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Journal of Nutritional Biochemistry 25 (2014) 104–110

Journal of Nutritional Biochemistry

RESEARCH ARTICLES

Docosahexaenoic acid inhibited the Wnt/β-Catenin pathway and suppressed breast cancer cells in vitro and in vivo☆

Meilan Xue[a,*], Qing Wang[b], Jinglan Zhao[c], Liyan Dong[d], Yinlin Ge[a], Lin Hou[a], Yongchao Liu[a], Zheng Zheng[a]

[a]Department of Biochemistry and Molecular Biology, Medical College, Qingdao University, Qingdao, Shandong Province, 266021, China
[b]Department of Ophthalmology, Affiliated Hospital of Qingdao University, Qingdao 266003, China
[c]Qingdao Center Medical Group, Shandong, China
[d]Center for Disease Control and Prevention, Qingdao, China

Received 8 March 2013; received in revised form 2 July 2013; accepted 9 September 2013

Abstract

N-3 fatty acids (FAs) are essential FAs necessary for human health and are known to possess anticancer properties. However, the relationship between *n*-3 FAs and β-catenin, one of the key components of the Wnt signaling pathway, in mouse breast cancer remains poorly characterized. In this study, 4T1 mouse breast cancer cells were exposed to a representative *n*-3 FA, docosahexaenoic acid (DHA), to investigate the relationship between *n*-3 FAs and the Wnt/*β*-catenin signaling pathway *in vivo* and *in vitro*. *In vitro* studies showed that DHA strongly inhibited cell growth, and induced G1 cell cycle arrest both in 4T1 mouse breast cells and MCF-7 human breast cells. DHA reduced *β*-catenin expression and T cell factor/lymphoid-enhancing factor reporter activity in 4T1 mouse breast cells. In addition, DHA down-regulated the expression of downstream target genes such as c-*myc* and cyclinD1. *In vivo*, therapy experiments were conducted on Babl/c mice bearing breast cancer. We found that feeding mouse the 5% fish oil-supplemented diet for 30 days significantly reduced the growth of 4T1 mouse breast cancer *in vivo* through inhibition of cancer cell proliferation as well as induction of apoptosis. Feeding animals a 5% fish oil diet significantly induced down-regulation of *β*-catenin in tumor tissues with a notable increase in apoptosis. In addition, fish oil-supplemented diet decreased lung metastases of breast cancer. These observations suggested that DHA exerted its anticancer activity through down-regulation of Wnt/*β*-catenin signaling. Thus, our data call for further studies to assess the effectiveness of fish oil as a dietary supplement in the prevention and treatment of breast cancer.

Keywords: Docosahexaenoic acid; Breast cancer; Fish oil; *β*-catenin; Wnt signaling; Tumor-bearing mouse

1. Introduction

Numerous studies suggested that activation of the Wnt/*β*-catenin signaling pathway plays an important role in human tumor genesis [1,2]. The levels of WNTs or other components of WNT pathway are known to be altered in 50% of breast cancer cases [3]. A hallmark of the Wnt/*β*-catenin signaling activation is the stabilization of cytosolic *β*-catenin, which enters the nucleus to activate Wnt target genes by binding transcription factors of the T-cell factor/lymphoid-enhancing factor (TCF/LEF) family [4]. Mutations that activate the Wnt/*β*-catenin pathway generally affect *β*-catenin phosphorylation and stability [5]. A variety of Wnt/*β*-catenin target genes have been identified, including those that regulate cell proliferation and apoptosis, thus mediating cancer initiation and progression [6–8].

N-3 fatty acids (FAs) are long-chain polyunsaturated FAs. The principal dietary source of eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) is from oily cold-water fish [9]. Currently, the western diet contains a disproportionally high amount of *n*-6 FAs and low amount of *n*-3 FAs, and the resulting high *n*-6/*n*-3 ratio is thought to contribute to cardiovascular disease, inflammation and cancer. Studies in human populations have linked high consumption of fish or fish oil to reduced risk of colon, prostate and breast cancer [10,11].

A number of biological effects that could contribute to cancer suppression by *n*-3 FAs have been suggested [12,13]. These effects include alterations in the properties of cancer cells (proliferation, invasion, metastasis and apoptosis) as well as those of host cells (inflammation, immune response and angiogenesis). Many signaling pathways are involved in these effects of *n*-3 FAs, including protein kinase C, ras, ERK 1/2 and NF-*κ*B [14–17]. However, the molecular mechanisms that account for these signaling effects are not completely understood.

This work was completed with the support of the Youth Science Fund project of the National Natural Science Foundation of China (81300790) and the Development project of Shandong Province medical science and technology (2013WS0262).

☆ Conflict of Interest Statement: All authors of this article have no any financial and personal relationships with other people or organisations.

* Corresponding author. Department of Biochemistry and Molecular Biology, Medical College, Qingdao University, Dengzhou Roud 38#, Qingdao, Shandong Province, 266021, China. Tel.: +08-0532-82991209.

E-mail address: snowml@126.com (M. Xue).

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http://dx.doi.org/10.1016/j.jnutbio.2013.09.008

To the best of our knowledge, the relationship between *n*-FAs and β-catenin, one of the key components of the Wnt signaling pathway, in mouse breast cancer remains poorly characterized. In this study, we sought to investigate the effect of *n*-FAs on β-catenin protein and protein expressions of c-*myc* and cyclinD1. Furthermore, the role of β-catenin in *n*-3 FA-mediated growth inhibition in 4T1 mouse breast cancer cells was studied *in vivo*. We believe that a better understanding of the mechanism of the anticancer actions of *n*-3 FAs would conduce to the development of new cancer therapeutic strategies involving the use of fish oil as a dietary supplement.

2. Materials and methods

2.1. Cell culture and MTT assay

The 4T1 mouse breast cancer cells and MCF-7 human breast cancer cells were both purchased from Shanghai Life Science of Chinese Academy of Sciences. The cells were routinely maintained in 1:1 (v/v) mixture of DMEM high glucose (Hyclone, Beijing, China) and 10% (vol/vol) fetal bovine serum (Gibco BRL, Grand Island, NY, USA), 37°C in a tissue culture incubator with 5% CO_2 and 98% relative humidity. The cells were placed in six-well plates and cultured as normal. The exponentially growing cells were used throughout the experiments.

The 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT, Sigma, St. Louis, MO, USA) assay was performed as described elsewhere [18]. In brief, cells were cultured in a 96-well plate at a density of 1×10^5 cells per ml. The cells were then treated with 25, 50 and 100-μM DHA or EPA (Sigma Chemical Co., St. Louis, MO, USA). After 3 days, the cells were treated with 20-μl MTT (5 mg/ml). The cultures were then re-incubated for an additional 4 h. After removal of the supernatant, 150-μl DMSO was added to each well to dissolve the crystals completely, and then, the absorbance was measured at 490 nm using an ELISA Reader (Bio-Tek Instruments, Inc., Winooski, VT, USA). The results are expressed as the percentage of inhibition that produced a reduction in absorbance by DHA treatment compared to the untreated controls.

2.2. Apoptosis analysis

The annexin V-FITC apoptosis detection kit was used for the apoptosis assay (Invitrogen, Carlsbad, CA, USA). The cells (1×10^6 cells/ml) were treated with 25, 50 and 100-μM DHA for 48 h. Cells were harvested by trypsinization, washed twice with PBS, and resuspended in 500 μL of binding buffer. Cell suspensions were then incubated with 5 μL of annexin V-FITC and 5 μL of propidium iodide (PI) for 10 min at room temperature in the dark. The cells were evaluated immediately by flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA).

2.3. Cell cycle analysis

The cells were treated with 25, 50 and 100-μM DHA for 48 h. Cells were harvested by trypsinization, washed twice with cold PBS, and then fixed with 70% cool ethanol for 2 h. After washing in cold PBS three times, cells were stained with PI (Cycle TEST PLUS DNA Reagent Kit, Becton-Dickinson, Franklin Lakes, NJ, USA). A 96-μm pore size nylonmesh was used to filter cells on the next day, and a total of 10,000 stained nuclei were analyzed with a FACScan flow cytometer with CellQuest software.

2.4. Luciferase reporter activity

4T1 cells were seeded and allowed to achieve 80% confluence in six-well plates. The cells were transiently transfected with 1 μg per well of TCF/LEF-Luc by using Lipofectamine Plus transfection reagents (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. After transfection, the cells were treated with 25, 50 and 100-μM DHA for 48 h. Cell lysates were prepared using 1× reporter lyses buffer (Promega, Madison, WI, USA). Luciferase activity was measured as described elsewhere [19], by using an AutoLumat LB953 Luminometer (Berthold, Stevenage, UK) and using the luciferase assay system from Promega. The relative luciferase activity was calculated after normalization of cellular proteins. All values are expressed as the percentage of activity relative to basal activity.

2.5. Western blot analysis

4T1 cells were seeded in six-well plates and treated with 25, 50 and 100-μM DHA. After 48 h, the cells were treated with 2% SDS (10-mM EDTA, 50-mM Tris base, 10% SDS, pH 8.0) and boiled at 100°C for 10 min. Protein concentrations were measured using the BCA protein assay. Briefly, 50-μg samples of protein were loaded on 12% SDS-PAGE gels, transferred to PVDF membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK) and blocked with 5% nonfat milk in TBST buffer (20-mM Tris–HCl, 120-mM NaCl, 0.1% Tween for 1 h). The rabbit polyclonal antibody for β-catenin, mouse monoclonal antibody for c-*myc* and rabbit polyclonal antibody for cyclinD1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). After three washes (5 min each) with TBST (TBS containing 0.1% Tween 20), the membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. After three washes (5 min each) with TBST, proteins were visualized using the enhanced chemiluminescence method (Amersham Pharmacia Biotech, Buckinghamshire, UK).The expression levels were normalized to β-actin.

2.6. Tumor growth in mice

All experimental procedures were conducted in conformity with the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No.85-23, revised 1985). The study protocol was approved by the Review Committee for the Use of Human or Animal Subjects of Medical College of Qingdao University.

Female outbreed Babl/c mice at 4 weeks of age were purchased from Shandong Laboratory Animal Center. Sixteen mice were housed in wire-top cages with sawdust bedding in a clean, air-conditioned room at a temperature of 26°C and a relative humidity of 50%. After approximately 1 week of acclimatization after arrival, the mice were randomized into two groups (8 animals per group). We injected 5×10^4 4T1 cells suspended in 50 μl of PBS into the mammary fat pad of each mouse. When tumors reached 8~10 mm^2 in size, the mice were fed with the experimental diets. One group was treated with the control diet (containing 5% corn oil), and the other group was fed the fish oil-supplemented diet (containing 5% fish oil). These diets contained similar quantities of carbohydrates, protein, lipids, vitamins and minerals (summarized in Table 1), and the only difference is the types of lipids (i.e., corn oil vs. fish oil). Both diets were stored in sealed containers at 4°C to reduce spontaneous lipid peroxidation.

Animal body weight and tumor size were measured and recorded. Tumor size was measured every 2 days in two perpendicular dimensions (a=length, b=width) with a vernier caliper and the size recorded as a volume (mm^3) as calculated by $a * b^2/2$. A tumor growth curve was then constructed, and data were presented as mean±S.E. After 30 days of tumor treatment, the mice were euthanized, and their tumors were excised and weighed. The tumor specimens were fixed in 4% formaldehyde, embedded in paraffin and cut in 4 μm sections for immunohistochemical analysis.

For enumeration of pulmonary metastatic nodules, the metastases appeared as discrete white nodules on the black surface of lungs insufflated and stained with a 15% solution of India ink and then bleached by Fekette's solution.

2.7. Analysis of plasma and tissue FA levels

Plasma and breast tissues were harvested from each animal, snap-frozen in liquid nitrogen and stored in a −80°C freezer until analysis. The measurement of linoleic acid (LA), arachidonic acid (AA), EPA and DHA in plasma and tissues were conducted as described elsewhere [20]. Briefly, total lipids from plasma were extracted using the chloroform:methanol mixture (2:1, v/v), dried under a stream of nitrogen and transmethylated using boron trifluoride in methanol (14 g/L). FA methyl esters were extracted from the mixture with pentane containing 0.05% butylated hydroxytoluene. One microliter of transmethylated sample was injected into the Agilent gas chromatography 6890N linked with the 5975B mass spectrometer. Capillary column HP5-MS (30 m×0.25 mm, film thickness 0.25 μm) was used for separation and helium as the carrier gas. The column oven temperature was set at 120°C, ramped to 250°C at 3°C/min, then ramped to 300°C at 10°C/min and held at 300°C for 5 min.

2.8. Immunohistochemistry and in situ TUNEL assay for apoptotic cells

Immunohistochemical analysis of β-catenin expression was performed according to the procedure described elsewhere [21]. The primary antibody is polyclonal rabbit anti-murine β-catenin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Apoptotic cell death in paraffin-embedded tumor tissue sections was examined using the TdT-FragEL

Table 1
Composition of the experimental diets used in this study

	Control diet	Fish oil diet
Casein (g/kg)	200.0	200.0
DL-Methionine (g/kg)	3.0	3.0
Sucrose (g/kg)	500.0	500.0
Corn starch (g/kg)	150.0	150.0
Corn oil (g/kg)	50.0	
Fish oil (g/kg)		50.0
Cellulose (g/kg)	50.0	50.0
Mineral mix, AIN-76 (170915) (g/kg)	35.0	35.0
Vitamin mix, AIN-76A (40077) (g/kg)	10.0	10.0
Choline bitartrate (g/kg)	2.0	2.0
Ethoxyquin, antioxidant (g/kg)	0.01	0.01

The mice were fed with the experimental diets. One group was treated with the control diet (containing 5% corn oil), and the other group was fed with the fish oil-supplemented diet (containing 5% fish oil). These diets contained similar quantities of carbohydrates, protein, lipids, vitamins and minerals, and the only difference is the types of lipids (i.e., corn oil vs. fish oil).