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ORIGINAL PAPER

Oxidative stress in plasma from maple syrup urine disease patients during treatment

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Abstract Maple Syrup Urine Disease (MSUD) is an autossomal recessive metabolic disorder caused by a deficiency of branched-chain α-keto acid dehydrogenase complex activity leading to accumulation of the branched-chain amino acids leucine, isoleucine and valine and their corresponding branched-chain α-keto acids. Affected patients usually present hypoglycemia, ketoacidosis, convulsions, poor feeding, coma, psychomotor delay and mental retardation. Considering that the pathophysiology of MSUD is still poorly understood, in this study we evaluated some parameters of oxidative stress, namely thiobarbituric acid-reactive substances (TBARS), total antioxidant reactivity (TAR) and total antioxidant status (TAS) in plasma from treated MSUD patients presenting high and low plasma leucine levels. We verified a significant increase of TBARS (lipid peroxidation) and a decrease of TAR (capacity to rapidly react with free radicals) in plasma from treated MSUD patients with low and with high plasma levels of leucine compared to the control group. It was also verified that TAS (quantity of tissue antioxidants) was not altered in plasma from treated MSUD patients with low and high blood leucine levels. Finally, we found no correlation between leucine, valine and isoleucine levels with the various parameters of oxidative stress. These results are indicative that increased lipid oxidative damage and decreased antioxidant defenses occur in plasma of MSUD patients and that the accumulating branched-chain amino acids are probably not directly associated to oxidative stress in this disorder.

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Introduction

Maple Syrup Urine Disease (MSUD) is an inherited disorder affecting the metabolism of branched-chain amino acids (BCAA) leucine (Leu), isoleucine (Ile) and valine (Val). The activity of the branched-chain α-keto acid dehydrogenase complex (BCKAD) is deficient in MSUD leading to tissue accumulation of BCAA (Leu, Ile e Val), as well as their corresponding transaminated branched-chain α-keto acids (BCKA) α-ketoisocaproate, α-keto-β-methylvalerate and α-ketoisovalerate (Chuang and Shih 2001; Treacy *et al.* 1992).

The clinical and biochemical phenotypes of MSUD patients are heterogeneous. Patients can be divided into five phenotypes ranging from the classical form with a neonatal onset to milder variants with later onset (Chuang and Shih 2001; Schadewaldt and Wendel 1997). Individuals with MSUD usually present poor feeding, convulsions, ketoacidosis, apnea, hypoglycemia, coma, ataxia, psychomotor delay and mental retardation, as well as generalized edema and hypomyelination/demyelination on magnetic resonance imaging studies of the central nervous system (CNS) (Chuang and Shih 2001; Schönberger *et al.* 2004). Therapy for this disease is based on a natural protein restricted diet with low BCAA supplemented with a semi-synthetic formula of essential amino acids, vitamins and minerals. This treatment minimizes the accumulation of the toxic metabolites and contributes to the survival of the affected individuals, but do not prevent a variable degree of neurological dysfunction evidenced by developmental delay and mental retardation whose pathogenesis is poorly known (Chuang and Shih 2001). Leucine and/or α-ketoisocaproate are thought to be the main neurotoxic metabolites in this disorder once their increased concentrations have been associated to the appearance of neurological symptoms (Chuang and Shih 2001; Snyderman *et al.* 1964).

Free radicals and oxidative stress have been associated with a large number of diseases including various neurodegenerative disorders, epileptic seizures, demyelination and dementia (Halliwell 1994; Reznick and Packer 1993; Przedborski *et al.* 1996; Ben-Menachem *et al.* 2000). This may occur because the CNS is highly susceptible to oxidative damage due to the relatively low activity of antioxidant defenses, high iron content, high lipid content, specially unsaturated fatty acids, and high oxygen consumption (Halliwell and Gutteridge 2001). Oxidative stress has been also observed in some inborn errors of intermediary metabolism, and this has been attributed to the accumulation of toxic metabolites which are able to induce excessive free radical generation (Colome *et al.* 2000; Wajner *et al.* 2004). We cannot also exclude the possibility that the restricted diets used to treat patients with metabolic disorders may decrease the tissue antioxidant defenses since they potentially deplete essential nutrients involved in the antioxidant defenses (Halliwell and Gutteridge 2001).

With regards to MSUD, experimental animal studies have demonstrated that lipid peroxidation is stimulated in vitro by the BCAA and their respective BCKA in brain homogenates of rats (Fontella *et al.* 2002). It was also verified that these metabolites reduce in vitro the cerebral capacity to modulate the damage associated to increased free radical production (Bridi *et al.* 2003; Bridi *et al.* 2005a) and that the increased

lipid peroxidation induced in vitro in cerebral cortex of rats by leucine is attenuated by the free radicals scavengers ascorbic acid, α-tocopherol, gluthatione and superoxide dismutase (Bridi *et al.* 2005b).

On the other hand, we recently verified that the antioxidant status and lipid peroxidation were significantly altered in plasma of MSUD patients at diagnosis, suggesting the involvement of oxidative stress in the pathogenesis of this disease (Barschak *et al.* 2006). In the present study, we evaluated various parameters of oxidative stress in blood from treated MSUD patients showing high and low blood leucine levels, in order to test whether this amino acid could be involved in the oxidative stress. The parameters analyzed were thiobarbituric acid-reactive substances (TBARS), total antioxidant reactivity (TAR) and total antioxidant status (TAS).

Materials and methods

Reagents

All chemicals were of PA purity and were purchased from Sigma (St. Louis, MO, USA) except thiobarbituric acid that 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), TBARS was measured in a spectrofluorimeter (Hitachi F2000) and TAS in a double-bean spectrophotometer with temperature control (Hitachi U-2001).

Subjects

Plasma specimens from ten treated MSUD patients with the classic form and five age matched controls were used to evaluate the parameters of oxidative stress. The patients were aged between 15 days and 4 months at diagnosis and followed a treatment that consisted of a natural protein restricted diet with low BCAA and supplemented with a semi-synthetic formula of essential amino acids, vitamins and minerals. The patients ingested the following amounts of Leu (before 12 months of age: 40–80 mg kg^{-1} day^{-1}; after 1 year of age: 275–535 mg/day), Ile (before 12 months of age: 20–50 mg kg^{-1} day^{-1}; after 1 year of age: 165–325 mg/day) and Val (before 12 months of age: 20–60 mg kg^{-1} day^{-1}; after 1 year of age: 190–375 mg/day). Plasma samples obtained from MSUD patients under treatment were divided into two groups depending on blood Leu levels. In group I plasma Leu levels were lower than 100 μmol/l (36.3±17.1 μmol/l, treatment duration was 18.6±12.9 months), whereas in group II plasma Leu levels were higher than 600 μmol/l (1,314±914 μmol/l, treatment duration was 17.2±20.1 months). The control group corresponded to healthy age matched individuals (leucine 158±37.6 μmol/l; isoleucine 76.5±18.0 μmol/l; valine 260±39.8 μmol/l) (Table 1).

The present study was approved by the Ethical Committee of Hospital de Clínicas de Porto Alegre, RS, Brazil (protocol number 04-256). The parents of the patients included in the present study gave informed consent.

Table 1 Plasma concentrations of leucine (Leu), isoleucine (Ile) and valine (Val) in controls and MSUD patients during treatment

	Leu (μmol/l)	Ile (μmol/l)	Val (μmol/l)
Controls	158±16.8	76.5±8.0	260±17.7
MSUD patients group I	36.3±7.66	112±42.2	341±169
MSUD patients group II	1,314±408*	198±62.6	384±154

Values represent mean±SE (n=5)
*p<0.01, different from controls (ANOVA followed by the Duncan multiple range test)

Plasma preparation and amino acids determination

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 and plasma was removed by aspiration and frozen at −80°C until analysis. Blood amino acids levels were measured by HPLC (Joseph and Marsden 1986), with slight modifications (Wajner *et al*. 2000).

Thiobarbituric acid-reactive substances (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 reduce 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 is proportional to their concentration (Miller *et al.* 1993; Yu and Ong 1999). The results were expressed in millimole per liter.

Protein determination

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

Statistical analysis

Results were analyzed by one-way ANOVA, followed by the Duncan multiple range test when appropriated (Altman 1991). Only significant F values are shown in the text. Correlations of plasma Leu, Val and Ile levels with TBARS, TAR or TAS were carried out using the Pearson correlation coefficient. 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 measurement was significantly increased in plasma from MSUD patients both with low (group I) and high (group II) Leu levels, as compared to the control group [$F(2,12)=4.289$, $p<0.05$]. These data indicate that lipid peroxidation is stimulated in plasma from MSUD patients.

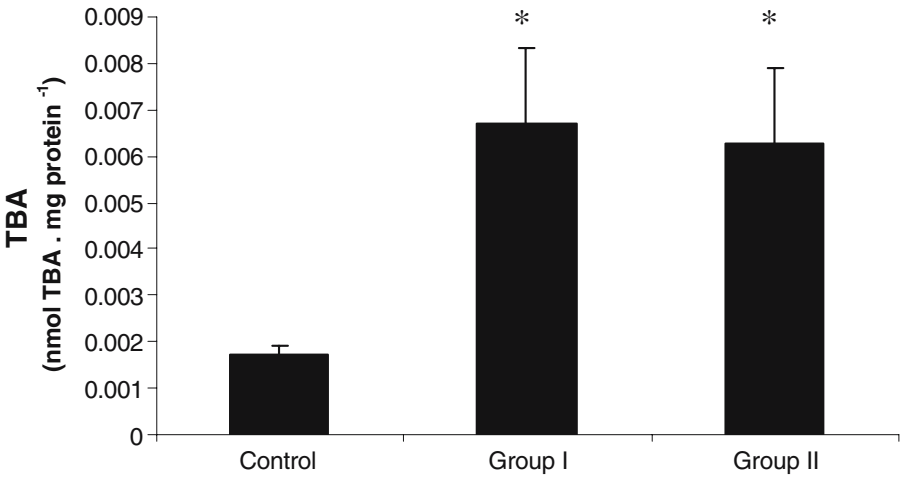

Fig. 1 Comparison between thiobarbituric acid reactive substances (TBARS) in plasma from MSUD patients during treatment and controls. Data represent the mean±SE ($n=5$). *Single asterisk* $p<0.05$, compared to controls (ANOVA, followed by the Duncan multiple range test). Group I: low plasma leucine levels; group II: high plasma leucine levels

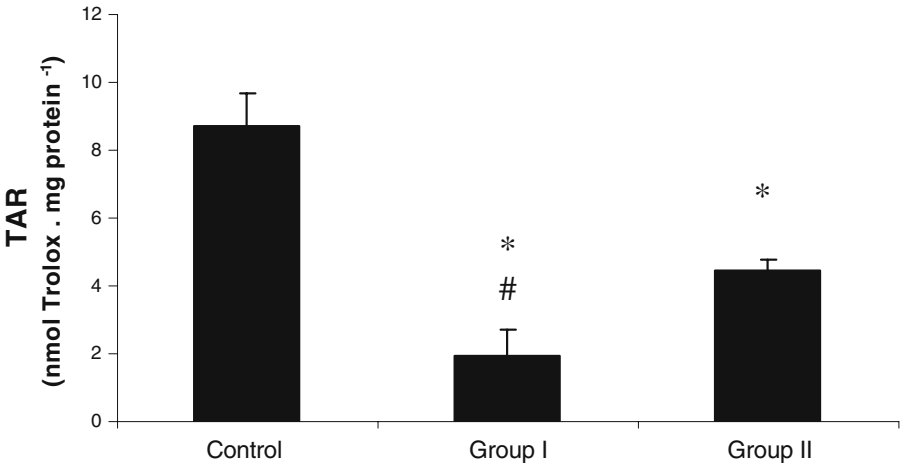

Fig. 2 Comparison between total antioxidant reactivity (TAR) in plasma from MSUD patients during treatment and controls. Data represent the mean±SE (n=5). *Single asterisk* $p<0.001$, compared to controls (ANOVA, followed by the Duncan multiple range test). *Pound sign* $p<0.001$, compared to group II (ANOVA, followed by the Duncan multiple range test). Group I: low plasma leucine levels; group II: high plasma leucine levels

Furthermore, TAR measurement was significantly reduced in plasma of MSUD patients independently of the concentration of Leu (high and low Leu levels), as compared to the control group [$F(2,12)=30.196$, $p<0.001$] (Fig. 2). It can be also observed in the figure that group I is different from group II. Considering that TAR measurement reflects the tissue capacity to react with free radicals, these results

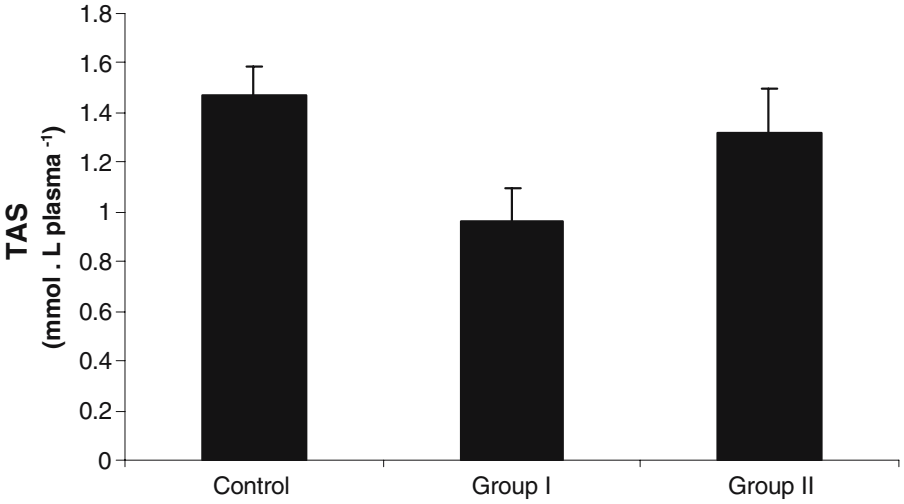

Fig. 3 Comparison between total antioxidant status (TAS) in plasma from MSUD patients during treatment and controls. Data represent the mean±SE (n=5). No significant differences between means were found (ANOVA). Group I: low plasma leucine levels; group II: high plasma leucine levels

suggest a deficient capacity of plasma from treated MSUD patients to modulate the damage associated with the enhanced production of reactive species.

We also observed that TAS measurement, which represents the quantity of tissue antioxidants, was not altered in plasma from treated MSUD patients (group I and group II), as compared to the control group [$F(2,12)=3.352$, $p>0.05$] (Fig. 3).

Finally we found no correlation between Leu, Ile and Val blood levels and TBARS, TAR and TAS measurements (results not shown).

Discussion

MSUD treatment is directed to minimize the accumulation of the toxic metabolites BCAA and BCKA and decisively contributes to the survival of affected individuals. However, some patients present a variable degree of developmental delay and mental retardation that are attributed to a poor adherence to the restricted diet (Chuang and Shih 2001).

Although the mechanisms underlying the pathophysiology of the brain damage in MSUD are still poorly understood, high plasma levels of Leu and its ketoacid derivative α-ketoisocaproate have been correlated with the appearance of neurological symptoms (Chuang and Shih 2001). Furthermore, it has been demonstrated that the metabolites accumulating in MSUD cause impairment of energy metabolism by inhibiting the electron transport chain (Sgaravatti *et al.* 2003) and creatine kinase activity (Pilla *et al.* 2003), induce neuronal apoptosis (Jouvet *et al.* 2000), convulsions (Coitinho *et al.* 2001), impairment of neurotransmitter synthesis and function (Zielke *et al.* 1996; Tavares *et al.* 2000), induce alterations of myelin synthesis or degradation (Treacy *et al.* 1992; Tribble and Shapira 1983; Taketomi *et al.* 1983) and also reduce the uptake of essential amino acids by the brain (Araújo *et al.* 2001).

Human and animal studies have indicated that metabolites accumulating in various inborn errors of metabolism induce excessive free radical production and reduce the tissue antioxidant defenses (Colome *et al.* 2000; Wajner *et al.* 2004). In this context, oxidative stress has been demonstrated in patients with phenylketonuria (Sirtori *et al.* 2005) and adrenoleukodystrophy (Vargas *et al.* 2004) and leucine and α-ketoisocaproate were shown to stimulate in vitro lipid peroxidation and reduce the cerebral antioxidant reactivity in cerebral homogenates from young rats (Fontella *et al.* 2002; Bridi *et al.* 2003; Bridi *et al.* 2005a).

We have recently verified that plasma from MSUD patients at diagnosis present increased lipid peroxidation and decreased antioxidant reactivity, indicating the involvement of oxidative stress in the pathogenesis of this disease (Barschak *et al.* 2006). In order to extend this investigation and better understand the involvement of oxidative stress in the pathophysiology of MSUD, in the present study we measured TBARS, TAR and TAS in plasma from MSUD patients with high and low levels of leucine and compared to plasma from normal age matched individuals. We also investigated whether alterations of those parameters were correlated with plasma leucine concentrations, as well as, isoleucine and valine.

We demonstrated that TBARS measurement was significantly increased in plasma from treated MSUD patients with both high and low leucine levels. Since TBARS reflects the formation of malondialdehyde, an end product of membrane fatty acid

peroxidation (Halliwell and Gutteridge 2001; Esterbauer and Cheeseman 1990), these data suggest that patients under treatment present increased lipid oxidative damage (lipid peroxidation) independently of plasma Leu levels. Furthermore, we did not find a correlation between plasma concentrations of leucine, valine and isoleucine with the lipid peroxidation parameter TBARS. Taken together these observations, it may be presumed that leucine and the other BCAA are not directly associated to free radical production in MSUD.

We also observed that TAR measurement, which represents the capacity of a tissue to modulate the damage associated with an increased production of free radicals, reflecting the quality of non enzymatic antioxidants (Lissi *et al.* 1995), was significantly decreased in plasma from treated MSUD patients and that this decrease was not dependent on the plasma concentrations of leucine. In addition, TAS (total antioxidant status) measurement, which corresponds to the total quantity of tissue non enzymatic antioxidants, was not altered plasma of MSUD patients presenting low and high leucine levels. It may be therefore presumed that the dietetic treatment (low protein diet) contributed to reduce the tissue antioxidants in these patients since strict protein ingest may secondarily deplete essential substances involved in the antioxidant system, like minerals, vitamins and selenium. We believe that lack of nutrients may be involved in the reduction of antioxidant defenses observed in our present study since patients with low blood leucine levels (more adherent to treatment) would theoretically have greater nutrient deficiency than those with high plasma leucine concentrations and consequently much lower TAR, what was observed in ours patients. These observations, allied to the fact that no correlation was found between leucine, valine and isoleucine with TBARS, TAR and TAS values, indicate that other factors than the BCAA should be investigated to explain our present results.

In summary, taken together the present and previous findings (Barschak *et al.* 2006), it can be concluded that MSUD patients present increased lipid peroxidation and decreased antioxidant defenses at diagnosis and after treatment, which is strongly indicative that oxidative stress may be an underlying mechanism of tissue damage in this disorder. We also observed that the alterations observed were not associated to the plasma levels of leucine, suggesting that other factors, including the BCKA, which are primarily accumulated in these patients, are potentially responsible for the oxidative damage in this disorder. It could be therefore presumed that oxidative stress may contribute at least in part to the chronic progressive neurological damage observed in MSUD patients. Thus, it is desirable that more studies involving other oxidative stress parameters and a larger number of treated patients are carried out in order to better understand the contribution of oxidative stress in MSUD pathophysiology.

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