ω-3 polyunsaturated fatty acids inhibit the proliferation of the lung adenocarcinoma cell line A549 *in vitro*

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Abstract. ω-3 polyunsaturated fatty acids (n-3 PUFA), in particular the marine-derived forms eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have been demonstrated to affect cancer cell replication, the cell cycle and cell death. Epidemiological studies have also suggested diets rich in n-3 PUFA were inversely correlated with the development of cancer. In the present study, we explored the effects of DHA and EPA on the proliferation activity and apoptosis of the human lung adenocarcinoma cell line A549. A methyl thiazolyl tetrazolium (MTT) assay was used to detect cell proliferation, apoptosis was detected by flow cytometry and morphological analysis was determined by fluorescence microscopy and transmission electron microscopy. A549 cells were treated with different doses of DHA (40, 45, 50 and 55 μg/ml) or EPA (45, 50, 55 and 60 μg/ml) for 24, 48 and 72 h. The results demonstrated that DHA and EPA significantly suppressed the proliferation of A549 cells and induced apoptosis of A549 cells in a dose- and time-dependent manner. The apoptotic phenomenon was also confirmed by fluorescence microscopy and transmission electron microscopy. Furthermore, compared with the control, the formation of autophagosomes was clearly enhanced in DHA- or EPA-treated cells. In conclusion, DHA and EPA inhibited the proliferation of A549 cells and induced cell apoptosis and autophagy, which may provide new safe and effective options for the treatment of lung cancer in the future.

Introduction

Lung cancer is the leading cause of cancer-related mortality worldwide (1) and ~75-85% of lung cancers are non-small cell lung cancer (NSCLC), which includes squamous cell carcinoma, adenocarcinoma and large cell carcinoma. Chemotherapy agents, including cisplatin and paclitaxel, are the main treatment measures for NSCLC, however, the side effects of chemotherapy are usually difficult to tolerate, particularly for elderly patients. Thus, new drugs which are safe and effective should be developed (2). Natural dietary agents consist of numerous bioactive compounds that have demonstrated great potential in preventing and treating a wide variety of diseases, including various types of cancer, the majority of which have been used as ancient traditional medicines (3). We consider this an interesting field worthy of exploration.

ω-3 polyunsaturated fatty acids (n-3 PUFA), in particular the marine-derived forms eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have been demonstrated to be natural, multipotent treatments for a wide variety of diseases. In previous decades there has been growing interest in the role of n-3 PUFA and their potential to prevent cancer development and progression (4,5). This was supported by a large number of *in vitro* experiments demonstrating the profound anti-tumour effects of n-3 PUFA by suppressing neoplastic transformation, angiogenesis and tumour cell growth (6-8). Using animal models, it has been repeatedly demonstrated that the growth of chemically induced cancer and of human cancer xenografts can be retarded or completely inhibited by the incorporation of n-3 PUFA in the diet (9,10). However, studies in lung cancer are not sufficient, therefore, in the present study, we explored the effects of DHA and EPA on the proliferation activity and apoptosis of the human lung adenocarcinoma cell line A549.

Materials and methods

Cells and reagents. Human lung cancer A549 cells were obtained from The Cell Bank of the Chinese Academy of Sciences (Shanghai, China). A549 cells were supplemented with 10% fetal bovine serum and antibiotics (100 U/ml of penicillin and 100 μg/ml of streptomycin). The cells were incubated in a humidified incubator under 5% CO_2 at 37˚C. DHA, EPA, dimethyl sulfoxide (DMSO), acridine orange (AO), ethidium bromide (EB) and methyl thiazolyl tetrazolium (MTT) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Annexin V-phycoerythrin (Annexin V-PE)

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and 7-amino-actinomycin D (7-AAD) were obtained from Millipore (Billerica, MA, USA).

MTT assay for the inhibition of cell growth. Cells were seeded at a density of 8x10³ cells in each well of the 96-well plates and incubated for 24 h. A series of concentrations of DHA (40, 45, 50 and 55 μg/ml) or EPA (45, 50, 55 and 60 μg/ml) were added to the wells for 24, 48 and 72 h. MTT (5 g/l, 20 μl/well) was added to each well and incubated at 37˚C for 4 h. DMSO was then added (150 μl/well) to each well to dissolve any crystals and the plates were agitated for 10 min. Absorbance values at 490 nm were detected by the microplate reader (Infinite M200; Tecan, Geneva, Switzerland). Cell growth inhibition was calculated using the formula: Cell growth inhibition rate (%) = [1 - A490 (experimental group)/A490 (control group)] x 100. Each experiment was repeated three times.

Apoptosis detected by flow cytometry. Cells were seeded at 3x10⁵ in each well of the 6-well plates and were incubated with DHA (45 and 50 μg/ml) or EPA (55 and 60 μg/ml) for 24 h, then cells were collected by trypsinization and washed with PBS. Following staining with Annexin V-PE and 7-AAD, respectively, the cells were immediately detected using flow cytometry (Millipore).

Morphological analysis using fluorescence microscopy. Cells were seeded at 5x10⁴ in each well of the 24-well plates and were incubated with DHA (45 and 50 μg/ml) or EPA (55 and 60 μg/ml) for 24 and 48 h. The cells were then harvested in an Eppendorf centrifuge tube, centrifuged for 5 min at 106 x g and suspended in PBS containing fluorescence dye AO/EB (AO and EB were at the concentration of 100 mg/l in PBS) (11). The cells were prepared and placed onto slides. Cell morphology was observed under a fluorescence microscope (IX71; Olympus, Tokyo, Japan) and images were captured.

Transmission electron microscope. Cells were seeded at 1x10⁵ in each well of the 6-well plates and incubated with DHA (50 μg/ml) or EPA (60 μg/ml) for 24 h. The cells were collected by trypsinization, washed with PBS, fixed in 2.5% glutaraldehyde at 4˚C for 2 h and then washed again twice with PBS. The material was dehydrated in a graded series of ethanol (50, 70, 80, 90 and 100%) and acetone for 15 min each and embedded in Epon 812. Ultrathin sections were stained with uranyl acetate and lead acetate, followed by an examination with a transmission electron microscope (TEM; JEM1002CXII; Hitachi, Tokyo, Japan).

Statistical analysis. The results are expressed as the mean ± standard deviation. SPSS 17.0 statistical software (SPSS Inc., Chicago, IL, USA) was used to analyze the results. One-way ANOVA, Dunnett's t-test and Pearson's correlation were used in the present study. All the tests performed were two-sided. P<0.05 was considered to indicate a statistically significant difference.

Results

n-3 PUFA inhibit the proliferation of A549 cells. A549 cells were treated with different doses of DHA (40, 45, 50 and 55 μg/ml) or EPA (45, 50, 55 and 60 μg/ml) for 24, 48 and 72 h. An MTT assay was used to examine the anti-proliferative effect of DHA/EPA on A549 cells. As shown in Fig. 1, DHA and EPA significantly suppressed the proliferation of A549 cells, in a dose- and time-dependent manner. The inhibitory rates of DHA and EPA on cell growth were 97.99±1.13 and 77.99±4.43%, respectively, when treated with high concentrations for 72 h.

n-3 PUFA induce apoptosis in A549 cells. A549 cells were treated with different doses of DHA (45 and 50 μg/ml) or EPA (55 and 60 μg/ml) for 24 h. Flow cytometry was used to assay the apoptosis by Annexin V-PE/7-AAD staining. Each concentration was measured three times. As shown in Fig. 2, DHA and EPA significantly induced apoptosis of A549 cells, in a dose-dependent manner. The early apoptosis rates of DHA and EPA on A549 cells were 14.68±1.81 and 14.46±1.63%, respectively, when treated with high concentrations.

Morphological changes of A549 cells induced by n-3 PUFA. Three types of cells can be recognized under a fluorescence microscope: live cells (green), live apoptotic cells (yellow) and dead cells by necrosis (red). When A549 cells were treated with DHA (45 and 50 μg/ml) or EPA (55 and 60 μg/ml) for 24 h (Fig. 3B and C) the morphological features of apoptotic cells, including cell surface protuberances and nuclear fragments, were identified by AO staining. Following 48 h, the typical apoptotic body appeared and the late apoptotic cells were observed by EB staining (Fig. 3E and F).

The apoptotic phenomenon was also demonstrated by transmission electron microscopy. When A549 cells were treated with DHA (50 μg/ml; Fig. 4B) or EPA (60 μg/ml; Fig. 4C) for 24 h, ultrastructure characteristics for the apoptotic cells included the condensation of nuclear chromatin and the degeneration of cytoplasmic organelles. The structure of the nuclear envelope partly disappeared in spermatogonia. The apoptotic bodies were observed in the cytoplasm. Furthermore, compared with the control (Fig. 4A), the formation of autophagosomes (double membrane structures which may have content in them) was clearly enhanced in DHA- and EPA-treated cells (Fig. 4D and E).

Discussion

Dietary fats have been known to be important in the etiology of cancer. A positive association between a high intake of fat and the incidence of breast, colon, pancreatic and prostate cancer has been demonstrated (12). However, such an association may be independent of the energy contents of the fats. Findings of recent studies have demonstrated that diets rich in n-3 PUFA were inversely correlated with the development of colorectal, prostate and breast cancer (13-15).

Mammals, including humans, cannot synthesize either the n-6 or the n-3 PUFA, thus, fatty acids containing these bonds are essential fatty acids and must be obtained in the diet. The n-3 PUFA may be consumed as linolenic acid, which is contained in various amounts in certain oils and in leafy green vegetables. Longer chain n-3 PUFA, mainly EPA and DHA, are found in fish and fish oils (16). While a large body of evidence indicates that n-6 PUFA promote the growth of tumour cells,
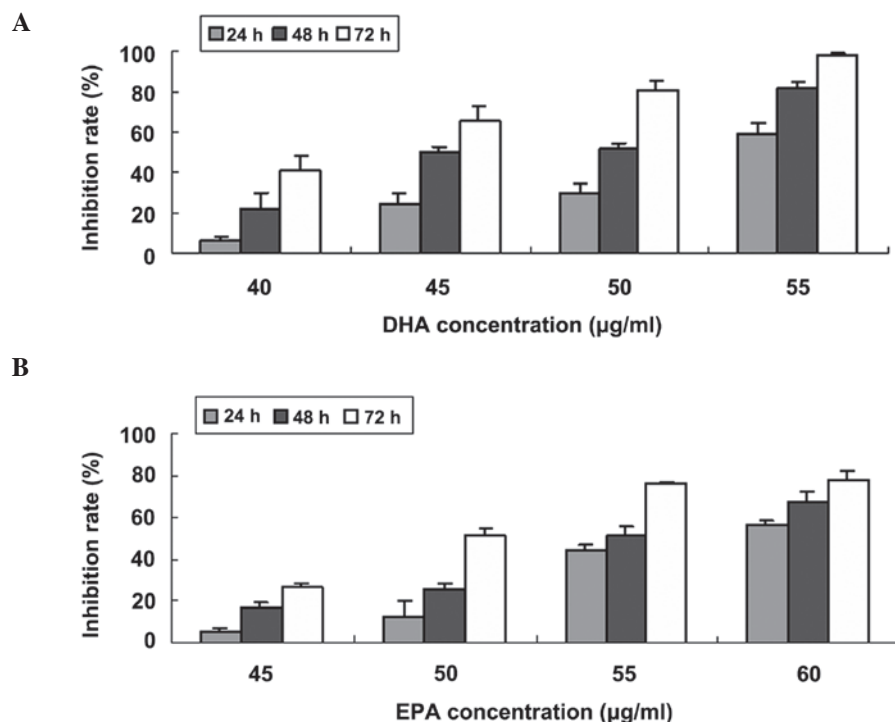
Figure 1. MTT assay for the cell growth inhibition of A549 cells. DHA and EPA significantly suppressed the proliferation of A549 cells, in a dose- and time-dependent manner. Growth inhibition rates of A549 cells treated with (A) DHA (40, 45, 50 and 55 μg/ml) for 24, 48 and 72 h and (B) EPA (45, 50, 55 and 60 μg/ml) for 24, 48 and 72 h, respectively. MTT, methyl thiazolyl tetrazolium; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid.
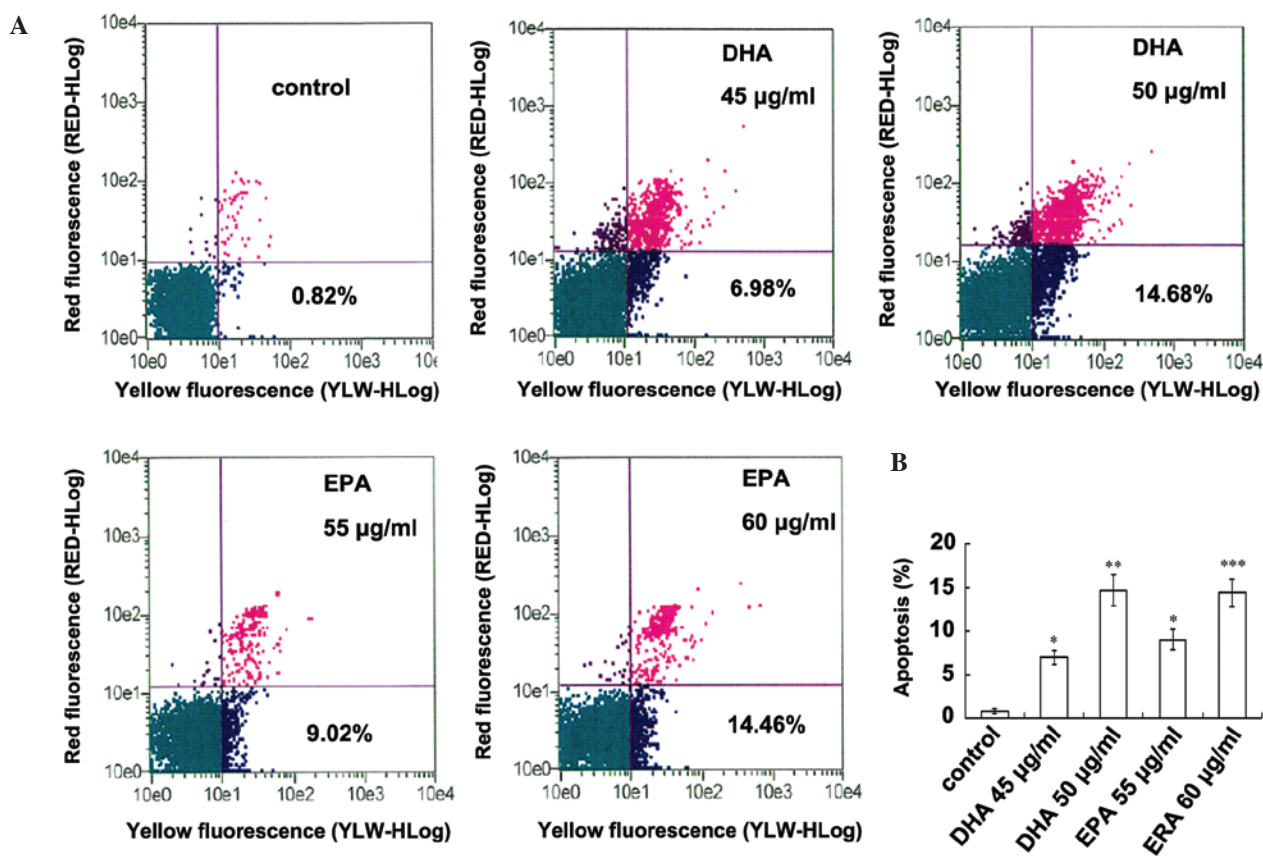

Figure 2. Flow cytometric analysis of apoptosis in A549 cells treated with different doses of DHA (45 and 50 μg/ml) or EPA (55 and 60 μg/ml) for 24 h. DHA and EPA significantly induced A549 cell apoptosis, in a dose-dependent manner. (A) Typical flow cytometric results, the x-axis indicates the Annexin V-positive populations and the y-axis indicates the 7-AAD-positive populations. (B) Histogram of the early apoptotic rates in each group. *vs. control, P<0.05; **vs. DHA 45 μg/ml, P<0.05; ***vs. EPA 55 μg/ml, P<0.05. DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; 7-AAD, 7-amino-actinomycin D.
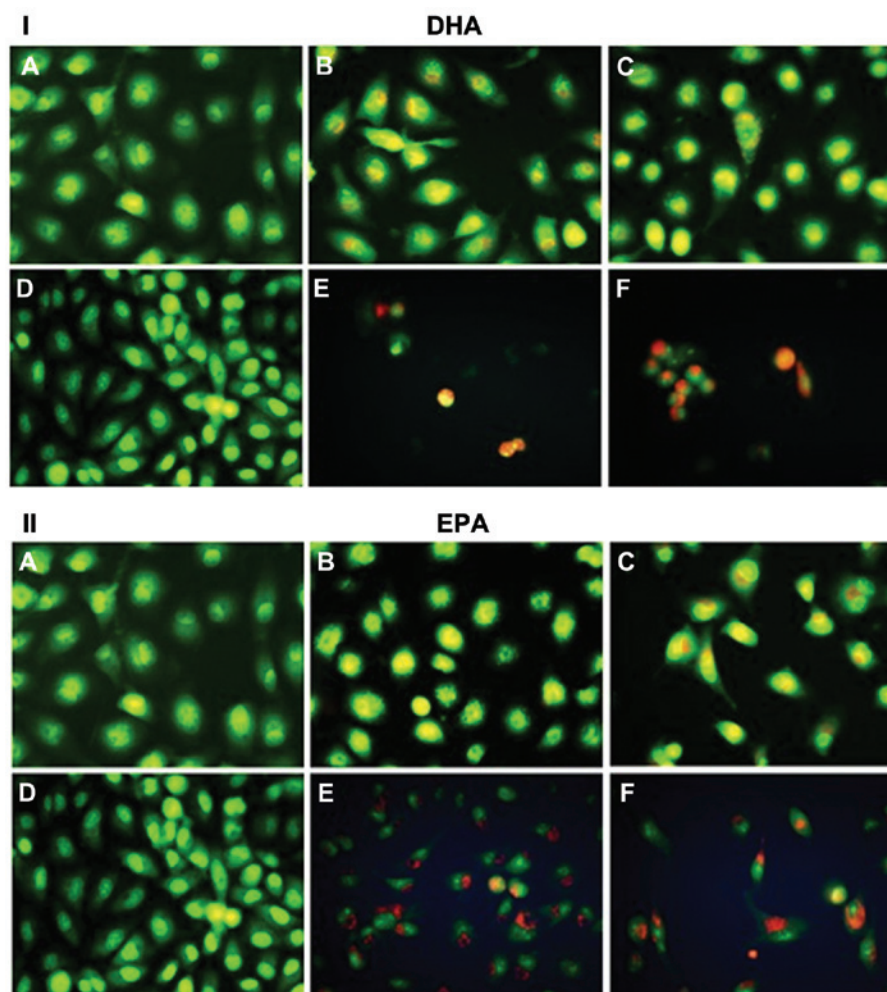
Figure 3. Morphological changes in A549 cells following treatment with (I) DHA or (II) EPA and staining by AO/EB. Three types of cells can be recognized under a fluorescence microscope: live cells (green), live apoptotic cells (yellow) and dead cells by necrosis (red). (A) Negative for 24 h, (B) treatment with 45 μg/ml of DHA (or 55 μg/ml of EPA) for 24 h, (C) treatment with 50 μg/ml of DHA (or 60 μg/ml of EPA) for 24 h, (D) negative for 48 h, (E) treatment with 45 μg/ml of DHA (or 55 μg/ml of EPA) for 48 h, and (F) treatment with 50 μg/ml of DHA (or 60 μg/ml of EPA) for 48 h. AO, acridine orange; EB, ethidium bromide; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid.
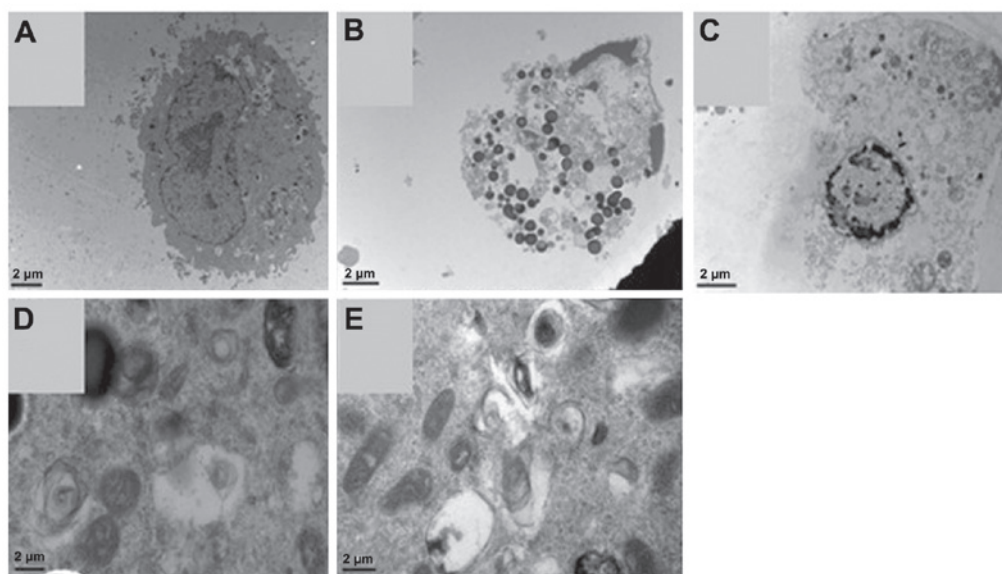

Figure 4. Ultrastructure changes under the transmission electron microscope. (B and C) The apoptotic phenomenon and (D and E) autophagosomes were clearly enhanced in DHA/EPA-treated cells. (A) Negative for 24 h, (B and D) treatment with DHA (50 μg/ml) for 24 h, (C and E) treatment with EPA (60 mg/ml) for 24 h. DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid.

n-3 PUFA have actually been demonstrated to inhibit breast, colon, prostate and melanoma cell proliferation (7,8,17,18). Supplementing the diet of tumor-bearing mice or rats with oils containing EPA or DHA has been demonstrated to slow the growth of various types of cancer, including lung (10,19,20), colon (21,22), mammary (23) and prostate (9). Additionally, a number of epidemiological studies suggest that fish consumption is significantly inversely associated with lung cancer risk and mortality (24-26). Furthermore, a combination of n-3 PUFA (2 g of fish oil, twice daily) and a COX-2 inhibitor (celecoxib, 200 mg) may ameliorate the symptoms and signs associated with systemic immune metabolic syndrome in advanced lung cancer (27). In the present study, the anti-proliferative effect of DHA or EPA on A549 cells was confirmed by an MTT assay, suggesting a potential therapeutic role of n-3 PUFA.

Apoptosis, or programmed cell death, is an essential component of cell number regulation in colonic epithelia and a crucial mechanism to prevent damaged or mutated cells from surviving and dividing, and thus contributing to carcinogenesis. The ability of n-3 PUFA to induce cancer cell apoptosis has been documented (28-30). Dietary supplementation with EPA resulted in a significant increase in crypt cell apoptosis in humans with a history of colorectal adenomas (31) as well as in normal rat colonic mucosa (32). As previously reported, EPA and DHA have also been demonstrated to induce apoptosis in the human lung cancer cell line A549, in the present study.

Autophagy is induced as a survival response to either growth factor or nutrient deprivation and it is also an important mechanism of tumor cell death. The autophagic process is characterized by the sequestration of bulk cytoplasm and organelles in double or multimembrane autophagic vesicles and their subsequent degradation by lysosomes (28). It has also been reported that DHA induces autophagy through p53-mediated AMPK/mTOR signaling and promotes apoptosis in human cancer cells harboring wild-type p53 (33). We revealed that DHA or EPA treatment induced the formation of autophagosomes in A549 cells, which confirmed that autophagy was associated with their anti-cancer mechanisms.

Numerous mechanisms have been suggested for the suppression of tumor cell growth by n-3 PUFA and new mechanisms are frequently reported as we gain additional knowledge regarding the regulation of gene expression by fatty acids. It has been recently documented that fish oil-derived fatty acids have anti-inflammatory or anti-proliferative activity through the reduction of COX-2 expression as well as the suppression of the formation of the proinflammatory lipid mediator prostaglandin E2 (34). It has been reported that DHA inhibits eicosanoid synthesis from arachidonic acid (AA), EPA is a better substrate for COX than AA and EPA competes more successfully than AA for COX activity (35). When activated, the transcription factor, nuclear factor-κB (NF-κB), inhibits programmed cell death or apoptosis. The n-3 PUFA can restore functional apoptosis by downregulating NF-κB (36), which in turn downregulates COX-2 expression. Furthermore, n-3 PUFA decrease the activation of oncogenic transcription factors Ras, transcription factor AP1 (37) and protein kinase C (38). It is likely that the suppression of tumor cell growth by n-3 PUFA is due to the combination of these mechanisms rather than to a single, unique activity.

In conclusion, typical n-3 PUFA, including DHA and EPA inhibit the proliferation of A549 cells and induce cell apoptosis and autophagy in a dose- and time-dependent manner. This may provide new safe and effective options for the treatment of lung cancer in the future.

Acknowledgements

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