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FAST TRACK

Omega-3 polyunsaturated fatty acids attenuate breast cancer growth through activation of a neutral sphingomyelinase-mediated pathway

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The effect of fish oils and their active omega-3 fatty acid constituents, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), were investigated on breast cancer growth. In *in vivo* experiments, mice were fed diets that were rich in either omega-3 (fish oil) or omega-6 (corn oil) fatty acids. Three weeks after implantation of MDA-MB-231 breast cancer cells, the tumor volume and weight were significantly lower ($p < 0.05$) for mice fed the omega-3 diets compared to those fed the omega-6 diets. Dietary fish oil also caused a 40% ($p < 0.05$) increase in neutral sphingomyelinase (N-SMYase) activity in the tumors. The tumor tissues from fish oil-fed animals expressed elevated p21 (waf1/cip1) mRNA, whereas tumor tissues from corn oil-fed animals exhibited undetectable levels of p21 expression. In *in vitro* experiments, at concentrations as low as 25 μM, DHA and EPA inhibited the growth of cultured MDA-MB-231 cells in a dose-dependent manner by 20–25% ($p < 0.05$). N-SMYase activity was also increased by 30–40% ($p < 0.05$) in the DHA- or EPA-treated cells in which an increase in ceramide formation was observed. DHA and EPA were both observed to enhance membrane bleb formation and also to induce the expression of p21. Omega-3 fatty acids-induced bleb formation and p21 expression were inhibited by the N-SMYase inhibitor GW4869, which also inhibited apoptosis by approximately 40% ($p < 0.05$). The results suggest that inhibition of breast cancer growth in nude mice by dietary fish oil and inhibition of breast cancer cell growth in culture by treatment with DHA and EPA is mediated by activation of N-SMYase.

Key words: breast cancer; docosahexaenoic acid; eicosapentaenoic acid; fish oil; sphingomyelinase; ceramide

The oils of certain cold-water fish have a well-documented role in inhibiting or preventing cancer. Epidemiologic evidence strongly links fish oil with low incidences of several cancers.[1–4] The anticancer properties of fish oils have been attributed to the omega-3 fatty acids, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). Proof of these fatty acids as anticancer agents has been substantiated by dietary studies on many types of animals, including humans and in cultured cells.[5–14] A number of studies have indicated that DHA's anticancer properties are not directly due to cytotoxicity but rather to the fatty acid's ability to induce apoptosis.[15–18] However, the molecular mechanism for the anticancer actions of omega-3 fatty acids remains unknown. Understanding the mechanistic effects of omega-3 lipids may aid in the development of new cancer therapies.

Numerous studies, including our own,[5,19–24] have linked fish oil to induction of apoptosis. We found that DHA activates sphingomyelinase (SMYase) activity in the plasma membrane of Jurkat leukemic cells, increasing ceramide levels.[23] SMYase is an enzyme that catalyzes the hydrolysis of sphingomyelin (SM) to ceramide. A variety of studies have shown that ceramide is ubiquitously produced during cellular stress and is associated with apoptosis.[25,26] To date, at least 7 classes of mammalian SMYases have been described, differing in subcellular location, pH optimums, cation dependence and roles in cell regulation.[27–29] However, only 2 forms of SMYases, distinguishable by their pH optima, are capable of initiating signal transduction.[30] The acid SMYase (pH optimum 4.5–5.0) is a cellular glycoprotein located in the acidic lysosomal compartment where it contributes to lysosomal SM turnover.[31] The neutral SMYase (N-SMYase; pH optimum 7.4) is a plasma membrane-bound enzyme[32,33] that has been implicated in mediating apoptosis in cells exposed to stressing agents. Substantial amounts of N-SMYase are proposed to reside in "lipid rafts."[34] Therefore, factors influencing the lipid composition of membranes can influence the activity and distribution of N-SMYase in "lipid rafts." We have demonstrated that DHA may alter lipid raft formation[35] and induce SMYase activity, leading to cell-cycle arrest in leukemic cells.[23] Furthermore, treating cells with synthetic short-chain ceramide has been shown to induce cell-cycle arrest and apoptosis.[36] Ceramide levels also change during progression through the cell cycle[37] and have been shown to enhance expression of p21 (waf1/cip1),[38] a cellular inhibitor of cdk2 kinase that is involved in cell-cycle arrest via hypophosphorylation of retinoblastoma protein (pRb).[39] We have previously shown that DHA-induced ceramide may regulate phosphorylation of pRb by inhibition of cyclin A/cdk-2 activities via increased expression of p21.[23] In our study described below, we investigated growth inhibition of breast cell xenografts by dietary fish oil in nude mice and the cellular effects of DHA and EPA in cultured breast cancer cells. Our data demonstrate that the long-chain omega-3 fatty acids inhibit breast cancer growth by activating N-SMYase, thereby generating ceramide.

Material and methods

Material

Human breast cancer MDA-MB-231 cells were obtained from ATCC (American Type Culture Collection, Manassas, VA). Nude mice (nu/nu) were purchased from Charles River Laboratories (Wilmington, MA). Dulbecco's Modified Eagle Medium (DMEM), penicillin, streptomycin and glutamine were from Invitrogen (Grand Island, NY). Fetal bovine serum was from BioWhittaker (Walkersville, MD). Docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), oleic acid (OA), linoleic acid (LOA) and fatty acid standards for gas chromatography (GC) were from Nu-Check Prep (Elysian, MN). Annexin V staining, cell death detection ELISA, WST-1 assay and the lactate dehydrogenase kits were

Abbreviations: DAPI, 4′, 6-Diamidino-2-phenylindole; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LOA, linoleic acid; N-SMYase, neutral-sphingomyelinase; OA, oleic acid; p21, waf1/cip1; PS, phosphatidylserine.

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TABLE I – EXPERIMENTAL DIETS

	n-6 diet	n-3 diet
Carbohydrates (% calories)	58.8	58.8
Starch (g/100 g)	25.0	25.0
Maltodextrins (g/100 g)	5.0	5.0
Sucrose (g/100 g)	30.0	30.0
Cellulose (g/100 g)	5.0	5.0
Protein (% calories)	19.6	19.6
Casein (g/100 g)	20.2	20.2
DI-methionine (g/100 g)	0.3	0.3
Lipid (% calories)	21.7	21.7
Corn oil (g/100 g)	10.0	1.0[1]
Fish oil (g/100 g)	0	9.0[1]
n-6/n-3 ratio	72.5	0.11[1]
Mineral mix (g/100 g)	3.45	3.45
Vitamin mix (g/100 g)	1.0	1.0
Vitamin E (g/100 g)	0.03	0.03
DHT (g/100 g)	0.02	0.02

[1]Differs between diets.

TABLE II – FATTY ACID COMPOSITION OF DIETARY OILS

Fatty acids	Corn oil (%)	Fish oil (%)
14:0		8.0
16:0	12.0	17.0
16:1		14.0
18:0	7.0	4.0
18:1n-9	22.0	26.0
18:2n-6	58.2	2.0
18:3n-3	0.8	3.0
18:4n-3		3.0
20:4n-6		1.0
20:5n-3		12.0
22:6n-3		10.0
Saturated fatty acids	19.0	29.0
Monounsaturated fatty acids	22.0	40.0
Total n-6 lipids	58.2	3.0
Total n-3 lipids	0.8	28.0
n-6/n-3 ratio	72	0.11

purchased from Roche Biochemicals (Indianapolis, IN). N-SMYase inhibitor, GW4869, was from Calbiochem (San Diego, CA). Hanks Balanced Salt Solution (HBSS), the fish oil (Manhaden) and all other reagents and chemicals were purchased from Sigma Chemical (St. Louis, MO).

Animal studies

Nude mice (nu/nu; Charles River Laboratories) were fed *ad libitum* corn oil (omega-6/omega-3 ratio of 72:1), balanced corn oil/fish oil (omega-6/omega-3 ratio of 1:1), or fish oil (omega-6/omega-3 ratio of 0.11:1) diets (Research Diets, New Brunswick, NJ) for 3 weeks prior to tumor implantation. Diets contained similar quantities of protein (59% of calories), carbohydrates (20% of calories), lipids (21% of calories), vitamins, and minerals as described in Table I. They only differed in the types of lipids (*i.e.,* corn and fish oil), and their fatty acids composition is described in Table II. Tumor xenografts were implanted by injecting subcutaneously 200 μl of MDA-MB-231 cells (1×10^6 cells) on the backs of animals, and the animals were returned to their corresponding diets for another 3 weeks. Tumor growth was monitored by measuring length and circumference of tumors by a flexible wire tape as described,[40] and tumor weight was determined at termination of the study after excising the tumor free of connective tissue. The tumor tissues were freeze-clamped in liquid nitrogen for later analysis.

Cell cultures

MDA-MB-231 breast cancer cells were grown in DMEM media containing 10% fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin at a density of 1×10^6 cells/ml for routine culture. For experimental purposes, cells were cultured at the cell density indicated and treated with fatty acids under serum-free conditions. The fatty acids EPA, DHA, OA and LOA were stored in ethanol under liquid nitrogen and diluted in ethanol just prior to use. The final concentration of ethanol ($< 0.1\%$) in the treated cultures did not induce any cytotoxic effects as measured by lactate dehydrogenase release and a WST-1 cell proliferation assay (results not shown).

Cell growth assay

The effect of the fatty acids on cell growth was determined using a WST-1 assay per the manufacturer's instructions (Roche Biosciences).

N-SMYase assay

Sphingomyelinase activity was measured by an Amplex Red sphingomyelinase assay kit (Molecular Probes, Eugene, OR). Briefly, the frozen tumor tissues were ground under liquid nitrogen and then homogenized in a reaction buffer containing 100 mM Tris-HCl (pH 7.5), 50 mM $MgCl_2$ and 0.1% Triton X-100. For measuring sphingomyelinase activities in MDA-MB-231 cells, DHA- or EPA-treated cells were lysed in 100 μl of lysis buffer [20 mM Tris-HCl (pH 7.5), 137 mM NaCl, 100 mM NaF, 2 mM Na_3VO_4, 10% v/v glycerol, 1% Nonidet P-40, 2 mM phenylmethanesulfonyl fluoride (PMSF), 1 mg/ml leupeptin, 0.15 units/ml aprotinin and 2.5 mM DIFP] for 10 min on ice. Protein concentrations in tumor homogenates, cell lysates and isolated membrane fractions (see below) were measured using a bicinchoninic acid (BCA) protein assay system (Pierce, Rockford, IL), and samples were diluted in reaction buffer for the assay in the presence of exogenous sphingomyelin (0.25 mM). The released phosphorylcholine from sphingomyelinase activity was measured by the sequential activity of alkaline phosphatase and choline oxidase. The resultant release of H_2O_2 was quantified by measuring fluorescence intensities (excitation at 540 nm and emission at 590 nm) after reaction with the Amplex Red reagent as described in the manufacturer's protocol. Amount of sample protein (approximately 10 μg) resulted in a linear fluorescence intensity from approximately 1/5th to 1/10th of the positive control.

Analysis of p21 mRNA expression

RNA from tumor tissues or cultured cells was extracted by an RNA assay kit (Qiagen, Valencia, CA). The amount of RNA in an aqueous solution was determined by absorbance at 260 nm. Semi-quantitative RT-PCR was performed to determine the mRNA levels of p21 and GAPDH (loading control) using the Titan One Tube RT-PCR System (Roche Diagnostics). The primer sequences 5′CGG-TCC-CGT-GGA-CAG-TGA-GCA-G3′, 5′GTC-AGG-CTG-GTC-TGC-CTC-CG3′ were used for p21. The thermocycling parameters were composed of an initial cycle at 50°C for 30 min for reverse transcription of RNA into cDNA. The subsequent DNA amplification was performed with a thermocycling reaction consisting of 95°C for 180 sec followed by 30 cycles at 95°C for 60 sec, 55°C for 60 sec and 72°C for 60 sec.

Western blot analysis

After treatment with DHA or EPA, cell lysates were separated by SDS PAGE (10%), and then electro-blotted onto presoaked Immobilon-P membranes (Millipore, Bedford, MA) as described previously.[23] The membranes were blocked in 5% dry milk in TBS-T solution (50 mM Tris-HCl pH 7.5, 150 mM NaCl and 0.05% Tween-20) for 2 hr at room temperature. The blot was incubated with monoclonal anti-p21 or monoclonal anti-GAPDH antibodies (Santa Cruz Biotech, Santa Cruz, CA; 1:1,000) at 4°C overnight and detected using secondary anti-rabbit peroxidase-conjugated antibodies (Amersham Pharmacia Biotech, Buckinghamshire, UK; 1:2,000 in TTBS). The bands were detected using a chemiluminescence detection kit (Pierce).

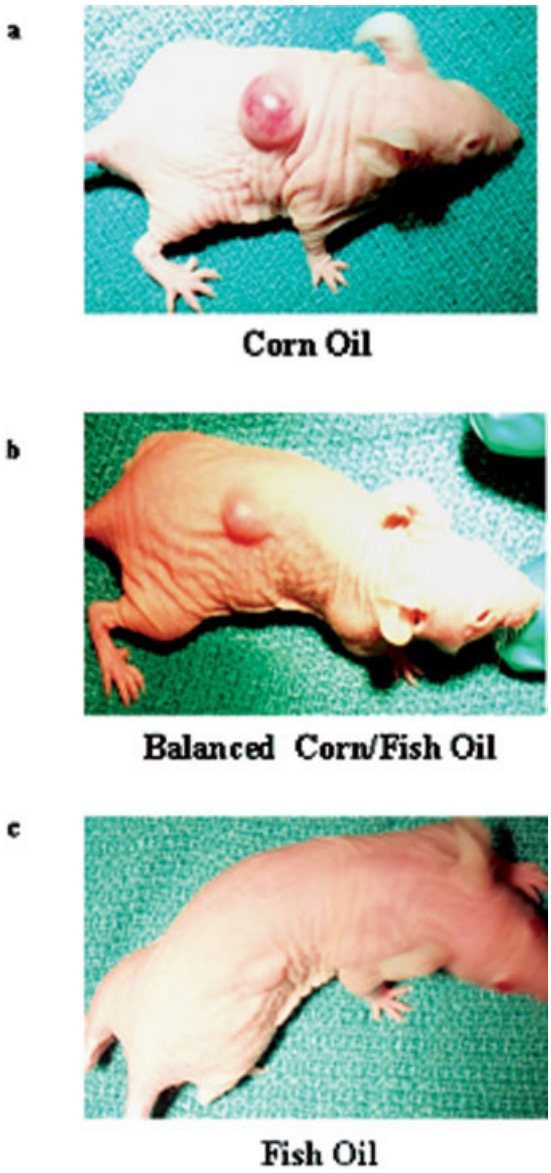

FIGURE 1 – Fish oil inhibits breast cancer growth in mice. Nude nice were maintained on *(a)* corn oil (omega-6/omega-3 ratio 72:1), *(b)* balanced corn/fish oil (omega-6/omega-3 ratio 1:1) or *(c)* fish oil (omega-6/omega-3 ratio 0.11:1) diets for 3 weeks prior to subcutaneous implantation of MDA-MB-231 cells (1×10^6 cells). The animals were then further fed on the corresponding diets for another 3 weeks. Results are representations from 6 mice in each group.

Immunohistochemistry

Ceramide and p21 formation were determined using immunohistochemistry.[41] After incubation with serum-free media containing DHA or EPA, the cells were fixed with 3% paraformaldehyde and then blocked with 1% BSA in PBS. Ceramide was detected using a specific anticeramide antibody (Alexis, Carlsbad, CA; clone MID 15B4; 1:200 dilution in blocking buffer) and assayed using an Alexa 488-labelled anti-mouse antibody (Molecular Probes; 1:200 dilution in blocking buffer), whereas p21 was detected using anti-p21 (Santa Cruz Biotech; 1:200 dilution in blocking buffer) and assayed using an Alexa-546-labelled mouse antibody. Presence of the nuclei was detected by DAPI stain. Cells were examined under a fluorescence microscope and pictures were taken using a MagnaFire digital camera (Optronics, Goleta, CA) for analysis.

TABLE III – EFFECT OF CORN OIL AND FISH OIL DIETS ON BREAST CANCER GROWTH IN NUDE MICE

Diet	Tumor volume (mm^3)	Surface area (mm^2)	Tumor weight (mg)
Corn oil	307.7 ± 7.0	76.9 ± 3.2	180 ± 10
Balanced corn/fish oil fed	114.5 ± 17.3*	49.8 ± 7.4*	130 ± 20*
Fish oil	75.5 ± 6.9*	38.9 ± 3.3*	90 ± 10*

Results are the mean ± SEM for 3 experiments. Results were analyzed by Student's *t*-test and 1-way ANOVA. Significant differences compared to the corn oil group are reported (*$p < 0.05$).

Cell death ELISA

Quantitative analysis of DNA fragmentation was carried out using a histone-based Death ELISA system (Roche) per the manufacturer's protocol. MDA-MB-231 cells (1×10^6/ml) in 6-well plates were incubated with fatty acids for 24 hr and then lysed. The nucleosomes containing fragmented DNA were captured by an immobilized antihistone antibody. The amounts of DNA fragments were then determined spectrophotometrically using a peroxidase-conjugated anti-DNA antibody.

Annexin V staining

Externalization of phosphatidylserine was evaluated using an annexin V staining kit (Roche Biochemicals) per the manufacturer's instructions. This kit can distinguish apoptotic and necrotic (dead) cells by propidium iodide (red) staining and FITC-conjugated annexin V (green) staining, respectively. The cells were visualized using a fluorescence microscope. The total and apoptotic cells were counted, and the percentage of cells exhibiting apoptosis was calculated.

Statistics

All experiments were performed at least 3 times each in triplicate and expressed as mean ± SE. Comparisons were done using a Student's *t*-test and 1-way ANOVA. Significance was defined as $p < 0.05$.

Results

*Inhibition of breast cancer tumors by dietary fish oil (*in vivo *studies)*

The effect of dietary fish oil on breast cancer growth was investigated in nude mice. Results demonstrate that mice fed a corn oil diet rich in omega-6 fatty acids exhibited substantial tumor growth (Fig. 1*a*). In contrast, mice fed on the omega-6/omega-3-balanced diet exhibited reduced cancer growth (Fig. 1*b*). Tumor growth in mice fed the omega-3-rich fish-oil diet had by far the smallest tumors (Fig. 1*c*). Because the tumors were asymmetric, quantification was achieved by measuring tumor volume, surface area and mass. Data shown in Table III indicate that at 3 weeks after tumor implantation, tumor volume was approximately 60% lower ($p < 0.05$), whereas tumor surface area and weight were approximately 30–40% lower ($p < 0.05$) in mice maintained on the balanced diet (omega-3/omega-6, 1:1) compared to mice on the corn oil diet (omega-6 enriched). The largest reduction in tumor volume was noted for the fish-oil diet (omega-3 enriched), where tumor volume was approximately 75% lower ($p < 0.05$), and tumor surface area and weight were approximately 50% lower ($p < 0.05$) compared to animals maintained on corn oil diets.

Tumor tissue isolated from mice maintained on each of the 3 diets was then analyzed for N-SMYase activity. Data in Figure 2*a* show that N-SMYase activity was higher by approximately 40% ($p < 0.05$) in the tissues from mice raised on the omega-3-containing diets (fish oil and balanced fish/corn oil) compared to those on the high omega-6 corn oil diet.

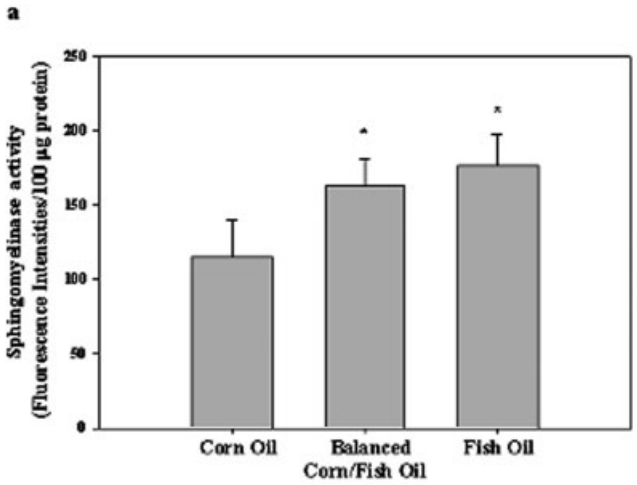

FIGURE 2 – Fish oil enhances N-SMYase activity and p21 expression in tumor tissues. Mice implanted with breast cancer cells as described in Figure 1 were sacrificed and their tumor tissues were isolated. *(a)* N-SMYase activity was assayed as described in Material and Methods. Results are the mean ± SEM for 3 experiments. Results were analyzed by Student's *t*-test and 1-way ANOVA. Significant differences compared to the corn oil group are reported ($*p < 0.05$). *(b)* For p21 expression, RNA from tumor tissues were extracted and RT-PCR was performed to determine the mRNA levels of p21 and GAPDH (loading control) using the Titan One Tube RT-PCR System as described in Material and Methods. Negative control (-C) was muscle tissue from a normal mouse; positive control (+C) was RNA isolated from HCT101 $p21^+$ cells. Results are shown for 3 tumor tissues for each group.

Tumor tissues were further analyzed for p21 expression using semiquantitative RT-PCR. Results presented in Figure 2*b* demonstrate that expression of p21 mRNA was upregulated by fish oil. mRNA expression of p21 in mice maintained on the fish oil diet was induced, whereas p21 was not detected in tumor tissues of corn-oil-diet-fed mice.

*Effects of omega-3 fatty acids on breast cancer cells in culture (*in vitro *studies)*

We next evaluated whether the long-chain polyunsaturated omega-3 fatty acids commonly found in fish oils (DHA and EPA) can affect MDA-MB-231 breast cancer cells *in vitro*. The dietary fish oil (Menhaden, Sigma Chemical) employed in these studies was first analyzed for its fatty acid content by gas chromatography. The fish oil was shown to have 150 mg of DHA and 160 mg of EPA per g of oil (Table II). Both DHA and EPA were then tested on cultured MDA cells, where both fatty acids similarly inhibited growth in a dose-dependent manner (Fig. 3). At concentrations as low as 25 µM, both fatty acids inhibited cell growth by approximately 25–30% ($p < 0.05$), and the inhibitory effects of

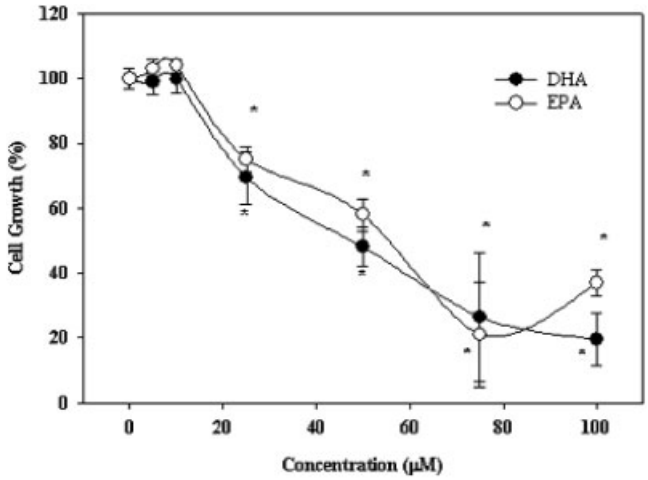

FIGURE 3 – Dose-dependent effect of omega-3 fatty acids on breast cancer cell growth. Cells (1×10^4 per well) were seeded in a 96-well plate overnight and then treated with varying concentrations of DHA or EPA in serum-free medium for 24 hr. Cell growth was assayed using a WST-1 assay as described in Material and Methods. Results are the mean ± SEM for 3 experiments. Results were analyzed by Student's *t*-test and 1-way ANOVA. Significant differences compared to the corn oil-fed group are reported ($*p < 0.05$).

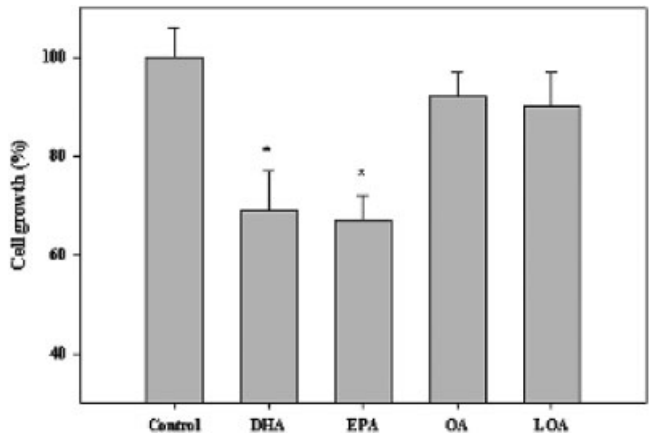

FIGURE 4 – Effect of different fatty acids on cell growth. MDA-MB-231 cells were treated with 25 µM concentrations of DHA, EPA, oleic acid (OA) or linoleic acid (LOA) and cell growth was assayed as described in Figure 4. Results are the mean ± SEM for 3 experiments. Results were analyzed by Student's *t*-test and 1-way ANOVA. Significant differences compared to the control are reported ($*p < 0.05$).

DHA and EPA progressively increased with increasing concentration to a maximum inhibition at 80 µM by approximately 80% ($p < 0.05$) after 24 hr of incubation.

We further tested whether other long-chain unsaturated fatty acids had effects similar to DHA and EPA on breast cancer growth. Results shown in Figure 4 demonstrate that at a concentration where DHA and EPA significantly inhibited MDA growth (25 µM), oleic acid, an omega-9 fatty acid, and linoleic acid, an omega-6 fatty acid, had only a minimal effect on growth. However, a concentration > 100 µM of OA or LA resulted in a similar effect as of DHA or EPA at 25 µM. Therefore, at lower concentrations, inhibition of cancer cell growth was not a general consequence of any long-chain fatty acid but rather is unique to the long-chain polyunsaturated omega-3 fatty acids, whereas at higher concentrations all fatty acids are generally cytotoxic (detergent effect).

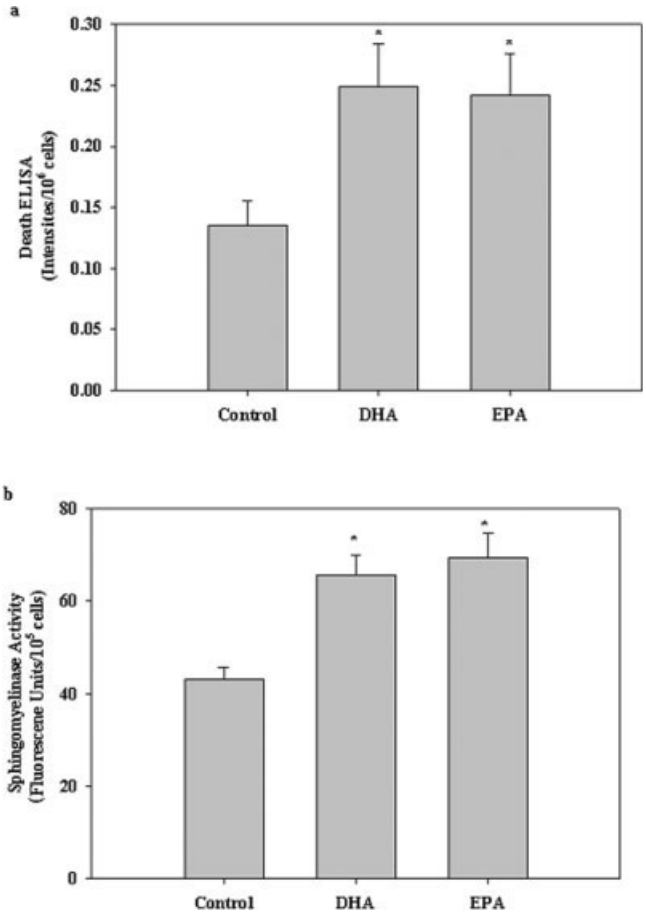

FIGURE 5 – Effects of omega-3 fatty acids on apoptosis and N-SMYase activity in breast cancer cells. MDA-MB-231 cells (1×10^6/ml) were grown in 6-well plates and treated with 25 μM DHA or EPA as described in Figure 4. Apoptosis *(a)* was assayed using a cell death ELISA kit, whereas N-SMYase activity *(b)* was assayed using an Amplex Red kit described in Material and Methods. Results are the mean ± SEM for 3 experiments. Results were analyzed by Student's *t*-test and 1-way ANOVA. Significant differences compared to the control are reported (*$p < 0.05$).

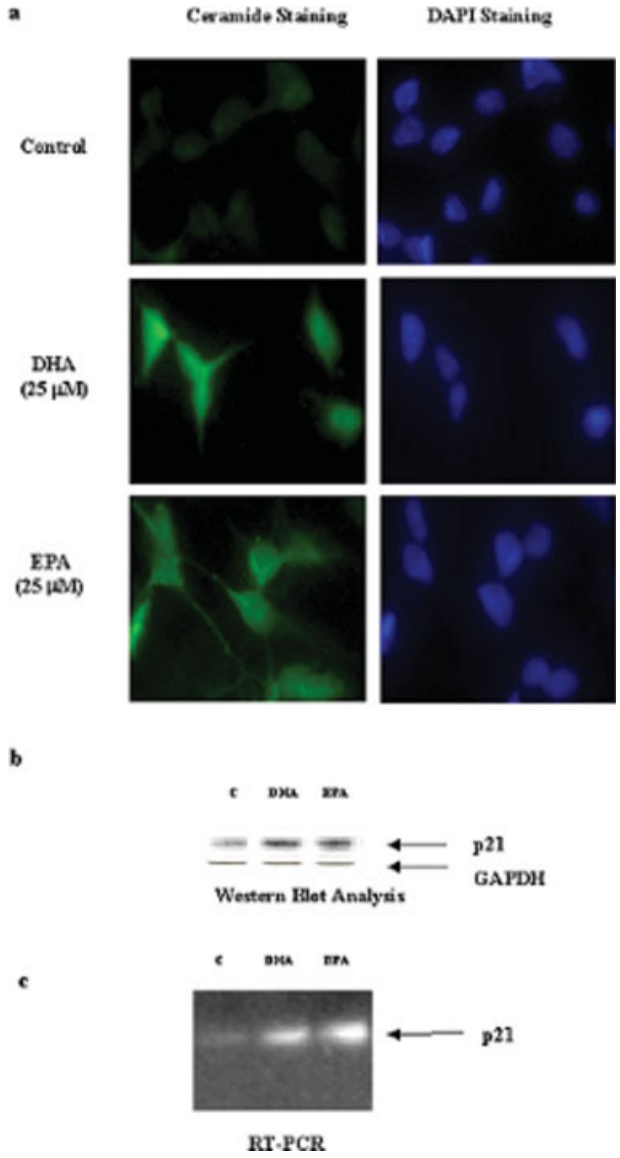

FIGURE 6 – Omega-3 fatty acids induce ceramide formation and p21 expression in breast cancer cells. MDA-MB-231 cells (1×10^4/ml grown in 4-well chamber slides or 1×10^6/ml grown in T75 flasks) were treated with 25 μM DHA or EPA as described in Figure 4. Generation of ceramide *(a)* was determined immunohistochemically using an anticeramide antibody (green fluorescence) as described in Material and Methods. Blue DAPI staining was used to visualize nuclei. Expression of p21 protein was analyzed by Western analysis *(b)* using GAPDH as a loading control as described in Material and Methods, whereas expression of p21 mRNA was analyzed by RT-PCR *(c)* as described in Figure 3. Results are representative of 3 experiments in each section.

We further examined whether growth inhibition of MDA cells was due to induction of apoptosis. Results presented in Figure 5*a* demonstrate that both DHA and EPA at 25 μM induce apoptosis by 70–75% ($p < 0.05$) as is evident from enhanced DNA fragmentation. Furthermore, N-SMYase activity was increased by 30–40% ($p < 0.05$) in cells treated with DHA or EPA compared to those of untreated (control) cells (Fig. 5*b*). The increase in N-SMYase activity induced by the omega-3 fatty acids was further analyzed by assaying ceramide formation, the product of sphingomyelin hydrolysis. The noticeable generation of ceramide was observed in MDA cells upon DHA or EPA treatment (Fig. 6*a*) compared to that of control cells. We also analyzed the effect of DHA and EPA on p21 expression by both RT-PCR and by Western analysis. Results presented in Figure 6*b* indicate that expression of p21 protein was increased approximately 2.5–3-fold ($p < 0.05$) in DHA- or EPA-treated cells compared to control cells. Similarly, DHA and EPA also increased expression of p21 mRNA in the same cells (Fig. 6*c*).

Effect of omega-3 fatty acids on membrane structure

One of the noticeable effects of DHA on MDA cells was the induction of plasma membrane blebs. Results shown in Figure 7*a* demonstrate substantial changes in appearance (bleb formation) of the MDA cell surface caused by increasing concentrations (0–100 μM) of DHA. The MDA cells were then stained with annexin V for the presence of externalized phosphatidylserine. Figure 7*b* shows that cells incubated in 50 μM DHA demonstrated extensive bleb formation, and these blebs appear to have aggregated phosphatidylserine on the surface (the membrane blebs and annexin V are colocalized). Similar effects on membrane bleb formation were also observed upon treatment with EPA (data not shown). In addition, bleb formation appears to be related to the DHA-induced N-SMYase activity reported in Figure 5*b*. Results presented in

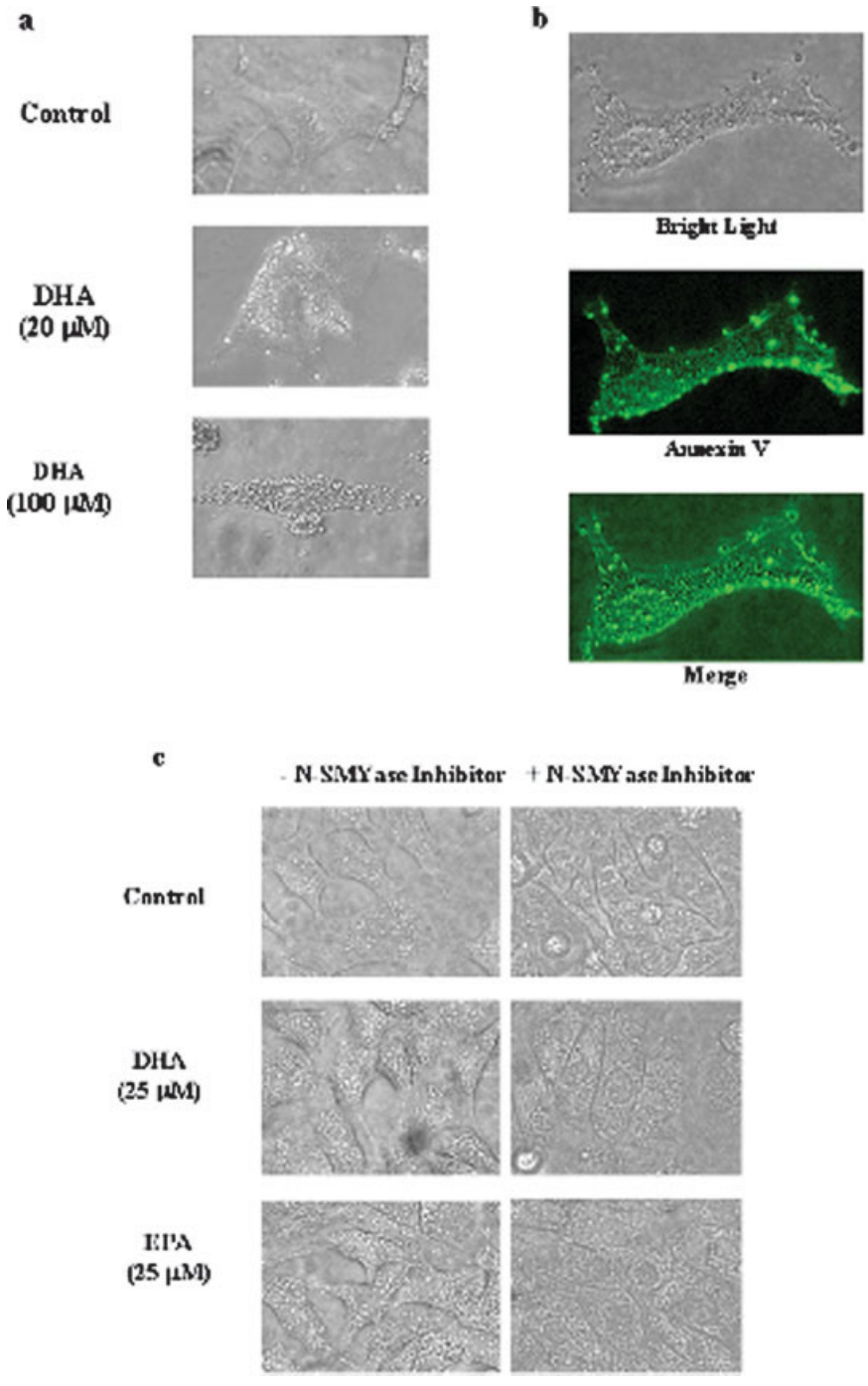

FIGURE 7 – Effect of omega-3 fatty acids on membrane bleb formation. MDA-MB-231 cells (1×10^4/ml) were grown in 4-well chamber slides and treated with varying concentrations of DHA as described in Figure 2. Cells were observed under a microscope using 400× magnification *(a)*. The blebs were assayed for externalized phosphatidylserine in cells treated with 50 μM DHA by using an annexin V binding kit as described in Material and Methods *(b)*. Inhibition of omega-3 fatty acid-induced membrane bleb formation was observed in the presence or absence of 20 μM GW4869, a N-SMYase inhibitor *(c)*. Cells were observed under the microscope using 20× magnification. Results are representative of 3 experiments in each section.

Figure 7*c* indicate that the DHA-induced membrane bleb formation was inhibited by the N-SMYase inhibitor GW4869.

Inhibition of omega-3 fatty acid-induced p21 expression and apoptosis by N-SMYase inhibitor

The involvement of N-SMYase in DHA- or EPA-induced p21 expression was further investigated using immunohistochemistry. Figure 8*a* indicates that expression of p21 protein was induced in DHA- and EPA-treated cells as is evident by enhanced fluorescence intensities. This DHA- or EPA-induced expression of p21 was markedly diminished by approximately 50–60% in the presence of N-SMYase inhibitor.

Involvement of N-SMYase in DHA- or EPA-induced apoptosis was also investigated. Data depicted in Figure 8*b* indicate that in the presence of the N-SMYase inhibitor, DHA- and EPA-induced apoptosis in MDA cells was inhibited by approximately 40–50% ($p < 0.05$).

Discussion

Breast cancer is one of the most frequently diagnosed nonskin cancers and the second most common cause of cancer death in women.[42] An estimated 215,990 new cases of breast cancer were expected in 2004. Epidemiologic evidence links fish oil consump-

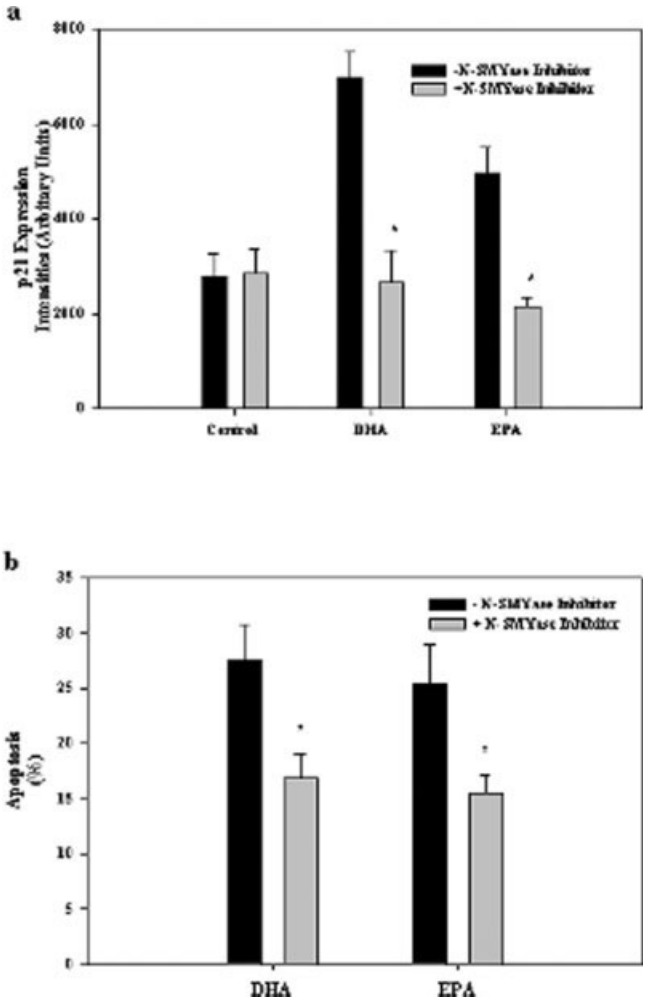

FIGURE 8 – Inhibition of p21 expression and apoptosis by the N-SMYase inhibitor. *(a)* MDA-MB-231 cells (1×10^4/ml) were grown in 4-well chamber slides and then treated with 25 μM DHA or EPA for 24 hr in the presence or absence of 20 μM GW4869. Cells were then fixed and expression of p21 was detected using immunohistochemical methods as described in Material and Methods. Expression of p21 was quantified by densitometric analysis using a KODAK Image Station 2000MM (Eastman Kodak Company, Rochester, NY). *(b)* MDA-MB-231 cells in 6-well plates were incubated for 24 hr with 25 μM DHA or EPA in the presence or absence of 20 μM GW4869. Plates were centrifuged at 800*g* in a Beckman J series centrifuge to deposit floating cells at the bottom for analysis, and the supernatant was carefully removed. Quantitative analysis of apoptosis was performed by using an annexin V staining kit as described in Material and Methods. The dead and necrotic cells exhibit red fluorescence, whereas apoptotic cells fluoresce green. The total and apoptotic cells were counted and the percentage of cells exhibiting apoptosis was calculated. Results are the mean ± SEM for 3 experiments. Results were analyzed by Student's *t*-test and 1-way ANOVA. Significant differences between groups are reported (*$p < 0.05$).

tion (rich in the omega-3 fatty acids DHA and EPA) with a low incidence of several types of cancer.[1–4] The anticancer role of omega-3 fatty acids has also been substantiated with dietary studies using many types of animals (including humans) and numerous different cell lines, including breast cancer.[8,10,19,20] In the study reported here, we investigated the effects of long-chain polyunsaturated omega-3 fatty acids on breast cancer cells both *in vivo* and *in vitro*.

Our *in vivo* studies, shown in Figures 1 and 2 and Table III, demonstrate that increasing the ratio of omega-3 to omega-6 fatty acids in the diet inhibits development of transplanted breast cancer cells in nude mice. Similar animal models to study the growth of transplanted breast cancer cells have been widely described.[5] The oils used for preparing the diets were examined for lipid peroxidation products as previously described[24] and tested for apoptosis on breast cancer cells. As previously reported for Jurkat cells, our data indicate that levels of lipid peroxidation products in oils did not correlate with the extent of apoptosis. It therefore appears that the oils but not the oxidized products are responsible for the cytotoxic effects in breast cancer cells. The purpose of our study was to link the omega-3 fatty acids abundant in dietary fish oil (DHA and EPA) to inhibition of breast cancer cell proliferation *in vivo* and to investigate one possible mode of action, namely the effect on N-SMYase.

Our studies were then extended to cultured MDA-MB-231 cells, in which we initially investigated whether the constituents of fish oil—DHA and EPA—could inhibit breast cancer proliferation *in vitro*. Data presented in Figure 3 indicate that both DHA and EPA were equally effective in inhibiting MDA-MB-231 cell growth. Similar inhibition was not observed with either oleic acid, an omega-9 fatty acid that is the most abundant fatty acid in animal tissues, or linoleic acid, an omega-6 fatty acid abundant in common vegetable oils (Fig. 4), indicating that inhibition is not just a general property of all long-chain fatty acids. The data presented in Figure 5*a* indicate that inhibition of cancer cell growth was likely due to induction of apoptosis by the omega-3 fatty acids.

We further evaluated a possible signaling pathway that may be responsible for regulating tumor growth by omega-3 fatty acids. Our previous studies suggest that DHA inhibits the growth of Jurkat leukemic cells partially through activation of N-SMYase, increased ceramide formation and enhanced expression of p21.[23] To link the breast cancer studies to our previous work with Jurkat leukemic cells, we monitored the activity of N-SMYase and p21 expression both *in vivo* (nude mice) and *in vitro* (cultured MDA-MB-231 cells). Results shown in Figure 2 demonstrate that tumors from mice fed a corn oil (omega-6)-based diet have considerably lower N-SMYase activity than mice fed on diets containing significantly higher omega-3 fatty acid levels. Similarly, treatment of cultured MDA-MB-231 cells with DHA or EPA enhanced N-SMYase activity (Fig. 5*b*), resulting in increased ceramide formation (Fig. 6*a*) and enhanced p21 expression as analyzed by Western blot and immunohistochemical analysis (Fig. 6*b*) and confirmed by RT-PCR (Fig. 6*c*). RT-PCR in our present study was performed as an extra measure to confirm increased expression of p21 in the presence of DHA or EPA. Although products generated after 30 cycles do not indicate a quantitative measure of p21 mRNA expression in these cells, they clearly demonstrate differences between control and treated cells.

Ceramide-induced activation of cellular N-SMYase-mediated signaling pathways in response to stress has been previously reported by Hannun *et al.*[43] and Kolesnick *et al.*[44] The N-SMYase pathway is known to be activated by various factors including heat, ischemia/reperfusion, oxidants, tumor necrosis factor-alpha (TNFα), Fas ligand, vitamin D3, IL-1 and α- interferon and initiates cellular events leading to cell death or apoptosis.[45,46] Hydrolysis of SM by N-SMYase generates ceramide, a lipid that is regarded as a "universal component of apoptosis."[47–49] About 70% of cellular SM is present in the outer leaflet of plasma membranes, primarily in lipid rafts, where it probably serves to stabilize rafts by interacting with cholesterol and phospholipids containing saturated fatty acids.[50,51] Several studies suggest that N-SMYase is generally localized in plasma membranes.[32,52] Activation of cells by TNF has been shown to induce translocation of N-SMYase from detergent-resistant membrane fractions (lipid rafts) to detergent-soluble (nonraft) fractions, resulting in enhanced activity.[34] In a preliminary study (data not shown), we have also demonstrated that DHA treatment decreases N-SMYase activity in the detergent-resistant (raft) fractions, whereas it increases activity in the detergent-soluble (nonraft) fractions. These results suggest that omega-3 fatty acids induce changes in

the plasma membrane composition and structure of MDA-MD-231 cells, affecting N-SMYase activity by either translocating the enzyme from detergent-resistant to detergent-soluble fractions or by directly affecting N-SMYase activity (conformational change) in these fractions. Although interesting, these results need to be verified using specific antibodies against N-SMYase, which are under development in our laboratory.

We have previously demonstrated that DHA causes substantial changes in the domain structure of model membranes and isolated plasma membranes[35] and that incorporation of DHA instigates cellular changes leading to apoptosis.[23] The experiments reported here further suggest that the addition of DHA causes visible changes in the plasma membrane as indicated by the appearance of membrane blebs with externally exposed PS (annexin V binding) (Fig. 7*a,b*). Furthermore, DHA-induced membrane bleb formation and p21 expression was inhibited by the N-SMYase inhibitor GW4869, indicating that ceramide generation is involved in this process. In fact, blebbing and externalization of PS are hallmarks of the execution phase of apoptosis[53] and are believed to be related to ceramide generation. In agreement with these findings, we observed that the N-SMYase inhibitor also reduced DHA- and EPA-induced apoptosis (Figure 8).

In conclusion, the data presented here strongly indicate a relationship between the omega-3 fatty acids DHA and EPA, tumor growth suppression, membrane structure, N-SMYase activity, ceramide formation, p21 expression and apoptosis. Our results suggest that modulation of the N-SMYase-ceramide pathway represents a potential pathway for treatment of breast cancer.

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