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Neurochemical evidence that phytanic acid induces oxidative damage and reduces the antioxidant defenses in cerebellum and cerebral cortex of rats

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ABSTRACT

Aims: In the present work we investigated the in vitro effects of phytanic acid (Phyt), that accumulates in Refsum disease and other peroxisomal diseases, on important parameters of oxidative stress in cerebellum and cerebral cortex from young rats.
Main methods: The parameters thiobarbituric acid-reactive substances levels (TBA-RS; lipid peroxidation), carbonyl formation and sulfhydryl oxidation (protein oxidative damage) and the concentrations of the most important nonenzymatic antioxidant defense reduced glutathione (GSH) were determined.
Key findings: It was observed that Phyt significantly increased TBA-RS levels in both cerebral structures. This effect was prevented by the antioxidants α-tocopherol and melatonin, suggesting the involvement of free radicals. Phyt also provoked protein oxidative damage in both cerebellum and cerebral cortex, as determined by increased carbonyl content and sulfhydryl oxidation. Furthermore, Phyt significantly diminished the concentrations of GSH, while melatonin and α-tocopherol treatment totally blocked this effect. We also verified that Phyt does not behave as a direct acting oxidant, since Phyt did not oxidize commercial solutions of GSH and reduced cytochrome *c* to Phyt in a free cell medium.
Significance: Our data indicate that oxidative stress is elicited in vitro by Phyt, a mechanism that may contribute at least in part to the pathophysiology of Refsum disease and other peroxisomal disorders where Phyt is accumulated.

Introduction

Adult Refsum disease is a disorder caused by phytanoyl-CoA hydroxylase (PHYH) deficiency and biochemically characterized by plasma and tissue accumulation of phytanic acid (Phyt) (200 μM to 1000 μM) (Wanders et al. 1993; Zomer et al. 2000). Phyt also accumulates in other peroxisomal disorders, including rhizomelic chondrodysplasia punctata type I (RCP), Zellweger syndrome, neonatal and X-linked adrenoleucodystrophy (X-ALD) and infantile Refsum disease (Gould et al. 2001; Brosius and Gartner 2002).

Adult Refsum disease is clinically characterized by progressive peripheral neuropathy, retinitis pigmentosa and cerebellar ataxia, although other symptoms including sensorineural hearing loss, ichthyosis, skeletal malformations and cardiac abnormalities may also be manifested (Wanders et al. 2001).

The major pathophysiological abnormalities observed in peroxisomal disorders include abnormalities in neuronal migration or differentiation, defects in the formation or maintenance of central white matter and postdevelopmental neuronal degeneration (Gould et al. 2001).

It was seen that reduction of dietary phytol leads to a decrease of Phyt levels and delays the progression of the symptoms in patients affected by Refsum disease (Eldjarn et al. 1966; Gibberd et al. 1979; Masters-Thomas et al. 1980; Hungerbuhler et al. 1985; Ferdinandusse et al. 2008), suggesting that Phyt is neurotoxic.

However, the exact mechanisms underlying the pathogenesis of the brain damage found in diseases where Phyt accumulates is not yet well established, though there is some evidence demonstrating that Phyt provokes mitochondrial dysfunction (Ronicke et al. 2009). In this context, it was shown that Phyt acts as a mitochondrial uncoupler, inhibits electron flow through the respiratory chain and the adenine nucleotide exchange across the inner mitocondrial membrane in mitochondria and synaptossomes from rat brain (Schonfeld et al. 2004; Reiser et al. 2006). Furthermore, this branched-chain fatty acid decreases ATP synthesis, the mitochondrial membrane potential and NAD(P)H content in digitonin-permeabilized fibroblasts (Komen et al. 2007). In turn, Reiser and colleagues (2006) observed that Phyt causes

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a remarkable increase of cytosolic Ca^{2+} levels, decreases the mitochondrial membrane potential and induces superoxide generation and cell death in mitochondria from rat whole brain and also in rat hippocampal astrocytes. It was then proposed that the cytotoxic action of Phyt is mainly due to reactive oxygen species (ROS) generation associated with respiratory chain inhibition (Schonfeld and Reiser 2008). Furthermore, a further recent study demonstrated that Phyt induces Ca^{2+} increase, mitochondrial depolarization, superoxide generation and cell death in hippocampal neurons, astrocytes and oligodendrocytes (Ronicke et al. 2009). However, there is so far no evidence that Phyt provokes lipid and protein oxidative damage in the brain.

Considering that the pathophysiology of the brain damage in Refsum disease and other disorders where Phyt accumulates is not fully established and practically nothing has been described on the effect of Phyt in cerebellum and cerebral cortex, in the present study we investigated the in vitro effects of Phyt at concentrations found in Refsum disease on important parameters of oxidative stress, namely thiobarbituric acid-reactive substances (TBA-RS) levels, carbonyl formation, sulfhydryl oxidation and glutathione (GSH) levels in cerebellum and cerebral cortex from young rats.

Materials and methods

Animals and reagents

Wistar male rats of 30 days of life (80–100 g) obtained from the Central Animal House of the Department of Biochemistry, ICBS, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS — 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 constant temperature (22 °C ± 1 °C) colony room, with free access to water and 20% (w/w) protein commercial chow (SUPRA, Porto Alegre, RS, Brazil). The experimental protocol was approved by the Ethics Committee for animal research of the Federal University of Rio Grande do Sul, Porto Alegre, Brazil and followed the "Principles of Laboratory Animal Care (NIH publication 85-23, revised 1985). All efforts were made to minimize the number of animals used and their suffering.

Chemicals were purchased from Sigma (St. Louis, MO, USA). Phyt solution was prepared on the day of the experiments in the incubation medium used for each technique and pH was adjusted at 7.4. The final concentrations of the acid in the medium ranged from 1 to 500 μM. In some experiments antioxidants were added to the incubation medium at the following final concentrations: 10 μM Trolox (TRO), 1000 μM melatonin (MEL), the combination of superoxide dismutase (SOD) plus catalase (CAT) (20 mU/mL each), 1000 μM GSH, 1000 μM N-acetylcysteine (NAC) and 750 μM N^{ω}-nitro-L-arginine (L-NAME). TBA-RS, carbonyl formation, sulfhydryl content and oxidation of cytochrome *c* were measured with a double-beam Hitachi U-2001 spectrophotometer with temperature control. GSH levels were measured in a Hitachi F-2000 fluorescence spectrophotometer.

Sample preparation and incubation

On the day of the experiments the rats were sacrificed by decapitation without anaesthesia and the brain was rapidly excised on a Petri dish placed on ice and the blood and external vessels were carefully removed. The olfactory bulbs, pons, medulla and striatum were discarded, and the cerebellum and cerebral cortex were dissected, weighed and separately homogenized in 10 volumes (1:10, w/v) of 20 mM sodium phosphate buffer, pH 7.4 containing 140 mM KCl. The homogenates of each brain structure were centrifuged at 750 g for 10 min at 4 °C to discard nuclei and cell debris (Evelson et al. 2001). The pellet was discarded and cerebellar or cortical supernatants, corresponding to a suspension of mixed and preserved organelles, including mitochondria, were separated and incubated at 37 °C for 1 h with Phyt (1-500 μM). In some experiments antioxidants were used to test their effects on Phyt-induced alterations of lipid peroxidation (TBA-RS) or of glutathione levels in rat cerebral cortex supernatants. Cortical supernatants were co-incubated for 1 h with 500 μM Phyt and either 1000 μM MEL, 10 μM TRO, a combination of SOD plus CAT (20 mU/mL each), 1000 μM GSH, 1000 μM NAC or 750 μM L-NAME. The chosen doses of the antioxidants, including TRO and MEL, were based on previous data described in the literature (Kolker et al. 2001; Latini et al. 2003) and corresponded to the highest concentrations that were not able to change per se the basal values of the parameters evaluated. Controls did not contain this fatty acid in the incubation medium. Immediately after incubation, aliquots were taken to measure TBA-RS, carbonyl content, sulfhydryl oxidation and GSH concentrations.

Thiobarbituric acid-reactive substances (TBA-RS)

TBA-RS was determined according to the method of Esterbauer and Cheeseman (1990). Briefly, 300 μL of cold 10% trichloroacetic acid were added to 150 μL of pre-treated cortical or cerebellar supernatants and centrifuged at 300 × *g* for 10 min. Three hundred μL of the supernatants were transferred to a pyrex tube and incubated with 300 μL of 0.67% TBA in 7.1% sodium sulphate on a boiling water bath for 25 min. The tubes containing the mixture were allowed to cool on running tap water for 5 min. The resulting pink-stained TBA-RS was determined in a spectrophotometer at 532 nm. A calibration curve was performed using 1,1,3,3-tetramethoxypropane, and each curve point was subjected to the same treatment as supernatants. TBA-RS values were calculated as nmol/mg protein and represented as percentage of control.

Protein carbonyl content

PCF (protein carbonyl formation), a marker of protein oxidative damage, was measured spectrophotometrically according to Reznick and Packer (1994). One hundred microliters of the aliquots from the incubation were treated with 400 μL of 10 mM 2,4-dinitrophenylhidrazine (DNPH) dissolved in 2.5 N HCl or with 2.5 N HCl (blank) and left in the dark for 1 h. Samples were then precipitated with 500 μL 20% TCA and centrifuged for 5 min at 10,000 × *g*. The pellet was then washed with 1 mL ethanol: ethyl acetate (1:1, V/V) and dissolved in 550 μL 6 M guanidine prepared in 2.5 N HCl at 37 °C for 5 min. The difference between the DNPH-treated and HCl-treated samples (blank) was used to calculate the carbonyl content determined at 365 nm. The results were calculated as nmol of carbonyl groups/mg of protein and represented as percentage of control, using the extinction coefficient of $22{,}000 \times 10^6$ nmol/mL for aliphatic hydrazones.

Sulfhydryl content

This assay is based on the reduction of 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) by thiols, generating a yellow derivative (TNB) whose absorption is measured spectrophotometrically at 412 nm (Aksenov and Markesbery 2001). Briefly, 0.1 mM DTNB was added to 120 μL of cerebellar or cortical supernatants. This was followed by a 30 min incubation at room temperature in a dark room. Absorption was measured at 412 nm. The sulfhydryl content is inversely correlated to oxidative damage to proteins. Results were reported as nmol TNB/mg protein and represented as percentage of control.

Reduced glutathione (GSH) content

GSH concentrations were measured according to Browne and Armstrong (1998). Aliquots from the incubation were diluted in 20 volumes (1:20, v/v) of 100 mM sodium phosphate buffer pH 8.0, containing 5 mM EDTA. One hundred μL of this preparation were

incubated with an equal volume of o-phthaldialdehyde (1 mg/mL methanol) at room temperature during 15 minutes. Fluorescence was measured using excitation and emission wavelengths of 350 nm and 420 nm, respectively. Calibration curve was prepared with standard GSH (0.0001 – 1 mM). GSH concentrations were calculated as nmol/mg protein and represented as percentage of control.

Oxidation of thiol groups and cytochrome c

Thiol group oxidation was tested by measuring the concentrations of a commercial solution of GSH (150 μM) after exposing this solution to 500 μM Phyt for 60 min in a medium devoid of brain supernatants. N-Ethylmaleimide (NEM 150 μM), a classical oxidant of sulfhydryl groups, was used as a positive control. After exposition, 7.4 mM o-phthaldialdehyde (1 mg/mL) was added to the vials, the mixture was incubated at room temperature during 15 min. Fluorescence was finally measured using excitation and emission wavelengths of 350 nm and 420 nm, respectively.

We also verified whether 500 μM Phyt were able to oxidize a commercial solution of cytochrome *c* (0.7 g/L), which was previously reduced by sodium borohydride (40 mM). Cytochrome *c* oxidation was assayed by measuring the absorbance decrease at 550 nm in a medium devoid of brain supernatants.

Protein determination

Protein content was determined in cortical and cerebellar supernatants by the method of Lowry and colleagues (1951), using bovine serum albumin as a 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. All statistical 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

Phyt induces lipid peroxidation

Fig. 1A shows that TBA-RS values were significantly increased in cerebellum (up to 70%) [$F_{(4,25)}=30.336$; $P<0.001$] and cerebral cortex (up to 100%) [$F_{(4,25)}=86.268$; $P<0.001$] exposed for 1 h to Phyt. Most important, Phyt already provoked a significant increase of TBA-RS levels in cerebellum at very low doses, whereas only much higher doses caused significant effects in cerebral cortex.

The next set of experiments was carried out to evaluate the role of antioxidants on Phyt-induced increase of TBA-RS levels in cerebral cortex. Our results demonstrate that MEL totally prevented and TRO attenuated the lipid peroxidation induced by Phyt [$F_{(7,32)}=16.152$; $P<0.001$] (Fig. 1B). These results indicate that Phyt induces lipid oxidative damage in rat brain probably through induction of ROS.

Phyt provokes protein oxidative damage

Next, we evaluated the in vitro effect of Phyt on carbonyl formation and sulfhydryl oxidation in cerebellar and cortical supernatants. Fig. 2A shows that Phyt significantly increased carbonyl formation in cerebellum (up to 40%) [$F_{(4,20)}=5.606$; $P<0.01$] and cerebral cortex (up to 203%) [$F_{(4,15)}=29.500$; $P<0.001$]. We also observed that Phyt induced sulfhydryl oxidation in both structures (up to 53% in cerebellum) [Cerebellum: $F_{(4,25)}=23.278$, $P<0.001$] (up to 55% in cerebral cortex) [Cerebral cortex: $F_{(4,25)}=29.344$; $P<0.001$] in a dose-dependent fashion [Cerebellum: $\beta=-0.886$; $P<0.001$] [Cerebral cortex: $\beta=-0.890$; $P<0.001$] (Fig. 2B). Furthermore, only the highest dose (500 μM) of Phyt caused a significant increase of carbonyl content in cerebellum, whereas 100 μM already provoked a significant effect in cortical supernatants. The present data indicate that Phyt induces protein oxidative damage in rat brain.

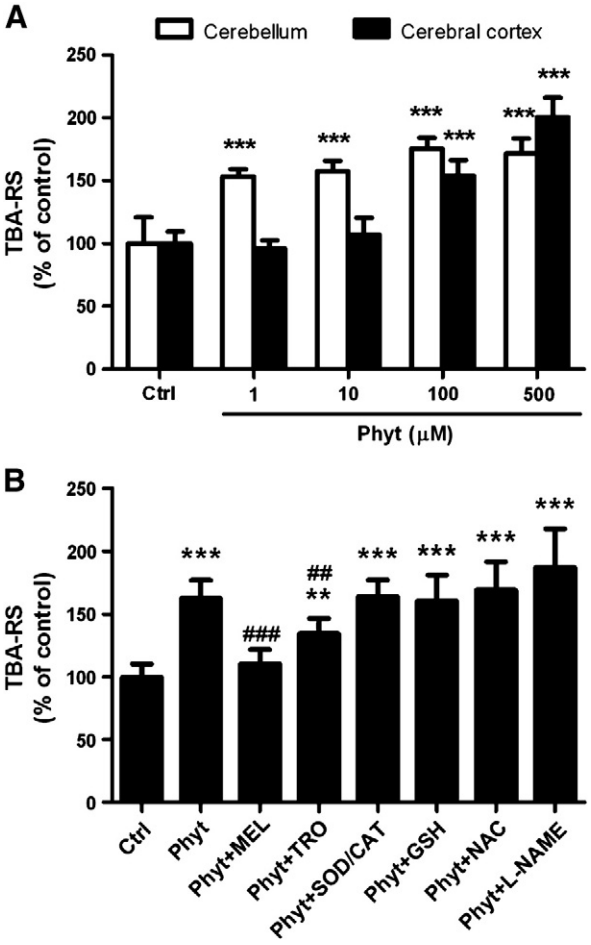

Fig. 1. In vitro effect of phytanic acid (Phyt) on thiobarbituric acid-reactive substances (TBA-RS) values in rat cerebellum and cerebral cortex. Cerebellar and cortical supernatants were incubated in the presence of Phyt (A). In some experiments antioxidants were used to test their effects on Phyt-induced increase of lipid peroxidation (TBA-RS) in rat cerebral cortex supernatants (B). Values are means ± standard deviation for six independent (animals) experiments performed in triplicate and are expressed as percentage of controls (Controls: TBA-RS levels [nmol/mg protein]: (A): cerebellum: 3.63 ± 0.74; cerebral cortex: 1.81 ± 0.18); (B): cerebral cortex: 2.01 ± 0.19. ** $P<0.01$, *** $P<0.001$, compared to control; ## $P<0.01$, ### $P<0.001$, compared to 500 μM Phyt (Duncan multiple range test).

Phyt diminishes non-enzymatic antioxidant defenses

The non-enzymatic antioxidant defenses were also investigated by assessing GSH levels. Fig. 3A shows that Phyt significantly diminished GSH levels in cerebellum (up to 44%) [$F_{(4,20)}=62.895$; $P<0.001$] and cerebral cortex (up to 49%) [$F_{(4,25)}=65.297$; $P<0.001$] in a dose dependent manner [Cerebellum: $\beta=-0.952$; $P<0.001$] [Cerebral cortex: $\beta=-0.955$; $P<0.001$].

We also tested whether TRO, MEL and SOD plus CAT could prevent Phyt-induced decrease of GSH content in cerebral cortex. The results demonstrate that MEL and TRO totally prevented the decrease of GSH content caused by Phyt [$F_{(4,25)}=13.573$; $P<0.001$] (Fig. 3B).

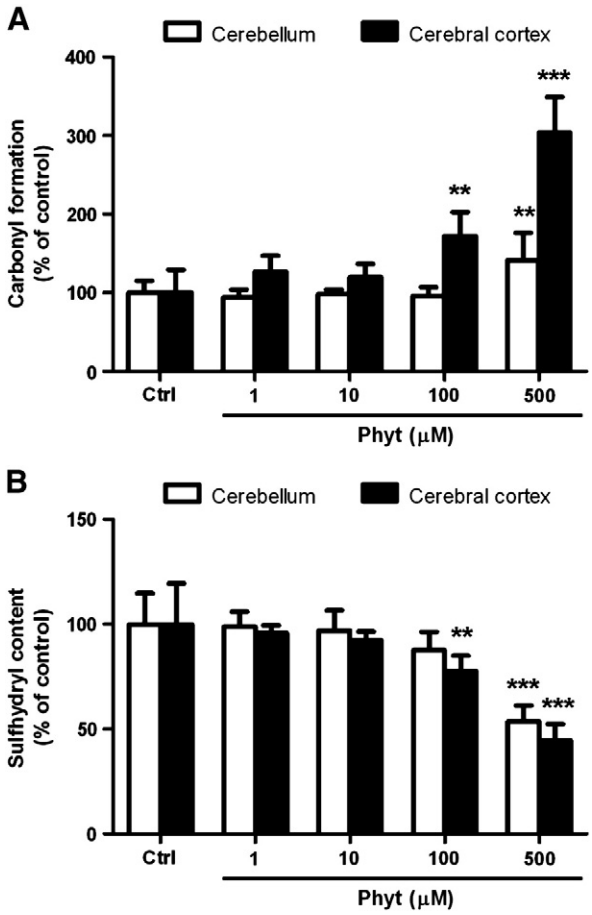

Fig. 2. In vitro effect of phytanic acid (Phyt) on carbonyl formation (A) and sulfhydryl oxidation (B) in rat cerebellum and cerebral cortex. The supernatants were incubated in the presence of Phyt. Values are means ± standard deviation for four to six independent (animals) experiments performed in triplicate and are expressed as percentage of controls (Controls: carbonyl content [nmol/mg protein] (A): cerebellum: 0.95 ± 0.32; cerebral cortex: 0.40 ± 0.12; sulfhydryl content [nmol/mg protein] (B): cerebellum: 81.8 ± 12; cerebral cortex: 61.9 ± 13). ** $P<0.01$, *** $P<0.001$, compared to control (Duncan multiple range test).

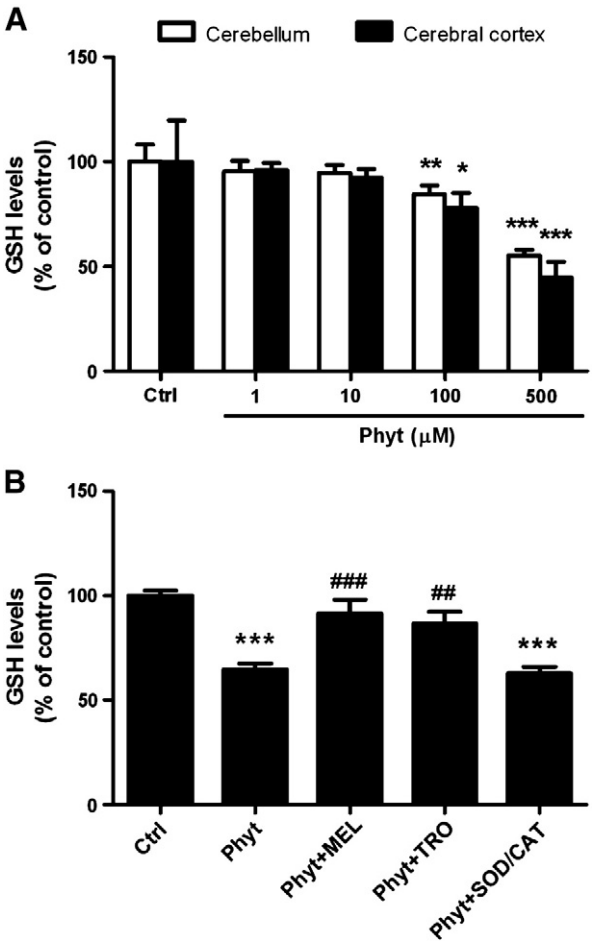

Fig. 3. In vitro effect of phytanic acid (Phyt) on reduced glutathione (GSH) levels in rat cerebellum and cerebral cortex. Cerebellar and cortical supernatants were incubated in the presence of Phyt (A). In some experiments antioxidants were used to test their effects on Phyt-induced decrease of GSH levels in rat cerebral cortex supernatants (B). Values are means ± standard deviation for five to six independent (animals) experiments performed in triplicate and are expressed as percentage of controls (Controls: GSH levels [nmol/mg protein]: (A): cerebellum: 6.95 ± 0.52; cerebral cortex: 6.19 ± 0.22; (B): cerebral cortex: 4.01 ± 0.24). * $P<0.05$, ** $P<0.01$, *** $P<0.001$, compared to control; ## $P<0.01$, ### $P<0.001$, compared to 500 μM Phyt (Duncan multiple range test).

Phyt does not behave as a direct oxidant

Finally, we investigated whether Phyt could by itself act as an oxidant agent. We therefore exposed commercial solutions of GSH or cytochrome *c* to 500 μM Phyt in the absence of cerebral tissue. Fig. 4 shows that Phyt per se did not modify GSH levels, whereas N-ethylmaleimide (NEM, 150 μM) exposition (positive control) markedly oxidized GSH. We also verified that Phyt was not able to oxidize a cytochrome *c* commercial solution reduced by sodium borohydride in a cell free medium (results not shown). Taken together, these data indicate that Phyt does not behave as a direct oxidant.

Discussion

The accumulation of the saturated branched-chain fatty acid Phyt is characteristic of peroxisomal fatty acid oxidation defects, particularly Refsum disease. Although neurological symptoms are severe and cerebellum is specially affected, the exact mechanisms underlying the neuropathology of these disorders remain to be elucidated. However, some reports have suggested that Phyt causes mitochondrial dysfunction (Schonfeld et al. 2004; Reiser et al. 2006; Komen et al. 2007), while other reports revealed that this compound induces reactive oxygen species generation and cell death in cultured hippocampal neural cells (Kahlert et al. 2005; Ronicke et al. 2009). It was also presumed that the cytotoxic effect of Phyt is mainly due to ROS generation associated with respiratory chain inhibition (Schonfeld and Reiser 2008). The present study investigated the in vitro effects of Phyt on lipid and protein oxidative damage and on the major nonenzymatic antioxidant defense

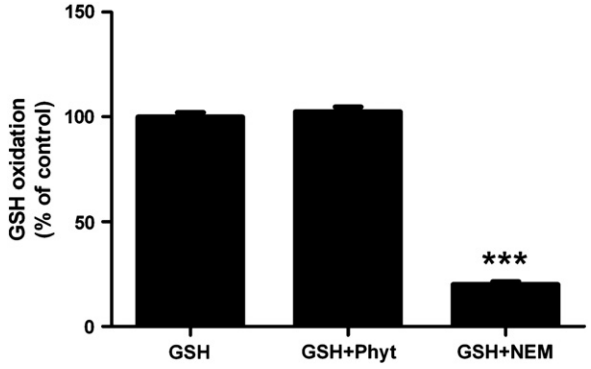

Fig. 4. Effect of phytanic acid (Phyt) on the oxidation of a commercial solution of reduced glutathione (GSH) in the absence of brain tissue. Values are means ± standard deviation for three independent experiments performed in triplicate and are expressed as percentage of controls (Control [fluorescence units]: 5969 ± 365). GSH content was measured in the absence of brain tissue. NEM = N-ethylmaleimide (positive control). *** $P<0.001$ compared to controls (Duncan multiple range test).

(GSH) in cerebellum and cerebral cortex of young rats in the hope to clarify whether oxidative stress is an important mechanism of brain damage caused by Phyt. Clarifying the molecular mechanisms and actions of Phyt is expected to lead not only to new physiochemical findings, but also clues to unknown information processing systems controlling the brain functions, which may promote the understanding of the cause pathology of neurological disorders and the development of new treatment methods in disorders in which Phyt accumulates. The doses of Phyt (1–500 μM) utilized in the in vitro assays were at the range of those appearing in plasma of patients affected by Refsum disease (up to 1 mM) (Wierzbicki et al. 2003; Ferdinandusse et al. 2008).

We first verified that Phyt provoked an increase of TBA-RS levels, which reflects the amount of malondialdehyde formation, an end product of membrane fatty acid peroxidation (Halliwell and Gutteridge 2007), in cerebellum and also in cerebral cortex but at higher doses. We also observed that lipid peroxidation induction caused by Phyt was attenuated by TRO and totally prevented by MEL, indicating that peroxyl and hydroxyl radicals were mainly involved in this effect since trolox and melatonin scavenge respectively these radicals. Otherwise, it is presumed that reactive nitrogen species and superoxide or hydrogen peroxide radicals were not involved in the lipid oxidative damage exerted by Phyt in the brain since the nitric oxide inhibitor L-NAME and the combination of SOD plus CAT did not modify the Phyt-induced increase of TBA-RS values. Interestingly, it was previously found that Phyt activates the inducible nitric oxide synthase (NOS) in vascular smooth muscle cells (Idel et al. 2002). It is therefore conceivable that the isoform found in vascular cells has different activating properties, as compared to the neuronal NOS which is well expressed in the brain.

Phyt also elicited an increase in carbonyl formation and sulfhydryl oxidation, indicating that this fatty acid provokes protein oxidative damage. We also observed that relatively to carbonyl formation, cerebral cortex was affected at a higher degree than the cerebellum. Carbonyl groups (aldehydes and ketones) are mainly produced by oxidation of amino acid side chains (especially Pro, Arg, Lys, and Thr) of proteins or from the reaction of reducing sugars or their oxidation products with lysine protein residues (Dalle-Donne et al. 2003). Although protein carbonyls are usually caused by ROS-mediated protein damage, we cannot exclude the possibility that aldehydes resulting from lipid peroxidation also induced carbonyl generation (Dalle-Donne et al. 2003). Otherwise, it should be stressed that protein sulfhydryl groups from cysteine residues can be oxidized to form disulfide, potentially altering the redox state of proteins and leading to their inactivation (Kuhn et al. 1999).

With regard to the antioxidant defense system, Phyt similarly reduced the total content of GSH in cerebellum and cerebral cortex that was completely blocked by TRO and MEL, implicating hydroxyl and peroxyl radicals, reinforcing the role of these radicals on Phyt-induced oxidative damage. Furthermore, Phyt was not able to oxidize both sulfhydryl groups from a commercial purified GSH solution and a commercial solution of reduced cytochrome *c* in the absence of brain tissue (cell free system), indicating that it does not behave as a direct oxidant. Taken together our data showing that TRO and MEL abolished Phyt-induced increase of lipid peroxidation and decrease of the major tissue antioxidant defense, and that Phyt does not behave as a direct pro-oxidant, it may be suggested that its effects are mediated by ROS generation.

Recent studies have suggested that Phyt increases superoxide generation by interacting with complexes I and III of the respiratory chain (Reiser et al. 2006; Schonfeld and Wojtczak 2007). Our present data demonstrated that Phyt induction of lipid and protein oxidative damage and reduction of the concentration of the major cerebral antioxidant defense (GSH) in cerebellum and cerebral cortex was probably mediated by the generation of peroxyl and hydroxyl radicals. We cannot at the present establish the mechanisms by which this fatty acid elicited reactive oxygen species. However it is conceivable that peroxyl radicals, an end product of lipid oxidation, were produced by the attack of the most toxic radical hydroxyl to lipid membranes (Delanty and Dichter 1998; Halliwell and Whiteman 2004; Halliwell and Gutteridge 2007). Hydroxyl radical is mainly produced by the Fenton reaction from hydrogen peroxide, which is formed from superoxide (Adam-Vizi 2005). Superoxide originated in the mitochondria mainly from a blockage of the respiratory chain has a very short half-life, being dismutated by manganese SOD (Mn-SOD) in the mitochondrial matrix or by copper/zinc SOD (Cu,Zn-SOD) in the intermembrane space and the cytosol giving rise to the formation of H_2O_2. Although the combination of SOD plus CAT, which scavenges superoxide and hydrogen peroxide respectively, did not prevent the pro-oxidant effects of Phyt in our experimental conditions, we cannot completely rule out a secondary effect of Phyt primarily inhibiting the electron flow through the respiration chain and generating superoxide. It is conceivable that the dismutation of superoxide by endogenous SOD followed by the Fenton reaction generating hydroxyl radical that occurs at a high rate possibly avoided superoxide detection in our experiments (Adam-Vizi 2005), rendering the prevention of Phyt-induced TBA-RS increase and reduction of GSH levels by the combination of SOD plus CAT impossible. However, more investigation is needed to clarify these pathways.

Regarding the toxicity of Phyt towards the studied brain structures, we found that lipid peroxidation occurred at very low Phyt concentrations in the cerebellum, whereas protein oxidation was more marked in the cerebral cortex. Although we do not have at the present an exact explanation for these differential effects, it is conceivable that cerebellum was more vulnerable to Phyt lipid pro-oxidant effect due to its high lipid content relatively to the cerebral cortex (Bazan et al. 1971). Otherwise, we do not know the reasons by which cerebral cortex was more vulnerable to protein oxidation caused by Phyt, as compared to the cerebellum.

Our results are in accordance with previous findings showing increase in ROS generation in hippocampal neural cell cultures (Kahlert et al. 2005; Ronicke et al. 2009). We used a large spectrum of oxidative stress parameters and demonstrated that Phyt induces lipid and protein oxidative damage and reduces the major brain antioxidant defense (Halliwell 1992), strongly indicating that this branched-chain fatty acid accumulated in Refsum disease and other peroxisomal disorders elicits oxidative stress in cerebellum and cerebral cortex possibly by other mechanisms than blockage of the respiratory chain (Halliwell and Gutteridge 2007). Interestingly, the brain has low cerebral antioxidant defenses compared with other tissues (Halliwell 1992), a fact that makes this tissue more vulnerable to increased reactive species. Furthermore, the brain has also a high content of iron, facilitating the Fenton reaction and leading to hydroxyl radical generation (Halliwell and Gutteridge 2007).

We cannot establish the pathophysiological significance of our present results since brain concentrations of Phyt in Refsum disease are unknown. However, considering that patients with adult Refsum disease usually show plasma Phyt concentrations above 200 μM, which may reach up to 1000 μM (Wierzbicki et al. 2003; Ferdinandusse et al. 2008), and that the significant alterations of the oxidative stress parameters elicited by Phyt occurred at these levels, it may be presumed that Phyt-induced pro-oxidant effects as here demonstrated may be of pathological relevance. It should be also stressed that increase in plasma concentration of Phyt is correlated with the severity of the disease and Phyt decrease with improvement of the symptoms (Eldjarn et al. 1966; Gibberd et al. 1979; Masters-Thomas et al. 1980; Hungerbuhler et al. 1985; Ferdinandusse et al. 2008), indicating that this fatty acid is neurotoxic.

Conclusion

In conclusion, the present data strongly indicate that oxidative stress is induced by Phyt in brain of young rats. In case the present in vitro findings are confirmed in vivo in animal experiments and also in

tissues from patients affected by Refsum disease, it is tempting to speculate that reactive species may contribute, at least in part, to the pathophysiology in this disorder. Finally, it may be proposed that the administration of antioxidants should be considered as an adjuvant therapy to specific diets or to other pharmacological agents for these patients.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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