Omega-3 polyunsaturated fatty acids exert anti-oxidant effects through the nuclear factor (erythroid-derived 2)-related factor 2 pathway in immortalized mouse Schwann cells

Yasuaki Tatsumi1, Ayako Kato1, Kazunori Sango2, Tatsuhiro Himeno3, Masaki Kondo3, Yoshiro Kato3, Hideki Kamiya3, Jiro Nakamura3, Koichi Kato1*

1Laboratory of Medicine, Aichi Gakuin University School of Pharmacy, Nagoya, Aichi, 2Diabetic Neuropathy Project, Department of Sensory and Motor Systems, Tokyo Metropolitan Institute of Medical Science, Tokyo, and 3Department of Internal Medicine, Division of Diabetes, Aichi Medical University School of Medicine, Nagakute, Aichi, Japan

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*Correspondence
Koichi Kato
Tel: +81-52-757-6778
Fax: +81-52-757-6799
E-mail address: kkato@dpc.agu.ac.jp

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INTRODUCTION
Diabetic peripheral neuropathies, which are the most prevalent chronic complications that affect patients with diabetes, decrease quality of life and increase morbidity1. The metabolic mechanisms responsible for diabetic complications as a result of hyperglycemia include oxidative stress upregulation2, protein kinase C abnormalities3, non-enzymatic glycation endproduct increase4 and polyol pathway hyperactivity4. Oxidative stress is specifically considered the final common pathway of cellular injury in hyperglycemic conditions5.

Reportedly, omega-3 polyunsaturated fatty acids (ω-3 PUFAs), such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) occurring in fish oil, decreased cardiovascular events in clinical studies of Eskimos7. Recent large-scale...
studies also reported that ω-3 PUFAs reduce the risk of cardiovascular disease. Several studies have shown that ω-3 PUFAs have anti-inflammatory and anti-oxidant effects. DHA upregulates anti-oxidant enzymes in human umbilical vein endothelial cells (HUVECs), and both DHA and EPA can prevent H₂O₂-induced cytotoxicity in 3T3-L1 adipocytes. Furthermore, there is evidence that DHA and EPA can decrease oxidative stress in patients with type 2 diabetes. Despite interest in the anti-oxidative actions of ω-3 PUFAs, their effects on neural cells, such as Schwann cells, have not been elucidated.

Nuclear factor (erythroid-derived 2)-related factor 2 (Nrf2) exerts significant cytoprotective effects on oxidative stress through the Nrf2–anti-oxidant response element (ARE) pathway. Nrf2 pathway activation initiates the transcriptional regulation of ARE-dependent expression of diverse anti-oxidants and phase II detoxification enzymes, including heme oxygenase-1 (H-1) and nicotinamide adenine dinucleotide (phosphate) H quinone oxidoreductase 1 (Nqo1). Furthermore, the overexpression of Nrf2 reportedly inhibits apoptosis that is induced by high glucose in Schwann cells, and the transplantation of Nrf2-overexpressing Schwann cells can recover nerve functions in diabetic animals. Therefore, Nrf2 has become a therapeutic target in diabetic peripheral neuropathy.

Schwann cells are glial cells of the peripheral nervous system, and support neurons and maintain the structural and functional integrity of nerves. In diabetes, Schwann cells themselves undergo hyperglycemic insults and the supporting function is disrupted, which results in peripheral nerve dysfunctions. The disruption of Schwann cell mitochondrial function in connection with glial support can cause primary neuronal degeneration, indicating that Schwann cell dysfunction has direct effects on neuronal function. Thus, Schwann cells and their cell line, such as immortalized mouse Schwann (IMS32) cells, are extensively applied for in vitro models of diabetic neuropathy.

We investigated whether ω-3 PUFAs might induce the expression of the anti-oxidant enzymes through the Nrf2 pathway and suppress the oxidative stress-induced Schwann cell death.

**METHODS**

The present study was an in vitro study and ethics approval was unnecessary.

**Reagents**

Bovine serum albumin (BSA) was obtained from BBI Solutions (Cardiff, UK). DHA, EPA and catalase assay kits were purchased from Cayman (Ann Arbor, MI, USA). Low-glucose Dulbecco’s modified Eagle’s medium, tert-butyl hydroperoxide (tBHP) and MTT were purchased from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum was purchased from Gibco (Poisley, UK). Anti-Ho-1 antibody was purchased from Assay Design (Ann Arbor, MI, USA). Anti-Nqo1 antibody was obtained from Abcam (Cambridge, UK). Anti-Nrf2 antibody, anti-β-actin and anti-lamin A/C antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The glutathione assay kit was purchased from OxisResearch (Foster City, CA, USA). CellROX Deep Red reagent was purchased from Invitrogen (Carlsbad, CA). Other reagents and chemicals were obtained from standard suppliers.

**Cell culture**

Immortalized mouse Schwann (IMS32) cells were willingly provided by Professor Kazuhiko Watabe (Kyorin University, Tokyo, Japan). IMS32 cells were cultured in low-glucose Dulbecco’s modified Eagle’s medium containing 5% fetal bovine serum at 37°C in 5% CO₂/95% air.

**Fatty acid preparation**

DHA and EPA were separately prepared as complexes with BSA. DHA and EPA (75 μmol/L) were dissolved in ethanol and gradually solubilized in 2.6 mmol/L fatty acid-free BSA solution. BSA-conjugated fatty acids were lysed in Dulbecco’s modified Eagle’s medium at the final desired concentrations.

**MTT assay**

IMS32 cells were cultured in 96-well plates. To identify the effects of DHA and EPA on tBHP-induced cytotoxicity, the cells were exposed to DHA (2.5–25 μmol/L) or EPA (2.5–25 μmol/L) for 16 h, followed by incubation with 50 μmol/L tBHP for 6 h. Cell viability was measured by conventional MTT assay. Cells were treated with 0.5 mg/mL MTT in the medium for 3 h. The medium was then discarded, the formazan product was solubilized by dimethyl sulfoxide and the absorbance at 570 nm was determined using a microplate reader. Values are presented as percentages of cell survival. Absorbance of the control cells was set at 100%.

**Quantitative real-time reverse transcription polymerase chain reaction**

Total RNA was prepared using the Nucleospin RNA kit (Macherey-Nagel GmbH & Co., KG Düren, Germany). Single-stranded complementary deoxyribonucleic acid was prepared from 0.5 μg total ribonucleic acid (RNA) using the PrimeScript RT reagent kit (Takara Bio, Shiga, Japan). Quantitative analyses of heme oxygenase-1 (Ho-1), nicotinamide adenine dinucleotide [phosphate] H quinone oxidoreductase 1 (Nqo1), catalase (Cat), superoxide dismutase (Sod) and glutathione peroxidase (Gpx) messenger RNAs (mRNAs) were carried out using real-time reverse transcription polymerase chain reaction with the Takara Dice thermal cycler (Takara Bio, Shiga, Japan). The primer pair sequences were as follows: mouse Ho-1, 5’-CCTCAGTGGAGGAAATCATC-3’ and 5’-CCTCAGGAGGAGCCTTACATACA-3’; mouse Nqo1, 5’-TATCCTCCGGAGTCACTCTGAG-3’ and 5’-TCCTGAGCTTCCAGCTTCTTCA-3’; mouse Cat, 5’-GAACGAGAGGAGGAAAC-3’ and 5’-TGAATCTTGGCACGCTTTCC-3’; mouse Sod, 5’-CAATGTTGGGAGACATATT-3’.
Assessment of cat activity
Cat activity was determined using a catalase assay kit (Cayman), according to the instructions.

Intracellular glutathione and glutathione disulfide
Glutathione (GSH) and glutathione disulfide (GSSG) content were determined using a Bioxytech GSH/GSSG-412 colorimetric assay kit (OxisResearch), according to the instructions.

Measurement of reactive oxygen species
Reactive oxygen species were determined using CellROX Deep Red reagent (Invitrogen) according to the manufacturer’s instructions.

Cell transfection and luciferase assay
The ARE-luciferase reporter plasmid (pNL[NlucP/ARE/Hygro]) was purchased from Promega (Madison, WI, USA). Cells were cotransfected with 0.2 µg of luciferase expression plasmid and 0.05 µg of pGL 4.53 (luc2/PGK) plasmid (Promega) using Lipofectamine 3000 (Invitrogen, Carlsbad, CA) for 24 h to normalize the transfection efficiency. Cells were then exposed to DHA or EPA for 12 h, and Firefly and Renilla luciferase activities were measured by the Dual Luciferase Reporter assay system (Promega).

Statistical analysis
Data are presented as the mean ± standard error for the indicated number of experiments. Statistical analyses were carried out by one-way analysis of variance (ANOVA), and the Tukey–Kramer correction for multiple comparisons. The statistical significance of differences between the control (BSA only) and DHA or EPA-treated cells was analyzed using one-way ANOVA followed by Dunnett’s multiple-comparison test. P-values <0.05 were considered to show statistical significance.

RESULTS
Preventive effects of DHA and EPA on oxidative stress-induced cytotoxicity
As ω-3 PUFAs are known to exert anti-oxidant effects in HUVECs and adipocytes, we evaluated whether ω-3 PUFAs also have anti-oxidative effects in IMS32 cells. tBHP was used to induce oxidative stress. Treatment with 5–50 µmol/L tBHP for 6 h elicited cell toxicity in a dose-dependent manner.
The cytoprotective effects of DHA and EPA against tBHP were investigated in IMS32 cells. DHA or EPA pretreatment for 16 h significantly prevented tBHP-induced cytotoxicity in a dose-dependent manner (Figure 1b,c; black bars). Neither DHA nor EPA alone altered cell viability compared with the BSA control (Figure 1b,c; white bars).

Figure 2 | Dose-dependent effects of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) on the messenger ribonucleic acid levels of Ho-1, Nqo1 and Catalase in immortalized mouse Schwann (IMS32) cells. IMS32 cells were cultured with the indicated (a–e) DHA or (f–j) EPA concentration for 6 h. Total ribonucleic acid was purified and quantified by real-time reverse transcription polymerase chain reaction. Each value represents the mean ± standard error of three experiments. **P < 0.01 compared with the bovine serum albumin control. BSA, bovine serum albumin; Gapdh, glyceraldehyde 3-phosphate dehydrogenase; Gpx, glutathione peroxidase; Ho-1, heme oxygenase-1; Nqo1, nicotinamide adenine dinucleotide (phosphate) H quinone oxidoreductase 1; Sod, superoxide dismutase.
Effects of DHA and EPA on anti-oxidant enzyme expression

We measured the mRNA levels of various anti-oxidant enzymes to explore the protective mechanisms of DHA and EPA against oxidative stress. Both DHA and EPA increased the mRNA levels of \( \text{Ho-1} \), \( \text{Nqo1} \) and \( \text{Cat} \) in a dose-dependent manner (Figure 2a–c,f–h); however, DHA and EPA treatment did not have any effect on the mRNA levels of \( \text{Sod} \) and \( \text{Gpx} \) (Figure 2d,e,i,j). The mRNA levels of \( \text{Ho-1} \) increased maximally between 3 and 6 h after treatment with 7.5 \( \mu \)mol/L DHA (Figure 3a) or 25 \( \mu \)mol/L EPA (Figure 3b), whereas the mRNA levels of \( \text{Nqo1} \) and \( \text{Cat} \) showed maximal increases at 12 h (Figure 3c–f). Furthermore, DHA or EPA treatment for 12 h significantly enhanced \( \text{Ho-1} \) protein levels (Figure 4a,b,d,e). Similarly, treatment with DHA for 12–24 h significantly enhanced the Nqo1 protein level (Figure 4a,c).

Effects of DHA and EPA on catalase activity

Because we previously reported that either DHA or EPA could increase the mRNA levels of \( \text{Cat} \), we then measured the

![Figure 3](image-url)
catalase activity in IMS32 cells. DHA or EPA treatment for 12 h significantly enhanced the catalase activity by 1.7- and 1.6-fold, respectively, compared with the controls (Figure 5).

**Effects of DHA and EPA on redox homeostasis**

As both DHA and EPA could induce anti-oxidant enzymes in IMS32 cells, we measured the GSH and GSSG content to...
determine whether the protective effects of DHA and EPA were involved in GSH metabolism. Figure 6a–c shows the intracellular GSH and GSSG content, and the GSH/GSSG ratio, respectively. DHA or EPA treatment for 12 h significantly enhanced the intracellular GSH content by 1.5- and 2.7-fold, respectively, compared with the controls (Figure 6a); however, the GSSG content remained unchanged after either DHA or EPA treatment (Figure 6b). The GSH/GSSG ratio was enhanced after either DHA or EPA treatment compared with the controls (Figure 6c). Treatment with 50 μmol/L tBHP for 90 min significantly increased reactive oxygen species compared with the BSA control. The increase in reactive oxygen species by 50 μmol/L tBHP was suppressed by pretreatment with 7.5 μmol/L DHA or 25 μmol/L EPA for 16 h (Figure 6d).

**Effects of DHA and EPA on Nrf2 activation**

Nrf2 is a key transcription factor responsible for the upregulation of anti-oxidant enzymes, including Ho-1, Nqo1 and Cat. To investigate whether Nrf2 is activated by DHA or EPA, we measured the ARE-luciferase activity and Nrf2 translocation into nuclei in IMS32 cells. Luciferase activities induced by DHA and EPA were evaluated by the ARE-luciferase expression plasmid in IMS32 cells. DHA and EPA significantly increased the ARE-luciferase activities by 2.8- and 3.1-fold, respectively, compared with the controls (Figure 7a). Nrf2 translocation into the nuclei was confirmed by western blotting of the nuclear and cytosolic fractions. DHA or EPA treatment for 3 and 6 h significantly increased Nrf2 in the nuclear fraction (Figure 7b,d). Conversely, DHA or EPA treatment for 3 and 6 h significantly decreased Nrf2 in the cytosolic fraction (Figure 7c,e). These data suggest that DHA or EPA can both increase Nrf2 nuclear translocation and that Nrf2 binds to ARE.

**DISCUSSION**

In the present work, we showed that ω-3 PUFAs exert anti-oxidant effects on IMS32 cells. ω-3 PUFAs stimulate Nrf2 translocation from the cytoplasm into the nucleus, where it binds to ARE and initiates the expression of anti-oxidant enzymes, including Ho-1, Nqo1 and Cat, in IMS32 cells. Furthermore, we identified the preventive effects of ω-3 PUFAs against the oxidative stress-induced cell death. The present findings are consistent with recent studies suggesting that the anti-oxidant effects are induced by ω-3 PUFAs in HUVECs and 3T3-L1 adipocytes. ω-3 PUFAs suppress inflammatory responses by inhibiting the arachidonate cascade in neutrophils, and the stimulation of the G protein-coupled receptor 120 by DHA exerts anti-inflammatory effects in macrophages. Recent studies report the anti-oxidant effects of 4-hydroxy-2-hexanal (4-HHE), resolvin E1 and protectin D1, which are intracellular metabolites of ω-3 PUFAs. We also examined the effects of 4-HHE on the mRNA levels of anti-oxidant enzymes and found that 4-HHE increases the mRNA levels of Ho-1, Nqo-1 and Cat (data not shown). Future studies should investigate whether ω-3 PUFAs are involved in the arachidonate cascade, the G protein-coupled receptor 120 pathway or 4-HHE.
DHA and EPA can induce the expression of the same anti-oxidant enzymes, including Ho-1, Nqo1 and Cat; however, DHA concentrations that induce the expression of these enzymes differ from those of EPA (Figure 2). DHA increases the mRNA levels of Ho-1, Nqo1 and Cat at 7.5 μmol/L. In contrast, EPA increases only the mRNA levels of Cat at 7.5 μmol/L, whereas 25 μmol/L EPA is required to increase that of Ho-1 and Nqo1. It has been suggested that DHA induces anti-oxidant enzymes more potently than EPA. The differences between DHA and EPA might result from differences in their metabolism. Studies have reported that DHA and EPA increase the mRNA levels of Ho-1 and Nqo1 in HUVECs and 3T3-L1 cells, respectively; however, the increase in the mRNA levels of Cat was confirmed in both cell types. The present results showed that both DHA and EPA can increase the mRNA levels of Cat, Ho-1 and Nqo1 in neural cells. We suggest that differences in the induction of anti-oxidant enzyme expression might depend on the cell or tissue type being examined.

Both DHA and EPA can induce the expression of Ho-1 mRNA within 3 h, peaking at 6 h. Furthermore, the expression of Nqo1 mRNA was increased at 6 h and peaked at 12 h (Figure 3). In our results, mRNA time lapse and DHA or EPA protein expression was different. As there are several processes for transcription or translation, the time for transcription or translation is known to vary, and we speculate that these processes differ between DHA and EPA. The time required for ω-3 PUFAs to be metabolized into 4-HHE might have reportedly caused this time lag. The different induction times might be responsible for the difference in timing of anti-oxidant effects after Nrf2/ARE pathway activation.

The present results showed that the levels of GSH, an anti-oxidant, were increased by both DHA and EPA (Figure 6). Glutathione was synthesized by glutamate cysteine ligase modifier subunit (GCLM) and glutathione reductase (GR). Lee et al. reported that DHA prevents cell death induced by paraquat in dopaminergic SN4741 cells. These protective effects against paraquat-induced oxidative stress suggest that DHA increases the mRNA levels of GR and GCLM through Nrf2, and enhances the accumulation of intracellular GSH. We found the same results for GCLM protein (data not shown). Therefore, DHA and EPA increase GSH levels, possibly enhancing their preventive effects against oxidative stress in Schwann cells.

We showed that DHA and EPA increased ARE-luciferase activity by 2.8- and 3.1-fold, respectively, compared with controls (Figure 7). Ishikado et al. reported that DHA increased ARE-luciferase activity by 50-fold in HUVECs; the extent of the increase was much higher than that seen in the present results. The difference in the extent of the ARE-luciferase activity induction might be due to low plasmid transfection efficiency in neural cells. In the present study, DHA and EPA both showed a weak induction of ARE-luciferase activity in IMS32 cells; however, we confirmed that both DHA and EPA can induce Nrf2 translocation, increase the mRNA levels of Ho-1, Nqo1 and Cat, and stimulate Ho-1 protein levels. Collectively, DHA and EPA exert anti-oxidant effects through the Nrf2/ARE pathway in IMS32 cells.

Nerve conduction velocity and nerve blood flow can be restored by DHA administration in diabetic rats. DHA entered the sciatic nerve in an in vivo study. Additionally, Na⁺, K⁺-ATPase activity of red blood cells was elevated by DHA. Recently, ω-3 PUFAs supplementation for 12 months was shown to increase the corneal nerve fiber length by 29% in patients with type 1 diabetes. We speculate that the induction of anti-oxidant enzymes in neural cells contribute, at least partly, to improving diabetic neuropathy observed in previous studies.

Previously, DHA and EPA were used at high concentrations (75–100 μmol/L). The maximum concentrations of a single oral dose of 2 g ethyl ω-3 fatty acid capsules (comprising EPA and DHA) are as follows: 58.1 μg/mL EPA (192 μmol/L) and 115.0 μg/mL DHA (350 μmol/L). In the present study, 25 μmol/L EPA or 7.5 μmol/L DHA protected neural cells from oxidative stress. The 25 μmol/L EPA and 7.5 μmol/L DHA doses used in the present study were approximately 1/8 and 1/45 of the maximum concentrations of a single oral dose of EPA and DHA capsules, respectively. Therefore, DHA or EPA concentrations used in the present study might be able to exert sufficient anti-oxidant effects in peripheral nerves.

Diabetic neuropathy progresses through multiple mechanisms, including oxidative stress upregulation, protein kinase C abnormalities, increased non-enzymatic glycation end-products, and polyol pathway activation. Of these, oxidative stress is recognized as the most significant common pathway of these pathogenic mechanisms.

Hyperglycemia-enhanced oxidative stress is exacerbated by a concomitant reduction in endogenous anti-oxidant defenses. Genetic variations and polymorphisms in endogenous anti-oxidant enzymes are associated with an increased diabetic neuropathy. Thus, the enhancement of endogenous anti-oxidant enzymes in nerve tissues might help prevent diabetic neuropathy.

In the present study, we showed that both DHA and EPA can prevent cell death by inducing numerous anti-oxidants in Schwann cells. The present findings suggest that the enhancement of anti-oxidative defenses facilitated by ω-3 PUFAs might have clinical values in diabetic neuropathy treatment.

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Figure 7 | Activation of nuclear factor (erythroid-derived 2)-related factor 2 (Nrf2) by docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) in immortalized mouse Schwann (IMS32) cells. (a) IMS32 cells were cotransfected with a reporter plasmid (pNL[NLucP/ARE/Hygro]) and a control plasmid (pGL4.33[luc2/PGK]). After transfection, IMS32 cells were cultured with 7.5 μmol/L DHA or 25 μmol/L EPA for 12 h. Each value represents the mean ± standard error of three experiments. **P < 0.01 compared with the bovine serum albumin (BSA) control. IMS32 cells were cultured with (b,c) 7.5 μmol/L DHA or (d,e) 25 μmol/L EPA for the indicated times. Nuclear and cytosolic proteins were used for western blotting analyses. Each value represents the mean ± standard error of three experiments. *P < 0.05, **P < 0.01 compared with each BSA control. ARE, anti-oxidant response element.
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