Role of autophagy in the ω-3 long chain polyunsaturated fatty acid-induced death of lung cancer A549 cells

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Abstract. The present study identified that ω-3 long chain polyunsaturated fatty acids (ω-3 PUFAs), docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) demonstrate anti-proliferative effects in lung cancer A549 cells. MTS and cytotoxicity assays were conducted to confirm that ω-3 PUFAs induced cell death. Autophagy-associated gene and signaling pathways were also detected. Microtubule-associated protein light chain 3 (LC3) expression was found to be increased subsequent to treatment with DHA and EPA, and the expression of LC3-II was particularly increased. mRFP-GFP-LC3 fluorescence staining and p62 expression levels were used to detect autophagic flux. The present results indicate that DHA and EPA block autophagic flux, suggesting autophagosome accumulation. Subsequent to treatment with DHA and EPA, which interfered with autophagosomes, the expression of Beclin 1 was significantly decreased, while the expression of phosphorylated Akt and phosphorylated mammalian target of rapamycin was significantly increased. Therefore, DHA and EPA exert anti-proliferative effects by inhibiting autophagy in A549 cells, which highlights the potential of DHA and EPA for use in the prevention or treatment of lung cancer.

Introduction

Lung cancer is the leading cause of cancer-associated mortality worldwide (1), and the five-year survival rate remains extremely poor (2). Overall, ~75-85% of lung cancers cases are non-small cell lung cancer (NSCLC), which includes squamous cell carcinoma, adenocarcinoma and large cell carcinoma. The treatment of lung cancer involves the use of medical therapies such as surgery, radiation, chemotherapy and palliative care (3) in an attempt to successfully treat or reduce the adverse impact of malignant neoplasms originating in lung tissue. Chemotherapeutic agents are the main treatment measures for NSCLC, but the side-effects are usually difficult to tolerate (4).

Fish oils are an excellent source of long-chain ω-3 polyunsaturated fatty acids (ω-3 PUFAs), including eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Fish oil supplements are increasingly recognized by clinical studies to be useful for the treatment of a variety of human afflictions, including cancer (5). Numerous studies reveal evidence for the capability of ω-3 PUFAs to decrease proliferation, exert a pro-apoptotic effect and inhibit angiogenesis in several in vitro models of colon cancer (6-9).

A previous study indicated that DHA and EPA inhibit the proliferation of A549 cells and induce apoptosis, with autophagy also being observed under transmission electron microscopy (10). Autophagy is a type of programmed cell death and it is an important process that is involved in various human pathologies. Previous studies suggest that autophagy is important in the regulation of cancer development and progression and also in determining the response of tumor cells to anticancer therapy (11-14). Several cell signaling pathways are implicated in regulating autophagy, including the phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) pathway (15,16). The present study aimed to investigate the role of autophagy on DHA- and EPA-induced cell death in A549 cells, and the associated Akt/mTOR signaling pathway.

Materials and methods

Cell and reagents. The human non-small cell lung carcinoma A549 cell line was obtained from The Cell Bank of Chinese Academy of Sciences (Shanghai, China). The cells were grown in modified RPMI-1640 medium (HyClone, Waltham, MA, USA) supplemented with 10% heat-inactivated fetal...
bovine serum at 37°C in a humidified incubator with a 5% CO2 atmosphere. DHA, EPA and 3-methyladenine (3-MA) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

MTS was obtained from Promega (Madison, Wisconsin, USA). A lactate dehydrogenase (LDH) kit was obtained from Jiancheng Bioengineering Institute (Nanjing, China). Lipofectamine 2000 reagent and Opti-MEM I reduced serum medium were obtained from Life Technologies (Carlsbad, CA, USA). The pCDNA3.1-mRFP-GFP-LC3 plasmid was provided by the Biomedical Research Center of Sir Run Run Shaw Hospital (Hangzhou, Zhejiang, China). Rabbit monoclonal primary antibodies against human mTOR (catalog no. 2983), phosphorylated-mTOR [p-mTOR (Ser2448); catalog no. 5536], Akt (catalog no. 4691), p-Akt (Ser473; catalog no. 8457), human microtubule-associated protein 1 light chain 3 isoform B (LC3B; catalog no. 3868), p62 (catalog no. 8025) and β-actin (catalog no. 8457) were obtained from Cell Signaling Technology (Danvers, MA, USA). A biocinchoninic acid (BCA) protein assay kit was obtained from Beyotime Institute of Biotechnology (Haimen, Jiangsu, China), and a protein extraction kit was obtained from KeyGEN (Nanjing, China).

MTS assay for cell viability. The cells were seeded at a density of 2.5x10⁴ cells/well in six-well plates and incubated for 24 h. DHA (50 µg/ml) or EPA (60 µg/ml) was added to the wells for 24 h, while the control group was administered with complete medium, as previously described (17). MTS solution was added to each well and incubated at 37°C for 0.5-2 h. Absorbance values were detected at 490 nm using a microplate reader (SynergyHT2, BioTek, Winooski, VT, USA). Cell viability was calculated based on the following formula:

\[
\text{Cell viability (\%)} = \frac{A_{490 \text{ experimental}}}{A_{490 \text{ control}}} \times 100
\]

Each experiment was repeated three times.

LDH detected by enzyme-labeling. The cells were seeded at a density of 2.5x10⁴ cells/well in six-well plates and were incubated with 50 µg/ml DHA or 60 µg/ml EPA for 24 h, then the culture supernatant was collected and added to a 96-well plate. The LDH cytotoxicity kit was used according to the manufacturer's instructions. Briefly, the standard liquid, matrix buffer, coenzyme I, dinitrophenylhydrazine and sodium hydroxide solution were combined and incubated at 37°C. Absorbance values were detected at 450 nm using a microplate reader (SynergyHT2, BioTek, Winooski, VT, USA). Cell viability was calculated based on the following formula:

\[
\text{LDH activity} = \frac{(A_{450 \text{ experimental}} - A_{450 \text{ control}})}{(A_{450 \text{ standard}} - A_{450 \text{ blank}})} \times 0.2 \text{ mmol/l} \times 1000
\]

Each experiment was repeated three times.

Plasmid transfection and morphological analysis. To analyze autophagic flux, the A549 cells were transfected with an mRFP-GFP-LC3 plasmid. The A549 cells were seeded at a density of 2x10⁵ cells/well in six-well plates. At 90-95% confluency, the cells were transfected using Lipofectamine 2000, Opti-MEM I reduced serum medium and the pCDNA3.1-mRFP-GFP-LC3 plasmid, according to the manufacturer's instructions, for 42 h. Following the addition of 50 µg/ml DHA or 60 µg/ml EPA, the cells were cultured for 24 h. Subsequent to washing twice with phosphate buffered saline, the cells were examined by fluorescence microscopy (BX51, Olympus, Tokyo, Japan).

Western blot analysis. Following treatment with 50 µg/ml DHA, 60 µg/ml EPA and 5 mM 3-MA, the cells were lysed in cell lysis buffer with phenylmethylsulfonyl fluoride, phosphatase inhibitor and protease inhibitor, according to the instructions of the manufacturer of the protein extraction kit. The protein concentration in the lysate was quantitated using a BCA protein assay kit. Equal amounts of protein for each sample were separated by SDS-PAGE and transferred to a polyvinylidene fluoride membrane (EMD Millipore, Billerica, MA, USA). Subsequent to blocking with 5% non-fat milk for 1 h, the membranes were incubated with monoclonal primary antibodies against mTOR, p-mTOR (Ser2448), Akt, p-Akt (Ser473), Beclin-1, LC3B, p62 and β-actin overnight at 4°C. The membranes were then incubated with a horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Cell Signaling Technology; catalog no. 7074) for 2 h. The bands were detected using ECL (Amresco LLC, Solon, OH, USA). The protein levels were quantitated by densitometry using Gel-Pro Analyzer software (Media Cybernetics, Inc., Rockville, MD, USA).

Statistical analysis. All experiments were repeated at least three times. Statistical analyses were performed using SPSS software, version 17.0 (SPSS, Inc., Chicago, IL, USA). The data are expressed as the mean ± standard deviation and were analyzed using one-way analysis of variance. P<0.05 was considered to indicate a statistically significant difference.

Results

ω-3 PUFAs inhibit the proliferation of A549 cells. A549 cells were treated with 50 µg/ml DHA or 60 µg/ml EPA for 24 h. The MTS assay was used to examine the anti-proliferative effect of DHA/EPA on A549 cells. As shown in Fig. 1A, the experimental groups demonstrated significantly decreased cell proliferation compared with the control group. The cell viability rates in the DHA and EPA groups were 26.1±1.3 and 30.72±1.66%, respectively, when treated for 24 h (P<0.01 vs. control).

ω-3 PUFAs induce cytotoxicity in A549 cells. LDH is located in the cytoplasm and is released when the membrane is damaged. Therefore, the degree of adherent cell damage may be evaluated through the LDH levels in the supernatant. The higher the optical density (OD) was, the more LDH was released, and therefore there was elevated cytotoxicity. As shown in Fig. 1B, the LDH OD values subsequent to treatment were 0.08±0.006, 0.192±0.014 and 0.217±0.01 for the control, DHA and EPA groups, respectively; the values for the DHA and EPA groups differed significantly from that of the control.
EPA and DHA. Consumption of EPA and DHA is associated with a decreased risk of breast (6,21,22), colon (8), prostate, and kidney (23) cancers.

Discussion

The major bioactive components of fish oil are the long-chain ω-3 PUFAs, which contain EPA and DHA. Consumption of EPA and DHA is associated with a decreased risk of breast (6,21,22), colon (8), prostate, and kidney (23) cancers. However, there have been few studies into the association...
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between ω-3 PUFAs and lung cancer. As lung cancer is the leading malignant tumor in humans, with 75-85% of cases involving NSCLC (24), the A549 cell line was chosen for the present study.

The MTS assay performed to assess the cell viability indicated that DHA and EPA demonstrated antiproliferative effects in the A549 cells. In addition, the LDH activity of the culture supernatant was increased subsequent to treatment with DHA or EPA, due to cell membrane damage. Overall, these results are consistent with and confirm the results of a previous study (10).

Autophagic vacuoles have previously been observed under transmission electron microscopy (10). During the formation of autophagosomes, the LC3-I cytoplasmic form is cleaved and lipidated to generate the membranous LC3-II form (25,26). To determine the form of LC3 that is affected by the presence of DHA and EPA, western blot analysis was used to detect LC3-I and LC3-II levels. The results revealed higher levels of LC3, particularly LC3-II, leading to a significantly increased LC3-II/LC3-I ratio subsequent to treatment with ω-3 PUFAs compared with the control group (P<0.05). An increased LC3-II to LC3-I ratio indicates an increased quantity of autophagosomes. Autophagosome accumulation may result from either the increased formation of autophagosomes or from the blockage of autophagic degradation (27).

Beclin 1 is the mammalian ortholog of the yeast Vps30/Apg6 gene, which is required for autophagosome formation, and is monoallelically deleted in a high percentage of human carcinomas (28‑30). Previous studies have revealed that the promotion of the expression of Beclin 1 through reduced autophagy demonstrates anticancer effects (25,26,31). The present study indicated that Beclin 1 expression levels were significantly decreased subsequent to treatment with DHA and EPA, which suggests that ω-3 PUFAs inhibit autophagosome formation in A549 cells.

There is conclusive evidence that the PI3K/Akt/mTOR pathway represents the major regulatory mechanism of autophagy (32). Numerous studies have revealed that the inhibition of the PI3K/Akt/mTOR signaling pathway activates autophagy and induces cell death (33,34). Selective inhibition of the Akt/mTOR signaling pathway reduces the level of macrophages and stabilizes vulnerable atherosclerotic plaques.
plaques by promoting macrophage autophagy (35). The addition of a PI3K inhibitor overcomes cellular resistance to mTORC1 inhibitors, regardless of PTEN status, and therefore substantially expands the molecular phenotype of tumors likely to respond (16). Coordinated inhibition of the mTOR and autophagy pathways promotes apoptosis and may be a novel therapeutic paradigm for the treatment of melanoma (36). Targeted therapy using the PI3K/mTOR inhibitor NVP-BEZ235 significantly enhances doxorubicin-induced apoptosis in neuroblastoma cells (37).

However, certain studies report that activation of the PI3K/Akt/mTOR signaling pathway inhibits autophagy and induces cell death (38,39). mTOR is a highly conserved serine/threonine kinase that is involved in the regulation of cell responses to altered nutrition conditions, and is also involved in a number of energy-associated regulatory pathways (40). Unc-51 like autophagy activating kinase 1 (ULK1) and mammalian autophagy related 13 (mAtg13) are downstream signaling targets of mTOR. When the activity of mTOR is inhibited, the ability of mTOR to inhibit the phosphorylation of ULK1 and mAtg13 is decreased. Therefore, ULK1, mAtg13 and focal adhesion kinase family interacting protein of 200 kD are phosphorylated, which leads to autophagosome formation (41). By contrast, the activation of mTOR has been found to inhibit autophagosome formation. Inhibition of autophagy may enhance matrine-induced apoptosis in human hepatoma cells (42). The human papillomavirus-host cell interaction stimulates the PI3K/Akt/mTOR pathway and inhibits autophagy, which benefits the viral infection (43). In the present study, western blot analysis revealed significantly increased levels of p-Akt and p-mTOR subsequent to treatment with DHA and EPA, indicating activation of this signaling pathway and a decrease in autophagy signal in the early stage. The inhibitor of PI3 K 3-MA inhibits the upstream Akt pathway, leading to a reduction in p-Akt and p-mTOR expression levels. The addition of DHA and EPA also decreased the total expression levels of Akt and mTOR, as demonstrated by western blot analysis.
During autophagolysosome formation, an increased quantity of autophagosomes may indicate accumulation and interfere with the formation of autophagolysosomes (44). mRFP-GFP-LC3 is used to distinguish early and late autophagosomes. The GFP tag is rapidly suppressed in the acidic environment of the autophagolysosome, leaving only the mRFP tag detectable (45,46). Fig. 2B shows the appearance of yellow fluorescence in DHA- and EPA-treated A549 cells, indicating decreased autophagolysosome formation and diminished autophagic flux.

Autophagic flux can also be measured by decreased p62 protein level, since p62 acts as an autophagosomal cargo receptor for ubiquitinated proteins, which is degraded in the autophagolysosome (47). The addition of DHA and EPA to A549 cells for 24 h increased p62 levels, suggesting a block in the autophagic flux.

In conclusion, the present study has revealed that DHA and EPA exert anti-proliferative and cytotoxic effects on A549 cells. DHA and EPA interfere with autophagosome formation by activating the Akt/mTOR signaling pathway, thereby reducing the autophagy signal prematurely and decreasing the Beclin 1 expression level. DHA and EPA also block the maturation of autophagy and diminish autophagic flux. Inhibition of autophagy promotes cell death in A549 lung cancer cells. The present data identify a plausible mechanism by which DHA and EPA exert potent anticancer activity, and suggest the potential application of ω-3 PUFAs as a tool or viable drug in anticancer treatments.

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