Neuroscience 304 (2015) 122–132

REACTIVE NITROGEN SPECIES MEDIATE OXIDATIVE STRESS AND ASTROGLIOSIS PROVOKED BY *IN VIVO* ADMINISTRATION OF PHYTANIC ACID IN CEREBELLUM OF ADOLESCENT RATS: A POTENTIAL CONTRIBUTING PATHOMECHANISM OF CEREBELLAR INJURY IN PEROXISOMAL DISORDERS

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Abstract—Phytanic acid (Phyt) accumulates in various peroxisomal diseases including Refsum disease (RD) and Zellweger syndrome (ZS). Since the pathogenesis of the neurological symptoms and especially the cerebellar abnormalities in these disorders are poorly known, we investigated the effects of *in vivo* intracerebral administration of Phyt on a large spectrum of redox homeostasis parameters in the cerebellum of young rats. Malondialdehyde (MDA) levels, sulfhydryl oxidation, carbonyl content, nitrite and nitrate concentrations, 2′,7′-dichlorofluorescein (DCFH) oxidation, total (tGS) and reduced glutathione (GSH) levels and the activities of important antioxidant enzymes were determined at different periods after Phyt administration. Immunohistochemical analysis was also carried out in the cerebellum. Phyt significantly increased MDA and nitric oxide (NO) production and decreased GSH levels, without altering tGS, DCFH oxidation, sulfhydryl oxidation, carbonyl content and the activities of glutathione peroxidase (GPx), superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR) and glucose-6-phosphate dehydrogenase (G6PD). Furthermore, immunohistochemical analysis revealed that Phyt caused astrogliosis and protein nitrosative damage in the cerebellum. It was also observed that the NO synthase inhibitor N_{ω}-Nitro-L-arginine methyl ester (L-NAME) prevented the increase of MDA and NO production as well as the decrease of GSH and the immunohistochemical alterations caused by Phyt, strongly suggesting that reactive nitrogen species (RNS) were involved in these effects. The present data provide *in vivo* solid evidence that Phyt disrupts redox homeostasis and causes astrogliosis in rat cerebellum probably mediated by RNS production. It is therefore presumed that disequilibrium of redox status may contribute at least in part to the cerebellum alterations characteristic of patients affected by RD and other disorders with Phyt accumulation. © 2015 Published by Elsevier Ltd. on behalf of IBRO.

Key words: phytanic acid, peroxisomal disorders, redox homeostasis, astrogliosis, cerebellum.

INTRODUCTION

Phytanic acid (3,7,11,15-tetramethylhexadecanoic acid, Phyt) is derived from chlorophyll, which is generated in the gastrointestinal system of ruminants by chlorophyll break, releasing phytol which is then converted into Phyt. Dairy products (milk, cheese) and red meat (cow and sheep) are rich sources of Phyt. This fatty acid accumulates in various peroxisomal disorders, such as Refsum disease (RD), Zellweger syndrome (ZS), rhizomelic chondrodysplasia punctata type I and α-methylacyl-CoA racemase deficiency (Wierzbicki, 2007; Van Veldhoven, 2010; Wanders et al., 2011; Braverman et al., 2013). RD is an autosomal recessive lipid-storage disorder caused by deficient activity of peroxisomal phytanoyl-CoA hydroxylase (EC 1.14.11.18) that converts phytanoyl-CoA into 2-hydroxy-phytanoyl-CoA (Reiser et al., 2006). The blockage of this pathway results in accumulation of Phyt in tissues and body fluids of the affected patients that may reach up to 5 mM in plasma (normal individuals up to 30 μM) (Schönfeld and Struy, 1999; Wanders et al., 2001; Al-Dirbashi et al., 2008).

The onset of RD symptoms is typically observed in late childhood or adolescence, but may occur from seven months to over 50 years (Wills et al., 2001;

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Abbreviations: CAT, catalase; DCFH, 2′,7′-dichlorofluorescein; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; DMSO, dimethyl sulfoxide; DNPH, 2,4-dinitrophenylhydrazine; EDTA, ethylenediaminetetraacetic acid; G6PD, glucose-6-phosphate dehydrogenase; GFAP, glial fibrillary acidic protein; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; L-NAME, N_{ω}-Nitro-L-arginine methyl ester; MDA, Malondialdehyde; MK-801, dizocilpine; NMDA, N-methyl-D-aspartate; NO, nitric oxide; NOS, NO synthase; PAF, paraformaldehyde; PBS, phosphate buffered saline; Phyt, Phytanic acid; RD, Refsum disease; RNS, reactive nitrogen species; SOD, superoxide dismutase; ZS, Zellweger syndrome.

http://dx.doi.org/10.1016/j.neuroscience.2015.07.028
0306-4522/

Verhoeven and Jakobs, 2001). Retinitis pigmentosa, chronic neuropathy and cerebellar ataxia are common clinical features. Retinal degeneration is an early sign of the disease, and the neurological symptoms are generally accompanied by a slowly progressive peripheral neuropathy, manifested by motor weakness and muscular wasting (Gould et al., 2001; Kahlert et al., 2005). Patients can also present anosmia, sensorial hearing loss, ichthyosis, skeletal alterations and cardiomyopathy. It is of note that cerebellar ataxia is a prominent clinical manifestation of RD, affecting more than half of the patients, being usually more severe than muscular weakness and sensory loss. Histopathological abnormalities including alterations of Purkinje cells in the molecular layer of the cerebellum are commonly seen in RD, highlighting that this cerebral structure is highly vulnerable in this disorder (Chow et al., 1992; Wanders et al., 2001; Wierzbicki et al., 2002). This is in line with the observations drawn from the genetic mouse models of ZS and RD, showing abnormal histogenesis with reduced granule neuron population, loss and delayed Purkinje cell dendrite development and astrocytosis in the cerebellum (Faust, 2003; Ferdinandusse et al., 2008).

However, the pathophysiology of the brain and particularly cerebellum alterations in peroxisomal disorders with accumulation of Phyt is still poorly known, although the progression of the symptoms may be blocked by reduction of the plasma concentrations of this fatty acid. Thus, it was observed that reduction of dietary Phyt intake decreases Phyt levels and ameliorates the polyneuropathy and ataxia, besides slowing down the progression of the other clinical manifestations in patients with RD, suggesting potential neurotoxic effects for Phyt (Masters-Thomas et al., 1980; Baldwin et al., 2010; Rüether et al., 2010; Kohlschütter et al., 2012). In this scenario, experimental *in vitro* studies demonstrated that Phyt causes oxidative stress, cell death and impairs mitochondrial function in neural tissues (Kahlert et al., 2005; Reiser et al., 2006; Schönfeld and Reiser, 2006; Schönfeld et al., 2006; Busanello et al., 2010; Leipnitz et al., 2010; Busanello et al., 2013a,3b; Nagai, 2015).

Although Phyt provokes mitochondrial dysregulation in neural cells, the exact neurotoxic effects of this fatty acid and its role *in vivo* on the cerebellum redox homeostasis have not been yet evaluated. Therefore, in the present study we investigated the ex vivo effects of intracerebellar administration of Phyt on a large spectrum of important parameters of redox homeostasis to evaluate lipid oxidation, protein oxidation, enzymatic and non-enzymatic antioxidant defenses, as well as the production of reactive oxygen and nitrogen species. Immunohistochemical analysis was also carried out in cerebellum to assess oxidative stress markers and astrogliosis. Our objective was to mimic an acute situation of high brain Phyt concentrations, such as those observed during catabolic states like fasting that are known to precipitate acute symptoms in patients with Refsun's disease and are biochemically characterized by significant increases of Phyt levels in tissues including the brain due to rapid mobilization of this fatty acid mainly from adipocytes and hepatocytes (Wierzbicki et al., 2003).

EXPERIMENTAL PROCEDURES

Animals and reagents

Thirty-day-old Wistar male rats 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 7–19 h) in air conditioned atmosphere with constant temperature (22 °C ± 1 °C), in a colony room with free access to water and a 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 Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil (protocol number 28057), 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, as well as to minimize their suffering.

Reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA), unless otherwise stated. Phyt solution (40 mM) was prepared in phosphate-buffered saline (PBS) containing 0.5 % dimethyl sulfoxide (DMSO), whereas control solution was made with PBS containing 0.5 % DMSO, and the pH was adjusted to 7.4 with NaOH. In brief, we first dissolved commercial Phyt in 50 μL DMSO (stock solution1600 mM). Five μL were taken from the flask and diluted with 195 μL of PBS and then the working solution (40 mM) was heated in water bath and vigorously shaken for about 30 min in vortex until complete dissolution. Aminoadipic acid was also dissolved in PBS containing 0.5 % DMSO to give a final concentration of 40 mM. N_{ω}-Nitro-L-arginine methyl ester (L-NAME) and saline solution (NaCl 0.9%) were prepared in water and the pH adjusted to 7.4.

Intracerebellar administration of Phyt

Rats were deeply anesthetized with ketamine plus xylazine (75 and 10 mg/kg i.p., respectively). The animals were placed in the stereotaxic apparatus and one small hole was drilled into the skull. Two microliters of Phyt (80 nmol) or vehicle (controls) were slowly injected into the cerebellum over 2 min via a needle connected by a polyethylene tube to a 10 μL Hamilton syringe. The needle was left in place for another 3 min, so that the total procedure lasted 5 min. We injected a similar amount and volume of aminoadipic acid into the cerebellum of some animals and measured biochemical parameters 4 h afterward. The coordinates for intracerebellar injection were 9.5 mm posterior to the bregma and 3.5 mm ventral from dura (Paxinos and Watson, 1997). The correct position of the needle was tested by injecting 2 μL of methylene blue (4% in NaCl 0.9%) followed by histological analysis. In some experiments animals were pre-treated intracerebellarly with L-NAME (0.2 nmol) 30 min before the administration of Phyt.