie *et al*., 2003; Halliwell and Gutteridge, 2001b; Kohen *et al*., 2000). However, KIC (up to 2.0 mM) does not modify the cyclic voltammogram of Fe or Cu ions (data not shown), indicating a lack of effect of KIC on the redox cycling of these ions. This lack of activation was also observed when we analyze LDL oxidation promoted by Cu(II) or Fe(III)/ascorbate (unpublished results). In these systems, no evidence of a pro-oxidant effect of KIC was observed.

Although the mechanisms of brain damage in MSUD are still uncertain, it has been considering that KIC and its precursor leucine, which achieve 5.0 mM concentrations in tissues of MSUD patients, are most toxic metabolites in this disease (Chuang and Shih, 2001; Saudubray *et al*., 1991; Snyderman *et al*., 1964, 1984). Our present results, showing that KIC provokes the most significant effects on several of the oxidative stress parameters, strengthen this view.

Though it is difficult to extrapolate our in vitro findings to the in vivo human condition, in case the effects here detected also occur in the brain of patients affected by MSUD, it is possible that they may contribute, at least in part, to the neurological dysfunction characteristic of this disease. It should be emphasized that the BCKAs accumulating in MSUD easily cross the blood-brain barrier (Yudkoff *et al*., 1994) and that the concentrations of these substances, particularly KIC, encountered in this disease are similar to those used in our assays (Zielke *et al*., 1996). In addition, further investigations should be conducted in animals to study the in vivo influence of these substances on the production of specific free radicals, as well as human studies in plasma, erythrocytes and skin fibroblasts from MSUD patients to confirm our in vitro findings. Finally, on the basis of previous and the present study showing that the metabolites accumulating in MSUD induce free radical formation and oxidative damage, it is tempting to speculate that antioxidants may represent a potential adjuvant therapy to the MSUD affected patients, especially during crises, when the levels of the BCAAs and BCKAs increase dramatically.

## ACKNOWLEDGMENTS

This work was supported by grants from Excellence Nuclei Program (PRONEX), Brazilian National Research Council (CNPq), FAPERGS, PROPESQ/UFRGS, and the International Brain Research Organization (IBRO).

## REFERENCES

Aebi, H. (1984). Catalase in vitro. *Methods Enzymol*. **105:**121–126.

Annopkumar-Dukie, S., Lack, B., McPhail, T.N., Lambat, Z., Maharaj, D., and Daya, S. (2003). Indomethacin Reduces Lipid Peroxidation in Rat Brain Homogenate by Binding $Fe^{2+}$. *Metab Brain Dis*. **18**(1):1–9.

Araújo, P., Wassermann, G.F., Tallini, K., Furlanetto, V., Vargas, C.R., Wannmacher, C.M.D., Dutra-Filho, C.S., Wyse, A.T.S., and Wajner, M. (2001). Reduction of large neutral amino acid levels in plasma and brain of hyperleucinemic rats. *Neurochem. Int*. **38:**529–537.

Aspée, A., and Lissi, E.A. (2000). Kinectics and mechanism of the chemiluminescence associated with the free radical-mediated oxidation amino acids. *Luminescence* **15:**273–282.

Bridi, R., Araldi, J., Sgarbi, M.B., Testa, C.G., Durigon, K., Wajner, M., and Dutra-Filho, C.S. (2003). Induction of oxidative stress in rat brain by the metabolite accumulating in maple syrup urine disease. *Int. J. Dev. Neurosci.* **21:**327–332.

Chuang, D.T., and Shih, V.E. (2001). Maple syrup urine disease (branched-chain ketoaciduria). In (C.R. Scriver, A.L. Beaudet, W.L. Sly, and D. Valle, eds.), *The Metabolic and Molecular Bases of Inherited Disease*, 8th edn., McGraw-Hill, New York, pp.1971–2005.

Cleeter, M.W., Cooper, J.M., and Shapira, A.H. (1992). Irreversible inhibition of mitocondrial complex I by 1-methyl-4-phenylpyrydinium: Evidence for free radical involvement. *J. Neurochem.* **58:**786–789.

Danner, D.J., and Elsas, L.J. II (1989). Disorders of branched chain amino acid and keto acid metabolism. In (C.R. Scriver, A.L. Beaudet, W.L. Sly, and D. Valle, eds.), *The Metabolic Basis of Inherited Disease*, 6th edn., McGraw-Hill, New York, pp. 671–692.

Dodd, P.R., Wiliams, A.L., Gundlach, A.L., Harper, P.A.W., Healy, P.J., Dennis, J.A., and Johnston, G.A.R. (1992). Glutamate and $\gamma$-aminobutyric acid neurotransmitter systems in the acute phase of maple syrup urine disease and citrullinemia encephalopathies in newborn calves. *J. Neurochem.* **59:**582–590.

Esterbauer, H., and Cheeseman, K.H. (1990). Determination of aldehydic lipid peroxidation products: Malonaldehyde and 4-hydroxynonenal. *Methods Enzymol.* **186:**407–421.

Evelson, P., Travacio, M., Repetto, M., Escobar, J., Llesuy, S., and Lissi, E.A. (2001). Evaluation of total reactive antioxidant potential (trap) of tissue homogenates and their cytosols. *Arch. Biochem. Biophys.* **388:**261–266.

Fontella, F.U., Gassen, E., Pulrolnik, V., Wannmacher, C.M.D., Klein, A.B., Wajner, M., and Dutra-Filho, C.S. (2002). Stimulation of lipid peroxidation in vitro in rat brain by metabolites accumulating in maple syrup urine disease. *Metab Brain Dis.* **17:**47–54.

González-Flecha, B., Llesuy, S., and Boveris, A. (1991). Hydroperoxide-initiated chemiluminescence: An assay for oxidative stress in biopsies of heart, liver and muscle. *Free Radic. Biol. Med.* **10:**93–100.

Halestrap, A.P., Brand, M.D., and Denton, R.M. (1974). Inhibition of mitochondrial pyruvate transport by phenylpyruvate and $\alpha$-ketoisocaproate. *Biochim. Biophys. Acta* **367:**102–108

Halliwell, B., and Gutteridge, J.M.C. (2001a). Detection of free radicals and others reactive species: Trapping and fingerprinting. In (B. Halliwell, and J.M.C. Gutteridge, eds.). *Free Radicals in Biology and Medicine*, Oxford University Press, Oxford, UK, pp. 351–425.

Halliwell, B., and Gutteridge, J.M.C. (2001b). The chemistry of free radicals and related "reactive species." In (B. Halliwell and J.M.C. Gutteridge, eds.). *Free Radicals in Biology and Medicine*, Oxford University Press, Oxford, UK, pp. 37–104.

Jouvet, P., Rustin, P., Taylor, D.L., Pocock, J.M., Felderhoff-Mueser, U., Mazarakis, N.D., Sarraf, C., Joashi, U., Koszma, M., Greewood, K., Edwards, A.D., and Mehmet, H. (2000). 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* **11:**1919–1932.

Kohen, R., Vellaichamy, E., Hrbac, J., Gati, I., and Tirosh, O. (2000). Quantification of the overall reactive oxygen species scavenging capacity of biological fluids and tissues. *Free Radic. Biol. Med.* **28**(6):871–879.

Land, J.M., Mowbray, J., and Clark, J.B. (1976). Control of pyruvate and $\beta$-hydroxybutyrate utilization in rat brain mitochondria and its relevance to phenylketonuria and maple syrup urine disease. *J. Neurochem.* **26:**823–830.

Lissi, E., Pascual, C., and Del Castillo, M.D. (1992). Luminol luminescence induced by 2,2′-azo-bis-(2-amidinopropane) thermolysis. *Free Radic. Res.* **17:**299–311.

Lissi, E., Salim-Hanna, M., Pascual, C., and Del Castillo, M.D. (1995). Evaluation of total antioxidant potential (TRAP) and total antioxidant reactivity from luminol-enhanced chemiluminescence measurements. *Free Radic. Biol. Med.* **18:**153–158.

Lissi, E.A., Cáceres, T., and Videla, L.A. (1988). Visible chemiluminescence from rat brain homogenates undergoing autoxidation. II. Kinetics of the luminescence decay. *Free Radic. Biol. Med.* **4:**93–87.

Llesuy, S.F., Milei, J., Molina, H., Boveris, A., and Milei, S. (1985). Comparison of lipid peroxidation and myocardial damage induced by adriamycin and 4′-epiadriamycin in mice. *Tumori* **71:**241–249.

Lowry, O.H., Rosebrough, N.J., Lewis-Farr, A., and Randall, R.J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:**265–275.

Nyhan, W.L. (1984). Maple syrup urine disease: branched-chain ketoaciduria. In (W.L. Nyhan, ed.), *Abnormalities in Amino Acid Metabolism in Clinical Medicine*, Appleton-Century-Crofts, Norwalk, pp. 21–35.

Ogier de Baulny, H., and Saudubray, J.M. (2002). Branched-chain organic acidurias. *Semin. Neonatol.* **7**(1):65–74.

Prensky, A.L., and Moser, H.W. (1967). Changes in the amino acid composition of proteolipids of white matter during maturation of the human nervous system. *J. Neurochem.* **14:**117–121.

Rice, M.E., and Russo-Menna, I. (1998). Differential compartmentalization of brain ascorbate and glutathione between neurons and glia. *Neuroscience* **82:**1213–1223.

Saudubray, J.M., Ogier, H., Billette de Vilmeur, T., Bonnefont, J.P., Lyonnet, S., Herve, F., Munnich, A., Rabier, D., Coude, M., and Charpentier, C. (1991). Inborn errors of the metabolism of branched-chain amino acids. In (J. Schuab, F. van Hoof, H.L. Vis, eds.), *Inborn Errors of Metabolism*, Raven Press, New York, pp. 137–153.

Schadewaldt, P., and Wendel, U. (1997). Metabolism of branched-chain amino acids in maple syrup urine disease. *Eur. J. Pediatr.* **156:**S62–S66.

Schapira, A.H.V. (1998). Human complex I defects in neurodegenerative diseases. *Biochim. Biophys. Acta* **1364:**261–270.

Sgaravatti, A.M., Rosa, R.B., Schuck, P.F., Ribeiro, C.A.J., Wannacher, C.M.D., Wyse, A.T.S., Dutra-Filho, C.S., and Wajner, M. (2003). Inhibition of brain energy metabolism by the α-keto acids accumulating in maple syrup urine disease. *Biochim. Biophys. Acta* **1639:**232–238.

Snyderman, S.E., Goldstein, F., Sansaricq, C., and Norton, P.M. (1984). The relationship between the branched chain amino acids and their α-ketoacids in maple syrup urine disease. *Pediatr. Res.* **18:**851–853.

Snyderman, S.E., Norton, P.M., and Roitman, E. (1964). Maple syrup urine disease with particular reference to diet therapy. *Pediatrics* **34:**454–472.

Tavares, R.G., Santos, C.E.S., Tasca, C., Wajner, M., Souza, D.O., and Dutra-Filho, C.S. (2000). Inhibition of glutamate uptake into synaptic vesicles of rat brain by the metabolites accumulating in maple syrup urine disease. *J. Neurol. Sci.* **181:**44–49.

Tipton, K.F., and Singer, T.P. (1993). Advances in our understanding of the mechanisms of neurotoxicity of MPTP and related compounds. *J. Neurochem.* **61:**1191–1206.

Treacy, E., Clow, C.L., Reade, T.R., Chitayat, D., Mamer, O.A., and Scriver, C.R. (1992). Maple syrup urine disease: Interrelationship between branched chain amino-, oxo-, and hydroxyacids; implications for treatment; association with CNS dysmelination. *J. Inherit. Metab. Dis.* **15:**121–135.

Wendel, A. (1981). Glutathione peroxidase. *Methods Enzymol.* **77:**325–332.

Yudkoff, M., Daikhin, Y., Lin, Z.P., Nissim, I., Stern, J., Pleasure, D., and Nissim, I. (1994). Interrelationships of leucine and glutamate metabolism in cultured astrocytes. *J. Neurochem.* **62:**1192–1202.

Zielke, H.R., Huang, Y., Tildon, J.T., Zielke, C.L., and Baab, P.J. (1996). 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.* **18:**420–425.

Zielke, H.R., Zielke, C.L., Baab, P.J., and Collins, R.M. (2002). Large neutral amino acids auto exchange whwn infused by microdialysis into the rat brain: Implications for maple syrup urine disease and phenylketonuria. *Neurochem. Int.* **40:**347–354.