Protective Effect of Nervonic Acid Against 6-Hydroxydopamine-Induced Oxidative Stress in PC-12 Cells

Hiroki Umemoto1#, Saika Yasugi2#, Shinji Tsuda1, Minoru Yoda1, Takashi Ishiguro1, Naoko Kaba2, and Tatsuki Itoh2*

1 Technical Department, Foods Division, Miyoshi Oil and Fat Co. Ltd., 4-66-1, Horikiri, Katsushika-ku, Tokyo 124-8510, JAPAN
2 Department of Food Science and Nutrition, Faculty of Agriculture, Kindai University, 3327-204, Nakamachi, Nara-shi, Nara 631-8505, JAPAN

Abstract: Increased oxidative stress in the human brain is observed in neurodegenerative diseases such as Parkinson’s disease (PD) and Alzheimer’s disease (AD), and is considered to be a major cause of progression of these disease states. A very long-chain fatty acid, nervonic acid (NA), is the main fatty acid found in various sphingolipid species in the central nervous system. NA plays an important role in forming the plasma membrane’s lipid bilayer and in maintaining normal myelin function. In this study, we examined the neuroprotective effect of NA against rat pheochromocytoma (PC-12) cells stimulated by 6-hydroxydopamine (6-OHDA), which served as a cell model of PD. PC-12 cells were pre-treated with different concentrations of NA for 48 h then subsequently co-treated with NA and 6-OHDA for 48 h to induce cellular oxidative stress. Cell viability was significantly increased by pre-treatment with a very low concentration of NA. The level of malondialdehyde, a marker of lipid peroxidation, was significantly decreased in NA-treated cells. The expression levels of superoxide dismutases (Mn SOD and Cu/Zn SOD) and γ-glutamylcysteine synthetase (GCLC), responsible for the synthesis of glutathione, were significantly increased, indicating that pre-treatment with NA activated the cellular antioxidant defense system. These results suggest that NA may play a role as a neuroprotective mediator in the brain.

Key words: nervonic acid, oxidative stress, PC-12 cell, Parkinson’s disease, antioxidant

1 Introduction

The human brain utilizes about 20% of the oxygen supply in the body, with between 1% and 2% of O2 consumed converted to reactive oxygen species (ROS)1). The brain is particularly susceptible to damage caused by ROS because of its high proportion of lipids and polyunsaturated fatty acids compared with the proportion of these molecules in other organs. Oxidative stress in the brain induces cell damage and death and has been implicated in the progression of several neurodegenerative diseases, including Alzheimer’s disease (AD) and Parkinson’s disease (PD)1). PD is the most common neurodegenerative-linked movement disorder and is characterized by a loss of dopaminergic neurons in the substantia nigra. It has been proposed that this loss of dopaminergic neurons leads to the depletion of dopamine and subsequent release of excess acetylcholine from the striatum; as a result, information transfer from the motor command centers in the cerebral cortex is interrupted2). The mechanisms of dopaminergic neuron degeneration in the PD brain are complex and are yet to be fully elucidated. However, increased levels of oxidized lipid, protein, DNA, and RNA have been observed in postmortem brains of patients with PD. Accumulating evidence indicates that increased oxidative stress in the brain is related to progression and further neurodegeneration in PD.

Many studies have investigated ways to reduce oxidative stress in the brain of patients with PD to alleviate or prevent the disease. Oxidative stress can be defined as a disequilibrium between the levels of ROS and the ability of a biological system to detoxify these reactive intermediates using endogenous antioxidants3–6). The mechanisms for the generation of increased ROS in PD are recognized as being due to the abnormal metabolism of dopamine, decreased levels of endogenous antioxidants such as glutathione and superoxide dismutase (SOD), or high levels of iron
or calcium in the substantia nigra pars compacta. Therefore, enhancing the biological antioxidant system is considered to be an attractive approach for the inhibition of pathological progression and prevention of pathogenesis in PD.

Here, we focused on nervonic acid (NA, cis-15-tetracosenoic acid, C24:1), a major fatty acid in the central nervous system (CNS). NA is a very long chain monounsaturated fatty acid that binds to several sphingolipid species which constitute white matter and myelin sheath in the human brain. NA is biosynthesized at the same time as myelination occurs, from the prenatal to the neonatal phase, and is essential for neural transmission in the CNS. In demyelinating diseases, such as X-adrenoleukodystrophy (X-ALD) and multiple sclerosis (MS), impairment of NA and the accumulation of very long chain saturated fatty acids (e.g., C24:0 and C26:0) in the sphingolipids of myelin sheath are seen in the postmortem brain. The shift in sphingolipid acyl chain length leads to changes in membrane fluidity and an increase of oxidative stress and subsequent demyelination. Lorenzo’s oil (a 4:1 mixture of glyceryl trioleate and glyceryl triruculate, precursors of NA) has been used in diet therapy for patients with X-ALD. Sass et al. reported that Lorenzo’s oil inhibited ELOVL1, an enzyme responsible for the synthesis of very long chain fatty acids and subsequently involved in the reduction of very long chain saturated fatty acid levels and the elevation of NA levels in sphingomyelin. These changes in sphingomyelin composition probably contribute to normalizing membrane fluidity and reducing cellular oxidative stress. Thus, NA may play a role as an antioxidant mediator in human brain cells.

The purpose of this study was to examine the anti-oxidative and anti-inflammatory effects of NA in cultured PD model cells. Rat phaeochromocytoma (PC-12) cells stimulated by 6-hydroxydopamine (6-OHDA) to induce cellular oxidative stress served as a pathological cell model of PD. We evaluated if the NA had a dose-dependent effect against these cells.

2 Experimental Procedures

2.1 Materials

Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), penicillin, and streptomycin were purchased from Gibco (CA, USA). Dimethyl sulfoxide (DMSO) and 6-OHDA were purchased from Nacalai tesque (Kyoto, Japan). NA (>99% grade) was obtained from Nu-Chek Prep (MN, USA). The MDA assay kit was purchased from Wako Pure Chemical Industries (Osaka, Japan). ISOGEN™ was purchased from Nippon Gene (Tokyo, Japan). The Takara RNA Kit™ was produced by Takara Bio (Shiga, Japan). Amersham ECL Prime western blotting detection reagent™ was purchased from GE Healthcare (IL, USA).

2.2 Cell culture and treatment

PC-12 cells were cultured in DMEM medium supplemented with 10% FBS, 20 U/mL penicillin, and 20 μg/mL streptomycin at 37°C in a 5% CO2 humidified incubator. When the cells reached 90% confluence, they were harvested using 0.025% trypsin and seeded into a 96-well plate at 2 × 10^4 cells/mL. The protective effect of NA in PC-12 cells was evaluated by either pre-treatment or cotreatment with 6-OHDA. For the pre-treatment procedure, NA dissolved in DMSO was added to the medium at various concentrations (0.001, 0.01, 0.1, 1, or 10 μM) and pre-cultured at 37°C for 48 h. Then, the medium was replaced with fresh medium and treated with an equivalent amount of NA and 50 μM of 6-OHDA at 37°C for 48 h. For the cotreatment procedure, the cells were treated with NA (0.05, 0.1, 0.5, or 1 μM) and 50 μM of 6-OHDA, then cultured at 37°C for 48 h. For the negative control group, the cells were treated with 50 μM of 6-OHDA only. All tests were carried out in triplicate or quadruplicate.

2.3 MTT Assay

Cell viability was measured using the methythiazole tetrazolium (MTT) assay. MTT labeling solution was prepared at a concentration of 5 mg/mL in sterile PBS. After 6-OHDA treatment of the PC-12 cells, each medium was replaced with fresh medium. Then, 50 μL of MTT solution was added to each well and the plates were incubated at 37°C for 1.5 h. To dissolve the formazan salt that formed, 50 μL of MTT solution was added to each well and the plates were incubated at 37°C for 48 h. The level of malondialdehyde (MDA) was quantified as a marker of lipid peroxidation. Cultured PC-12 cells were washed with PBS buffer and homogenized using an ultrasonic processor. The suspension was centrifuged at 12,000 × g for 15 min, and the supernatant was subjected to the MDA assay kit, i.e. 250 μL of the supernatant was added to a 1.5 mL Eppendorf tube, then 10 μL BHT solution, 250 μL 1 M phosphoric acid solution, and 250 μL TBA solution were added to the tube. The tube was incubated at 60°C for 1 h and then the absorbance at 514 nm was measured.

2.4 Quantitation of MDA

The level of malondialdehyde (MDA) was quantified as a marker of lipid peroxidation. Cultured PC-12 cells were washed with PBS buffer and homogenized using an ultrasonic processor. The suspension was centrifuged at 12,000 × g for 15 min, and the supernatant was subjected to the MDA assay kit, i.e. 250 μL of the supernatant was added to a 1.5 mL Eppendorf tube, then 10 μL BHT solution, 250 μL 1 M phosphoric acid solution, and 250 μL TBA solution were added to the tube. The tube was incubated at 60°C for 1 h and then the absorbance at 514 nm was measured.

2.5 Quantitative real-time PCR

The gene expression levels of Bax (an apoptosis promoter) and Bcl-2 (an apoptosis inhibitor) were evaluated using quantitative real-time PCR (qRT-PCR). Cultured PC-12 cells were washed with PBS buffer, and total RNA was extracted using ISOGEN™ containing phenol and guanidine thiocyanate. The concentration of RNA was determined by measuring the ratio of the absorption (260/280 nm) and adjusting it to 100 ng/μL. Reverse transcription to cDNA and
PCR amplification were performed using a Takara RNA PCR kit according to the manufacturer’s instructions. Total RNA (1 μL) was reverse transcribed in 9 μL of RT Master Mix (Takara Bio, Japan), containing MgCl₂, Ex Taq buffer, dNTP, RNase inhibitor, reverse transcriptase XL, and the primers. The reaction was performed using a Thermo Hybaid PCR Express cycler (Thermo Scientific) and the cycle was carried out as follows: 55°C for 30 min, 99°C for 5 min, and 5°C for 5 min. The subsequent PCR reaction was carried out using 20 μL PCR Master Mix (Takara Bio), containing MgCl₂, Ex Taq buffer, Takara Ex Taq HS, and the primers. Initial denaturation was carried out at 94°C for 2 min and one cycle was as follows: 94°C for 0.5 min, 60°C for 0.5 min, and 72°C for 1.5 min. Each of the primers, corresponding to Bax, Bcl-2, and GAPDH (internal control), is shown in Table 1. The PCR products were analyzed by electrophoresis in 2% agarose gel stained with ethidium bromide, using an Image Quant LAS-4000 mini system (Fujiﬁlm, Japan).

2.6 Western blotting and immunoassay

The protein expression levels of Caspase-3, Cox-2, Cu/Zn superoxide dismutase (Cu/Zn SOD), Mn superoxide dismutase (Mn SOD), and γ-glutamylcysteine synthetase (GCLC) were examined using western blotting. Cultured PC-12 cells were washed with PBS buffer, and total protein was extracted using RIPA buffer. The suspension was centrifuged, and the supernatants were subjected to the BCA protein assay kit to determine protein content. Total protein (10 μg) was separated by SDS-PAGE followed by transfer to a PVDF membrane. The PVDF membrane was precipitated in each of the following solvents for 1 h; 5% skim milk in tris-buffered saline containing 0.05% of tween 20 (TBST) for GAPDH, Caspase-3, Cox-2, Cu/Zn SOD, and Mn SOD, or 1% BSA in TBST for GCLC. The membrane was incubated with respective primary antibodies, including GAPDH (1:10,000), Caspase-3 (1:500), Cox-2 (1:1,000), Cu/Zn SOD (1:5,000), Mn SOD (1:2,000), and GCLC (1:1,000), overnight at 4°C. Blots were washed three times with TBST and incubated with respective secondary antibodies (anti-IgG) for 1 h. The immunoreactive bands were visualized using Amersham ECL Prime western blotting detection reagent and analyzed using the Image Quant LAS-4000 mini system.

2.7 Statistical analysis

All data were expressed as the mean ± SD. Statistical comparisons were performed using one-way ANOVA followed by a Tukey’s post hoc test. P values < 0.05 were considered to be statistically significant.

3 Results

3.1 Effect of NA on 6-OHDA-induced neurotoxicity

DMSO used for the resolution of 6-OHDA and NA had no toxic effect on PC-12 cell viability compared with cell viability in the control group (Fig. 1). Treatment of PC-12 cells with NA alone also showed no statistically significant differences in cell viability compared with that of the control group. However, PC-12 cell viability markedly decreased with increasing concentrations of 6-OHDA. The concentration of 6-OHDA (50 μM) at which cell viability was 70% after 48 h was used for further study.

The protective effect of NA against oxidative stress in PC-12 cells was evaluated by either pre-treatment or co-treatment with 6-OHDA. Following co-treatment, the addition of NA did not recover cell viability, suggesting that NA itself had no direct protective effect against 6-OHDA generating oxidative stress (Fig. 2(a)). However, cells pre-treated with a very low concentration of NA (0.001, 0.01, or 0.1 μM) followed by co-treatment with an equivalent amount of NA and 50 μM of 6-OHDA showed significantly higher viability than the 6-OHDA only group (p < 0.05, Fig. 2(b)). High doses of NA (1 and 10 μM) did not result in any changes in cell viability.

3.2 Effects of NA on cellular oxidative stress

Cellular oxidative stress was evaluated using PC-12 cells pre-treated with 0.01 μM NA followed by co-treatment with NA and 6-OHDA. The level of MDA was significantly increased in the 6-OHDA group compared to that in the control group (p < 0.01, Fig. 3). Although the NA group showed a higher MDA level than the control group (p <
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Cell viability (as determined by an MTT assay) of cells cultured with NA and without 6-OHDA for 48 h. Data are shown as mean ± SD.

Fig. 2 Cell viability (as determined by an MTT assay) of cells (a) co-treated with NA and 6-OHDA for 48 h, and (b) pre-treated with NA for 48 h followed by co-treatment with NA and 6-OHDA for 48 h. Data are shown as mean ± SD. # $p < 0.05$ compared with the control group. * $p < 0.05$ compared with the 6-OHDA group.

Fig. 3 The levels of cellular MDA pre-treatment with 0.01 μM NA for 48 h followed by co-treatment with NA and 6-OHDA for 48 h. GAPDH was used as an internal control. Data are shown as mean ± SD. ## $p < 0.01$ compared with the control group; * $p < 0.05$ compared with the 6-OHDA group.

0.01), the level was significantly reduced compared with that in the 6-OHDA group, suggesting that the cellular damage induced by 6-OHDA was alleviated by the addition of NA ($p < 0.05$, Fig. 3).

The gene expression levels of Bax and Bcl-2 were examined using qRT-PCR. GAPDH was used as an internal control. In this study, no change was observed in the levels of expression of either gene (Figs. 4(a), (b)). The expression levels of an apoptosis-related enzyme, Caspase-3, and an inflammation-related enzyme, Cox-2, were examined by western blotting. The NA group showed a tendency toward decreased levels of Caspase-3, but this was not statistically significant (Fig. 4(c)). The levels of Cox-2 were significantly reduced compared with in the 6-OHDA alone group, suggesting that the 6-OHDA induced inflammatory was alleviated in the NA group ($p < 0.05$, Fig. 4(d)).

Superoxide dismutases (SOD), such as Mn SOD and Cu/Zn SOD, catalyze the dismutation of superoxide radicals and are important form of antioxidant defense in the human brain. Mn SOD expressed in mitochondria was significantly increased in the NA group compared with its expression in the control ($p < 0.01$) and the 6-OHDA groups ($p$
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**Fig. 4** The transcript levels of apoptosis related genes, (a) Bax, (b) Bcl-2, (c) Caspase-3, and (d) Cox-2 pre-treated with 0.01 μM NA for 48 h followed by co-treatment with NA and 6-OHDA for 48 h. GAPDH was used as an internal control. Data are shown as mean ± SD. # p<0.05 compared with the control group; * p<0.05 compared with the 6-OHDA group.

**Fig. 5** The expression levels of superoxide dismutase enzyme (a) Mn SOD and (b) Cu/Zn SOD, and (c) GCLC enzyme pre-treated with 0.01 μM NA for 48 h followed by co-treatment with NA and 6-OHDA for 48 h. GAPDH was used as an internal control. Data are shown as mean ± SD. # p<0.05, ## p<0.01 compared with the control group; *p<0.05, **p<0.01 compared with the 6-OHDA group.
<0.05, Fig. 5(a)). Cu/Zn SOD, which is expressed in the cytosol, was also significantly increased in the NA group compared with its level in the control ($p<0.01$) and 6-OHDA groups ($p<0.05$, Fig. 5(b)). Furthermore, the level of GCLC, which is the rate limiting enzyme of glutathione synthesis, was significantly increased in the NA group compared with its level in both the control and 6-OHDA groups ($p<0.01$, Fig. 5(c)).

4 Discussion

Increased oxidative stress in the brain is considered to be a major factor in the progression of neurodegenerative diseases such as AD and PD. Many studies have investigated ways to reduce the damage caused by ROS by using edible ingredients, such as antioxidants (e.g. quercetin\textsuperscript{12}, rutin\textsuperscript{13}, and resveratrol\textsuperscript{14}), omega-3 fatty acids\textsuperscript{15}, coenzyme Q10\textsuperscript{16}, and selenium\textsuperscript{17}. We recently found that NA has potential anti-oxidative and neuroprotective effects in human cells.

In this study, we examined the protective effect of NA against 6-OHDA-induced oxidative stress in PC-12 cells. Although co-treatment of PC-12 cells with 6-OHDA and NA showed no difference in cell viability compared with the viability of the group treated with 6-OHDA alone, treatment with NA prior to 6-OHDA treatment resulted in a significant increase in cell viability. The level of MDA decreased in NA pre-treated cells, suggesting that cellular oxidative stress was decreased in this group. The expression levels of Mn SOD, Cu/Zn SOD, and GCLC also significantly increased in this group, suggesting that pre-treatment of NA upregulated the ROS elimination pathway.

NA has been identified in several seed oils, including those of \textit{Lunaria annua} and \textit{Acer truncatum}. In a previous study, we reported a method for the production of NA-rich, single-cell oil fermented by a filamentous fungus, \textit{Mortierella capitata} RD000969\textsuperscript{18}. NA is a major fatty acid present in the sphingomyelin of myelin sheath and plays an essential role in signal transmission in the CNS. Impairment of NA provision has been found in the postmortem brains of individuals with demyelinating diseases such as X-ALD and MS and has been suggested as a cause of pathogenesis in these diseases\textsuperscript{19}. Experiments using EAE mice, an animal model for MS, revealed that during acute inflammation, NA synthesis was inhibited with a shift to the synthesis of proinflammatory arachidonic acid\textsuperscript{20}. \textit{In vitro} assays of NA-containing fish oil added to cultured human oligodendrocyte precursor cells led to an enhanced maturation rate of the cells and increased synthesis of sphingomyelin. In addition, NA-containing oil was shown to inhibit the secretion of several proinflammatory factors while promoting growth factor synthesis, which is important for the regeneration of the CNS\textsuperscript{21}. Therefore, it is suggested that dietary therapy with NA-rich oil may be beneficial for these neurodegenerative diseases\textsuperscript{22}. NA accumulation in sphingomyelin is important for cerebral myelination and normal development of the brain\textsuperscript{23}. Babin \textit{et al.}\textsuperscript{24} showed a remarkable increase in NA in red blood cell membranes at between 32- and 37-weeks gestational age. Following birth, the concentration of NA continues to dramatically increase, in parallel with myellogenesis\textsuperscript{25}. Newborn infants obtain NA from breast milk. The NA concentration in human breast milk markedly decreases as lactation stages progress; its concentration in colostrum has been found to range from two- to six-fold higher than the concentration in mature milk\textsuperscript{26}. A low level of NA is present in infant formula; therefore, it has been suggested that the content of NA in infant formula for newborns or premature infants should be increased\textsuperscript{27–29}. Recently, decreased levels of NA in erythrocyte membranes were observed in individuals with psychotic disorder\textsuperscript{30}. The pathophysiology of schizophrenia may involve a disturbance in myelin development and/or myelin maintenance, resulting in compromised connectivity between neurons and brain regions. Decreased levels of NA could reflect myelin abnormalities. Therefore, NA is strongly correlated with the correct functioning of myelin and the CNS, and it may be effective in preventing such diseases.

Although PD is primarily considered as a disease of the gray matter, recent investigations suggest that alterations in the microstructure of white matter may accompany this disease’s progress\textsuperscript{31}. White matter microstructural alterations are considered to be a cause of typical neurodegeneration, including reduced fractional anisotropy and increased mean, axial, and radial diffusivities. Moreover, according to Abbott \textit{et al.}\textsuperscript{32}, decreased levels of ceramide and sphingomyelin and a shift in the sphingolipid acyl chain composition towards shorter length were observed in the postmortem brains of patients with PD. The levels of long-chain ceramides (C16:0, C18:0, and C18:1) significantly increased, with a complementary decrease in very long-chain ceramides (particularly NA) in the brain. C24 sphingolipids play an important role in membrane microdomain function. In HeLa cells, the knockdown of ELOVL1, which is responsible for C24 sphingolipid synthesis, showed a significant decrease in C24 sphingomyelin and an increase in C16 sphingomyelin levels compared with the levels in the control group; furthermore, the activity of the src-family tyrosine kinase LYN, which is important for membrane microdomain function, was significantly inhibited\textsuperscript{33}. Under ELOVL1 knockdown conditions, apoptosis induced by UV irradiation, cisplatin, or C6 ceramides significantly increased\textsuperscript{34}. Changes in the composition of sphingolipid chain length may affect susceptibility to stimuli-induced apoptosis by affecting the properties of cell membranes, such as lipid microdomain/raft formation.

In the present study, changes in sphingolipid composi-
tion in NA-treated PC-12 cells were not analyzed and the mechanisms by which oxidative stress was reduced were not clarified. Saito et al.\textsuperscript{30} reported that exogeneous NA was incorporated into cellular sphingolipid moieties when the cells were incubated in NA-containing medium. In our study, NA may have been incorporated in cellular sphingolipids. Next, we plan to analyze the composition of sphingolipids treated with NA. Future studies will be necessary to elucidate the relationship between increased oxidative stress and changes in sphingolipid composition in the brain cells of individuals with PD.

5 Conclusion

In conclusion, our results showed that cell viability was significantly increased in NA-treated cells, suggesting NA had a potential activity to upregulate the ROS elimination pathway. In the cells, the expression levels of SOD (Mn SOD and Cu/Zn SOD) and GCLC were significantly increased. These results suggest that NA may play a role as a neuroprotective mediator in the brain.

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