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Oxidative stress in patients with phenylketonuria

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Abstract

Phenylketonuria (PKU) is an autossomal recessive disease caused by phenylalanine-4-hydroxylase deficiency, which is a liver-specific enzyme that catalyzes the hydroxylation of L-phenylalanine (Phe) to L-tyrosine (Tyr). The deficiency of this enzyme leads to the accumulation of Phe in the tissues and plasma of patients. The clinical characterization of this disease is mental retardation and other neurological features. The mechanisms of brain damage are poorly understood. Oxidative stress is observed in some inborn errors of intermediary metabolism owing to the accumulation of toxic metabolites leading to excessive free radical production and may be a result of restricted diets on the antioxidant status. In the present study we evaluated various oxidative stress parameters, namely thiobarbituric acid-reactive species (TBA-RS) and total antioxidant reactivity (TAR) in the plasma of PKU patients. The activities of the antioxidant enzymes catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) were also measured in erythrocytes from these patients. It was observed that phenylketonuric patients present a significant increase of plasma TBA-RS measurement, indicating a stimulation of lipoperoxidation, as well as a decrease of plasma TAR, reflecting a deficient capacity to rapidly handle an increase of reactive species. The results also showed a decrease of erythrocyte GSH-Px activity. Therefore, it is presumed that oxidative stress is involved in the pathophysiology of the tissue damage found in PKU.

Keywords: Phenylketonuria; Oxidative stress; Free radical

1. Introduction

Phenylketonuria (PKU) is an autossomal recessive disease caused by deficiency of phenylalanine-4-hydroxylase, which is a liver-specific enzyme that catalyzes the hydroxylation of L-phenylalanine (Phe) to L-tyrosine (Tyr) in the presence of the cofactor, tetrahydrobiopterin (BH_4). The deficiency of this enzyme leads to the accumulation of Phe in the tissues and plasma of patients. The incidence in Caucasians is approximately 1:10,000 [1].

High Phe levels interfere with the production of the neurotransmitters dopamine and noradrenaline [2]. Phe decreases the availability of tryptophan (Trp) and Tyr [3] and causes serotonin and catecholamine depletion in PKU [4] thus influencing brain function. Additionally, high Phe concentrations were found to influence several mechanisms such as neural excitability, axonal conduction [1,5] and synaptic transmission velocity [6]. It was also demonstrated that Na^+,K^+-ATPase activity is reduced in the synaptic plasma membrane of animal models of PKU [7,8].

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Therapy is based on a low-Phe diet by eliminating high-protein foods, enabling children with PKU to develop normally [9]. As a substitute for proteins, PKU patients consume an artificial amino acid mixture that does not contain Phe; such mixtures are enriched with vitamins and minerals [10]. Unfortunately, many PKU patients do not adhere strictly to this diet, resulting in uncontrolled high Phe blood levels. In PKU, Phe plasma concentrations may reach 400–1800 μmol/L and are harmful especially during the first year of life [5,11,12]. This condition leads to severe retardation of intellectual development, neuropsychiatric symptoms and seizures [13].

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, neoplasic and neurodegenerative diseases including Parkinson's disease, Alzheimer's disease, strokes, multiple sclerosis, epilepsy, etc. [14–17]. The brain has relatively low levels of antioxidant defenses, high lipid content, specially unsaturated fatty acids and cathecolamines, which are highly susceptible to reactive oxygen species attack.

Oxidative stress was observed in some inborn errors of intermediary metabolism owing to the accumulation of toxic metabolites leading to excessive free radical production [18]. Restricted diets also alter the antioxidant status in some inborn errors of metabolism [19,20]. In this context, oxidative stress has been demonstrated in animal models of hyperphenylalaninemia and PKU [21–23].

Our objective in the present study was to evaluate various parameters of oxidative stress such as thiobarbituric acid-reactive species (TBA-RS) and total antioxidant reactivity (TAR) in the plasma and the activities of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase (CAT) in erythrocytes from PKU patients in order to verify whether free radicals may be involved in the pathophysiology of the tissue damage in these patients.

2. Material and methods

2.1. Patients and controls

A total of 20 PKU patients aged between 2 and 20 years was used to evaluate the parameters of oxidative stress. Samples (plasma from 20 patients and erythrocytes from 4 patients) were obtained at the time of the diagnosis of the index cases in our laboratory, which consisted of the determination of increased plasma levels of Phe by a fluorimetric method [24]. For analysis were used samples whose plasma levels of Phe were at least 600 μmol/L and the mean value for the PKU samples was 1160 μmol/L. The period between blood collection and analysis was always less than 2 weeks. Plasma and erythrocytes were also obtained from healthy age matched individuals used as the control group. Samples were kept frozen until analysis.

2.2. Reagents

All chemicals were of PA purity and were purchased from Sigma (St. Louis, MO) except by TBA, which was purchased from Merck (Darmstadt, Germany). TAR was assayed using a beta liquid scintillation spectrometer (Wallac model 1409). TBA-RS and antioxidant enzyme activities were measured with a double-bean spectrophotometer with temperature control (Hitachi U-2001).

2.3. Erythrocytes and plasma preparation

Erythrocytes and plasma were prepared from whole blood samples obtained from fasting individuals (controls and PKU patients) by venous puncture with heparinized vials. Whole blood was centrifuged at $1000\times g$, plasma was removed by aspiration and frozen at −80 °C until determination. Erythrocytes were washed three times with cold saline solution (sodium chloride 0.153 mol/L). Lysates were prepared by the addition of 100 μL of washed erythrocytes to 1 mL of distilled water and frozen at −80 °C until the determination of the antioxidant enzyme activities.

For antioxidant enzyme activity determination, erythrocytes were frozen and thawn three times, centrifuged at $13500\times g$ for 10 min. The supernatant was diluted to approximately 0.5 mg/mL of protein.

2.4. Thiobarbituric acid-reactive species (TBA-RS)

Thiobarbituric acid-reactive species (TBA-RS) were determined according to the method described by Esterbauer and Cheeseman (1990) [25]. Briefly, 300 μl of 10% trichloroacetic acid was added to 150 μl of plasma and centrifuged at $1000\times g$ for 10 min at 4 °C. Three hundred microliters of the supernatant was transferred to a test tube and incubated with 300 μl 0.67% thiobarbituric acid (in 7.1% sodium sulfate) at 100 °C 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. Calibration curve was performed using 1,1,3,3-tetramethoxypropane subjected to the same treatment as that of the supernatants. TBA-RS were calculated as nmol TBA-RS/mg protein.

2.5. Total antioxidant reactivity (TAR)

TAR, which represents the quality of the tissue antioxidants, was determined by measuring the luminol chemiluminescence intensity induced by z,z′-azo-bis-(2-amidinopropane) (ABAP) according to the method of Lissi et al. (1992) [26]. The background chemilumines-

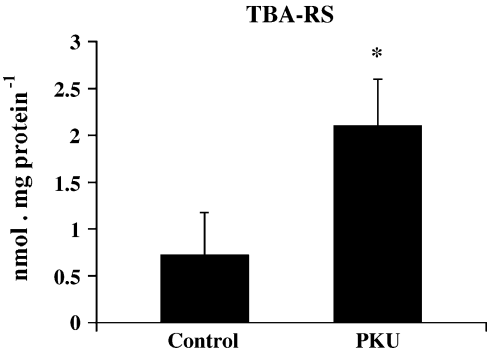

Fig. 1. Plasma thiobarbituric-acid reactive species (TBA-RS) from PKU patients and controls. Data represent the mean±S.D. (n=20). Difference from control, *P<0.05 (Student's t test for non-paired samples).

cence was measured by adding 4 mL of 2 mM ABAP (in 0.1 M glycine buffer, pH 8.6) into a glass scintillation vial. Fifteen microliters of luminol (4 mM) was added to each vial and the chemiluminescence was measured. This was considered to be the basal value. Ten microliters of 10 μM Trolox or plasma was then added and the chemiluminescence was measured during 60 s. The Trolox or supernatant 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.

2.6. Antioxidant enzyme activities

2.6.1. Catalase assay (CAT)

CAT activity was assayed by the method of Aebi (1983) [27] measuring the absorbance decrease at 240 nm in a reaction medium containing 20 mM H_2O_2, 10 mM potassium phosphate buffer, pH 7.0, and 0.1–0.3 mg protein/ml. One unit of the enzyme is defined as 1 μmol of H_2O_2 consumed per minute and the specific activity is reported as units per milligram of protein.

2.6.2. Glutathione peroxidase (GSH-Px)

GSH-Px was measured by the method of Wendel (1981) [28] using *tert*-butyl-hydroperoxide as substrate. The activity was determined monitoring the NADPH disappearance at 340 nm in a medium containing 2 mM glutathione, 0.15 U/ml glutathione reductase, 0.4 mM azide, 0.5 mM *tert*-butyl-hydroperoxide and 0.1 mM NADPH. One GSH-Px unit is defined as 1 μmol of NADPH consumed per minute and the specific activity is represented as units per mg protein.

2.6.3. Superoxide dismutase (SOD)

SOD activity was determined using the RANSOD kit (Ransox, Antrim, United Kingdom). 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 per mg protein.

2.7. Protein determination

Protein concentrations were determined by the method of Lowry et al. (1951) [29], using bovine serum albumin as standard.

2.8. Statistical analysis

Data are expressed as mean±standard deviation. The Student's t test for non-paired samples was used to compare results from controls and PKU 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.

3. Results

TBA-RS, a parameter of lipid peroxidation, was determined in the plasma of PKU patients. Fig. 1 shows that TBA-RS measurement was significantly increased from 0.7195 to 2.0975 nmol/mg protein (290%) [t(12)=5.849, P<0.05] in the plasma of PKU patients. These results strongly indicate that lipid peroxidation is stimulated in the PKU.

TAR measurement, which is a measure of the tissue capacity to react with free radicals, was markedly reduced from 2.1030 to 0.9220 nmol/mg protein (56%) [t(6)=3.341, P<0.05] in the plasma of PKU patients (Fig. 2). These data indicate a deficient capacity to modulate the damage associated with the enhanced production of reactive species in the PKU.

Next, we examined the activities of the antioxidant enzymes CAT, GSH-Px and SOD in erythrocytes (Table 1). A significant decrease from 0.866 to 0.243 mU/mg protein (70%) of erythrocyte GSH-PX activity [t(6)=44.01,

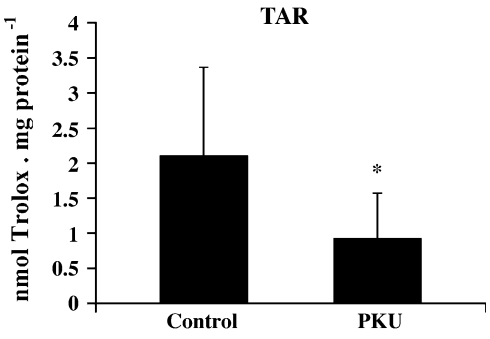

Fig. 2. Plasma total antioxidant reactivity (TAR) from PKU patients and controls. Data represent the mean±S.D. (n=20). Difference from control, *P<0.05 (Student's t test for non-paired samples).

Table 1
Antioxidant enzyme activities in erythrocytes from control and PKU patients

Antioxidant enzyme	Control (n=4)	PKU patient (n=4)
CAT	3.225±0.210	3.48±0.231
GSH-Px	0.866±0.027	0.243±0.087*
SOD	1.265±0.2086	1.215±0.2641

CAT: catalase (pmol/mg prot); GSH-Px: glutathione peroxidase (mU/mg prot); SOD: superoxide dismutase (U/mg prot). Data represent the mean±S.D. One U is defined as 1 μmol of NADPH consumed per minute for GSH-Px and 50% of produced chromogen inhibition for SOD. Difference from control, *P<0.05 (non-paired Student's t test).

P<0.05] (Table 1) was verified in the PKU group compared to the controls. In contrast, the activities of CAT and SOD in PKU patients showed no significant difference from the controls.

4. Discussion

Neurological symptoms and brain abnormalities are characteristic of patients with high Phe plasma levels. However, very little is known about the pathomechanisms involved in the tissue damage of this disorder. In the present study we investigated various parameters of oxidative stress in plasma and erythrocytes from PKU patients.

We demonstrated a significant increase of TBA-RS in the plasma of these patients. Considering that TBA-RS reflects the amount of malondialdehyde formation, an end product of membrane fatty acid peroxidation [30], our data indicate that lipid peroxidation is induced in the plasma of PKU patients, probably secondary to free radical generation.

A significant decrease of TAR measurement was also observed, reflecting a deficient capacity to rapidly handle an increase of reactive species. It should be noted that TAR 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 reflects the quality of antioxidants (given by its reactivity) [31]. This is in agreement with some studies demonstrating that the total antioxidant status is lower in PKU patients than in control children [19,20]. It was also demonstrated that ubiquinone-10 [10,32] and α-tocopherol [10] are found in lower concentrations in PKU patients. The possible responsible mechanism for this deficiency is a decrease of ubiquinone synthesis secondary to the increase of Phe plasma levels since Phe regulates the mevalonate pathway [1].

In this study, we also verified a decrease of erythrocyte glutathione peroxidase (GSH-Px) activity in the PKU patients. These results probably cannot be explained by the deficiency of selenium which is essential for this enzyme activity since the patients used in the present investigation were not under protein or Phe dietary restricted therapy. In this context, it has been previously reported that GSH-Px activity is decreased in treated PKU patients and that this activity is well correlated with plasma selenium levels [20]. On the other hand, other investigators found that GSH-Px activity is normal in erythrocytes from PKU patients under selenium supplementation, whereas catalase activity was mildly (8%) reduced and negatively correlated with plasma Phe levels [19]. These differences in antioxidant enzyme activities may possibly be attributed to the distinct samples (non-treated and treated PKU patients) and on the clinical status of the patients utilized in the various studies.

GSH-Px functions as a part of the antioxidant system to protect membranes and essential proteins from the potentially damaging effects of reactive oxygen and lipid peroxides [33,34]. This is very important in erythrocytes given that these cells are by nature highly susceptible to oxidative stress because their membranes are rich in polyunsaturated fatty acids and because the cellular content of oxygen and iron are high [30].

Therefore, taken together our present data showing a significant increase of TBA-RS levels (lipoperoxidation) and a diminution of TAR (capacity to react with free radicals) in plasma and erythrocyte GSH-Px activity, and considering that an unbalance between the total antioxidant defenses and the reactive species formed in the tissues are indicative of oxidative stress [35], it is proposed that free radical generation is involved in the pathophysiology of the tissue damage found in PKU.

Oxidative stress has been demonstrated in animal models of PKU. In this context, it has been observed that experimental hyperphenylalaninemia provokes oxidative stress in rat brain [21] and also that lipid peroxidation was significantly increased in the brain, whereas the ratio glutathione/glutathione disulfite was decreased and glucose-6-phosphate dehydrogenase and catalase activities were increased in the erythrocytes of an PKU animal model [22]. In addition, it has been shown that maternal hyperphenylalaninemia induces significant morphological damage in pup rat brain and cerebellum and that the biomolecular oxidative damage was prevented by the antioxidants melatonin, vitamin C and vitamin E [23].

At this point it should be emphasized that the brain has low cerebral antioxidant defenses compared with other tissues [36], a fact that makes this tissue more vulnerable to increased reactive species. There are considerable evidences that oxidative stress is implicated in the pathophysiology of common neurodegenerative disorders such as Parkinson's disease, Alzheimer's disease, multiple sclerosis, as well as in epileptic seizures and demyelination [15,37,38]. Therefore, in case the same results of oxidative stress achieved in plasma from PKU patients also occur in the brain, it may be presumed that oxidative stress also compromises the brain, similar to what occurs in other neurodegenerative disorders.

Our results should, however, be taken with caution and confirmed with a higher number of patients and with other techniques to measure oxidative stress since we used specimens from only a few patients. In this context, CFS

specimens may be useful in certain circumstances to evaluate whether the brain is also a target for reactive species, as for example in non-responsive PKU patients. If the present results are confirmed, we may conclude that oxidative stress contributes at least in part to the severe neurological dysfunction found in PKU. As a perspective of continuation of this work, we propose to study the oxidative stress in treated PKU patients with high and low Phe plasma levels.

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