Experimental hyperphenylalaninemia provokes oxidative stress in rat brain

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Abstract

Tissue accumulation of L-phenylalanine (Phe) is the biochemical hallmark of human phenylketonuria (PKU), an inherited metabolic disorder clinically characterized by mental retardation and other neurological features. The mechanisms of brain damage observed in this disorder are poorly understood. In the present study we investigated some oxidative stress parameters in the brain of rats with experimental hyperphenylalaninemia. Chemiluminescence, total radical-trapping antioxidant potential (TRAP), superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) activities were measured in the brain of the animals. We observed that chemiluminescence is increased and TRAP is reduced in the brain of hyperphenylalaninemimic rats. Similar data were obtained in the in vitro experiments using Phe at various concentrations. CAT activity was significantly inhibited by Phe in vitro and in vivo, whereas GSH-Px activity was reduced in vivo but not in vitro and SOD activity was not altered by any treatment. The results indicate that oxidative stress may be involved in the neuropathology of PKU. However, further studies are necessary to confirm and extend our findings to the human condition and also to determine whether an antioxidant therapy may be of benefit to these patients. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Phenylketonuria; Phenylalanine; Brain; Central nervous system; Oxidative stress; Free radical

1. Introduction

Phenylketonuria (PKU) is the most frequent inherited disorder of amino acid metabolism. PKU is caused by a severe deficiency of hepatic phenylalanine hydroxylase activity resulting in accumulation of L-phenylalanine (Phe) and its metabolites in blood and other tissues [1]. Permanent brain damage in this disease has been associated with increased Phe levels during critical periods of brain development [2,3]. Unless diagnosed and treated very early in infancy, this condition leads to severe retardation of intellectual development, neuropsychiatric symptoms and seizures. Although long-term therapy with a Phe-restricted diet may prevent these symptoms [4], intellectual and school performance depend on good die-
tary compliance, which may be difficult to achieve [5]. Therefore, it would be desirable to develop alternative therapeutic approaches that could counteract the toxic effects of Phe, mainly in patients with poor compliance [6].

Some mechanisms have been proposed to explain the pathophysiology of the neurological dysfunction characteristic of PKU. Severe reduction in the amount of myelin, retarded synapse formation and decreased dendritic arborization have been described in human and experimental PKU [7,8]. Phe affects brain protein synthesis due to polysome disaggregation and inhibition of translation initiation [9,10]. In addition, Phe disturbs the transport of various neutral amino acids into the brain, possibly reducing their availability for protein and neurotransmitter synthesis [11-15]. We have previously demonstrated some biochemical alterations in the brain of hyperphenylalaninemic rats chronically treated with daily administration of Phe plus α-methyl-DL-phenylalanine (MePhe), a phenylalanine hydroxylase inhibitor. These alterations include reduced Na⁺,K⁺-ATPase activity in the synaptic plasma membrane [16,17], decreased high molecular mass neurofilament subunit (NF-H) content and increased phosphorylation of the 150 kDa neurofilament subunit (NF-M) and tubulins present in the cytoskeletal fraction [18]. Finally, brain energy metabolism is impaired by Phe in vitro [19], a fact that is also observed in patients [20]. However, despite these studies, the mechanisms by which Phe causes brain damage are poorly understood to date.

Oxidative stress is an important event that has been related to the pathogenesis of some diseases affecting the central nervous system such as neurodegenerative disorders, epileptic seizures, demyelination (multiple sclerosis), dementia, and others [21,22]. This is understandable since the central nervous system is highly sensitive to oxidative stress due to its high oxygen consumption, its high iron and lipid contents, especially polyunsaturated fatty acids, and the low activity of antioxidant defenses [23].

Several authors have reported low selenium levels in plasma of hyperphenylalaninemic patients probably secondary to the protein restricted diet imposed to these children in order to achieve normal plasma Phe levels [24-27]. A more recent evaluation of hyperphenylalaninemic patients found that, although a normal plasma level of selenium was seen in these patients, a significantly lower activity of erythrocyte GSH-Px was detected [26]. Considering that selenium is essential to GSH-Px activity, selenium supplementation of the diet of these patients should be an adjunct therapy in situations of selenium deficiency. Another study demonstrated that PKU patients present elevated products of lipid peroxidation in plasma and diminished activities of plasma and erythrocyte GSH-Px [28]. These findings indicate that other factors may influence GSH-Px activity in PKU. Moreover, decreased serum ubiquinone-10 levels were recently observed in PKU patients [29]. Therefore, oxidative stress may be related to brain damage in PKU, but the exact mechanisms underlying these effects remain to be elucidated.

In the present study we investigated various parameters of oxidative stress in the brain of rats subjected to chemically induced hyperphenylalaninemia (HPA), namely chemiluminescence, total radical-trapping antioxidant potential (TRAP), superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) activities. We also examined the in vitro effects of Phe on the same parameters in the brain of 6-day-old rats. The results revealed alterations of various of these parameters, indicating that oxidative stress may be one of the mechanisms by which Phe provokes brain damage in PKU. Further studies on the oxidative stress status of PKU patients appear to be worthwhile in order to determine whether an antioxidant therapy may be of benefit to these patients.

2. Materials and methods

2.1. Reagents and equipment

All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, USA) except for the RAN-SOD kit which was purchased from Randox (USA). Phe and MePhe were dissolved on the day of the experiment in distilled water for in vitro assays, and in saline for subcutaneous administration with the pH adjusted to 7.4 when necessary. Chemiluminescence and TRAP were assayed using a beta liquid scintillation spectrometer (Packard Tricarb 2100TR and Wallac model 1409, respectively) and the enzyme
activities were measured with a double-beam spectrophotometer with temperature control (Hitachi U-2001).

2.2. Animals

Six- to 14-day-old Wistar rats bred in the Department of Biochemistry, UFRGS, were used. Rats were kept with dams while receiving the drugs until they were killed. The dams had free access to water and to a standard commercial chow (Germani, Porto Alegre, RS, Brazil) containing 20.5% protein (predominantly soybean), 54% carbohydrate, 4.5% fiber, 4% lipids, 7% ash and 10% moisture. Temperature was maintained at 24 ± 1°C, with a 12:12-h light/dark cycle. The Principles of Laboratory Animal Care (NIH publication no. 85-23, revised 1985) were followed in all the experiments.

2.3. Tissue preparation

On the day of the experiments, animals were killed by decapitation without anesthesia and the brain was immediately removed. The olfactory bulbs and pons/medulla were discarded. The rest of the brain (forebrain or cerebrum) was weighed and kept chilled until homogenization. The brain was homogenized in the incubation medium used for each technique and centrifuged at 1000 \( \times \) g for 10 min at 4°C, and the supernatant was immediately used for the measurements. For the enzyme activity determinations, the brain tissue was kept frozen at \(-74°C\) for up to 1 week.

2.4. Chemically induced HPA

We induced HPA in rats as described by Wyse et al. [16,17]. Suckling animals received subcutaneous injections of 2.1 \( \mu \)mol Phe plus 1.6 \( \mu \)mol MePhe per gram body weight. The substances were dissolved in saline by heating. Controls received equivalent volumes of saline. The acute model consisted of one injection of MePhe on the sixth day of life and Phe on the seventh day (after a 24-h interval). To produce the chronic HPA model, rats received the drugs from the sixth to the 13th day. The same doses utilized in the acute treatment were used. MePhe was administered once a day and Phe two times daily (with a 9-h interval). Rats were killed 14 h after the last injection of Phe. Treated animals achieved maximal plasma Phe levels 30 min after the subcutaneous injection (3.6 ± 0.2 mM). Maximal brain levels were achieved 60 min after Phe administration (1 ± 0.1 mmol/kg wet weight tissue). Brain Phe concentrations returned to normal levels 14 h after treatment (0.15 ± 0.01 mmol/kg wet weight tissue).

2.5. In vitro experiments

Brain homogenates were incubated for 1 h at 37°C in the presence of Phe or MePhe, and controls were incubated with medium only. The final concentrations of each substance in the incubation medium ranged from 0.5 to 5 mM. After incubation, aliquots were taken to measure chemiluminescence and TRAP. To test the action of Phe on the enzymatic antioxidant defenses (CAT, SOD and GSH-Px), the amino acid was added to the incubation medium at the time of assay of each enzyme activity without previous incubation with tissue homogenate.

2.6. Chemiluminescence

Samples were assayed for chemiluminescence in a dark room by the method of Gonzalez-Flecha et al. [30]. Incubation flasks contained 3.5 ml of medium consisting of 20 mM sodium phosphate (pH 7.4) and 140 mM KCl. The background chemiluminescence was measured and 0.5 ml of homogenate immediately added. Chemiluminescence was measured for 30 min at room temperature. The background chemiluminescence was subtracted from the total value and the results are represented as cps per mg protein.

2.7. Total radical-trapping antioxidant potential (TRAP)

TRAP represents the total antioxidant capacity of the tissue and was determined by measuring the luminol chemiluminescence intensity induced by 2,2'-azo-bis(2-aminopropane) (ABAP) [31] at room temperature. Tissue was homogenized 1:10 (w/v) in 0.1 M glycine buffer, pH 8.6, which was also used to prepare the other solutions. Four ml of 10 mM ABAP was added to the vial and the background chemiluminescence was measured. Ten \( \mu \)l of 4 mM
luminol was then added and the chemiluminescence was measured. This was considered to be the initial value. Ten µl of 80 µM Trolox or homogenates was added and chemiluminescence was measured until it reached the initial levels. The addition of Trolox or tissue homogenate to the incubation medium reduces the chemiluminescence. The time necessary to return to the levels present before the addition was considered to be the induction time. The induction time is directly proportional to the antioxidant capacity of the tissue and was compared to the induction time of Trolox. The results are reported as nmol of trolox per mg protein.

2.8. Catalase assay

CAT activity was assayed by the method of Aebi [32] which is based on the disappearance of H$_2$O$_2$ at 240 nm. Brain tissue was homogenized 1:10 (w/v) in 10 mM potassium phosphate buffer, pH 7.4. One unit is defined as 1 µmol of hydrogen peroxide consumed per minute and the specific activity is reported as units per mg protein.

2.9. Superoxide dismutase assay

The assay of SOD activity was carried out with the RANSOD kit (Randox). Cerebral tissue was homogenized 1:10 (w/v) in 10 mM potassium phosphate buffer, pH 7.4. 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. 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 mg protein.

2.10. Glutathione peroxidase assay

GSH-Px activity was measured by the method of Wendel [33], except for the concentration of NADPH which was adjusted to 0.1 mM after previous tests performed in our laboratory. Tissue was homogenized 1:10 (w/v) in 10 mM potassium phosphate buffer, pH 7.6. tert-butyl-Hydroperoxide was used as substrate. NADPH disappearance was monitored with a spectrophotometer at 340 nm. One GSH-Px unit is defined as one µmol of NADPH consumed per minute and specific activity is represented as units per mg protein.

2.11. Protein determination

Protein was measured by the method of Lowry et al. [34] using bovine serum albumin as standard.

2.12. Statistical analysis

Data were analyzed by the Student’s t-test or by one-way analysis of variance followed by the Duncan multiple range test when the F value was significant. All analyses were performed using the Statistical Package for the Social Sciences (SPSS) software.

3. Results

3.1. Chemically induced HPA

The effects of acute and chronic administration of Phe plus MePhe on TRAP and chemiluminescence were initially studied. Fig. 1 shows that TRAP was significantly decreased in brain homogenates from rats acutely ($t(12) = 4.95; P < 0.01$) and chronically treated ($t(12) = 4.19; P < 0.01$) with Phe plus MePhe.
as compared to control rats injected with saline. In contrast, chemiluminescence in brain homogenates from rats submitted to the acute \((t(10) = 3.87; P < 0.01)\) and chronic \((t(10) = 4.46; P < 0.01)\) HPA model was significantly increased compared to saline-treated animals (Fig. 2).

Furthermore, brain CAT activity (Fig. 3) was not altered in the acute HPA model \((t(10) = 0.33; P > 0.05)\) but was strongly reduced in chronically treated rats \((t(10) = 2.76; P < 0.05)\). In turn, the activity of SOD in the homogenates was not affected by the acute \((t(12) = 0.52; P > 0.05)\) or chronic HPA model \((t(12) = 1.79; P > 0.05)\), as shown in Fig. 4. Finally, rats subjected to the acute \((t(12) = 5.43; P < 0.01)\) and chronic \((t(12) = 3.54; P < 0.01)\) treatments showed a significant decrease in brain GSH-Px activity (Fig. 5).

### 3.2. In vitro experiments

Next, we tested the in vitro effect of Phe at concentrations resembling those found in PKU patients on TRAP and chemiluminescence (Table 1). We observed that Phe at concentrations of 1 mM and higher significantly diminished the antioxidant capacity (TRAP) of brain homogenates after 1 h incubation \((F(4,25) = 3.60; P < 0.05)\) and stimulated chemiluminescence \((F(4,25) = 15.60; P < 0.001)\) in brain homogenates from 6-day-old rats. Moreover, we also observed a significantly inverse correlation between chemiluminescence and TRAP \((r^2 = -0.79; P < 0.01)\), as illustrated in Fig. 6. These results
strongly indicate that Phe increases the in vitro oxidative stress in brain from young rats. To study the mechanisms by which Phe reduces the antioxidant capacity when administered to rats, we tested the effect of this amino acid on the activity of the principal enzymes responsible for the antioxidant defense in the brain homogenates from 6-day-old rats (Table 2). CAT activity was strongly inhibited by Phe at all concentrations used \((F(4,15) = 90.36; P < 0.001)\), whereas the activities of SOD \((F(4,15) = 1.26; P > 0.05)\) and GSH-Px \((F(4,30) = 1.17; P > 0.05)\) were not altered by the presence of Phe in the incubation medium.

We also studied the in vitro effect of a high concentration \((5 \text{ mM})\) of MePhe, the inhibitor of phenylalanine hydroxylase activity used together with Phe to induce experimental HPA in rats, on these parameters. The results obtained for five rats (mean ± S.D.) revealed that MePhe does not alter the antioxidant capacity (control: \(9.37 ± 0.9 \text{ nmol trolox/mg protein; MePhe } 8.79 ± 0.68 \text{ nmol trolox/mg protein; } t(8) = 1.33; P > 0.05\)) or the chemiluminescence (control: \(2816 ± 223 \text{ cps/mg protein; MePhe } 2930 ± 184 \text{ cps/mg protein; } t(8) = 0.75; P > 0.05\)), suggesting that MePhe by itself does not interfere with the oxidative stress parameters measured.

4. Discussion

Phenylketonuric patients are clinically characterized by marked neurological dysfunction which is usually related to tissue Phe levels. Therefore, early institution of a therapy based on a Phe-restricted diet was developed by Bickel and coworkers in 1953 and proved to effectively prevent the mental retardation of affected individuals [35–37]. These observations and the findings that Phe derivatives do not reach toxic levels in brain indicate that Phe is the main neurotoxic agent in PKU [1,38]. However, although neurologic symptoms predominate, the underlying mechanisms responsible for brain damage seem to be multiple and have not been completely elucidated. Furthermore, despite the apparently appropriate Phe-restricted diet, some patients present poor intellectual and school performance for reasons not yet understood, a fact indicating that new therapeutic approaches should be devised to treat phenylketonuric patients [6,40–42].

Increased plasma malondialdehyde levels, as well as reduced blood selenium content and GSH-Px activity, have been recently described in hyperphenylalaninemic patients [26,28]. However, no symptoms observed in these patients could be directly attributed

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<td><strong>In vitro effect of L-phenylalanine on total radical-trapping antioxidant potential (TRAP) and chemiluminescence in brain homogenates from 6-day-old rats</strong></td>
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<td><strong>L-Phenylalanine concentration (mM)</strong></td>
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Results are mean ± S.D. for experiments performed in duplicate. TRAP is calculated as nmol of trolox per mg protein and chemiluminescence is calculated as cps per mg protein. *P < 0.05, **P < 0.01 compared to control (Duncan multiple range test).

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<td><strong>In vitro effect of L-phenylalanine on antioxidant enzyme activities in brain homogenates from 6-day-old rats</strong></td>
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<td><strong>Enzyme activities (units/mg protein)</strong></td>
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<td><strong>L-Phenylalanine concentration (mM)</strong></td>
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<td><strong>GSH-Px ((n = 7))</strong></td>
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Results are mean ± S.D. for experiments performed in duplicate. One CAT unit is defined as 1 μmol of H₂O₂ consumed per minute. One SOD unit is defined as 50% inhibition of red formazan formation. One GSH-Px unit is defined as 1 μmol of NADPH consumed per minute. *P < 0.01 compared to control (Duncan multiple range test).
to selenium deficiency [28]. Furthermore, the low activities of erythrocyte GSH-Px observed in many hyperphenylalaninemic patients were not altered by the selenium-supplemented formulas employed, suggesting the absence of a cause/effect relationship between plasma selenium levels and erythrocyte GSH-Px activity [26]. The same authors also observed that neurological disturbances were more frequent in these patients than in those with normal GSH-Px activity.

Another interesting result is that ubiquinone-10, a substance with antioxidant action, is found at decreased concentrations in serum of PKU patients [29].

We decided to study a few oxidative stress parameters in brain of rats subjected to the widely used HPA model based on the administration of Phe plus MePhe, a phenylalanine hydroxylase inhibitor [43,44]. This Phe analogue has been tested by others on many neurochemical parameters and was considered a low-toxic agent compared to the other phenylalanine hydroxylase inhibitor, p-chlorophenylalanine [44].

In this study, we have demonstrated that chemiluminescence is significantly increased and TRAP is reduced in brain of hyperphenylalaninemic rats, indicating that chemical treatment with Phe plus MePhe induced oxidative stress in the animals. Since the in vitro experiments revealed that MePhe does not alter these parameters and that Phe increased chemiluminescence and reduced TRAP in vitro in brain homogenates, we presume that Phe induces free radical production and compromises the total antioxidant capacity of the nervous tissue. Moreover, the alteration of both parameters in a similar fashion is strongly indicative of oxidative stress since this process is defined as the imbalance between free radical production and antioxidant defenses. At this point it should be emphasized the low cerebral antioxidant defenses [23], a fact that makes this tissue more vulnerable to increases of reactive oxygen species. In fact, an increasing number of pathological situations involving the CNS, such as neurodegenerative disorders, seizures, ischemia/reperfusion, and dementia, have been associated with oxidative stress [22].

The enzymes CAT, SOD and GSH-Px are considered to be the main enzymatic defenses of the brain against free radical production. We therefore tested the effects of the acute and chronic HPA models and also the in vitro effect of Phe on these enzyme activities. CAT activity was significantly inhibited by Phe both in vitro (up to 77%) and in vivo in the chronic HPA model (55%), but not in the acute one. Regarding the mechanisms by which Phe inhibits CAT activity, the in vitro findings probably indicate a direct interaction of the amino acid with the enzyme inhibiting its activity. On the other hand, apart from acting directly on the enzyme, speculatively Phe could also have an indirect effect in chronic in vivo studies at the transcription level, blocking CAT enzyme synthesis, or otherwise increasing its degradation.

SOD activity was not affected in vivo or in vitro by Phe, a fact indicating the specificity of the amino acid effect. On the other hand, Phe inhibited GSH-Px activity in the chronic and acute in vivo model of HPA, but not in vitro. These findings suggest an indirect action of Phe, not present in the homogenates, on this enzyme activity. Again, one possibility could be an action of Phe on the enzyme turnover affecting the synthesis and/or the degradation processes of GSH-Px. Considering the results of Phe action on CAT and on GSH-Px activities as a whole, we may presume that Phe administration gives rise to a high concentration of hydrogen peroxide in the brain of the animals, secondarily leading to brain damage [45].

The present results strongly indicate that Phe stimulates oxidative stress in the brain of hyperphenyla-
laninemic rats and are in agreement with other findings observed in PKU patients. These findings include increased products of lipid peroxidation found in plasma and reduced activity of GSH-Px in plasma and erythrocytes of PKU patients [26,28]. Furthermore, normal SOD activity was observed in these patients [46], which is also in line with our results.

The concentrations of Phe at which the effects were caused in this study were similar to those observed in PKU patients [1,39,42,47,48]. Even so, it is difficult to extrapolate our findings to the human condition. However, if the effects here detected also occur in the brain of phenylketonuric patients, it is possible that they may contribute, at least in part, to the acute and chronic neurological dysfunction characteristic of this disease. Therefore, the institution of antioxidant therapy in PKU may be of interest to test whether the affected patients will benefit from this therapy which could possibly be an adjuvant to the commonly used Phe-restricted diet.

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