Metab Brain Dis (2006) 21:279–286
DOI 10.1007/s11011-006-9030-5

ORIGINAL PAPER

Evidence that oxidative stress is increased in plasma from patients with maple syrup urine disease

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Received: 17 January 2006 / Accepted: 8 May 2006 /
Published online: 8 November 2006

Abstract Maple syrup urine disease (MSUD) or branched-chain α-keto aciduria (BCKA) is an inherited disorder caused by a deficiency of the branched-chain α-keto acid dehydrogenase complex (BCKAD) activity. The blockage of this pathway leads to tissue accumulation of the branched-chain amino acids (BCAA) leucine, isoleucine and valine and their respective keto-acids. The clinical features presented by MSUD patients include ketoacidosis, convulsions, coma, psychomotor delay and mental retardation. The mechanism of brain damage in this disease is still poorly understood. However, an increase in lipid peroxidation *in vitro* in cerebral cortex of young rats as well as a decrease in the antioxidant defenses has been previously observed. In the present work we evaluated different oxidative stress parameters, named reactive species of thiobarbituric acid (TBARS), total antioxidant reactivity (TAR) and total antioxidant status (TAS) in plasma of MSUD patients in order to evaluate whether oxidative stress is involved in this disorder. We verified a marked increase of plasma TBARS measurements, which is indicative of increased lipid peroxidation, as well as a decrease on plasma TAR reflecting a deficient capacity to efficiently modulate the damage associated with an increased production of reactive species. In contrast, TAS was not changed indicating that the total content of antioxidants in plasma of patients affected by MSUD was not altered.

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These results suggest that free radical generation is elicited in MSUD and is possibly involved in the pathophysiology of the tissue damage found in this disorder.

Keywords Maple syrup urine disease · Oxidative stress · Lipid peroxidation · Antioxidant defenses

Introduction

Maple syrup urine disease (MSUD) or branched-chain α-keto aciduria (BCKA) is an autosomal recessive metabolic disorder caused by a severe deficiency of the branched-chain α-keto acid dehydrogenase complex (BCKAD) activity. The blockage in this enzyme complex leads to tissue accumulation of the branched-chain amino acids (BCAA) leucine, isoleucine and valine as well as their corresponding branched chain α-keto acids (BCKA) α-ketoisocaproate, α-keto-β-methylvalerate and α-ketoisovalerate, respectively (Chuang and Shih, 2001; Treacy *et al*., 1992).

The major clinical features presented by MSUD patients include ketoacidosis, hypoglycemia, poor feeding, apnea, ataxia, convulsions, coma, psychomotor delay and mental retardation. Magnetic resonance imaging studies have demonstrated generalized edema and hypomyelination/demyelination in central nervous system (CNS) of MSUD patients (Chuang and Shih, 2001; Schönberger *et al*., 2004).

MSUD presents heterogeneous molecular and clinical phenotypes range from a severe classic form with neonatal onset to milder variant forms with later onset, and presenting different residual enzyme activity (Chuang and Shih, 2001; Schadewaldt and Wendel, 1997).

The aim of MSUD treatment is to keep the BCAA plasma concentrations in the normal range, protecting the brain from injury. The treatment consists of a low protein diet and a semi-synthetic formula restricted in BCAA and supplemented with essential amino acids. Metabolic intoxication may cause a fatal outcome in untreated patients (Chuang and Shih, 2001; Danner and Elsas, 1989).

The mechanisms of the neurological symptoms presented by MSUD patients are still poorly understood. However, considering that increased concentrations of leucine and/or α-ketoisocaproate were associated with the appearance of neurological symptoms, these compounds seem to be the main important neurotoxic metabolites in MSUD (Chuang and Shih, 2001; Snyderman *et al*., 1964). Furthermore, it has been demonstrated that the metabolities accumulating in MSUD cause impairment of energy metabolism by inhibiting the electron transport chain (Sgaravatti *et al*., 2003) and creatine kinase activity in rat brain (Pilla *et al*., 2003). Other investigators demonstrated that the BCAA and/or BCKA that accumulate in MSUD provoke neuronal apoptosis (Jouvet *et al*., 2000), as well as convulsions (Coitinho *et al*., 2001), impairment of neurotransmitter synthesis and function (Zielke *et al*., 1996; Tavares *et al*., 2000), myelin alteration (Treacy *et al*., 1992; Tribble and Shapira, 1983; Taketomi *et al*., 1983) and reduced uptake of essential amino acids by the brain (Araújo *et al*., 2001).

Free radicals seem to be involved in a large number of human diseases. Increasing evidence has shown that damage caused by free radicals is an important contributing factor in chronic-inflammatory, vasculary, neoplastic and neurodegenerative diseases (Halliwell, 1994; Reznick and Packer, 1993; Przedborski *et al*., 1996; Bem-Menachem *et al*., 2000).

Oxidative stress has been observed in some inborn errors of intermediary metabolism owing to the accumulation of toxic metabolites which leads to excessive free radical production (Colome *et al*., 2000). Restricted diets utilized to treat patients affected by metabolic disorders may result in a low antioxidant status (Colome *et al*., 2000).

Recently, it was demonstrated that the BCAA and their respective BCKA that accumulate in MSUD stimulate *in vitro* lipid peroxidation in brain homogenates of rats (Fontella *et al*., 2002). It was later demonstrated that these compounds, particularly leucine and α-ketoisocaproate, not only stimulate *in vitro* lipid peroxidation but also reduce the cerebral capacity to modulate the damage associated with the increased free radical production (Bridi *et al*., 2003, 2005a). Furthermore, it was shown that the increased lipid peroxidation induced by leucine could be attenuated by the free radicals scavengers ascorbic acid, α-tocopherol, gluthatione and superoxide dismutase (Bridi *et al*., 2005b).

The aim of the present work was to evaluate some parameters of oxidative stress, namely thiobarbituric acid-reactive substances (TBARS), total antioxidant reactivity (TAR) and total antioxidant status (TAS), in plasma of MSUD patients at the time of diagnosis in order to verify whether free radicals could be involved in the pathophysiology of this disease.

Material and methods

Patients and controls

Plasma from five MSUD patients (classic form) aged between 15 days and 4 months at diagnosis were used to evaluate the parameters of oxidative stress. The most common clinical features presented by these patients were convulsions, hypoglycemia, poor feeding, ketoacidosis and psychomotor delay. Samples were obtained at the time of the diagnosis, which was made by increased plasma levels of leucine ($2{,}346.1 \pm 810.7$ μmol/L), isoleucine (304.8 ± 185.2 μmol/L) and valine (456.5 ± 275.1 μmol/L) by HPLC method (Joseph and Marsden, 1986). Control group was composed of healthy age matched individuals (leucine 158.33 ± 37.63 μmol/L; isoleucine 76.54 ± 18.02 μmol/L; valine 260.73 ± 39.79 μmol/L).

Reagents

All chemicals were of PA purity and were purchased from Sigma (St. Louis, MO, USA) except for thiobarbituric acid, which was purchased from Merck (Darmstadt, Germany) and a kit for TAS measurement that was purchased from Randox Laboratories (Antrim, United Kingdom). TAR was assayed using a beta liquid scintillation spectrometer (Wallac model 1409) and TBARS was measured with a spectrofluorimeter (Hitachi F2000).

Plasma preparation

Plasma was prepared from whole blood samples obtained from fasting individuals (controls and MSUD patients) by venous puncture with heparinized vials. Whole blood was centrifuged at 1,000*g*. Plasma was removed by aspiration and frozen at -80°C until determination.

Thiobarbituric Acid-Reactive Species (TBARS)

Thiobarbituric acid-reactive substances (TBARS) were determined according to the method described by Buege and Aust (1978). Briefly, 250 μL of 10% trichloroacetic acid were added to 125 μL of plasma, then 375 μl 0.67% thiobarbituric acid (in 7.1% sodium sulphate) were added and incubated at 100°C for 30 min. After the incubation, the mixture was extracted with 750 μL butanol. The resulting pink stained TBARS were determined in a spectrofluorimeter at 515 nm. Calibration curve was performed using 1,1,3,3-tetramethoxypropane subjected

to the same treatment as that of the samples. TBARS were calculated as nmol TBARS/mg protein.

Total Antioxidant Reactivity (TAR)

TAR, which represents the quality of the tissue antioxidants, was determined by measuring the luminol chemiluminescence intensity induced by 2,2′-azo-bis-(2-amidinopropane) (ABAP) according to the method of Lissi *et al*. (1992). The background chemiluminescence was measured by adding 4 mL of 2 mM ABAP (in 0.1 M glycine buffer, pH 8.6) 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 basal value. Ten microliters of 25–200 μM Trolox (curve calibration) or plasma was then added and the chemiluminescence was measured during 60 s. The Trolox and plasma addition reduces the chemiluminescence. The rapid reduction in luminol intensity is considered as a measure of the TAR capacity. TAR measurement was calculated as nmol Trolox/mg protein.

Total Antioxidant Status (TAS)

TAS, which represents the quantity of the tissue antioxidants, was determined by using a kit from RANDOX Laboratories. The plasma sample was incubated with ABTS (2,2′-azino-di-[3-ethylbenzthiazoline sulphonate]) plus a peroxidase (metmyoglobin) and H_2O_2 to produce the cation ABTS+. A relatively stable blue–green color occurred and was measured at 37°C at 600 nm. Antioxidants in the added sample cause suppression of this color production to a degree which was proportional to their concentration (Miller *et al*., 1993; Yu and Ong, 1999). The results were expressed in mmol/L plasma.

Protein determination

Protein concentrations were determined by the biuret method from Labtest® (Gornall *et al*., 1949), using albumin as standard.

Statistical analysis

The Student *t* test was used to compare results from controls and MSUD patients. A p value less than 0.05 was considered significant. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software in a PC-compatible computer.

Results

Figure 1 shows that TBARS was significantly increased in plasma of MSUD patients when compared to control group [$t(8) = -4.552$, $p < 0.01$], indicating that lipid peroxidation is stimulated in MSUD patients.

TAR measurement, which is a measure of the tissue capacity to react with free radicals, was markedly reduced [$t(8) = 3.021$, $p < 0.05$] in plasma of MSUD patients (Fig. 2). These results suggest a deficient capacity of plasma to modulate the damage associated with the enhanced production of reactive species in these MSUD patients. Finally, it was also observed that TAS measurement, which represents the quantity of the tissue antioxidants,

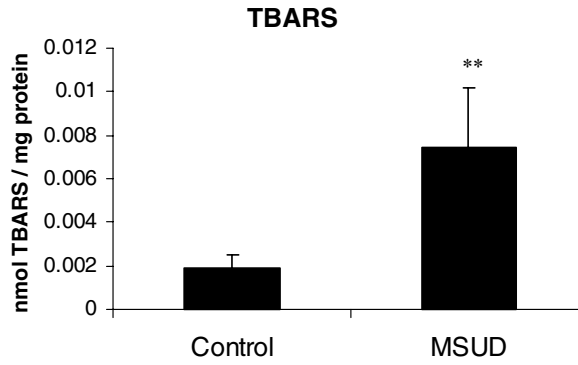

Fig. 1 Plasma thiobarbituric acid reactive species (TBARS) from MSUD patients and controls. Data represent the mean ± SD ($n = 5$). $^{**}p < 0.01$ (Student' t test for unpaired samples) compared to control

was not altered in plasma of MSUD patients [$t(6) = -0.713$, $p > 0.05$] (Fig. 3), suggesting that the total nonenzymatic antioxidant defenses were not altered.

Discussion

Neurological symptoms are frequent in MSUD patients and untreated patients normally have a fatal outcome (Chuang and Shih, 2001; Danner and Elsas, 1989). Leucine and/or α-ketoisocaproate are considered the main neurotoxic metabolites in these disease (Chuang and Shih, 2001; Snyderman *et al*., 1964). However, the mechanisms underlying the sequelae presented by these patients are not well understood.

It was previously demonstrated that the BCAA and BCKA accumulating in MSUD stimulate *in vitro* lipid peroxidation and reduce the antioxidant defenses in cerebral homogenates of young rats (Fontella *et al*., 2002; Bridi *et al*., 2003, 2005a). However, to our knowledge there is no report in the literature assessing whether oxidative stress occurs in tissues from MSUD patients. Therefore, in the present study we investigated some parameters of oxidative stress in plasma from these patients which were not under any therapy. So our results cannot be attributed to any medication.

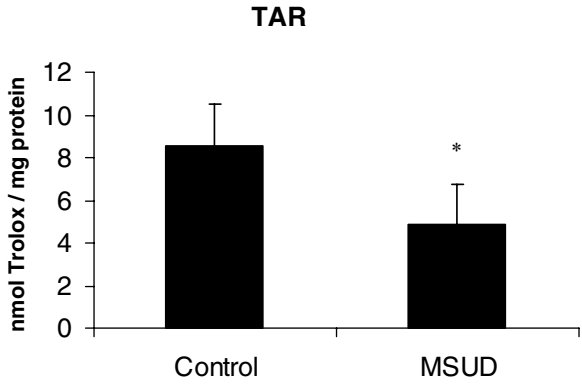

Fig. 2 Plasma total antioxidant reactivity (TAR) from MSUD patients and controls. Data represent the mean ± SD ($n = 5$). $^{*}p < 0.05$ (Student' t test for unpaired samples) compared to control

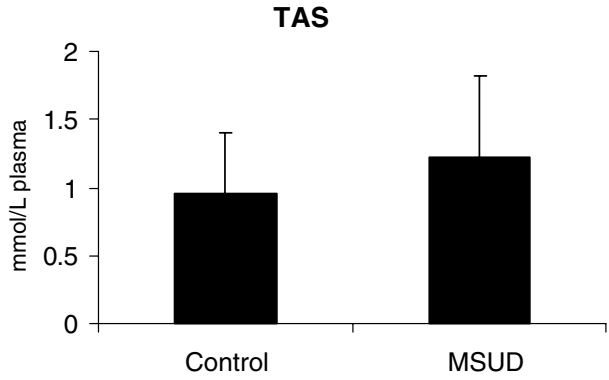

Fig. 3 Plasma total antioxidant status (TAS) from MSUD patients and controls. Data represent the mean ± SD ($n = 4$). No significant differences between means were found (Student' t test for unpaired samples)

We demonstrated a significant increase of TBARS in plasma from MSUD patients. Considering that TBARS reflects the content of malondialdehyde, an end product of lipid breakdown due to lipid peroxidation (Halliwell and Gutteridge, 2001; Esterbauer and Cheeseman, 1990), our data indicate that lipid peroxidation is induced in MSUD patients, probably secondary to free radical generation. Despite the fact that we did not find any decrease of total antioxidant defenses in plasma from these patients as indicated by TAS values TAR, which corresponds to a useful index of the capacity of a given tissue to modulate the damage associated with an increased production of free radicals and mainly reflects the quality of antioxidants (Lissi *et al*., 1995), was significantly decreased in the patients studied. These results indicate a deficient capacity of plasma from MSUD patients to rapidly handle an increase of reactive species. Considering that an imbalance between the total antioxidant defenses and the reactive species formed in the tissues are indicative of oxidative stress (Halliwell and Gutteridge, 2001), it is proposed that free radical generation is involved in the pathophysiology of the tissue damage found in MSUD.

Oxidative stress has been considered an important contributor to brain damage in neurodegenerative diseases, seizures and demyelination (Halliwell, 2001; Méndez-Álvarez *et al*., 2001; Karelson *et al*., 2001), probably because brain has relatively low levels of antioxidant defenses (Halliwell and Gutteridge, 2001), as well as high lipid content, specially unsaturated fatty acids, and iron that stimulates the Fenton reaction being therefore highly susceptible to reactive species attack. Taken together our present *in vivo* results and those demonstrating in studies *in vitro* with rats that the metabolities accumulated in MSUD cause a stimulation of lipid peroxidation and a reduction of brain antioxidant defenses, (Fontella *et al*., 2002; Bridi *et al*., 2003, 2005a), suggest that oxidative stress is probably involved in the pathophysiology of MSUD.

To our knowledge this is the first report demonstrating increased oxidative stress in patients affected by MSUD. Our results should, however, be taken with caution and confirmed with a higher number of patients and with other techniques to measure oxidative stress. In this context, CSF specimens may be useful in order to evaluate if the brain is also a target for reactive species. In case the present results are confirmed, we may conclude that oxidative stress contributes at least in part to the severe neurological dysfunction found in MSUD.

Acknowledgements This work was supported by grants from Brazilian National Research Council (CNPq), FAPERGS, and FIPE/HCPA—Brazil.

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