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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