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Original article

Phytanic acid induced neurological alterations in rat brain synaptosomes and its attenuation by melatonin

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ABSTRACT

Phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) (Phyt) is a saturated branched chain fatty acid which originates after the breakdown of chlorophyll molecule, phytol. It plays an important role in a variety of metabolic disorders with peroxisomal impairments. The aim of our investigation was to evaluate the adverse effects of Phyt on synaptic functions by using synaptosomal preparation of rat brain as an *in vitro* model and the possible protective role of melatonin against Phyt-induced neurotoxicity. Melatonin is an antioxidant, secreted by the pineal gland. Melatonin and its metabolites have neuroprotective effects on cellular stress, by reducing reactive oxygen species (ROS) and reactive nitrogen species (RNS). In the present investigation, synaptosomes prepared from rat brain were co-treated with melatonin (10 μM) and Phyt (50 μM) for 2 h. Co-treatment of Phyt with melatonin significantly restored the altered levels of protein carbonyl (PC) contents and lipid peroxidation (LPO). It also replenished the Phyt-induced alterations on the levels of non-enzymatic antioxidant defence reduced glutathione (GSH), enzymatic antioxidants such as catalase (CAT) and superoxide dismutase (SOD) and synaptosomal integral enzymes such as AChE, Na^+, K^+-ATPase and MAO. We observed that Phyt induced oxidative stress in synaptosomes as indicated by an elevation in the generation of ROS and melatonin was able to inhibit the elevated ROS generation. Moreover, the neurotoxic effects elicited by Phyt on NO level and membrane potential were totally prevented by the treatment of melatonin. The results of our investigation emphasize the potential use of melatonin as a nutraceutical and mitigatory agent against Phyt-induced oxidative stress.

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1. Introduction

The lipids that are present in dairy products and fish have various forms of branched chain fatty acids (BCFAs) (iso- and anteiso-) as minor dietary constituents. They also constitute as major fatty acids of gram-positive bacteria. Bacterial sources have been implicated with the ubiquitous presence of these fatty acids in food products [1]. There are several BCFAs which are said to be present in food, which may include 12-methyltridecanoic acid, 13-methyltetradecanoic acid, 14-methylhexadecanoic acid, 15- methylhexadecanoic acid, and 16- methylheptadecanoic acid [2]. Phytanic acid (Phyt), pristanic acid and valproic acid are the most important BCFAs. Phyt is a saturated BCFA and major constituent of the human diet, predominantly found in dairy products, meat and fish. It is a degradation product from the phytol side chain of chlorophyll [3]. Degradation of Phyt is known to occur mainly in peroxisomes via α-oxidation and in mitochondria via β-oxidation [4]. Due to its β-methyl group present at the 3-position of the carbon atoms, Phyt cannot be β-oxidized [5]. Alteration in the metabolism of Phyt may play an important role in the neurodegeneration but the exact mechanism behind it remains to be evaluated [6]. Phyt enhances the phospholipid mobility of membrane in the mitochondria to disrupt the conformational state of membrane proteins.

The physiology of cells might therefore be affected by multiple actions of Phyt. High concentrations of BCFAs such as Phyt in the tissues and plasma are thought to be involved in the etiology of a number of neurodegenerative disorders including Refsum disease [7]. A number of *in vitro* studies have indicated that Phyt is probably neurotoxic at high concentrations, and capable of

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destroying neurons [8]. The neurotoxicity of Phyt was elucidated by several investigators and one of the major mechanisms behind Phyt toxicity has been attributed to be oxidative stress [9]. Several reports have shown that Phyt also induces oxidative stress in brain mitochondria and exerts cytotoxic actions associated with alteration of mitochondrial energy homeostasis [10,11]. Other studies have also investigated that Phyt provokes iNOS dependent apoptosis in vascular smooth muscle cells [12] and induces lipid and protein oxidative damage and decreases the antioxidant defences in rat cerebellum and cerebral cortex [13].

A great number of antioxidants have shown a probable way to prevent the cellular injuries induced by oxidative stress by augmenting endogenous oxidative defence capacity through dietary or pharmacological intake of antioxidants [14,15]. Melatonin is an antioxidant and scavenger of free radical secreted by the pineal gland [16]. Numerous studies have provided strong evidence for its antioxidant properties and neuroprotective effects on neuronal cell degeneration [17–19]. Melatonin also affects the mitochondrial homeostatic mechanism and assists in maintaining cell function and survival [20]. It is reported that mitochondrial oxidative stress can be reduced through melatonin by improving ETC function and increased synthesis of ATP [21]. The efficacy of melatonin to treat neurological disorders by inhibiting the mitochondrial cell death pathways have been investigated in several studies [22,23]. In a variety of studies, melatonin has shown antioxidant, anti-inflammatory, immunoregulatory and anticancer properties [24,25]. Based on these observations, it has created our interest to evaluate the possible neurotoxic effects of BCFA such as Phyt in synaptosomes of rat brain.

Synaptosomes are isolated nerve terminal portion of the axons which contain neurotransmitters that diffuse across the synaptic cleft, relaying neuronal messages to the next cell. They behave as metabolically autonomous minicells and provide a good experimental *in vitro* model to evaluate the neurodegenerative process and peroxidative events in neuronal cells [26]. Synaptosomes have been used in various studies as a suitable model system for *in vitro* toxicity evaluation [27–29]. In the present study, we investigated neuroprotective effects of melatonin on oxidative stress induced by BCFA such as Phyt in rat brain synaptosomes.

2. Materials and methods

2.1. Chemicals

Acetylthiocholine iodide (ATC), 1-amino-2-naphthol,4-sulphonic acid (ANSA), benzylaminehydrochloride (BAHC), bovine serum albumin (BSA), butylated hydroxyl taulene (BHT), 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA), 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl terazoliumbromide (MTT) dye, 2,4-dinitrophenylhydrazine (DNPH), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), griess reagent, orthophosphoric acid (OPA), perchloric acid (PCA), phenylmethylsulfonylfluoride (PMSF), reduced glutathione (GSH), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), sodium azide, sulfosalicylic acid, tetramethylrhodamine ethyl ester perchlorate (TMRE) dye, thiobarbituric acid (TBA) and trichloroacetic acid (TCA) were purchased from Sigma-Aldrich (St Louis, MO, USA). Other routine chemicals used in this study were obtained from Hi-Media Labs (Mumbai, India). The antibody of rabbit anti-SNAP-25 was purchased from Santa Cruz Biotechnology (Santa Cruz, Taxes, USA) and the horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody was purchased from Cell Signaling (Beverly, MA, USA). Melatonin and phytanic acid were purchased from Sigma-Aldrich (St Louis, MO, USA).

2.2. Animals

In this study male Wistar rats (4–5 weeks old) weighing 150–180 g, were maintained under standard conditions in Central Animal House of Jamia Hamdard (Hamdard University), New Delhi, India. Rats were kept at temperature $22 \pm 2\,^{\circ}C$ with relative humidity at $65 \pm 10\%$ and at a photoperiod of 12 h light/dark cycle. Standard rodent food and water were supplied to the animals *ad libitum* prior to the start of the experiment.

2.3. Preparation of rat brain synaptosomes

The crude synaptosomal fractions were prepared by differential centrifugation by the method of Kanbak et al. [30]. After the male Wistar rats (160 ± 20 g) were sacrificed, the brain were quickly excised and kept on ice-cold plate immediately, washed in physiological saline solution and weighed. Thereafter, the brain tissue was homogenized in 10% (w/v) of an ice-cold solution of HEPES (10 mM) and sucrose (0.32 mM) and centrifuged at 600*g* for 10 min at 4 °C. After that the pellet was removed and supernatant was again centrifuged at 20,000*g* for 30 min at 4 °C. The cytosolic supernatant was discarded and resulting crude synaptosomal pellets were resuspended in radioimmunoprecipitation assay buffer (RIPA) containing (5.5 mM KCl, 1.0 mM $MgCl_2$, 115 mM NaCl, 1.0 mM NaH_2PO_4, 2 mM $CaCl_2$, 20 mM $NaHCO_3$ and 10 mM glucose, pH 7.2) mixed with protease inhibitor and 0.1 mM PMSF. The synaptosomal sample was immediately used for protein estimation, biochemical analysis and protein electrophoresis.

2.4. Experimental design

Brain synaptosomal preparations were freshly employed in the experiments. To investigate the neurotoxicity of Phyt under *in vitro* conditions, the synaptosomal fractions prepared from rat brain were incubated with different concentrations of Phyt (10, 20, 50 μM) for 2 h at 37 °C in a temperature controlled water bath. Based on the dose response results, the concentration of Phyt (50 μM) was chosen for further experiments and synaptosomal fractions were co-incubated with Phyt (50 μM) and different concentrations of melatonin (5, 10, 20 μM) for 2 h in same conditions. In order to determine the protective effects of melatonin against Phyt, the next sets of experiments were performed and the brain synaptosomal fractions were co-incubated with melatonin (10 μM) and Phyt (50 μM) for 2 h. The concentrations of Phyt and melatonin were based on previous published reports [9,22,31]. RIPA buffer was added to controls instead of Phyt and melatonin at the same volume. The stock and working solution were prepared in such a way that the same volume was added in the synaptosomal fractions of rat brain for incubation.

2.5. Western blot analysis of synaptosomal associated protein-25 (SNAP-25)

The characterization of brain synaptosomes was determined by the presence of SNAP-25 protein expression in synaptosomal fractions according to the method of Gil et al. [32]. 20–40 μg amounts of synaptosomal protein was resolved in 10–12% sodium dodecyl sulfate gel electrophoresis and transferred onto PVDF membranes. Briefly, protein was transferred at 50 mA for 1 h to PVDF membranes using Mini Trans-Blot Cell apparatus (Bio-Rad, Hercules, CA, USA). The procedure for immunodetection includes the transfer and blocking of the membrane for 1 h at room temperature with Phosphate-buffered saline-Tween (PBST) (150 mM NaCl, 10 mM KCl, 0.1 M NaH_2PO_4, 0.1 M KH_2PO_4, pH 7.4, and 0.05% Tween-20) containing 5% nonfat-dry milk. After that

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