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Original Article

Inhibitory Effect of Polyunsaturated Fatty Acids on Apoptosis Induced by Etoposide, Okadaic Acid and AraC in Neuro2a Cells

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Neuronal apoptosis is involved in neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease. An efficient means of preventing it remains to be found. Some n-3 polyunsaturated fatty acids (PUFAs) such as docosahexaenoic acid (DHA, 22:6n-3) and eicosapentaenoic acid (EPA, 20:5n-3) have been reported to be protective against the neuronal apoptosis and neuronal degeneration seen after spinal cord injury (SCI) [1]. However, it is unclear which kinds of PUFAs have the most potent ability to inhibit neuronal apoptosis and whether the simultaneous treatment of PUFAs inhibits the apoptosis. In the present study, we compared the abilities of various n-3- and n-6-PUFAs to inhibit the apoptosis induced after the administration of different apoptotic inducers, etoposide, okadaic acid, and AraC, in mouse neuroblastoma cells (Neuro2a). Preincubation with DHA (22:6n-3), eicosapentaenoic acid (EPA, 20:5n-3), α-linolenic acid (α-LNA, 18:3n-3), linoleic acid (LA, 18:2n-6), arachidonic acid (AA, 20:4n-3), and γ-linolenic acid (γ-LNA, 18:3n-6) significantly inhibited caspase-3 activity and LDH leakage but simultaneous treatment with the PUFAs had no effect on the apoptosis of Neuro2a cells. There were no significant differences of the anti-apoptotic effect among the PUFAs. These results suggest that PUFAs may not be effective for inhibiting neuronal cell death after acute and chronic neurodegenerative disorders. However, dietary supplementation with PUFAs may be beneficial as a potential means to delay the onset of the diseases and/or their rate of progression.

Key words: polyunsaturated fatty acid (PUFA), neurodegenerative disease, caspase, neuronal apoptosis, DHA

Nervous tissue has the second highest concentration of fatty acids after adipose tissue. The cell membrane consists of a double phospholipid layer incorporating both n-3 and n-6 long chain PUFAs (*e.g.*, EPA, DHA, and arachidonic acid (AA)). Both n-3 and n-6 long chain PUFAs play important roles in neuronal growth, the development of synaptic processing during neural cell interactions, and the

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expression of genes regulating cell differentiation and growth [2]. Long chain PUFA levels are particularly high in the retina and cerebral cortex. DHA can account for up to 50% of phospholipid fatty acids in these tissues, suggesting that it is heavily involved in neuronal and visual functions [2].

DHA has garnered much attention for its potential in treating certain diseases, cancers [3] and neurodegenerative disorders such as Parkinson's and Alzheimer's disease [4]. The anti-cancer action of DHA is mainly due to its apoptotic effects [5]. It also shows anti-apoptotic effects in neuronal cell lines and primary neurons [6–9]. Previous studies have shown the molecular mechanism of the anti-apoptotic effect of DHA. DHA exerts its protective effects due to its role as a neurotrophic factor, its activation of the ERK/MAPK pathway, or activation of some survival signaling cascades [6, 8, 10].

Previous studies showed that preincubation with some other PUFAs such as AA and linolenic acid (LNA, 18:3n-6) also had anti-apoptotic effects in neurons [1, 11, 12]. However, it is not known which kinds of PUFAs are most useful for the inhibition of neuronal apoptosis, since comparative studies of the anti-apoptotic effects of various PUFAs have not been done. Moreover, it is unclear whether PUFAs are effective against acute and chronic neurodegenerative disorders since the inhibitory effect of simultaneous treatment with PUFAs on neuronal apoptosis has not been studied.

Neuro2a cells are a murine neuroblastoma cell line that has proven to be a useful experimental model for studying neuronal apoptosis since its apoptosis can be controlled by various conditions, such as etoposide, okadaic acid, AraC or serum starvation. In the present study, we compared the anti-apoptotic effects of long chain PUFAs, including n-3 and n-6 PUFAs, in Neuro2a cells and examined the inhibitory effect of simultaneous treatment with PUFAs on the apoptosis of the cells.

Materials and Methods

Cell culture. Mouse neuroblastoma Neuro2a cells were cultured in DMEM (Invitrogen, San Diego, CA, USA) supplemented with 10% fetal calf serum (FCS) and penicillin/streptomycin at 37 °C in an atmosphere of 95% air and 5% CO_2. The cells were exposed to DHA or other fatty acids (Funakoshi, Tokyo, Japan) (FCS was reduced to 2%), etoposide (Sigma-Aldrich, St. Louis, MO, USA), okadaic acid (Sigma-Aldrich), or AraC (Nipponshinyaku, Tokyo, Japan) 24 h after plating. For the LDH activity assay, the cells were seeded into 24-well plates at a density of 6×10^4 cells/well. For the caspase-3 activity assay, the cells were seeded into T25 flasks at a density of 1.0×10^6 cells / 10 ml. For the DNA fragmentation assays, the cells were seeded into T25 flasks at a density of 1.5×10^6 cells / 10 ml.

Trypan blue staining. The viability of the Neuro2a cells was determined by staining with trypan blue. After the cells were stripped from the flask, they were stained with trypan blue and then counted with a hemocytometer.

Measurement of released LDH (lactate dehydrogenase) activity. The release of LDH from Neuro2a cells was measured as described previously [10]. Briefly, after treatment with various apoptotic inducers in the presence or absence of DHA for 48 h, the cells were centrifuged at 250 *g* for 10 min at room temperature. The supernatant (100 μl) was collected and then incubated with 3 ml of reaction buffer (16 mM KH_2PO_4, 84 mM K_2HPO_4, 2.4 mM NADH, and 23 mM sodium pyruvate, pH 7.5). The optical density, reflecting the reduction of NADH, which represents the amount of LDH released from Neuro2a cells, was measured at 340 nm with a Shimadzu-UV3000 (Shimadzu, Kyoto, Japan). The amount of LDH released was expressed as a percentage of the total pool of enzyme measured in negative control samples in which the cells were lysed with 1 % Triton X-100.

Measurement of caspase-3 activity. After treatment with various apoptotic inducers in the presence or absence of DHA, the cells were stripped from T25 flasks and washed with PBS (−) 2 times. The cells were then sonicated in extraction buffer (25 mM HEPES (pH 7.5), 5 mM EGTA, 1 mM $MgCl_2$, 5 mM DTT, 10 μg/ml pepstatinA, 10 μg/ml leupeptin, 1 mM PMSF and 0.1% CHAPS) for 30 sec on ice. After centrifugation at 16,000 rpm for 20 min at 4 °C, the supernatant was collected and preserved at −80 °C until use. Caspase-3 activity was determined by incubation of the supernatant (80 μg of protein) with 10 μM fluorogenic substrate Acetyl-DEVD-4-methyl-coumaryl-7-amide in caspase

assay buffer (25 mM HEPES (pH 7.5), 5 mM DTT, 10% sucrose, and 0.1% CHAPS). The release of 7-amino-4-methylcoumarin was detected by fluorometry, with excitation at 380 nm and emission at 460 nm.

Assay of DNA fragmentation. After 48 h of treatment with various inducers of apoptosis with or without DHA, the Neuro2a cells were harvested and lysed by incubation at 4 °C for 10 min in lysis buffer (10 mM Tris-HCl, 10 mM EDTA, and 0.5% Triton X-100, pH 7.5). The cell lysates were centrifuged at 15,000 rpm for 20 min. The supernatant was incubated with RNase A (400 μg/ml) at 37 °C for 1 h and then for an additional 30 min with proteinase K (400 μg/ml). Fragmented DNA was precipitated with isopropyl alcohol and was electrophoresed in 1.6% agarose gels. The DNA ladders were visualized and photographed under UV light.

Statistical analysis. Data were expressed as the mean ± s.e.m. Data were analyzed using Student's *t*-test to compare 2 conditions and one-way ANOVA followed by planned comparisons of multiple conditions, and $p < 0.05$ was considered to be significant.

Results

To optimize the effects of DHA, we examined the dose-dependent cell toxicity of DHA in Neuro2a cells (Fig. 1). Cell toxicity was assessed by the inhibition of cell growth and released LDH activity. Trypan blue staining revealed that none of the concentrations (10–50 μM) of DHA tested inhibited the cell growth at 24 h or 48 h of treatment (Fig. 1A). However, incubation with high concentrations of DHA (20 and 50 μM) for 72 h significantly inhibited the cell growth. High-dose DHA (20 and 50 μM) induced the LDH activity in the cells, whereas low-dose (5 and 10 μM) DHA had no effect on the induction of LDH activity (Fig. 1B). These results suggest that long-term treatment with high concentrations of DHA may cause cell toxicity such as the inhibition of cell growth and neuronal cell death, and that 10 μM of DHA is an optimal concentration for avoiding cell toxicity.

We next examined the dose-dependent ability of DHA to inhibit the induction of apoptosis after the administration of etoposide (10 μM), okadaic acid (40 nM), AraC (100 μM) or serum starvation in Neuro2a cells. The cells were preincubated with various concentrations (5, 10, 20 or 50 μM) of DHA for 24 h, and were then induced to undergo apoptosis by adding various chemicals or subjecting them to serum starvation. Preincubation with DHA attenuated the inhibition of cell growth induced after the administration of the chemicals (Fig. 2A). Each concentration of DHA tested (5–50 μM) significantly inhibited the ability of all of the chemicals to induce LDH activity (Fig. 2B). A low concentration (5 μM) of DHA had no effect on the inhibition of etoposide-induced caspase-3 activity, whereas 10 and 20 μM

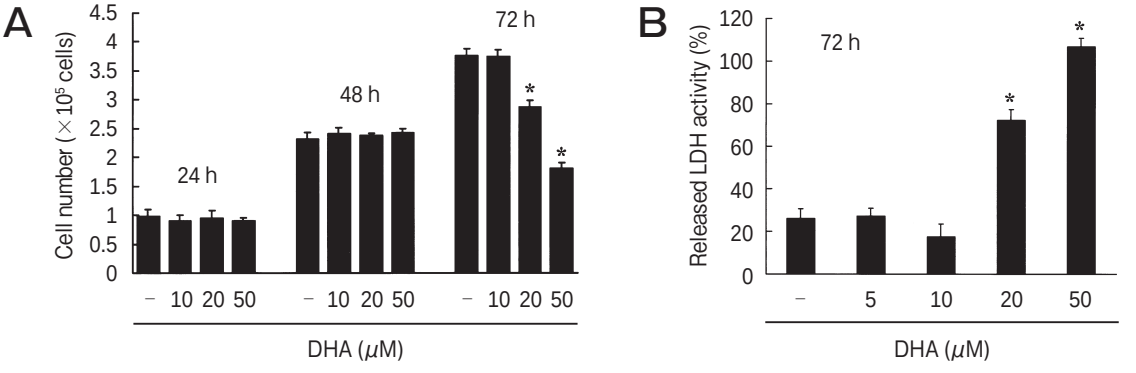

Fig. 1 Effect of various concentrations of DHA on cell growth (**A**) and cytotoxicity (**B**) in Neuro2a cells. **A**, Counting of living cells. Neuro2a cells were treated with DHA (0–50 μM) for 24 h, 48 h, or 72 h, and the number of living cells was counted after trypan blue staining as described in "Materials and Methods"; **B**, The cells were treated with DHA (0–50 μM) for 72 h and the released LDH activity was then measured. Data are shown as mean values (± s.e.m.) from 5 separate experiments. $^*p < 0.01$.

DHA inhibited the casapse-3 activity (Fig. 2 C). Each of these concentrations of DHA inhibited the induction of caspase-3 activity after the administration of other chemicals or serum starvation (Fig. 2 C). These results suggest that DHA (5–50 μM) inhibits neuronal apoptosis.

We next investigated whether simultaneous treatment with DHA inhibited chemically induced apoptosis in Neuro2a cells. The cells were simultaneously exposed to 10 μM DHA and each of various chemicals (etoposide, okadaic acid, or AraC or serum starvation conditions), and DNA fragmentation of the cells were examined 24 h after the treatment. All of these chemicals and serum starvation induced DNA fragmentation (Fig. 3). Simultaneous treatment with DHA did not affect the induction of DNA fragmentation by any of these chemicals, suggesting that the simultaneous treatment did not inhibit neuronal apoptosis. In contrast, preincubation with DHA inhibited the DNA fragmentation (Fig. 3).

Finally, we examined whether other PUFAs had inhibitory effects on neuronal apoptosis in Neuro2a cells. For this, the anti-apoptotic effects of DHA, EPA AA and LA were examined. As controls, the inhibitory effects of stearic acid (SA, C18:0), a saturated fatty acid, and oleic acid (OA, C18:1, n-9), a monosaturated fatty acid, on neuronal apoptosis were examined. SA showed no ability to inhibit the induction of apoptosis with any of the chemicals, (Figs. 4A and B). OA inhibited the release of LDH activity induced after the administration of each chemical (Fig. 4A). This fatty acid also inhibited

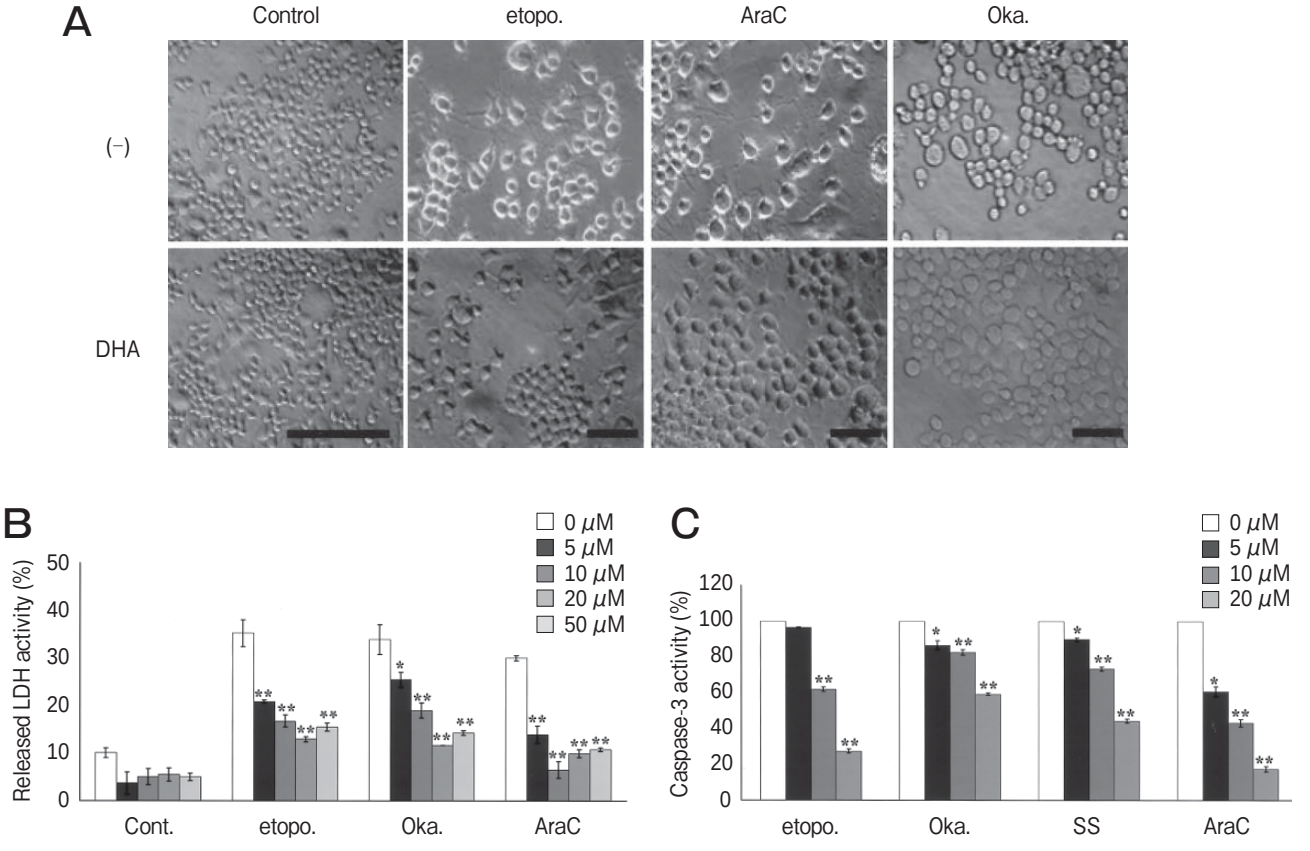

Fig. 2 Inhibitory effect of DHA on etoposide (etopo. 10 μM), okadaic acid (Oka., 40 nM), or AraC (100 μM)-induced apoptosis in Neuro2a cells. The cells were preincubated with DHA (10 μM) for 24 h and were then treated with each chemical for 12 h. **A**, Effect of DHA on inhibition of cell growth induced by each chemical. Bar = 100 μm; **B**, Effect of DHA on release of LDH activity. The LDH activity was measured 48 h after incubation with each inducer of apoptosis; **C**, Effect of DHA on caspase-3 activity. SS, serum starvation. n = 5 each. $^{*}p < 0.05$, $^{**}p < 0.01$.

caspase-3 activity, but the inhibitory effect was weaker than that of the PUFAs (Fig. 4B). All of the PUFAs tested (DHA, EPA, AA and LA) significantly inhibited both LDH activity and caspase-3 activity, and the inhibitory effect was not obviously different among the PUFAs (Fig. 4).

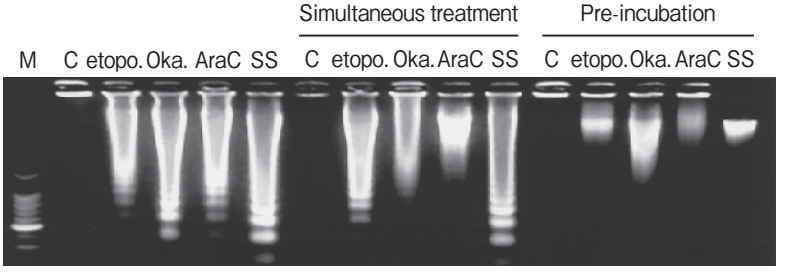

Fig. 3 Comparison of the effects of simultaneous treatment and pretreatment with DHA (10 μM) on DNA fragmentation of Neuro2a cells induced by various chemicals. The cells were treated with 10 μM DHA for 24 h (preincubation) or were simultaneously treated with DHA plus each inducer of apoptosis, etoposide (10 μM), okadaic acid (40 nM), AraC (100 μM) or serum starvation (simultaneous treatment). After 24 h, the cells were collected and DNA fragmentation in the cells was analyzed as described in "Materials and Methods".

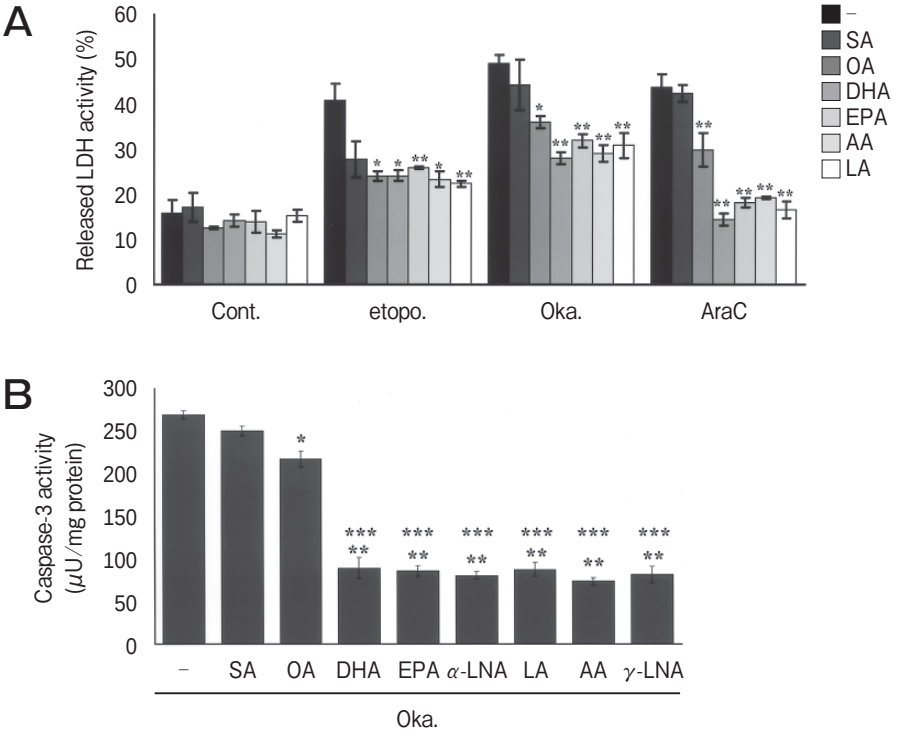

Fig. 4 Comparison of inhibitory effect of each PUFA on apoptosis in Neuro2a cells. The cells were treated with each PUFA at 10 μM, and were then induced to undergo apoptosis with various chemicals. SA, stearic acid (C18 : 0); OA, oleic acid (C18 : 1n-9); EPA, eicosapentaenoic acid (C20 : 5n-3); α-LNA, α-linolenic acid (C18 : 3n-3); LA, linoleic acid (C18 : 2n-6); AA, arachidonic acid (C20 : 4n-6); γ-LNA, γ-linolenic acid (C18 : 3n-6). **A**, Effect of PUFAs on release of LDH activity; **B**, Effect of PUFAs on caspase-3 activity. n = 5 each, $^{*}p < 0.05$ v.s. Cont., $^{**}p < 0.01$ v.s. Cont., $^{***}p < 0.01$ v.s. OA.

Discussion

In the present study, DHA (5–50 μM) had a protective effect against apoptosis in Neuro2a cells, but high concentrations of DHA (20 and 50 μM) caused cytotoxicity after 72 h of treatment (Fig. 1). The cytotoxicity of DHA toward the cells may be consistent with its anti-cancer effect that has been reported previously [5]. A previous study showed that 5 or 10 μM DHA did not have an anti-cancer effect, whereas 20 and 30 μM DHA induced the apoptosis of cancer cells and inhibited cell growth [5]. These results suggest that DHA has contradictory effects on cell growth and apoptosis, and that the effects are dose-dependent. DHA at the concentration of 5 or 10 μM may be most effective for the inhibition of neuronal apoptosis without cytotoxicity.

The molecular mechanism of the neuroprotective effect of DHA has not been elucidated. In the present study, neuronal apoptosis was induced after the administration of different apoptotic inducers. All these apoptotic inducers induce neuronal apoptosis by different mechanisms. Etoposide is an inhibitor of the enzyme topoisomerase II, blocking DNA re-ligation and leading to direct DNA damage [13]. AraC acid (Cytarabine) is an inhibitor of DNA polymerase-α and -β, and acts by inhibiting DNA chain elongation [14]. Okadaic acid is an inhibitor of protein phosphatase 1, 2A and induces increases of caspase-3 activity and DNA fragmentation through blocking dephosphorylation of some signal proteins [15]. However, all of these chemicals induced caspase activity and DHA inhibited the induction of caspase activity and apoptosis by these chemicals in Neuro2a cells. These results suggest that DHA may regulate the pathway of caspase activation. In the present study, other PUFAs in addition to DHA had protective effects against neuronal apoptosis, and the effect was not significantly different among the PUFAs examined. These results suggest that all n-3 and n-6 PUFAs may have anti-apoptotic effects through the regulation of the same signal pathway.

Neuronal apoptosis has been viewed to be involved in many neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease. Preincubation with PUFAs inhibited neuronal apoptosis, whereas simultaneous treatment failed to induce such inhibition. These results suggest that PUFAs may not be effective for inhibiting neuronal cell death after acute and chronic neurodegenerative disorders. However, dietary supplementation with PUFAs may be beneficial as a potential means of delaying the onset of the diseases and/or their rate of progression.

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