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Neuronal nitric oxide synthase phosphorylation induced by docosahexaenoic acid protects dopaminergic neurons in an experimental model of Parkinson's disease

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Abstract

Introduction. Docosahexaenoic acid (DHA) has been shown to have beneficial effects on Parkinson's disease (PD). The aim of this study was to investigate if the DHA acts on neurons of substantia nigra (SN) by phosphorylation of neuronal nitric oxide synthase (nNOS) in an experimental mouse model of PD.

Material and methods. An experimental model of PD was created by intraperitoneal injections (4 × 20 mg/kg) of the neurotoxin 1-methyl-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP). Three-month-old male C57BL/6 mice were randomly divided into four groups as follows: control (C), DHA-treated (DHA), MPTP-injected (MPTP) and DHA-treated and MPTP-injected (DHA + MPTP). DHA (36 mg/kg/day) was administered daily by gavage for four weeks. Motor activity of the mice was evaluated with pole, locomotor activity and rotarod tests. Caspase-3 activity, nitrate/nitrite and 4-hydroxynonenal (4-HNE) levels were determined by spectrophotometric assays. Immunohistochemistry was used to localize and assess the expressions of tyrosine hydroxylase (TH), nNOS and phospho-nNOS (p-nNOS) in SN.

Results. An increased return and total down time in the MPTP group was observed in the pole test, while DHA treatment decreased both parameters. The ambulatory activity, total distance and total locomotor activities were decreased in the MPTP group, whereas they were increased by DHA treatment. MPTP-treated animals exhibited shorter time on the rod test which was significantly increased by DHA treatment. DHA administration significantly decreased 4-HNE and nitrate/nitrite levels of SN supernatants and protected the TH (+) dopaminergic neurons of SN in the DHA + MPTP group compared to the MPTP group. DHA treatment significantly decreased nNOS and increased p-nNOS immunoreactivities in the DHA + MPTP group compared to the MPTP group.

Conclusions. These results indicate that DHA treatment protects dopaminergic neurons in SN *via* increasing nNOS serine 852 phosphorylation in the experimental mice model of PD. *(Folia Histochemica et Cytobiologica 2018, Vol. 56, No. 1, 27–37)*

Key words: Parkinson's disease; mice; protection; MPTP; DHA; nNOS phosphorylation; lipid peroxidation; caspase-3

Introduction

Parkinson's disease (PD) was first described in 1817 by Dr. James Parkinson; it is the most common neurodegenerative disease after Alzheimer's disease [1, 2]. Parkinson's disease results mainly from the death of dopaminergic neurons in the substantia nigra (SN).

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The definitive cause of PD is not entirely known but hypotheses such as some traumas [3], ageing [4], α-synuclein accumulation [5], genetic inclination and misfolded proteins [6], mitochondrial abnormalities [7], inflammation [8] and microglial activity [9] are being proposed.

One of the most important hypotheses of PD is the increased level of free radicals in SN. The formation of free radicals leads to the peroxidation of lipids [10]. Nitric oxide (NO) is a neurotransmitter in the nervous system that is synthesized by the enzyme nitric oxide synthase (NOS). It is known to produce nitrogen derivatives like nitrite and nitrate in the presence of oxygen and acts like a radical. It has also been shown that NO reacts with oxygen radicals and superoxides to form peroxynitrite ($ONOO^-$) [11]. It has been shown that the phosphorylation of different nNOS residues has different cellular effects. nNOS can be phosphorylated by various enzymes [12]. Being phosphorylated at serine 1412 increases nNOS activity [13], while nNOS is inhibited by phosphorylation at Ser847 [14].

Omega-3 polyunsaturated fatty acids (PUFAs) exhibit neuroprotective properties and represent a potential treatment for various kinds of neurodegenerative disorders [15]. It has been identified that the primary PUFA of the brain phospholipids is docosahexaenoic acid (DHA) which can affect nNOS and inducible NOS (iNOS) activities [16].

It is known that iNOS expression and nNOS activity increases in substantia nigra pars compacta (SNpc) after the administration of the neurotoxin 1-methyl-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) which is being used to create an experimental PD model [17]. In a previous study [18], it has been shown that nNOS plays a role in the death of dopaminergic neurons in SN. It was shown in the experimental PD models that DHA prevents the death of dopaminergic neurons containing tyrosine hydroxylase (TH) [19]. It is known that DHA affects neuronal cell membrane channels and intercellular signaling pathways activated by molecules such as adhesion proteins [20]. It was established that DHA has neuroprotective effects in experimental PD models [21]. However, it is not known whether the phosphorylation of nNOS at the serine 852 region that corresponds to Ser847 in the mouse and nNOS inhibition play a role in the protective effects of DHA in the experimental model of PD. This study was aimed to identify the role of DHA on nigral nNOS phosphorylation in an experimental mice model of PD and the results were supported by biochemical and motor parameters.

Materials and methods

Animals. Animal maintenance and treatment were carried out in accordance with the Institutional Animal Care and Use Committee at Akdeniz University Medical School, Antalya, Turkey. Male C57BL/6 mice (three months old, 25–30 g) were obtained from Akdeniz University Animal Care Unit. Animals were housed at an ambient temperature of 23 ± 2°C, in a 12 h light/dark cycle and were fed standard mice chow and tap water ad libitum up to day of the experiments.

Drug administration. One hundred male mice were randomly divided into 4 groups: control (C), DHA-treated (DHA), MPTP-injected (MPTP), DHA-treated and MPTP-injected (DHA + MPTP). DHA (36 mg/kg/day) was administered daily by gavage for four weeks to DHA and DHA + MPTP groups [22]. The experimental PD model was utilized by consecutive intraperitoneal (*i.p.*) injections of MPTP neurotoxin (4 × 20 mg/kg) at 2-h intervals on the 23rd day of the experimental schedule [17].

Assessment of behavioral activity

Measurement of motor activity. Motor activity of the mice was evaluated by "pole test" (as a marker for the intensity of bradykinesia, a typical symptom of PD) as well as the locomotor activity and rotarod tests [23–27]. All of the tests were performed on the 7th day after the injection of MPTP.

Pole test. The pole test is used to assess basal ganglia-related movement disorders in mice. To determine the degree of bradykinesia, the pole test was performed according to Ogawa *et al.* with minor modifications [23, 24]. Animals were placed head-up on top of a rough-surfaced iron pole (50 cm in length and 0.8 cm in diameter). The base of the pole is placed in a cage filled with bedding material. Sticking plaster was wrapped around the iron pole to increase traction. When placed on the pole, animals orient themselves downward and descend the length of the pole back into the cage. Trials were eliminated if the mouse jumped or slid down the pole rather than climbed down. After two days of training, animals were subjected to test trials. The time it took for the animal to turn (T-Turn) its head downwards (movement initiation) and t-turn + climb down the entire length of the pole (total turn time) was recorded. Averages of the best three measurements were taken as results.

Locomotor activity test. Locomotor activity was measured with an open-field activity monitoring system (MAY 9908 model Activity Monitoring System: Commat Ltd, Ankara, Turkey) [25]. Each of the eight locomotor activity chambers is a clear Plexiglas box (42 cm L × 42 cm W × 30 cm H) equipped with infrared photocells. These cages contain

fifteen photocell emitter and detector pairs that are mounted on opposite walls (2 cm above the chamber floor), and another 15 photocell pairs were located 8 cm above the floor. Breaks of the photocell beams were recorded by a computer system and the mouse's position in the chamber was calculated by the software at 0.1 s sensitivity. If the calculated locations were completely changed, this was expressed as ambulatory activity. Total distance moved and the total locomotor activity was recorded, too. For testing all parameters one mouse was placed in the center of the open field cage and its activity was recorded for 5 min. Before placing another animal, the open field was cleaned with 10% ethanol to attenuate odors.

Rotarod test. A rotarod test was used to determine the motor coordination and balance of the mice [26, 27]. For three consecutive days, mice were trained to the rotarod apparatus. On testing day, mice were habituated to the testing room for 30 min. Then they were placed on a rotating rod (model 7650 Rota-rod, Ugo Basile, Collegeville, PA, USA) that has rotating speed of 5, 10, 20, 30 or 40 rpm. The maximum duration of each trial was 5 min. The time that the mice fell off the rod was recorded. There were 5 min breaks between each recording speeds. Latency to fall was measured.

Tissue collection. After motor performance tests, at the end of the treatment period, mice were anesthetized with urethane (1 g/kg, *i.p.*) diluted in saline. Mice were perfused transcardially with heparinized saline and their brains were removed immediately, SN areas dissected and stored at −80°C for later biochemical analysis. For immunohistochemical (IHC) analysis, mice were perfused transcardially with heparinized saline and then with 10% formalin. Total brain tissues were removed and fixed in 10% formalin for 6 h and washed with tap water for approximately 2 h.

Immunohistochemistry. For the detection of TH, nNOS and phospho-nNOS (p-nNOS) immunoreactivities, paraffin sections were deparaffinized in xylene and rehydrated in a graded series of ethanol. Sections were immersed in 3% hydrogen peroxide in methanol for 15 min to block endogenous peroxidase activity. Slides were then incubated with a universal blocking reagent (TA-125-UB, LabVision Ultra V Block, Fremont, CA, USA) for 7 min at room temperature (RT). Afterwards, excess serum was drained and sections were incubated with primary antibodies; rabbit polyclonal anti-TH (1/800; #ab-112, Abcam, Cambridge, UK), rabbit polyclonal anti-nNOS (1/200; #ab-106417, Abcam) and rabbit polyclonal anti-nNOS (phospho S852, 1/200; #ab-58533, Abcam) in a humidified chamber overnight at 4°C. For negative controls, the primary antibodies were replaced by phosphate-buffered saline (PBS). After several washes in PBS, sections were incubated with biotinylated goat anti-rabbit IgG antibody (1/400; #BA-1000, Vector, Burlingame, CA, USA) for 30 min followed by incubation with LSAB streptavidin-peroxidase complex (Vector ABC kit, VECTOR, #PK400) for 45 min and were rinsed with PBS. Antibody complexes were visualized by incubation with diaminobenzidine (DAB) chromogen (TA-125-HD, DAB chromogen Substrate System, THERMO). Sections were counterstained with Mayer's hematoxylin (Dako, Glostrup, Denmark), dehydrated, mounted and examined by an Axioplan microscope (Zeiss, Oberkochen, Germany). The images were taken using a 5MP Canon A95 camera integrated to the microscope (Canon, Tokyo, Japan). TH immunoreactivity was evaluated using image-J analysis (NIH, Bethesda, MD, USA). Three randomly selected slides, each of ten different fields of SN were evaluated at 40× objective magnification. The distinct labeled cells with observed immunostaining were counted in every μm^2.

Biochemical measurements

Nitrate/nitrite assay. To determine total NO production in SN tissues, the levels of nitrate and nitrite that represent oxidized forms of NO, were determined in the SN supernatants by the use of nitrate/nitrite commercial colorimetric assay kit (CAYMAN-780001, Cayman Chemical Co, Ann Arbor, MI, USA). Whole SN tissues were weighed and homogenized in 20 ml of PBS per gram tissue on ice and centrifuged at 17,500 rpm for 20 min. Supernatants were collected and stored at –80°C. In this method, the nitrate in the SN samples was converted into nitrite by nitrate reductase, and the total nitrite levels were determined as the total nitrate/nitrite (NOx). Absorbance was read at 550 nm and nitrate/nitrite values were expressed as nanomole per mg protein.

4-hydroxynonenal (4-HNE) assay. (4-HNE)-His Adduct Elisa kit (OxiSelect HNE-STA-334, San Diego, CA, USA) was used to measure lipid peroxidation in SN tissue samples. The utilized protocol is an enzyme immunoassay developed for detection and quantitation of HNE-His protein adducts. Whole SN tissues were weighed and homogenized in 20 ml of PBS per gr tissue on ice and assayed for quantitation of HNE-His protein adduct according to manufacturer's instructions. The absorbance of samples were measured at 450 nm and the quantity of HNE adduct was expressed as ng per μg of tissue protein.

Caspase-3 activity assay. Caspase-3 activities in SN tissue samples were measured by quantifying the cleavage of acetyl-Asp-Glu-Val-Asp-p-nitroaniline (Ac-DEVD-pNa) with a colorimetric caspase-3 activity assay kit (Millipore-APT165, Darmstadt, Germany). Whole SN tissues were homogenized in 200 μL lysis buffer supplied with the kit and assayed according to the manufacturer's instruction. The absorbance was then measured at 405 nm in a microplate reader. The activity of the enzyme was calculated as units/mg protein and then expressed as fold increase.

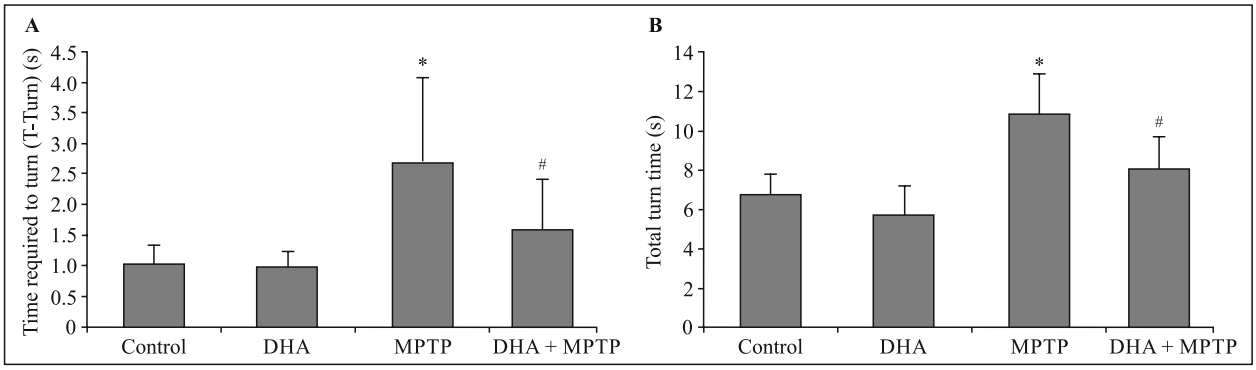

Figure 1. The effect of DHA on bradykinesia in an experimental model of Parkinson's disease measured by the pole test. Mice were treated with docosahexaenoic acid (DHA groups) for 4 weeks and a part of them was injected *i.p.* with neurotoxin MPTP (DHA + MPTP group) at the 23rd day as described in Methods. Some control mice were also treated with MPTP on the 23rd day (MPTP group). All animals were sampled after 4 weeks from the beginning of the experiment. **A.** The time it took for the animal to turn (T-Turn) its head downwards. **B.** T-Turn and climb down the entire length of the pole (total turn time) were shown in the graphs. Data are presented as means ± SEM, n = 20 in each group. *$p < 0.05$ *vs.* control group, #$p < 0.05$ *vs.* MPTP group.

Protein measurement. Protein concentrations were measured spectrophotometrically (Shimadzu RF-5500, Kyoto, Japan) at 595 nm by a modified Bradford assay [28] using Coomassie Plus reagent with bovine serum albumin as a standard (Pierce Chemical Company, Rockford, IL, USA).

Statistical analysis. The statistical analyses were conduct by using the SPSS v.23.0 (IBM Corp., Armonk, NY, USA). Data are expressed as mean ± SEM. Differences were analyzed *via* a one way analysis of variance (ANOVA) followed by Tukey's Post Hoc Test for normally distributed variables and Kruskal-Wallis followed up Mann-Whitney U test for non-normally distributed variables. P values less than 0.05 were considered as significant.

Results

Motor performances

Bradykinesia

Changes in bradykinesia (slow-down of movements) are presented in Figure 1. Time required to turn (T-Turn) (A) and total turn time (B) were determined for all groups. MPTP-injected mice showed elongated T-Turn and total turn time as compared with the control group. DHA treatment improved T-turn and total turn times in DHA + MPTP group compared to the MPTP group but the values were still above the control group levels.

Locomotor activity

MPTP-injected mice exhibited reduced ambulatory, total distance and total locomotor activity compared to the control group. These activities increased in the DHA + MPTP group compared to the MPTP group but did not reach the values of the control group (Fig. 2).

Rotarod

Rotarod performances were measured for five consecutive trials in all groups. It was seen that the time mice stayed on the rod rotating at 40 rpm decreased in the MPTP group when compared to the control group. The treatment with DHA significantly increased the time on the rod at 40 rpm in the DHA + MPTP group compared to the MPTP group. However, it did not reach level of the control group (Fig. 3).

The immunoreactivity of the studied proteins in the substantia nigra

Tyrosine hydroxylase immunoreactivity

Representative microphotographs of dopaminergic neurons that expressed TH immunoreactivity and the number of TH-immunostained cells are shown in Figure 4. The immunoreactivity for TH was observed in neuron bodies. The number of TH-immunoreactive (-ir) cells in the MPTP group was significantly lower (75%) compared to the control group. Dopaminergic neuron number significantly increased two-fold in the DHA + MPTP group compared to the MPTP group but it did not reach the level of the control group.

Neuronal nitric oxide synthase immunoreactivity

Representative microphotographs of nNOS immunoreactivity of dopaminergic neurons in SN are shown in Figure 5. nNOS immunoreactivity was weakly positive in neuron cytoplasm (arrowheads) in the control and

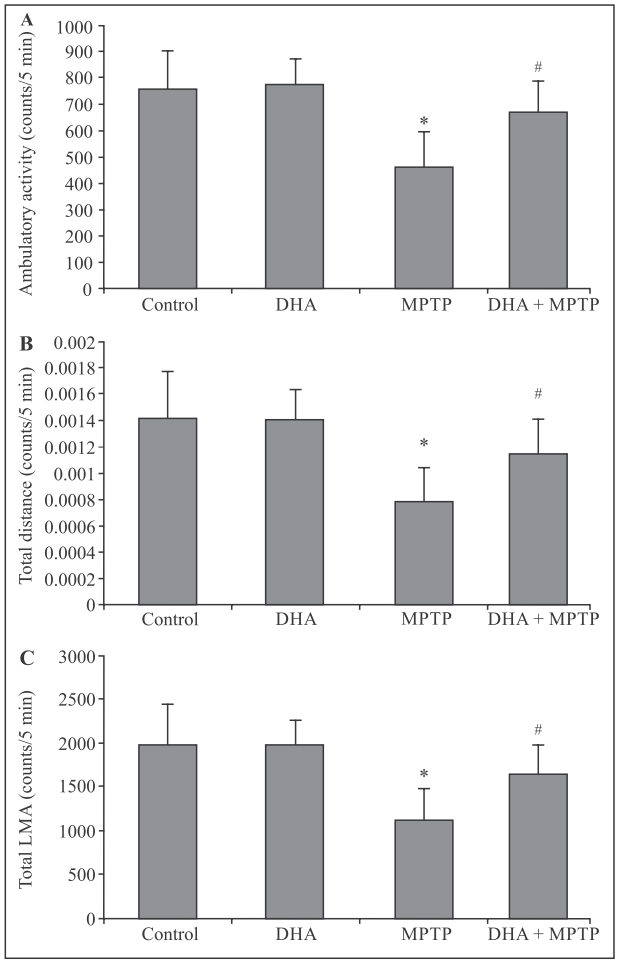

Figure 2. The effects of DHA on locomotor activities of mice in an experimental model of Parkinson's disease. The experiment was planned and groups of animals designed as described in the legend to Figure 1. **A.** Ambulatory Activity. **B.** Total Distance. C. Total Locomotor Activity (Total LMA) of experimental groups. Data are means ± SEM, n = 14 in each group. *p < 0.05 *vs.* control group, #p < 0.05 *vs.* MPTP group.

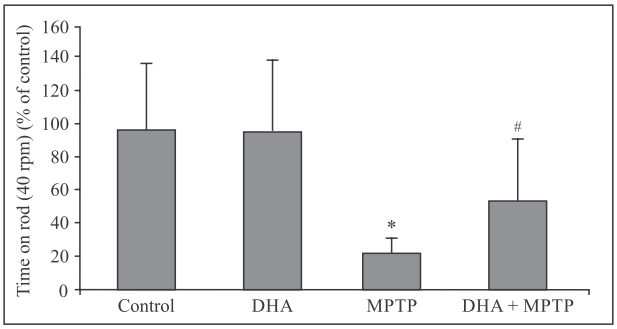

Figure 3. The effect of DHA on rotarod performances of mice in an experimental model of Parkinson's disease. The experiment was planned and groups of animals designed as described in the legend to Figure 1. Data are means ± SEM, n = 10 in each group. *p < 0.05 *vs.* control group. #p < 0.05 *vs.* MPTP group.

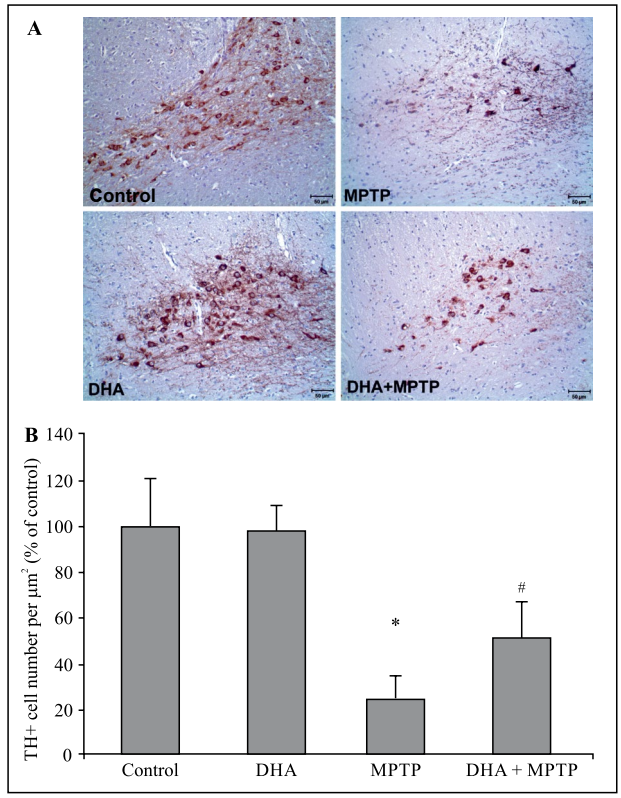

Figure 4. Tyrosine hydroxylase (TH)-immunoreactivity in dopaminergic neurons of substantia nigra in brains of mice in an experimental model of Parkinson disease. The experiment was planned and groups of animals designed as described in the legend to Figure 1. **A.** The number of TH (+) dopaminergic neurons was clearly decreased in the MPTP group compared to the control group. An increase in dopaminergic neuron numbers were seen in the DHA + MPTP group compared to the MPTP group. Magnifications are presented as 40×. Scale bars — 50 μm. **B.** The percentage of the number of TH (+) neurons in the experimental groups as compared to control mice. Data are means ± SEM, n = 5 in each group. *p < 0.05 *vs.* control group. #p < 0.05 *vs.* MPTP group.

DHA groups. nNOS showed strong immunoreactivity in the MPTP group (arrows). The reactions were clearly increased in the MPTP group compared to the control group. nNOS immunoreactivity was decreased in the DHA + MPTP group compared to the MPTP group (arrows) but it was still higher than in the control group.

Phosphorylated nNOS immunoreactivity

Coronal brain sections of SN were immunostained with p-nNOS antibody which is a phosphorylation marker of nNOS serine 852 region that corresponds to Ser847 in the mouse. In these sections, there was little immunoreactivity (arrowheads) in the control and DHA groups. p-nNOS expression was observed in the MPTP group but it was more prominent in the

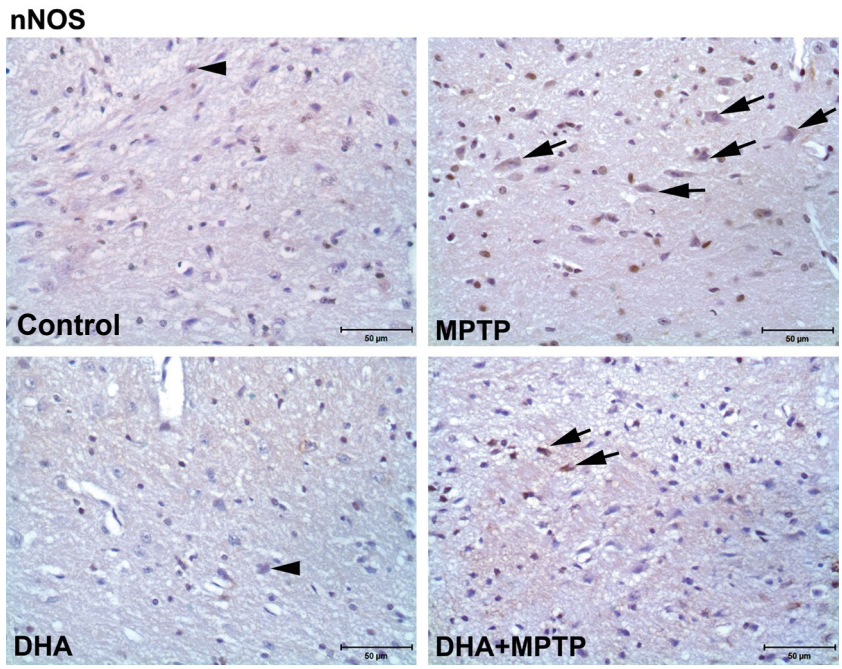

Figure 5. Neuronal nitric oxide synthase (nNOS)-immunoreactivity of dopaminergic neurons of substantia nigra in brains of mice in an experimental model of Parkinson disease. The experiment was planned and groups of animals designed as described in the legend to Figure 1. DHA treatment did not cause any difference in the expression of nNOS of dopaminergic neurons compared with the control group. Immunohistochemical localizations of nNOS were prominent in the cytoplasm of neurons with an increased expression in the MPTP and DHA + MPTP groups compared to the control group (arrows). However, DHA treatment decreased the nNOS expression in the DHA + MPTP group compared to the MPTP group (arrows). Magnifications, 40×; scale bars 50 μm; n = 5 in each group.

DHA + MPTP group compared to the MPTP group (Fig. 6, arrows).

The biochemical parameters in the substantia nigra

Nitrate/nitrite levels

The levels of nitrate and nitrite, the products of NO degradation, were higher in the MPTP group than in the control group. The treatment with DHA of MPTP-treated mice decreased nitrate/nitrite levels compared to the MPTP group without reaching the control group levels (Fig. 7).

4-HNE levels

4-HNE is an indicator of lipid peroxidation. The level of nigral 4-HNE increased in the MPTP group compared to the control group. DHA treatment decreased the level of nigral 4-HNE in the DHA + MPTP group compared to the MPTP group but it did not reach control levels (Fig. 8).

Caspase-3 activity

Caspase-3 activity levels are presented in Figure 9. Caspase-3 activity was higher in the MPTP group compared to the control group. DHA treatment had no effect on caspase-3 activity in the DHA + MPTP group compared to the MPTP group.

Discussion

In the present study, we evaluated the effects of DHA treatment on mice subjected to the experimental model of Parkinson's disease. The PD model was developed by MPTP injection, and we have found that DHA treatment has a restorative effect on locomotion. Additionally, the role of nNOS and its phosphorylation in the DHA's restorative effect on locomotor activity was investigated and the results were supported by biochemical and immunohistochemical findings. In the MPTP mouse model of PD, as a neurotoxin MPTP crosses the blood brain barrier (BBB). It is metabolized into 1-methyl 4-phenylpridinium (MPP^+) and selectively affects the mesencephalic dopaminergic neurons [29]. The MPTP dose defines the level of damage to dopaminergic neurons. While the MPTP technique fails when administered to rats, it is known that C57BL/6 mice are more susceptible to systemic MPTP administration than other murine strains. Thus, we used this model in our study and administered four doses consistent

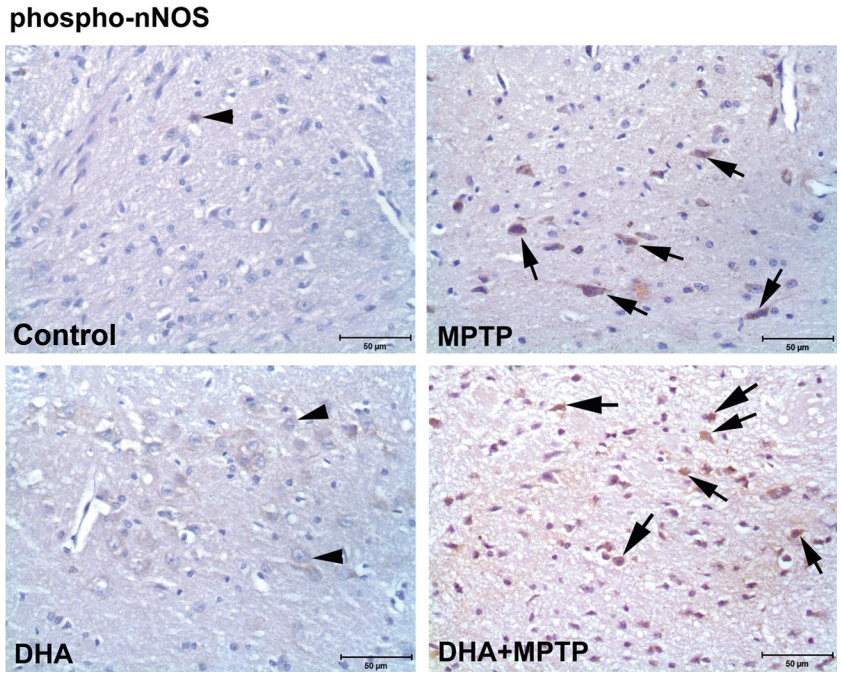

Figure 6. Phosphorylated neuronal nitric oxide synthase (p-nNOS)-immunoreactivity of dopaminergic neurons of substantia nigra in brains of mice in an experimental model of Parkinson disease. The experiment was planned and groups of animals designed as described in the legend to Figure 1. DHA treatment did not cause any difference in the expression of p-nNOS of neurons compared to the control group. Immunohistochemical localizations of p-nNOS were prominent in the cytoplasm of neurons with an increased expression in the MPTP and DHA + MPTP groups compared to the control group (arrows). However, DHA treatment caused p-nNOS expression to increase in the DHA + MPTP group compared to the MPTP group (arrows). Magnifications, 40×; scale bars 50 μm; n = 5 in each group.

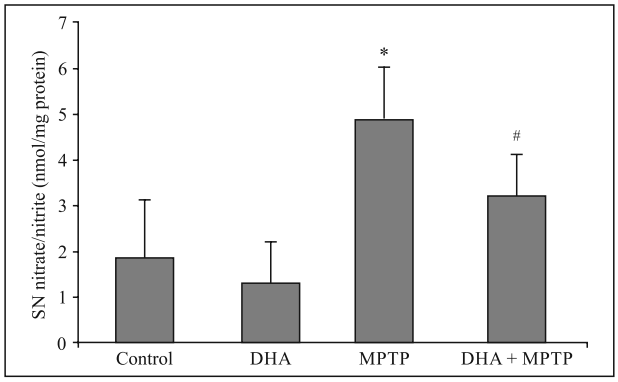

Figure 7. The effect of DHA on nitrate/nitrite levels that represent oxidized forms of NO in the supernatants of substantia nigra (SN) in an experimental model of Parkinson's disease. The experiment was planned and groups of animals designed as described in the legend to Figure 1. Data are means ± SEM, n = 6 in each group. *p < 0.05 *vs.* control group. #p < 0.05 *vs.* MPTP group.

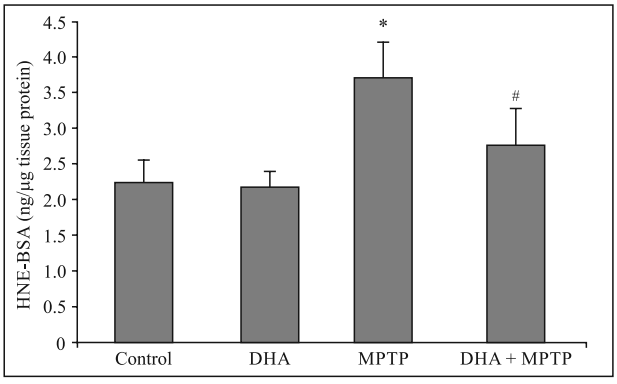

Figure 8. The effect of DHA on 4-hydroxynonenal (4-HNE) levels that represent lipid peroxidation in the supernatants of substantia nigra (SN) in an experimental model of Parkinson's disease. The experiment was planned and groups of animals designed as described in the legend to Figure 1. Data are means ± SEM, n = 6 in each group. *p < 0.05 *vs.* control group. #p < 0.05 *vs.* MPTP group.

with the literature (4 × 20 mg/kg) every two hours intraperitoneally [17].

It was shown that DHA, which is important for brain function, changes the composition of fatty acids in the brain's neuronal membranes when it is given as a supplement [30]. DHA is used at various concentrations (10–100 mg/kg/day) in neurobiological studies [31, 32]. We used the dose we found to be effective but not toxic as shown in our previous studies [33, 34].To identify successful implementation of PD in

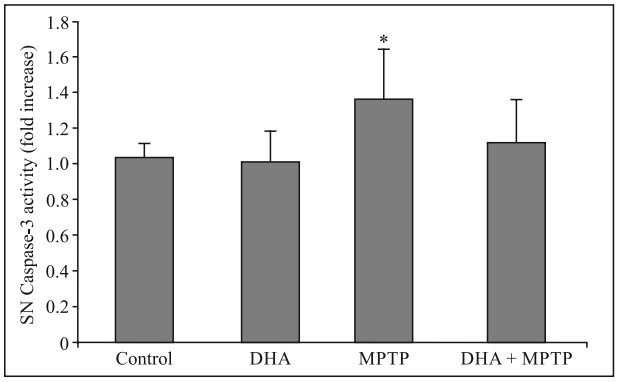

Figure 9. The effect of DHA on Caspase-3 activity levels that represent apoptosis in the supernatants of substantia nigra (SN) in an experimental model of Parkinson's disease. The experiment was planned and groups of animals designed as described in the legend to Figure 1. Data are means ± SEM, n = 6 in each group. *p < 0.05 *vs.* control group.

animals behavioral tests, such as bradykinesia levels, locomotor activity and motor coordination must be performed. One of the commonly used tests for this purpose is the measurement of bradykinesia by a pole test. In the present study, it was observed in the MPTP group that the initiation of movement was delayed and the time to go down was prolonged. We found that pretreatment with DHA before the injection of MPTP neurotoxin restored bradykinesia in the DHA + MPTP group. This is an important finding that underscores the restorative potential of DHA supplementation in this experimental model of PD. In this study, the locomotor activity test parameters (ambulatory activity, total distance and total locomotor activities) were decreased by MPTP injection. DHA administration significantly improved the performances of the MPTP intoxicated mice although it did not reach control levels. In a previous study, it has been shown that the administration of DHA (100 mg/kg) reduced dyskinesia and restored motor activity disorders in MPTP-intoxicated monkeys [31].

Rotarod test mainly involves the motor performance and balance of mice. In recent study, DHA administration improved these parameters in MPTP-intoxicated mice as it was seen in the other motor tests. In a previous study it was shown that the diet consisting of regular chow supplemented with 0.8% ethyl-eicosapentaenoate (E-EPA) prolongs the time on the rotarod in MPTP-injected (10 × 25 mg/kg) rats [35].

TH is the rate-limiting enzyme in the synthesis of catecholamines [36]. TH-immunoreactive cells are densely located in the SN and extend to the striatum. The loss of TH (+) neurons in the SN causes an estimated 70–80% depletion of dopamine (DA) in the striatum [37]. Therefore, TH is generally a phenotypic marker for identifying and counting dopaminergic neurons and has been often used as an indicator of dopaminergic neuron damage [38]. In an earlier study, it was shown that n-3 PUFA enriched diet exhibits neuroprotective effect against MPTP-induced neurotoxicity [19]. In the present study, when the histological slices were examined, it was seen that the number of neurons in SN decreased by 75% in the MPTP group and the neurons decreased by 50% in the DHA + MPTP group. Altogether, our data show that the experimental PD model was developed successfully and that DHA had a protective effect.

Nitric oxide is one of the many agents that causes the formation of free radicals and is a very reactive and unstable gas. It is a neurotransmitter in the basal ganglia of the central nervous system (CNS), and it is known that its synthesis increases in PD [39]. Previous studies have shown that the activity of iNOS and nNOS, which plays a role in the synthesis of NO, also has a role in the developing of neurodegeneration in PD [40]. Also the results of our study revealed that the amount of nitrate/nitrite (breakdown products of NO) commonly used as indicators of NO tissue levels, increased in the SN of MPTP group, most probably as a result of nNOS and iNOS induction by MPTP. In previous studies, it was found that the nitrate/nitrite levels increased in the MPTP or 6-OHDA-induced rat models of PD [41, 42]. In our study, the administration of DHA to the MPTP-treated mice decreased the nitrate/nitrite levels in substantia nigra. This might be related to the phosphorylation of nNOS or suppression of NO synthesis because of lower L-arginin entry into the cell. In previous studies it was proposed that omega-3 acids decrease the catalytic activity of nNOS through calcium-calmodulin-dependent protein kinases, which facilitate the phosphorylation of nNOS at serine 847 [43, 44]. Therefore, in our study, the suppressive effect of DHA on nitrate/nitrite levels may be associated with the serine 852 phosphorylation of nNOS which was shown to decrease since nNOS activity decreases when the protein is phosphorylated at the inhibition site of Ser847 [14]. For this reason, we used the serine 852 antibody that corresponded to Ser847 in the mouse to identify the effect of DHA on nNOS phosphorylation. Although nNOS phosphorylation was slightly expressed in the control and DHA groups, it was found that nNOS phosphorylation at serine 852 was increased in the DHA+MPTP group compared to the MPTP group. To our knowledge, there are no studies suggesting that the protective effect of DHA treatment in experimental PD may be mediated by nNOS phosphorylation, so this is an original and new finding.

The balance between free radical production and its neutralization by a complex antioxidant system is disturbed in SN of PD patients [45]. The high content of unsaturated fatty acids in the CNS makes this tissue susceptible to free radicals' attack. Free radicals cause oxidation of lipids in the cell membranes and lead to important changes in cell functions by forming lipid peroxidation products [46]. Because the free radicals have short half-lives and low concentrations and are highly reactive, they are difficult to measure [47]. In our study, the 4-HNE method has been used to identify the lipid peroxidation caused by oxidative damage. Oxidative stress leads to the degradation of very unstable lipid hydroperoxides by elimination reactions and the formation of secondary products such as aldehydes like cytotoxic 4-HNE [48]. Oxygen radicals are secondary toxic messengers of lipid peroxidation. It is known that lipid peroxidation and the 4-HNE level in SN increases in PD [49]. This increase intensifies the activation of caspases 3–8 what leads to apoptosis [50] and formation of α-synuclein aggregates [51]. In this study we found that the injection of MPTP increased nigral 4-HNE amount. These findings are consistent with the literature data [52, 53]. In our study, the administration of DHA decreased the content of 4-HNE in the substantia nigra of DHA+MPTP group. In a previous study, it was found that the gene expression of antioxidant enzymes are upregulated by omega-3 fatty acids and they downregulate the genes related with the production of reactive oxygen species (ROS) [54].

MPTP inhibits the respiratory chain in the mitochondria and increases superoxide generation [29]. It also activates NOS and causes nitrative stress [55]. The increase in free radicals leads to the release of mitochondrial cytochrome-c [56]. Seven copies of a heterodimer between apoptotic protease-activating factor 1 (Apaf-1) and cytochrome c (cytc) form apoptosome which leads to activation of initiator caspase-9 that activates caspase-3 [57]. The indicator of apoptosis is caspase-3 enzyme activity. Besides the increase of free radicals, lipid peroxidation is also effective in the initiation of apoptosis [58]. In our study, caspase-3 activity in the substantia nigra was increased after MPTP treatment. When DHA was administered, nigral caspase-3 activity was slightly suppressed, however, this decrease was not significant compared to the MPTP group.

However, it has to be stressed that DHA does not produce its effects only on above mentioned pathways: it is known that DHA affects the characteristics instead of the cellular membrane [59], retinoid X receptors [60], and the dopamine transporter (DAT) activity of the cellular membrane [61]. It was shown that PUFA produces its effects by decreasing the transformation of MPTP to MPP^+ and monoamine oxidase-B (MAO-B) activity [62].

Finally, in our study, it was identified that DHA has restorative effects on MPTP-induced motor activity disturbances, lipid peroxidation and apoptosis in the experimental mice model of PD. The serine 852 phosphorylation of nNOS may have a role in this protective effect. However, further studies are warranted to fully elucidate the protective mechanisms of DHA.

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