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Docosahexaenoic acid prevents paraquat-induced reactive oxygen species production in dopaminergic neurons via enhancement of glutathione homeostasis

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ABSTRACT

Omega-3 polyunsaturated fatty acid levels are reduced in the substantia nigra area in Parkinson's disease patients and animal models, implicating docosahexaenoic acid (DHA) as a potential treatment for preventing Parkinson's disease and suggesting the need for investigations into how DHA might protect against neurotoxin-induced dopaminergic neuron loss. The herbicide paraquat (PQ) induces dopaminergic neuron loss through the excessive production of reactive oxygen species (ROS). We found that treatment of dopaminergic SN4741 cells with PQ reduced cell viability in a dose-dependent manner, but pretreatment with DHA ameliorated the toxic effect of PQ. To determine the toxic mechanism of PQ, we measured intracellular ROS content in different organelles with specific dyes. As expected, all types of ROS were increased by PQ treatment, but DHA pretreatment selectively decreased cytosolic hydrogen peroxide content. Furthermore, DHA treatment-induced increases in glutathione reductase and glutamate cysteine ligase modifier subunit (*GCLm*) mRNA expression were positively correlated with glutathione (GSH) content. Consistent with this increase in *GCLm* mRNA levels, Western blot analysis revealed that DHA pretreatment increased nuclear factor-erythroid 2 related factor 2 (Nrf2) protein levels. These findings indicate that DHA prevents PQ-induced neuronal cell loss by enhancing Nrf2-regulated GSH homeostasis.

1. Introduction

Parkinson's disease (PD) is characterized by a progressive loss of dopaminergic neurons in the substantia nigra (SN) area [1]. Sporadic PD is linked to environmental factors including pesticides, solvents, metals, carbon monoxide, and herbicides. In particular, the herbicide paraquat (PQ) has been linked to an increased incidence of PD through an excessive production of reactive oxygen species (ROS) [2,3], which leads to oxidative challenges in the brain such as increased DNA damage, lipid peroxidation, and mitochondrial dysfunction [4]. Thus, maintaining a balance of intracellular ROS content is essential for preventing the progression of PD.

Docosahexaenoic acid (DHA) is the major omega-3 polyunsaturated fatty acid (ω-3 PUFA) found in the phospholipid fraction of the brain [5,6]. PUFA appears to be essential for cognition through its regulation of neuronal activity, as declines in membrane PUFA concentrations lead to cognitive impairment [7]. Several studies indicate that levels of DHA and other PUFAs are reduced in brain tissue of PD patients and animal models [8]. Importantly, DHA cannot be synthesized *de novo* in mammals due to the lack of a specific enzyme that converts ω-6 into ω-3 PUFA [9]. Therefore, a shortage of ω-3 PUFA in the brain must be offset through dietary intake. According to previous studies, administration of DHA has neuroprotective actions in animal models of PD [10,11]. Specifically, Bousquet et al. report that a diet high in ω-3 PUFA increases the number of tyrosine hydroxylase-immunoreactive neurons in the SN and dopamine content in the striatum following 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine treatment [12].

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However, the protective mechanism of DHA in models of PQ-induced PD needs to be clarified.

Nrf2 protects neurons from acute injury and counteracts ROS-mediated damage in neurodegenerative diseases. In response to oxidative stress, Nrf2 dissociates from its cytoplasmic inhibitor Keap1 and then translocates into the nucleus, where it binds to the antioxidant responsive elements in the promoters of target genes [13,14]. This leads to transcriptional induction of several cellular defense genes, such as glutathione biosynthetic enzymes (e.g., *GCLm* and *GCLc*) and GSH-dependent antioxidant enzymes (e.g., *glutathione peroxidase 2, glutathione S-transferases*).

The upregulation of antioxidant enzyme capacity through the administration of DHA ameliorates organelle injury and cellular toxicity [15,16]. For instance, DHA increases the activity of heme oxygenase-1 in rats with renal injury or cerebral ischemia. Also, administration of DHA induces the upregulation of thioredoxin and glutathione (GSH) in hippocampal cells, leading to an attenuation of amyloid beta-induced neuronal toxicity [17].

Based on previous studies, we expected that DHA may help prevent PQ-induced PD by enhancement of total GSH expression via DHA related Nrf2 induction. However, the relationship between DHA and antioxidant enzymes within the context of PD remains unclear. The aim of the present study was to test whether DHA can overcome PQ-induced ROS production by changing the antioxidant content of dopaminergic neurons, which would suggest a role for DHA as an alternative preventative therapy for PD.

2. Materials and methods

2.1. Reagents

DHA was purchased from Cayman Chemical (Ann Arbor, MI, USA). PQ and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma–Aldrich (St. Louis, MO, USA). MitoSOX™, dihydroethidium (DHE), dichlorofluorescein-diacetate (DCF-DA), anti-Nrf2 antibody, and mBCL were purchased from Invitrogen (Camarillo, CA, USA). Anti-β-actin rabbit antibody was purchased from Santa Cruz (Santa Cruz, CA, USA). Trizol was purchased from Invitrogen (Carlsbad, CA, USA).

2.2. Cell lines and culture conditions

The dopaminergic neuronal cell line SN4741 has been previously described [18]. SN4741 cells were maintained in RF medium, which was comprised of Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Camarillo, CA, USA) supplemented with 10% fetal bovine serum, 1% glucose, L-glutamine (2 mM), and penicillin–streptomycin. Cells were typically incubated at 33 °C with 5% CO_2 as described previously [18].

2.3. Cell viability and cytotoxicity

Cell viability was assessed by MTT assay as previously described [19]. Cells were cultured at 4.5×10^3 cells per well in 96-well cell culture plates. Cultured cells were treated with PQ (100–600 μM) for 24 h with or without pretreatment of 25 μM DHA, eicosapentaenoic acid (EPA), or arachidonic acid (AA) for 3 h. The treated cells were incubated with 0.5 mg/ml MTT solution for 1 h and then dissolved in DMSO and measured at 540 nm using a MultiSkan Ascent microplate reader (Thermo Electron Corporation, Bremen, Germany).

2.4. Detection of intracellular ROS

ROS generation was analyzed using the fluorescent dyes MitoSOX, DCF-DA, and DHE. Briefly, after exposure to 400 μM PQ for 24 h with or without 25 μM DHA pretreatment for 3 h, cells were incubated with one of the fluorescent dyes (10 μM) in Krebs-HEPES buffer (pH 7.4) at 33 °C for 20 min. Next, cells were washed with Hank's Balanced Salt Solution (HBSS) and identified by fluorescence microscopy (Olympus, Tokyo, Japan). Other cells were detached using 0.05% trypsin–EDTA, centrifuged in tubes, and resuspended in HBSS buffer (pH 7.4). Cells were identified using a FACScan (BD Bio-science, San Jose, CA, USA), and data analysis was performed using a FACSDiva (BD Bio-science, San Jose, CA, USA).

2.5. Changes in intracellular GSH content

Monochlorobimane (mBCl) is a specific probe that is used to measure intracellular GSH content in dopaminergic neurons [20]. Cultured cells were exposed to 400 μM PQ for 24 h in the presence or absence of 25 μM DHA pretreatment for 3 h. Treated cells were then exposed to 100 μM mBCl for 1 h in a buffer including 2 mM $CaCl_2$, 5 mM HEPES, 140 mM NaCl, 10 mM glucose, 6 mM KCl, 1 mM $MgCl_2$, and 100 μM mBCl at room temperature. Intracellular GSH content was identified by fluorescence microscopy.

2.6. Western blot analysis

SN4741 cells were cultured at 5×10^5 cells per well in 6-well cell culture plates. SN4741 cells were exposed to 400 μM PQ for 24 h with or without 25 μM DHA pretreatment for 3 h. Proteins were extracted in RIPA lysis buffer (100 mM Tris–HCl (pH 8.5), 200 mM NaCl, 5 mM EDTA, and 0.2% SDS). Quantification of protein levels was performed using the Bradford method [21]. Isolated protein (20 μg) was resolved using 12% SDS–PAGE and transferred onto polyvinylidene fluoride membranes, which were blocked with 5% skim milk in TBST (10 mM Tris–HCl (pH 7.6), 150 mM NaCl, and 0.1% Tween 20). The membranes were incubated with primary antibodies against Nrf2 (1:1000) and β-actin (1:5000), and proteins were detected with horseradish peroxidase-coupled secondary antibody according to the manufacture's protocol. Detection of antibody-labeled proteins was performed using an ECL chemiluminescence system (INtRON BioTechnology, Korea).

2.7. Real-time polymerase chain reaction (PCR) analysis

Total RNA was isolated using Trizol according to the manufacturer's instructions. Real-time quantitative PCR was performed using cDNA, SYBR Green PCR Master Mix (iCycleriQ Real-Time PCR Detection System; Bio-Rad), and specific primers. Primers and their sequences were as follows: *SOD1* F (5′-GAGACCTGGGCAATGTGACT-3′), *SOD1* R (5′-GTTTACTGCGCAATCCCAAT-3′), *SOD2* F (5′-CCGAGGAGAAGTACCACGAG-3′), *SOD2* R (5′-GCTTGATAGCCTCCAGCAAC-3′), *Catalase* F (5′-ACATGGTCTGGGACTTCTGG-3′), *Catalase* R (5′-CAAGTTTTGATGCCCTGGT-3′), *GR* F (5′-CACGACCATGATTCCAGATG-3′), *GR* R (5′-CAGCATAGACGCCTTTGACA), *GPx1* F (5′-GTCCACCGTGTATGCCTTCT-3′), *GPx1* R (5′-TCTGCAGATCGTTCATCTCG-3′), *GCLm* F (5′-TGGAGCAGCTGTATCAGTGG-3′), and *GCLm* R (5′AGAGCAGTTCTTT CGGGTCA-3′). All primers were designed by the Primer3 program with an appropriated size (less than 150 bp) for the Rotor-Gene 6000 real time instrument. Relative expression of these genes was quantified and normalized to 18s ribosomal RNA using Rotor-Gene 6000 real-time rotary analyzer software (Qiagen, CA, USA).

2.8. Statistical analysis

All results were obtained from at least three independent experiments. Statistical analysis was performed using GraphPad Prism (GraphPad Software Inc., San Diego, CA, USA). Data are