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Research Report

Pristanic acid promotes oxidative stress in brain cortex of young rats: A possible pathophysiological mechanism for brain damage in peroxisomal disorders

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ABSTRACT

Pristanic acid (Prist) is accumulated in various peroxisomal disorders characterized by severe neurological dysfunction whose pathogenesis is poorly understood. Since oxidative damage has been demonstrated in brain of patients affected by neurodegenerative disorders, in the present work we investigated the *in vitro* effects of Prist on important parameters of oxidative stress in cerebral cortex from young rats. Prist significantly increased malondialdehyde levels, reflecting an increase of lipid peroxidation. This effect was totally prevented by the free radical scavenger melatonin, suggesting the involvement of reactive species. Prist also provoked protein oxidative damage, as determined by increased carbonyl formation and sulfhydryl oxidation. Otherwise, it did not alter nitric oxide production, indicating that nitrogen reactive species were not implicated in the lipid and oxidative damage provoked by Prist. Furthermore, the concentration of glutathione (GSH), the major brain non-enzymatic antioxidant defense, was significantly decreased by Prist and this decrease was fully prevented by melatonin and attenuated by α-tocopherol. It is therefore presumed that Prist elicits oxidative stress in the brain probably via reactive oxygen species formation and that this pathomechanism may possibly be involved in the brain damage found in patients affected by peroxisomal disorders where Prist accumulates.

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Abbreviations: ANOVA, Analysis of variance; CAT, Catalase; DNPH, 2,4-Dinitrophenylhidrazine; DTNB, 2-Dithio-bis(2-nitrobenzoic acid); GSH, Glutathione; MEL, Melatonin; MRI, Magnetic resonance image; NAC, N-Acetylcysteine; NEM, N-Ethylmaleimide; L-NAME, N^{ω}-nitro-L-arginine methyl ester; PPARα, Peroxisome proliferator-activated receptor α; Prist, Pristanic acid; SOD, Superoxide dismutase; TBA-RS, Thiobarbituric acid-reactive substances; TCA, Trichloroacetic acid; TRO, Trolox; SPSS, Statistical Package for the Social Sciences

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1. Introduction

Pristanic acid (Prist) (2,6,10,14-tetramethylpentadecanoic acid), a branched-chain fatty acid derived from peroxisomal α-oxidation of phytanic acid, accumulates in various inherited peroxisomal disorders (Wanders et al., 2001). These disorders can be due to a single-protein defect or by peroxisome biogenesis disorders. The first group comprehends defects in which a single metabolic function is deficient, whereas the second is characterized by a deficiency of peroxisome biogenesis leading to an impairment of multiple peroxisome functions (Gould et al., 2001; Wanders et al., 2001; Brosius and Gartner, 2002). The frequency of these disorders is estimated in 1:20,000–1:100,000 births (Gould et al., 2001; Wanders et al., 2001; Brosius and Gartner, 2002). The highest concentrations of Prist occurs in D-bifunctional protein and α-methylacyl-CoA racemase deficiencies (single-protein defects), as well as in Zellweger syndrome (peroxisome biogenesis disorders) (Gould et al., 2001; Wanders et al., 2001; Brosius and Gartner, 2002; Johnson et al., 2003; Ronicke et al., 2009) achieving 100–300 μM in plasma of the affected patients (Zomer et al., 2000; Ferdinandusse et al., 2002).

The clinical presentation of these disorders is predominantly characterized by neurological symptoms, such as hypotonia, global developmental delay and seizures, although abnormal facial appearance, feeding difficulty and liver disease also occur (Gould et al., 2001; Wanders et al., 2001; Brosius and Gartner, 2002). The most common findings in magnetic resonance imaging (MRI) involve progressive white matter abnormalities and cortical atrophy (Gould et al., 2001; Wanders et al., 2001), whose pathophysiology is poorly known. However, it was recently shown that Prist increases the intracellular Ca^{2+} level and reduces the mitochondrial membrane potential, besides inducing reactive oxygen species production and cell death in hippocampal neurons, astrocytes and oligodendrocytes (Ronicke et al., 2009). Furthermore, Zomer and colleagues (2000) demonstrated that Prist is a naturally occurring ligand for the peroxisome proliferator-activated receptor α (PPARα), which plays an important role in the regulation of genes involved in lipid homeostasis. Therefore, Prist might possibly contribute to the pathology of peroxisomal disorders by activating PPARα when found at pathological concentrations.

In the present study we investigated the role of Prist on important biochemical parameters of oxidative stress, namely, thiobarbituric acid-reactive substances (TBA-RS) (lipid peroxidation), sulfhydryl content and carbonyl formation (protein oxidative damage), reduced glutathione (GSH) levels and nitric oxide production in cerebral cortex of young rats in the hope to clarify the underlying mechanisms inducing neurotoxic effects of this fatty acid.

2. Results

2.1. Prist induces lipid oxidation

The effect of Prist on lipid oxidation was investigated by assessing TBA-RS levels in rat brain. Fig. 1A shows that TBA-RS values were significantly increased (up to 45%) in cortical supernatants exposed for 1 h to Prist [$F_{(4,25)}=12.494$; $P<0.001$] in a dose-dependent manner [$\beta=0.768$; $P<0.001$]. Considering that TBA-RS reflects the amount of malondialdehyde formed in the medium, which is a product of lipid oxidation. These data suggest that Prist induces lipid oxidative damage.

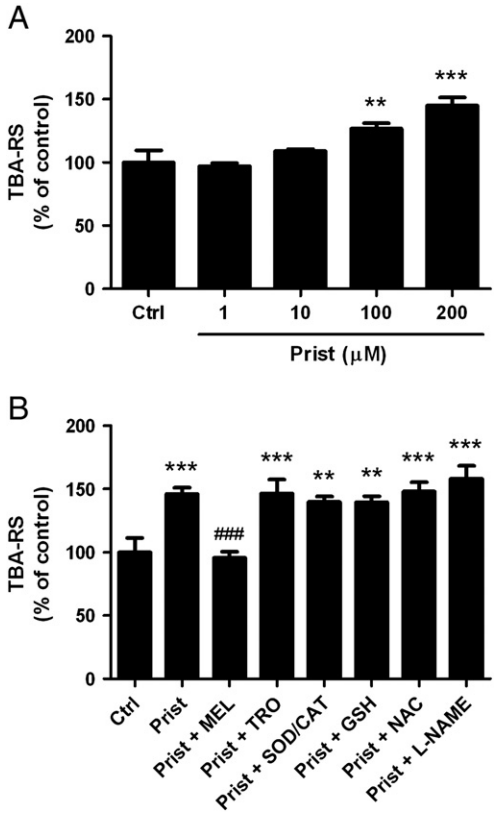

Fig. 1 – *In vitro* effect of pristanic acid (Prist) on thiobarbituric acid-reactive substances (TBA-RS) values in rat cerebral cortex (A). Cortical supernatants were incubated during 1 h in the presence of Prist at concentrations ranging from 1 to 200 μM (A). In some experiments 200 μM Prist was co-incubated during 1 h with the antioxidants 1.0 mM melatonin (MEL), 10 μM Trolox (TRO, soluble α-tocoferol), combination of 20 mU/mL superoxide dismutase (SOD) plus 20 mU/mL catalase (CAT), 1.0 mM reduced glutathione (GSH), 1 mM N-acetylcysteine (NAC) or 750 μM N^{ω}-nitro-L-arginine methyl ester (L-NAME) (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 (A): 2.70 ± 0.64 nmol/mg protein; (B): 3.28 ± 0.73 nmol/mg protein. $^{**}P<0.01$, $^{***}P<0.001$, compared to control; $^{###}P<0.001$, compared to 200 μM Prist (Duncan multiple range test).

We then evaluated the role of antioxidants on Prist-induced increase of TBA-RS levels. Cortical supernatants were co-incubated with the antioxidants MEL (1000 μM), TRO (10 μM), combination of SOD plus CAT (20 mU/mL each), GSH (1000 μM), NAC (1000 μM) or L-NAME (750 μM) in the presence of 200 μM Prist. Fig. 1B shows that MEL totally prevent Prist-induced *in vitro* lipoperoxidation [$F_{(7,24)}=8.805$; $P<0.001$]. These

results indicate that reactive species are involved in Prist-induced increase of lipid peroxidation.

2.2. *Prist induces protein oxidative damage*

The next set of experiments was carried out to evaluate the *in vitro* effect of Prist on carbonyl and sulfhydryl content in cortical supernatants from young rats, which are parameters that evaluate protein oxidative damage. Prist significantly increased carbonyl formation at 100 μM and higher concentrations (up to 87%) [$F_{(4,19)}=10.409$; $P<0.01$] (Fig. 2A). This branched-chain fatty acid also provoked an enhancement of sulfhydryl oxidation (up to 33%) [$F_{(4,25)}=18.877$; $P<0.001$] in a dose-dependent manner [$\beta=-0.860$; $P<0.001$] (Fig. 2B). Considering that carbonyl originates from the attack of free radicals to proteins and the sulfhydryl groups are oxidized by these reactive radicals, it is therefore presumed that Prist induces protein oxidative damage.

2.3. *Prist diminishes non-enzymatic antioxidant defenses*

The non-enzymatic antioxidant defenses were also investigated by assessing the concentrations of GSH, the naturally occurring antioxidant found in the brain, in the presence of Prist in cortical supernatants. It can be seen in Fig. 3A that Prist significantly diminished GSH levels (up to 28%) in a dose-dependent manner [$F_{(4,19)}=19.489$; $P<0.001$] [$\beta=-0.845$; $P<0.001$]. It is therefore concluded that Prist reduces the major brain antioxidant defense.

It was also tested whether the antioxidants MEL (1000 μM), TRO (10 μM), combination of SOD plus CAT (20 mU/mL each) or L-NAME (750 μM) could prevent Prist-induced decrease of GSH levels in cortical supernatants. Fig. 3B shows that MEL [$F_{(5,24)}=30.334$; $P<0.001$] fully prevented and TRO [$F_{(5,24)}=30.334$; $P<0.001$] attenuated Prist-induced decrease of GSH levels. The data indicate that Prist-elicited diminution of GSH concentrations occurred via reactive oxygen species.

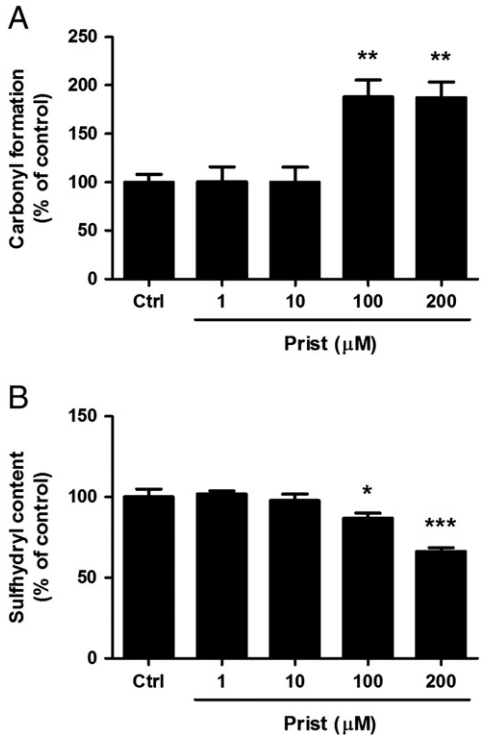

Fig. 2 – *In vitro* effect of pristanic acid (Prist) on carbonyl and sulfhydryl content in rat cerebral cortex. Cortical supernatants were incubated during 1 h in the presence of Prist at concentrations ranging from 1 to 200 μM. Values are means ± standard deviation for five to six independent (animals) experiments performed in triplicate and are expressed as percentage of controls (Controls: carbonyl content (A): 0.42 ± 0.07 nmol/mg protein; sulfhydryl content (B): 69.9 ± 8.3 nmol/mg protein). *P < 0.05, **P < 0.01, ***P < 0.001, compared to controls (Duncan multiple range test).

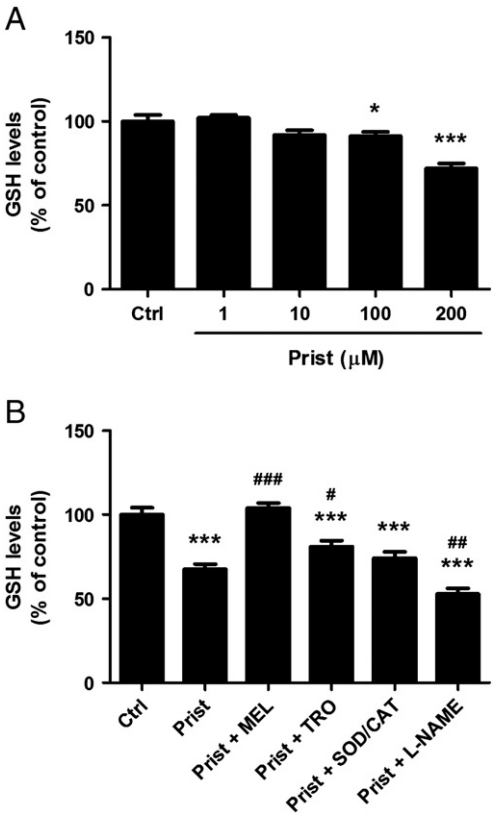

Fig. 3 – *In vitro* effect of pristanic acid (Prist) on reduced glutathione (GSH) levels (A) in rat cerebral cortex. Cortical supernatants were incubated during 1 h in the presence of Prist at concentrations ranging from 1 to 200 μM (A). In some experiments 200 μM Prist was co-incubated during 1 h with the antioxidants 1.0 mM melatonin (MEL), 10 μM Trolox (TRO, soluble α-tocoferol), combination of 20 mU/mL superoxide dismutase (SOD) plus 20 mU/mL catalase (CAT), 1.0 mM reduced glutathione (GSH), 1 mM N-acetylcysteine (NAC) or 750 μM N^{ω}-nitro-L-arginine methyl ester (L-NAME) (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 (A): 8.26 ± 0.67 nmol/mg protein; (B): 5.05 ± 0.47 nmol/mg protein). *P < 0.05, ***P < 0.001, compared to controls; #P < 0.05, ###P < 0.001, compared to 200 μM Prist (Duncan multiple range test).

2.4. Prist does not behave as a direct oxidant

In order to evaluate whether Prist could directly affect thiol groups in a cell free medium, we exposed a commercial GSH solution (150 μM) to 200 μM Prist for 1 h in the absence of brain supernatants. Fig. 4 shows that Prist per se did not modify GSH levels, whereas N-ethylmaleimide (NEM, 150 μM) (positive control) markedly oxidized GSH. The data clearly indicate that Prist does not behave as a direct oxidant.

2.5. Prist does not induce nitric oxide production

Finally, we assessed whether nitrogen reactive species were involved in Prist pro-oxidant effects by investigating the effect of Prist on nitrate and nitrite production. It can be seen in Table 1 that Prist did not induce nitrogen reactive species generation in cerebral cortex from young rats. Taken together these observations suggest that the pro-oxidant effects of Prist were mainly due to reactive oxygen species.

3. Discussion

Although patients affected by peroxisomal disorders present severe neurological dysfunction and cerebral cortex abnormalities, the pathogenesis of the brain damage in these diseases is poorly known. However, it is presumed that Prist, that is accumulated at high concentrations in these pathologies, may be involved in their neuropathology (Gould et al., 2001; Wanders et al., 2001; Brosius and Gartner, 2002). In this particular, it was recently demonstrated that Prist is cytotoxic to neurons, astrocytes and oligodendrocytes prepared from rat hippocampus (Wanders et al., 2001; Ronicke et al., 2009). Although the mechanisms of this toxicity were not well established, it was shown that Prist induces reactive species formation and impairs intracellular calcium homeostasis (Ronicke et al., 2009). In the present study we investigated the *in vitro* effects of Prist on important parameters of oxidative stress, by assessing lipid and protein oxidative damage, as well as the antioxidant defenses and nitric oxide content in cerebral cortex of young rats in order to clarify the pathophysiology of disorders in which Prist accumulates.

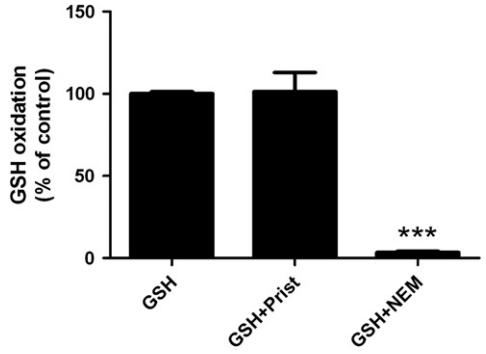

Fig. 4 – Effect of pristanic acid (Prist) on the concentrations of reduced glutathione (GSH) in the absence of brain tissue. A commercial GSH solution (150 μM) was incubated during 1 h in the presence of 200 μM Prist or NEM (150 μM). Values are means ± standard deviation for three independent experiments performed in triplicate and are expressed as percentage of controls (Control: 2341 ± 31.5 fluorescence units). 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).

Table 1 – Effect of pristanic acid (Prist) on nitric oxide production in rat cerebral cortex supernatants.

	Nitric oxide production		
	Control	100 μM	200 μM
Prist	100 ± 9.5	100 ± 5.4	99.1 ± 10

Values are means ± standard deviation of six independent experiments performed in triplicate and are expressed as percentage of controls (Control: [nmol/mg protein]: 2.8 ± 0.3). No significant differences were detected (one-way ANOVA).

We first observed that Prist significantly increased TBA-RS levels, reflecting an induction of malondialdehyde generation, an end product of membrane fatty acid peroxidation (Halliwell and Gutteridge, 2007). Therefore, it is presumed that Prist caused lipid peroxidation *in vitro*. As the Prist-induced lipid oxidative damage in cerebral cortex was totally prevented by the free radical scavenger MEL that mainly sequesters peroxyl and hydroxyl radicals, it is conceivable that this deleterious effect can be attributed to these oxygen reactive species.

Prist also provoked protein oxidation, as detected by a marked increase of carbonyl formation and sulfhydryl oxidation. In this context, it should be noted that carbonyl groups (aldehydes and ketones) are mainly formed by oxidation of protein side chains (especially Pro, Arg, Lys, and Thr), as well as by oxidative cleavage of proteins, or by the reaction of reducing sugars with lysine protein residues (Dalle-Donne et al., 2003). We cannot exclude the possibility that aldehydes resulting from lipid peroxidation may also induce carbonyl generation (Dalle-Donne et al., 2003). Otherwise, oxidation of protein sulfhydryl groups, especially from cysteine residues, gives rise to disulfide bonds, altering the redox state of proteins and potentially leading to their inactivation (Kuhn et al., 1999). Although the exact mechanisms by which Prist caused protein oxidation were not investigated, it is presumed that oxidative damage to proteins occurred through the attack of reactive species induced by this branched-chain fatty acid.

Besides causing lipid and protein oxidative damage, Prist significantly reduced the total content of GSH, which corresponds to the major endogenous antioxidant in the brain (Halliwell and Gutteridge, 2007). The reduction of GSH levels caused by Prist was fully prevented by MEL and attenuated by TRO, suggesting that this effect was probably mediated by the hydroxyl and peroxyl radicals, which are trapped by these antioxidants. Furthermore, Prist did not change the sulfhydryl content of a commercial solution of GSH in a cell free medium, indicating that it does not directly oxidize thiol groups. Considering that GSH is an important measurement of the antioxidant defenses of a tissue (Halliwell and Gutteridge, 2007), it can be therefore assumed that the rat cortical non-enzymatic antioxidant defenses were compromised by Prist.

L-NAME, a selective inhibitor of nitric oxide synthase activity, did not alter the increase of TBA-RS values and the

decrease of GSH levels caused by Prist. These data, allied to the fact that this fatty acid did not induce nitrogen reactive species formation, as determined by nitrates and nitrites generation, strongly indicate that Prist pro-oxidant effects (induction of lipid and protein oxidative damage and reduction of GSH levels) in cerebral cortex were probably mediated by the generation of reactive oxygen species, especially peroxyl and hydroxyl radicals. Regarding the peroxyl radical, which is an end product of lipid oxidation, it is conceivable that it was produced by the oxidative attack to lipid membranes (Delanty and Dichter, 1998; Halliwell and Whiteman, 2004; Halliwell and Gutteridge, 2007). Furthermore, the hydroxyl radical is mainly produced by the Fenton reaction from hydrogen peroxide, which is formed from superoxide (Adam-Vizi, 2005).

Our present data strongly indicate that Prist induces oxidative stress in rat brain, a deleterious cell condition that results from an imbalance between the total antioxidant defenses and the pro-oxidant effects in a tissue (Halliwell and Gutteridge, 2007). At this point, it should be emphasized that the brain has low cerebral antioxidant defenses and a high lipid and iron content compared with other tissues (Halliwell, 1992; Halliwell and Gutteridge, 2007), a fact that makes this tissue more vulnerable to increased reactive species.

We used cortical supernatants in our present study because these preparations are frequently used as model systems to evaluate important pro-oxidant and antioxidant parameters of oxidative stress (Cadenas et al., 1981; Gonzalez Flecha et al., 1991; Lores Arnaiz and Llesuy, 1993; Llesuy et al., 1994; Evelson et al., 2001; Halliwell and Gutteridge, 2007). In fact, tissue supernatants contain the whole cell machinery including preserved organelles such as mitochondria (the major source of free radical generation) and enzymes that are necessary for free radical production and scavenge (Stocks et al., 1974; Cadenas et al., 1981; Llesuy et al., 1994; Evelson et al., 2001; Dresch et al., 2009).

Although other studies with intact cell models are necessary to determine the involvement of Prist on the pathophysiology of diseases in which this fatty acid accumulates, it should be noted that the Prist-induced significant effects on the parameters of oxidative stress in brain cortex was achieved with concentrations of 100 μM, which are similar or even lower to those found in plasma of patients affected by various peroxisomal disorders (Zomer et al., 2000; Ferdinandusse et al., 2002). We have also to take into account that a considerable fraction of the supplemented exogenous Prist was possibly bound to proteins present in the incubation medium, leaving a smaller portion of this acid compound free to react and exert its effects.

On the other hand, we have recently described that phytanic acid (Phyt), which also accumulates in some peroxisomal disorders, provokes oxidative damage to lipids and proteins and reduces the non-enzymatic antioxidant defenses, besides impairing bioenergetics in rat brain (Busanello et al., 2010; Leipnitz et al., 2010). However, the oxidative effects exerted by Phyt were moderate and occurred with higher doses supplemented to the incubation medium as compared to those caused by Prist. This is in line with previous findings obtained in cultured neural cells demonstrating that induction of reactive oxygen species generation by Prist is greater than that provoked by Phyt (Ronicke et al., 2009).

In conclusion, to our knowledge, this is the first report showing that Prist that accumulates in some peroxisomal disorders provokes lipid and protein oxidative damage and diminishes the antioxidant defenses in the cerebral cortex. However, additional studies performed in intact neural cells and in animal models of peroxisomal disorders are required to confirm the role of oxidative stress in the pathophysiology of these diseases. In case the *in vitro* effects detected in the present study are confirmed *in vivo* and also in tissues from affected patients, it is tempting to speculate that the administration of antioxidants should be considered as an adjuvant therapy for these patients.

4. Experimental procedures

4.1. *Animals and reagents*

Wistar male rats of 30 days of life 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±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 1996). All efforts were made to minimize the number of animals used and their suffering.

All chemicals were purchased from Sigma (St. Louis, MO, USA). Prist solution was prepared on the day of the experiments in the incubation medium used for each technique and pH was adjusted to 7.4. TBA-RS, carbonyl formation, sulfhydryl content and nitric oxide production were measured with a double-beam Hitachi U-2001 spectrophotometer, whereas GSH levels were measured in a Hitachi F-2000 fluorescence spectrophotometer.

4.2. *Cerebral cortex supernatant preparation and incubation*

Rats sacrificed by decapitation without anaesthesia had their brain rapidly excised on a Petri dish placed on ice. The olfactory bulbs, pons, medulla, cerebellum, hippocampus and striatum were discarded, and the cerebral cortex was dissected, weighed and homogenized in 10 volumes (w/v) of 20 mM sodium phosphate buffer, pH 7.4, containing 140 mM KCl. Homogenates 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 the supernatant, a suspension of mixed and preserved organelles, including mitochondria, was pre-incubated at 37 °C for 1 h with Prist at concentrations of 1, 10, 100 or 200 μM. Controls did not contain this fatty acid in the incubation medium. Immediately after incubation, aliquots were taken to measure TBA-RS, carbonyl formation, sulfhydryl content and GSH levels. In some experiments, antioxidants were co-incubated with supernatants at the following final concentrations: 10 μM Trolox (TRO, soluble α-tocoferol), 1000 μM GSH, 1000 μM N-acetylcysteine (NAC), combination of 20 mU/mL superoxide dismutase (SOD) plus 20 mU/mL catalase (CAT), 750 μM N^{ω}-nitro-L-arginine methyl ester (L-NAME) and 1000 μM melatonin (MEL). The chosen concentrations of the antioxidants were those

capable to efficiently scavenge free radicals (Halliwell and Gutteridge, 2007).

4.3. *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-incubated cerebral cortex supernatants and centrifuged at 3000g for 10 min. Three hundred microliters of the pre-incubated 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.

4.4. *Protein carbonyl content*

Protein carbonyl formation, a marker of protein oxidative damage, was measured spectrophotometrically according to Reznick and Packer (1994). Two hundred microliters of the aliquots from the pre-treated supernatants 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 600 μL 20% trichloroacetic acid and centrifuged for 5 min at 9000*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 ºC 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 22000×10^6 nmol/mL for aliphatic hydrazones.

4.5. *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, 160 μL of pre-treated supernatant were incubated at 37 °C for 1 h with Prist. Then 30 μL of 10 mM DTNB, prepared in 0.2 M potassium phosphate solution, pH 8.0, was added. This was followed by 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/mg protein and represented as percentage of control.

4.6. *Reduced glutathione (GSH) content*

GSH concentrations were measured according to Browne and Armstrong (1998). Aliquots from the pre-treated supernatants were diluted in 20 volumes of (1:20, v/v) 100 mM sodium phosphate buffer, pH 8.0, containing 5 mM EDTA. One hundred microliters of this preparation were incubated with an equal volume of o-phthaldialdehyde (1 mg/mL methanol) at room temperature during 15 min. Fluorescence was measured using excitation and emission wavelengths of 350 and 420 nm, respectively. Calibration curve was prepared with standard GSH (0.001–1 mM) and the concentrations were calculated as nmol/mg protein and represented as percentage of control.

4.7. *Nitric oxide production*

Nitric oxide production was determined by measuring its derivatives nitrate (NO_3^-) and nitrite (NO_2^-) according to Miranda and colleagues (2001). Vanadium chloride (200 μL) was added to the tube containing 200 μL of Prist pre-treated cerebral cortex supernatants for complete reduction of nitrate to nitrite. Then, 200 μL of Griess reagent (a mixture of N-1-naphtylethylenediamine dihydrochloride and sulfanilamide) were added and the tube was incubated for 30 min at 37 °C in a water bath in a dark room. The resulting pink-stained pigment was determined in a spectrophotometer at 540 nm. A calibration curve was performed using sodium nitrate (2.5–100 μM), and each curve point was subjected to the same treatment as supernatants. Nitric oxide production values were calculated as nmol/mg protein and represented as percentage of control.

4.8. *Protein determination*

Protein content was determined in cerebral cortex supernatants by the method of Lowry and colleagues (1951), using bovine serum albumin as a standard.

4.9. *Statistical analysis*

Results are presented as mean ± standard deviation. Assays were performed in duplicate or triplicate and the mean or median was used for statistical calculations. Data was analyzed using one-way analysis of variance (ANOVA) followed by the post-hoc Duncan multiple range test when F was significant. Linear regression analysis was also used to test dose-dependent effects. Only significant *F* values are shown in the text. Differences between groups were rated significant at $P < 0.05$. All analyses were carried out in an IBM-compatible PC computer using the Statistical Package for the Social Sciences (SPSS) software.

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REFERENCES

Adam-Vizi, V., 2005. Production of reactive oxygen species in brain mitochondria: contribution by electron transport chain and non-electron transport chain sources. Antioxid. Redox Signal. 7, 1140–1149.

Aksenov, M.Y., Markesbery, W.R., 2001. Changes in thiol content and expression of glutathione redox system genes in the hippocampus and cerebellum in Alzheimer's disease. Neurosci. Lett. 302, 141–145.

Brosius, U., Gartner, J., 2002. Cellular and molecular aspects of Zellweger syndrome and other peroxisome biogenesis disorders. Cell. Mol. Life Sci. 59, 1058–1069.

Browne, R.W., Armstrong, D., 1998. Reduced glutathione and glutathione disulfide. Meth. Mol. Biol. 108, 347–352.

Busanello, E.N., Viegas, C.M., Moura, A.P., Tonin, A.M., Grings, M., Vargas, C.R., Wajner, M., 2010. In vitro evidence that phytanic acid compromises Na(+), K(+)-ATPase activity and the electron flow through the respiratory chain in brain cortex from young rats. Brain Res. 1352, 231–238.

Cadenas, E., Varsavsky, A.I., Boveris, A., Chance, B., 1981. Oxygen- or organic hydroperoxide-induced chemiluminescence of brain and liver homogenates. Biochem. J. 198, 645–654.

Dalle-Donne, I., Rossi, R., Giustarini, D., Milzani, A., Colombo, R., 2003. Protein carbonyl groups as biomarkers of oxidative stress. Clin. Chim. Acta 329, 23–38.

Delanty, N., Dichter, M.A., 1998. Oxidative injury in the nervous system. Acta Neurol. Scand. 98, 145–153.

Dresch, M.T., Rossato, S.B., Kappel, V.D., Biegelmeyer, R., Hoff, M.L., Mayorga, P., Zuanazzi, J.A., Henriques, A.T., Moreira, J.C., 2009. Optimization and validation of an alternative method to evaluate total reactive antioxidant potential. Anal. Biochem. 385, 107–114.

Esterbauer, H., Cheeseman, K.H., 1990. Determination of aldehydic lipid peroxidation products: malonaldehyde and 4-hydroxynonenal. Meth. Enzymol. 186, 407–421.

Evelson, P., Travacio, M., Repetto, M., Escobar, J., Llesuy, S., Lissi, E.A., 2001. Evaluation of total reactive antioxidant potential (TRAP) of tissue homogenates and their cytosols. Arch. Biochem. Biophys. 388, 261–266.

Ferdinandusse, S., Rusch, H., van Lint, A.E., Dacremont, G., Wanders, R.J., Vreken, P., 2002. Stereochemistry of the peroxisomal branched-chain fatty acid alpha- and beta-oxidation systems in patients suffering from different peroxisomal disorders. J. Lipid Res. 43, 438–444.

Gonzalez Flecha, B., Llesuy, S., Boveris, A., 1991. Hydroperoxide-initiated chemiluminescence: an assay for oxidative stress in biopsies of heart, liver, and muscle. Free Radic. Biol. Med. 10, 93–100.

Gould, S.J., Raymond, G.V., Valle, D., 2001. The peroxisome biogenesis disorders. In: Scriver, C.R., Beaudet, A.L., Valle, D. (Eds.), The metabolic and molecular bases of inherited disease. McGraw-Hill, New York, pp. 3181–3217.

Halliwell, B., 1992. Reactive oxygen species and the central nervous system. J. Neurochem. 59, 1609–1623.

Halliwell, B., Gutteridge, J.M.C., 2007. Free radicals in biology and medicine. In: Halliwell, B., Gutteridge, J.M.C. (Eds.), Cellular responses to oxidative stress: adaptation, damage, repair, senescence and death. Oxford University Press, Oxford, pp. 187–267.

Halliwell, B., Whiteman, M., 2004. Measuring reactive species and oxidative damage in vivo and in cell culture: how should you do it and what do the results mean? Br. J. Pharmacol. 142, 231–255.

Johnson, D.W., Trinh, M.U., Oe, T., 2003. Measurement of plasma pristanic, phytanic and very long chain fatty acids by liquid chromatography-electrospray tandem mass spectrometry for the diagnosis of peroxisomal disorders. J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 798, 159–162.

Kuhn, D.M., Aretha, C.W., Geddes, T.J., 1999. Peroxynitrite inactivation of tyrosine hydroxylase: mediation by sulfhydryl oxidation, not tyrosine nitration. J. Neurosci. 19, 10289–10294.

Leipnitz, G., Amaral, A.U., Zanatta, A., Seminotti, B., Fernandes, C.G., Knebel, L.A., Vargas, C.R., Wajner, M., 2010. Neurochemical evidence that phytanic acid induces oxidative damage and reduces the antioxidant defenses in cerebellum and cerebral cortex of rats. Life Sci. 87, 275–280.

Llesuy, S., Evelson, P., Gonzalez-Flecha, B., Peralta, J., Carreras, M.C., Poderoso, J.J., Boveris, A., 1994. Oxidative stress in muscle and liver of rats with septic syndrome. Free Radic. Biol. Med. 16, 445–451.

Lores Arnaiz, S., Llesuy, S., 1993. Oxidative stress in mouse heart by antitumoral drugs: a comparative study of doxorubicin and mitoxantrone. Toxicology 77, 31–38.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

Miranda, K.M., Espey, M.G., Wink, D.A., 2001. A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite. Nitric Oxide 5, 62–71.

Reznick, A.Z., Packer, L., 1994. Oxidative damage to proteins: spectrophotometric method for carbonyl assay. Meth. Enzymol. 233, 357–363.

Ronicke, S., Kruska, N., Kahlert, S., Reiser, G., 2009. The influence of the branched-chain fatty acids pristanic acid and Refsum disease-associated phytanic acid on mitochondrial functions and calcium regulation of hippocampal neurons, astrocytes, and oligodendrocytes. Neurobiol. Dis. 36, 401–410.

Stocks, J., Gutteridge, J.M., Sharp, R.J., Dormandy, T.L., 1974. Assay using brain homogenate for measuring the antioxidant activity of biological fluids. Clin. Sci. Mol. Med. 47, 215–222.

Wanders, R.J., Vreken, P., Ferdinandusse, S., Jansen, G.A., Waterham, H.R., van Roermund, C.W., Van Grunsven, E.G., 2001. Peroxisomal fatty acid alpha- and beta-oxidation in humans: enzymology, peroxisomal metabolite transporters and peroxisomal diseases. Biochem. Soc. Trans. 29, 250–267.

Zomer, A.W., van Der Burg, B., Jansen, G.A., Wanders, R.J., Poll-The, B.T., van Der Saag, P.T., 2000. Pristanic acid and phytanic acid: naturally occurring ligands for the nuclear receptor peroxisome proliferator-activated receptor alpha. J. Lipid Res. 41, 1801–1807.