The Journal of Nutrition
Biochemical, Molecular, and Genetic Mechanisms

(n-3) PUFA Alter Raft Lipid Composition and Decrease Epidermal Growth Factor Receptor Levels in Lipid Rafts of Human Breast Cancer Cells[1,2]

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Abstract

To determine the mechanism by which the (n-3) fatty acids (FA) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) decrease proliferation and induce apoptosis in MDA-MB-231 human breast cancer cells, we examined the effects of EPA and DHA on the lipid composition of lipid rafts as well as epidermal growth factor receptor (EGFR) raft localization and phosphorylation. (n-3) FA (a combination of EPA and DHA) inhibited ($P < 0.05$) the growth of MDA-MB-231 cells by 48–62% in the presence and absence, respectively, of linoleic acid (LA). More EPA and DHA were incorporated into lipid rafts isolated from MDA-MB-231 cells after treatment with (n-3) FA compared with cells treated with LA ($P < 0.05$). EPA and DHA treatment decreased ($P < 0.05$) lipid raft sphingomyelin, cholesterol, and diacylglycerol content and, in the absence of LA, EPA and DHA increased ($P < 0.05$) raft ceramide levels. Furthermore, there was a marked decrease in EGFR levels in lipid rafts, accompanied by increases in the phosphorylation of both EGFR and p38 mitogen-activated protein kinase (MAPK), in EPA+DHA-treated cells ($P < 0.05$). As sustained activation of the EGFR and p38 MAPK has been associated with apoptosis in human breast cancer cells, our results indicate that (n-3) FA modify the lipid composition of membrane rafts and alter EGFR signaling in a way that decreases the growth of breast tumors. J. Nutr. 137: 548–553, 2007.

Introduction

The role of dietary fat in breast cancer development is not well understood and remains controversial. The (n-3) fatty acids (FA)[5] from fish oils have been purported to reduce breast cancer risk and some epidemiological studies have suggested an inverse relation between breast cancer risk and fish or fish FA consumption (1). However, prospective cohort studies that examined the effects of (n-3) FA on breast cancer incidence yielded mixed results. The majority of these studies did not show a significant association between (n-3) FA consumption and breast cancer risk [reviewed in (2)], suggesting a limited role for (n-3) FA in breast cancer prevention when consumed at modest levels during adulthood.

Studies of the effects of (n-3) FA on breast cancer progression are limited in humans. However, a large body of research involving animal and cell culture models supports a role for the long-chain (n-3) PUFA eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in the inhibition of tumor growth promotion. When provided in the diet through fish oils, these FA inhibit the growth and metastasis of chemically induced and transplantable mammary tumors [reviewed in (1)]. DHA and EPA together and alone also inhibit the growth of breast cancer cell lines in vitro (3–5). The mechanism for how (n-3) PUFA inhibit breast cancer cell growth is not well understood. (n-3) PUFA are incorporated into membrane phospholipids of tumor cells when provided in the diet (6,7), but the biological impact of this has not been established. These FA may exert their growth-inhibitory effects on cancer cells by altering the plasma membrane composition and/or associated signaling events.

Recent findings in membrane biology suggest that the plasma membrane is composed of microdomains of saturated lipids that segregate together to form lipid "rafts." Lipid rafts have been operationally defined as cholesterol- and sphingolipid-enriched membrane microdomains resistant to solubilization by nonionic

[1] Supported by grants from the Natural Sciences and Engineering Research Council of Canada (C.J.F.), the Canadian Breast Cancer Foundation (C.J.F.), and the Canadian Institutes of Health Research (D.N.B.). P.D.S. is a recipient of scholarships from the Natural Sciences and Engineering Research Council of Canada, the Alberta Heritage Foundation for Medical Research, the University of Alberta Dissertation Fellowship, and the Andrew Stewart Memorial Graduate Prize. D.N.B. holds a Medical Scientist award from the Alberta Heritage Foundation for Medical Research.

[2] Supplemental Figure 1 is available with the online posting of this paper at jn.nutrition.org.

[5] Abbreviations used: DAG, diacylglycerol; DHA, docosahexaenoic acid; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; EPA, eicosapentaenoic acid; EPA60 + DHA40, 60 μmol/L EPA + 40 μmol/L DHA; EPA45 + DHA30 + LA75, 45 μmol/L EPA + 30 μmol/L DHA + 75 μmol/L LA; FA, fatty acid; LA, linoleic acid; LA75, 75 μmol/L LA; LA150, 150 μmol/L LA; MAPK, mitogen-activated protein kinase; MUFA, monounsaturated fatty acids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin.

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Manuscript received 15 October 2006. Initial review completed 2 November 2006. Revision accepted 6 December 2006.

detergents (traditionally Triton X-100) at low temperatures (8). Rafts are enriched in glycosylphosphatidylinositol-linked proteins (9) and also contain several signaling proteins, including the epidermal growth factor receptor (EGFR) (10). Such findings suggest that rafts play a role in cell signal transduction, perhaps by facilitating the association of signaling molecules (8). A subset of specialized rafts termed caveolae have also been described, which are flask-shaped invaginations in the plasma membrane that are enriched in the integral membrane protein, caveolin (8). Despite a large body of research supporting the lipid raft hypothesis, proof of the existence of rafts to date is based largely on biochemical and biophysical evidence. The inability to visualize rafts directly in cell membranes, as well as a lack of understanding of some basic properties (e.g. size, half-life), has led to controversy over their definition and very existence (9).

Recent research implicates a role for lipid rafts in the regulation of EGFR activation. Caveolae/rafts appear to function as negative regulators of EGFR tyrosine phosphorylation (11,12), with ligand binding and receptor activation leading to a migration of the EGFR out of caveolae/rafts (13,14). Although signaling through the EGFR is primarily thought to promote cell proliferation and survival, recent reports also implicate EGFR activation in the induction of apoptosis (15,16), including evidence from MDA-MB-231 human breast cancer cells (17).

There have been few studies examining the effects of (n-3) PUFA on the lipid and protein composition of membrane rafts. To our knowledge, the impact of (n-3) PUFA on lipid rafts in tumor cells has not been examined. Here, we report the effects of (n-3) PUFA on the lipid raft composition of MDA-MB-231 human breast cancer cells.

Materials and Methods

Cell culture. The culture of MDA-MB-231 cells and the preparation of FA-supplemented culture medium have been described previously (5).

Growth experiments. Preliminary experiments were conducted to determine an optimal concentration of linoleic acid (LA) to stimulate growth [75 μmol/L (LA75)] and appropriate concentrations of EPA and DHA to inhibit cell growth in the absence of LA [60 μmol/L EPA + 40 μmol/L DHA (EPA60 + DHA40)] and presence of LA [45 μmol/L EPA + 30 μmol/L DHA + 75 μmol/L LA (EPA45 + DHA30 + LA75)]. As we have described previously (5), the addition of LA (at 75 μmol/L or 50 μmol/L) to EPA60 + DHA40 was toxic to the cells, thus EPA and DHA at 45 μmol/L and 30 μmol/L, respectively, were used in combination with LA75. An additional control LA treatment [150 μmol/L (LA150)] was added to each experiment to ensure that the total FA concentration used in the EPA45 + DHA30 + LA75 treatment was not cytotoxic.

For lipid raft isolation, cells were seeded at 3×10^6 cells per flask (150 cm^2) in medium containing 40 mL/L fetal calf serum. After 48 h, the culture medium was replaced with fresh medium containing the experimental FA and 40 mL/L fetal calf serum and the cells were incubated for 72 h, during which time the medium was not changed. After 72 h, cells were harvested using trypsin-EDTA (Gibco Invitrogen) and viable cells were counted under a microscope after trypan blue staining (Sigma-Aldrich).

Separate growth experiments were conducted in 75-cm^2 flasks [per Schley et al. (5)] to obtain cells for whole cell FA composition, ceramide and diacylglycerol (DAG) concentrations, phosphorylated EGFR determination, and immunoblotting of p38 mitogen-activated protein kinase (MAPK). For analysis of EGFR and p38 MAPK phosphorylation, FA-treated cells were stimulated with 50 μg/L epidermal growth factor (EGF) (Sigma-Aldrich) for 15 min at 37°C prior to harvesting.

Lipid raft isolation. We developed a microscale method for the isolation of lipid rafts from mammary tumor cells, adapted from Brown and Rose (18) and Li et al. (19). Cells were lysed in 500 μL of ice-cold TNE (25 mmol/L Tris-Cl, pH 7.5, 150 mmol/L NaCl, 5 mmol/L EDTA) containing 10 mL/L Triton X-100 (VWR) and fresh protease and phosphatase inhibitors (Sigma-Aldrich). Cell lysates were passed through a 26½ G needle 10 times and incubated on ice for 30 min. Lysates were centrifuged at 200 × *g*; 2 min at 4°C and the supernatants (500 μL) were transferred to cooled, 5-mL ultracentrifuge tubes (Beckman Coulter). Lysates were mixed with 500 μL of ice-cold 80% w/v sucrose (800 g/L in TNE) and then overlaid with 2 mL of 30% w/v sucrose (300 g/L in TNE) and 2 mL of 5% w/v sucrose (50 g/L in TNE). Tubes were centrifuged at 268,000 × *g*; 8 h at 4°C in an Optima Max Ultracentrifuge (Beckman Coulter), MLS-50 rotor. To confirm the location of lipid rafts on the gradients, 6 fractions (800 μL each) were collected sequentially from the top of the gradient (fraction 1) to the bottom (fraction 6) and aliquots (10 μL) of each fraction were dot-blotted directly onto nitrocellulose membranes. Membranes were probed for the presence of the raft markers $G_{\alpha i}$ (using anti-$G_{\alpha i}$ antibody, Oncogene Research Products, CedarLane Laboratories) and GM1-ganglioside (using horseradish peroxidase–conjugated cholera toxin B subunit, Sigma-Aldrich) as well as the transferrin receptor (Zymed Laboratories, Inter Medico), a negative marker of lipid rafts. Both $G_{\alpha i}$ and GM1-ganglioside stained most intensely in fraction 3, which included the 5/30% interface where flocculent material could be seen (**Supplemental Fig. 1**). The transferrin receptor stained most intensely in fractions 4 and 5. This same pattern was observed with all FA treatments (results not shown). Based on these results, the visible, flocculent material at the 5/30% interface encompassing fraction 3 (~1 mL) was collected in subsequent experiments and was referred to as the lipid raft fraction. Raft fractions were diluted to 5 mL with ice-cold TNE and centrifuged at 268,000 × *g* for 30 min to pellet and concentrate the rafts.

FA composition analysis. Lipids from whole cells or pelleted rafts were extracted using a modified Folch procedure (20). The total phospholipid fraction was isolated from whole cells, as previously described (21). FA methyl esters were prepared from raft lipids or whole cell total phospholipids (20) and separated by automated GLC (Varian CP-3800) on an SGE BP20 column (60-m × 0.25-mm i.d., Varian) (20).

Phosphate assay. Lipids from pelleted rafts were extracted as above and individual phospholipid classes were separated by TLC (20). Phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), and sphingomyelin (SM) bands were scraped into phosphate-free test tubes. Phosphate was measured by a modified Bartlett assay for microscale lipid phosphate analysis (22). Absorbance was measured at 790 nm with a Molecular Devices SpectraMax PLUS 384 spectrophotometer. The phosphate content of each phospholipid was determined by reference to phosphate standards (1.5 mmol/L KH_2PO_4) and expressed as a percentage of the total phospholipid phosphorus.

Raft cholesterol quantitation. Lipids from pelleted rafts were extracted as above, dried under nitrogen, and resuspended in a 5-mL/L Triton X-100 solution in water. Cholesterol was assayed by an Amplex Red Cholesterol Assay kit (Molecular Probes, Invitrogen) according to the manufacturer's instructions. The same samples were also assayed in duplicate for total lipid phosphorus to normalize raft cholesterol to raft lipid phosphorus levels.

Ceramide and DAG quantitation. Lipids were extracted from rafts or whole cells, and ceramide and DAG concentrations were quantified by a DAG kinase assay (23). The same samples were also assayed in duplicate for total lipid phosphorus to normalize ceramide and DAG levels to lipid phosphorus levels.

Western blotting. Protein extracts were prepared from whole cells or pelleted rafts, as previously described (5). Extracts were assayed for protein concentration by bicinchoninic acid assay (Sigma-Aldrich) and western blotting was conducted as per Schley et al. (5). Primary antibodies to EGFR, p38 MAPK, and phospho-p38 MAPK were obtained from Cell Signaling Technology (CedarLane Laboratories). Horseradish peroxidase–conjugated anti-rabbit secondary antibody was from BD

Transduction Laboratories. Membranes were developed using an enhanced chemiluminescence detection kit and Hyperfilm enhanced chemiluminescence film from Amersham Biosciences.

EGFR phosphorylation. Phosphorylated EGFR was quantified using an Active EGF Receptor ELISA kit (Calbiochem, CedarLane Laboratories) according to the manufacturer's instructions. Samples were assayed for protein concentration by bicinchoninic acid assay and equal amounts of protein from each sample were assayed for phosphorylated EGFR.

Statistical analysis. All statistical analyses were conducted using the SAS statistical package, version 8 (SAS Institute). Results were analyzed by 1-way ANOVA, blocked by experiment/passage number, followed by a Tukey's test to identify significant ($P < 0.05$) differences between FA treatments. The phosphorylated EGFR data were not normally distributed and were log-transformed prior to statistical analysis.

Results

EPA and DHA decrease the growth of human breast cancer cells. Treatment of MDA-MB-231 cells with EPA and DHA in the absence of LA (i.e. EPA60 + DHA40) decreased ($P < 0.05$) the number of viable cells by 62% compared with the control treatment, LA75 (**Fig. 1**). Incubation of cells with (n-3) PUFA in the presence of LA (i.e. EPA45 + DHA30 + LA75) decreased ($P < 0.05$) the number of cells per flask by 48% compared with incubation with LA75 alone. Although untreated cells were not included in this study, we have shown previously that the growth of untreated cells does not differ from that of LA-treated cells in this system (*5*).

EPA and DHA alter the FA composition of lipid rafts. Treatment with EPA60 + DHA40 increased ($P < 0.05$) the concentrations of EPA, DHA, docosapentaenoic acid [22:5(n-3)], and total (n-3) PUFA in lipid rafts as well as in whole cell membrane phospholipids in MDA-MB-231 cells (**Table 1**) compared with treatment with LA75 or LA150. Treatment with EPA45 + DHA30 + LA75 increased ($P < 0.05$) all of these lipids in both rafts and whole cell phospholipids relative to treatment with LA75 or LA150. Enrichment of EPA, docosapentaenoic acid, DHA, and total (n-3) PUFA was lower ($P < 0.05$) in both rafts and whole cell phospholipids from cells incubated with EPA45 + DHA30 + LA75 rather than EPA60 + DHA40. The unsaturation index in rafts was increased ($P < 0.05$) by EPA60 + DHA40 treatment, but not EPA45 + DHA30 + LA75 treatment, relative to LA75 treatment. In general, lipid rafts had a lower concentration of both (n-3) and (n-6) PUFA and a lower unsaturation index, as well as a higher concentration of saturated FA, than whole cell phospholipids.

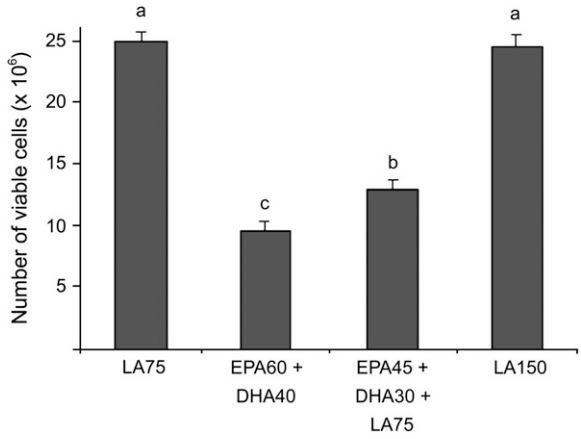

Figure 1 (n-3) PUFA reduce the growth of MDA-MB-231 human breast cancer cells. Bars represent means ± SEM, $n = 20$. Means without a common letter differ, $P < 0.05$.

EPA and DHA alter the phospholipid composition of lipid rafts. A mixture of EPA and DHA (EPA60 + DHA40) reduced ($P < 0.05$) the amount of raft SM (as a % of the total phospholipids) and increased ($P < 0.05$) the amount of raft PC compared with LA75, LA150, and EPA45 + DHA30 + LA75 (**Table 2**). Treatment with EPA45 + DHA30 + LA75 reduced ($P < 0.05$) raft SM content compared with LA150, but not compared with LA75 ($P < 0.05$), and did not affect raft PC levels compared with these 2 treatments.

EPA and DHA decrease raft cholesterol levels. Raft cholesterol levels were reduced ($P < 0.05$) by both (n-3) PUFA treatments compared with treatment with LA75 (Table 2). Cells

TABLE 1 Raft and whole cell phospholipid FA composition of PUFA-treated MDA-MB-231 cells[1,2]

	Lipid rafts				Whole cell phospholipids			
	LA75	EPA60 + DHA40	EPA45 + DHA30 + LA75	LA150	LA75	EPA60 + DHA40	EPA45 + DHA30 + LA75	LA150
FA	*g/100 g total FA*							
16:0	35.6 ± 1.9^{b}	43.4 ± 1.3^{a}	32.7 ± 1.8^{b}	33.0 ± 1.8^{b}	14.8 ± 0.2^{b}	24.0 ± 0.3^{a}	15.4 ± 0.9^{b}	13.7 ± 0.4^{b}
18:2(n-6)	16.0 ± 0.7^{b}	0.8 ± 0.1^{c}	17.8 ± 2.2^{ab}	22.0 ± 0.3^{a}	33.1 ± 0.8^{b}	1.7 ± 0.1^{c}	33.1 ± 0.7^{b}	38.8 ± 1.2^{a}
20:4(n-6)	2.8 ± 0.5^{a}	1.4 ± 0.2^{b}	1.2 ± 0.2^{b}	2.3 ± 0.2^{a}	6.1 ± 0.3^{a}	3.3 ± 0.1^{c}	2.9 ± 0.1^{c}	4.5 ± 0.3^{b}
20:5(n-3)	0.0 ± 0.0^{c}	7.4 ± 0.2^{a}	2.2 ± 0.5^{b}	0.1 ± 0.0^{c}	0.1 ± 0.0^{c}	15.8 ± 0.6^{a}	5.0 ± 0.5^{b}	0.1 ± 0.0^{c}
22:5(n-3)	0.5 ± 0.1^{c}	4.5 ± 0.4^{a}	1.9 ± 0.6^{b}	0.4 ± 0.0^{c}	0.8 ± 0.1^{c}	8.7 ± 0.3^{a}	3.4 ± 0.1^{b}	0.7 ± 0.1^{c}
22:6(n-3)	0.6 ± 0.1^{c}	5.2 ± 0.3^{a}	2.1 ± 0.5^{b}	0.6 ± 0.1^{c}	1.1 ± 0.1^{c}	8.4 ± 0.5^{a}	3.7 ± 0.3^{b}	1.0 ± 0.1^{c}
Σ (n-3) PUFA	1.4 ± 0.2^{c}	17.3 ± 0.5^{a}	6.4 ± 1.6^{b}	1.4 ± 0.0^{c}	2.0 ± 0.1^{c}	32.9 ± 1.2^{a}	12.1 ± 0.8^{b}	1.8 ± 0.2^{c}
Σ (n-6) PUFA	25.2 ± 1.6^{ab}	2.8 ± 0.2^{c}	20.7 ± 2.9^{b}	31.6 ± 0.9^{a}	49.8 ± 0.4^{b}	6.3 ± 0.2^{d}	40.1 ± 0.5^{c}	55.6 ± 0.7^{a}
Σ SFA	63.3 ± 2.7^{ab}	69.4 ± 1.6^{a}	61.0 ± 3.3^{b}	57.7 ± 1.1^{b}	35.6 ± 0.5^{b}	46.2 ± 1.0^{a}	36.1 ± 0.9^{b}	32.8 ± 0.7^{c}
Σ MUFA	10.1 ± 1.1	10.5 ± 0.9	11.9 ± 1.5	9.4 ± 0.4	12.7 ± 0.2^{b}	14.7 ± 0.4^{a}	11.6 ± 0.3^{c}	9.8 ± 0.1^{d}
Σ PUFA	26.6 ± 1.8^{ab}	20.2 ± 0.7^{b}	27.2 ± 4.5^{ab}	33.0 ± 0.9^{a}	51.8 ± 0.5^{b}	39.2 ± 1.1^{c}	52.2 ± 1.1^{b}	57.4 ± 0.7^{a}
Unsaturation index[3]	0.75 ± 0.1^{b}	1.11 ± 0.0^{a}	0.90 ± 0.1^{ab}	0.86 ± 0.0^{ab}	1.40 ± 0.0^{c}	2.08 ± 0.1^{a}	1.63 ± 0.0^{b}	1.44 ± 0.0^{c}

[1] Values are means ± SEM, $n = 3$–4. Means in a row (within either rafts or whole cells) with superscripts without a common letter differ, $P < 0.05$.
[2] FA not shown: 14:0, 15:0, 16:1(n-7), 17:0, 17:1(n-7), 18:0, 18:1(n-7), 18:1(n-9), 20:0, 20:1(n-7), 20:1(n-9), 20:2(n-6), 20:3(n-6), 22:0, 22:2(n-6), 22:4(n-6), 24:0, and 24: 1(n-9).
[3] Unsaturation index was calculated as the sum total of the number of double bonds per FA multiplied by its mol %.

TABLE 2 Phospholipid, cholesterol, ceramide, and DAG content of lipid rafts from PUFA-treated MDA-MB-231 breast cancer cells[1]

	FA treatment			
	LA75	EPA60 + DHA40	EPA45 + DHA30 + LA75	LA150
Phospholipids	*mol/100 mol phospholipid phosphorus*			
PE	14.6 ± 0.7^{ab}	13.0 ± 0.7^{b}	16.3 ± 0.2^{a}	14.8 ± 0.5^{ab}
PI	3.8 ± 0.2^{ab}	3.4 ± 0.1^{b}	4.2 ± 0.1^{ab}	4.4 ± 0.4^{a}
PS	7.3 ± 0.1	7.7 ± 0.8	7.5 ± 0.3	7.6 ± 0.2
PC	49.2 ± 0.8^{b}	57.0 ± 0.7^{a}	49.6 ± 1.2^{b}	46.7 ± 1.3^{b}
SM	25.0 ± 0.6^{ab}	19.0 ± 1.0^{c}	22.4 ± 1.2^{b}	26.5 ± 1.2^{a}
	pmol/nmol lipid phosphorus			
Cholesterol	229 ± 14^{a}	134 ± 15^{c}	185 ± 11^{b}	221 ± 17^{ab}
Ceramide	1.35 ± 0.12^{b}	2.65 ± 0.24^{a}	1.44 ± 0.18^{b}	1.55 ± 0.20^{b}
DAG	1.40 ± 0.07^{a}	0.84 ± 0.08^{c}	1.16 ± 0.08^{b}	1.26 ± 0.08^{ab}

[1] Values are means ± SEM, $n = 6$ except raft cholesterol, $n = 7$. Means in a row with superscripts without a common letter differ, $P < 0.05$.

treated with EPA60 + DHA40 had lower ($P < 0.05$) raft cholesterol levels than cells treated with EPA45 + DHA30 + LA75.

EPA and DHA increase raft ceramide levels and decrease raft DAG levels. Treatment with EPA and DHA, in the absence but not in the presence of LA, increased ($P < 0.05$) raft ceramide levels compared with both LA75 and LA150 (Table 2). Both (n-3) FA treatments decreased ($P < 0.05$) raft DAG levels compared with treatment with LA75 (Table 2). By contrast to the rafts, whole cell concentrations of ceramide and DAG were much higher (7.1–8.4 and 32.0–40.9 pmol/nmol lipid phosphorus, respectively), but EPA and DHA treatment did not affect these concentrations.

EPA and DHA decrease EGFR protein levels in lipid rafts and increase whole cell levels of phosphorylated EGFR. Both (n-3) PUFA treatments decreased EGFR protein levels in rafts of MDA-MB-231 cells compared with LA75 and LA150 treatments (**Fig. 2***A*). Total cellular EGFR protein levels did not differ among FA treatments (Fig. 2, *top*). The amount of phosphorylated EGFR in whole cells was increased ($P < 0.05$) by both (n-3) PUFA treatments compared with LA75 and LA150 treatments (Fig. 2, *bottom*).

EPA and DHA increase whole cell levels of phosphorylated p38 MAPK. Both (n-3) PUFA treatments increased whole cell levels of phosphorylated p38 MAPK compared with LA75 and LA150 treatments (**Fig. 3**). Whole cell levels of total p38 MAPK did not differ among FA treatments.

Discussion

(n-3) PUFA are readily incorporated into tumor cell membranes both in vitro and in vivo (3,6,7,24), but the biological impact of this has not been established. We hypothesized that (n-3) PUFA alter the properties of membrane lipid rafts as a mechanism to explain their growth-inhibitory effects on breast tumor cells. We observed a significant enrichment of both EPA and DHA into rafts of MDA-MB-231 breast cancer cells treated with either EPA60 + DHA40 or EPA45 + DHA30 + LA75. This is consistent with other reports demonstrating incorporation of (n-3)

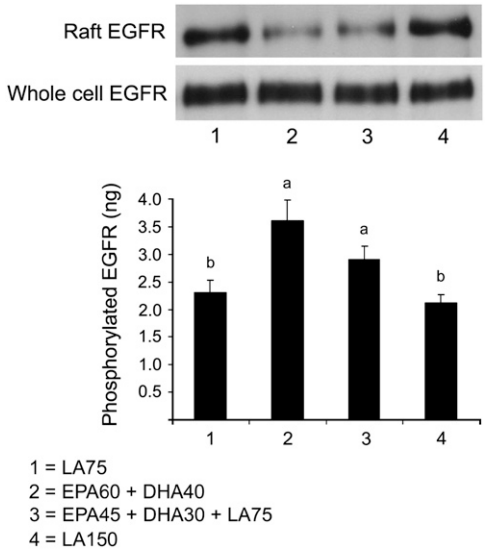

Figure 2 (n-3) PUFA treatment decreases EGFR levels in lipid rafts of MDA-MB-231 cells and increases whole cell EGFR tyrosine phosphorylation. (*Top*) Raft and whole cell EGFR protein levels. Blots shown are representative of 3 experiments. (*Bottom*) Whole cell levels of tyrosine phosphorylated EGFR. Bars represent means ± SEM, $n = 6$. Means without a common letter differ, $P < 0.05$.

PUFA into rafts of different cell types (Jurkat T cells, splenic T cells, colonocytes) both in vitro (25) and in vivo (26,27). Consistent with these studies, the concentration of EPA and DHA in rafts was less than in whole cell phospholipids. This was not unexpected considering the high degree of saturation of membrane rafts. Enrichment of (n-3) PUFA was lower in cells treated with EPA and DHA plus LA than in cells treated with EPA and DHA alone. This might be predicted due to the lower concentration of (n-3) PUFA in the EPA45 + DHA30 + LA75 treatment, as well as the inclusion of LA, which competes with EPA and DHA for incorporation into membrane phospholipids (24).

Overall, the levels of incorporation of (n-3) PUFA into raft phospholipids in the current study are consistent with in vivo levels of (n-3) PUFA incorporation into lipid rafts. In our study, (n-3) PUFA comprised 17 and 6% of the total raft FA following EPA+DHA treatment in the absence and presence of LA, respectively. In colonic caveolae from mice fed a 4 g/100 g fish oil diet, (n-3) PUFA comprised 6, 9, 18, and 28% of the total FA in PS, PC, PI, and PE, respectively (27). Similarly, in T cell rafts from mice fed 4 g/100 g fish oil, (n-3) PUFA comprised 11 and 16% of the total FA in PS and PE, respectively (26). The whole cell phospholipid FA composition of cells treated with EPA and DHA plus LA in this study is also consistent with the FA composition of mammary tumor cell phospholipids from animals fed fish oil. In this study, total (n-3) PUFA, EPA, and

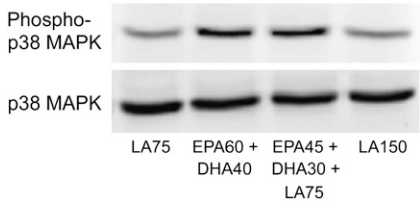

Figure 3 (n-3) PUFA treatment increases p38 MAPK phosphorylation in EGF-stimulated MDA-MB-231 cells. Blots shown are representative of 4 experiments.

DHA comprised 12, 5, and 4% of whole cell phospholipid FA, respectively, after treatment with EPA and DHA plus LA. A previous study from our laboratory showed that (n-3) PUFA made up 14% of total FA in PE in R3230AC mammary tumor cells from rats fed fish oil at 5 g/100 g of total fat (7). In addition, in MDA-MB-435 mammary tumor cells from mice fed either 8 g/100 g EPA or DHA, EPA, and DHA comprised 11 to 13% of the total FA (6). Thus, our enrichment levels of (n-3) PUFA in lipid rafts and whole cell phospholipids are consistent with those that are attainable through dietary manipulation, supporting the relevance of this research to a dietary model of mammary tumor growth inhibition.

Treatment with EPA60 + DHA40 led to a significant decrease in the proportion of SM in rafts as well as a significant increase in the proportion of raft PC, as compared with treatment with LA75. Only 1 other study, to our knowledge, has examined the effects of (n-3) PUFA on the phospholipid content of rafts and also found a decrease in the proportion of SM in rafts isolated from T cells of (n-3) PUFA-fed mice (26). We hypothesized that the decrease in SM induced by (n-3) PUFA might result from cleavage of SM to generate ceramide, which is believed to mediate anti-proliferative responses such as cell cycle arrest, apoptosis, and differentiation (28). Indeed, raft ceramide levels were significantly increased in cells treated with EPA60 + DHA40. These results support the hydrolysis of raft SM to ceramide, at least in cells that incorporate a relatively high level of (n-3) PUFA. A decrease in raft SM may have other importance as well, perhaps affecting the function or structure of rafts.

We observed a significant decrease in raft DAG in (n-3) PUFA-treated cells; the reasons for this are not clear. It is possible that the decreased expression of EGFR in rafts is reflected in lower formation of DAG through phospholipase Cγ or phospholipase D (29). Also, the increased presence of ceramide might block phospholipase D activation (30). Alternatively, the changes in DAG content could result from overall modification of physical characteristics of rafts induced by (n-3) PUFA.

Treatment with PUFA has been shown to displace signaling proteins from rafts in immune cells (25,31). We examined the effects of (n-3) PUFA on raft localization of the EGFR, which is often over-expressed in breast carcinomas and suggested to be involved in tumor cell progression (32). MDA-MB-231 cells express high levels of the EGFR (32) and were a good model to study EGFR raft modulation by (n-3) PUFA. We found that protein levels of the EGFR were reduced in lipid rafts of (n-3) PUFA-treated MDA-MB-231 cells. This effect was selective for rafts, because total cellular EGFR protein levels were not altered by (n-3) PUFA treatment. To determine whether raft displacement of the EGFR affected overall EGFR signaling, we measured whole cell levels of tyrosine phosphorylated EGFR and downstream p38 MAPK phosphorylation. We observed an increase in the levels of total cellular tyrosine phosphorylated EGFR in (n-3) PUFA-treated cells, which is in agreement with reports of EPA/DHA-induced EGFR phosphorylation or activation in vitro (33). Our results are also consistent with recent reports that indicate caveolae/rafts function as negative regulators of EGFR phosphorylation (11,12), with ligand binding and EGFR phosphorylation coinciding with EGFR migration out of caveolae/rafts (13,14).

Several recent studies have implicated membrane cholesterol as an important regulator of EGFR activity (11,12,34–36). Cholesterol depletion with methyl-β-cyclodextrin reduces EGFR levels in lipid rafts (11,12,36) and increases EGF binding to the EGFR (11,36), tyrosine phosphorylation of the EGFR (12,34–36), and EGFR kinase activity (36). In this study, (n-3) PUFA decreased raft cholesterol levels. This finding is consistent with other reports that show (n-3) PUFA reduce cholesterol levels in whole cell membranes (37) as well as in caveolae (27), perhaps due to steric incompatibility between cholesterol and (n-3) PUFA (27). The (n-3) PUFA-induced reduction of raft cholesterol observed in this study suggests a mechanism by which (n-3) PUFA treatment results in raft exclusion and phosphorylation of the EGFR and is consistent with the purported role of cholesterol in EGFR activation and raft localization.

Although it is generally thought that increased activity of, or signaling through, the EGFR promotes cell proliferation and survival in most cell types, recent reports indicate that increased EGFR phosphorylation can occur with the induction of apoptosis (15,16), including in MDA-MB-231 cells (17). Sustained EGFR phosphorylation has been associated with p38 MAPK activation (16,17,38), which promotes cancer cell apoptosis following initiation by several anti-cancer agents (39). Activated EGFR has also been shown to be required for activation of the CD95 death receptor system (16). Similar to these studies, the increased EGFR phosphorylation induced by (n-3) PUFA in this study did not appear to be associated with growth promotion but rather with growth inhibition, because EPA and DHA significantly reduced the number of viable MDA-MB-231 cells. Our previous work demonstrated that EPA and DHA, at these same concentrations, induced apoptotic cell death and decreased proliferation of MDA-MB-231 cells (5). Akt(Ser-473) phosphorylation, as well as nuclear factor kappa B DNA-binding activity, were also decreased by EPA and DHA treatment (5). Interestingly, decreased Akt(Ser-473) phosphorylation has also been observed with cholesterol depletion (34). These findings, along with the observed (n-3) PUFA-induced increase in p38 MAPK phosphorylation, suggest that in this study, increased EGFR phosphorylation does not promote growth but may be associated with the induction of apoptosis. Thus, it appears that the EGFR may play a role in both cell growth and cell death [reviewed in (40)]. In mammary tumor cells, the outcome of EGFR signaling (i.e. growth promotion vs. apoptosis) may depend on the concentration of EGFR/ErbB receptors in the cell (38).

(n-3) PUFA are generally regarded as safe compounds that are well tolerated and produce few side effects. Their effects on nontumorigenic cells have not been fully elucidated, but some studies suggest that when provided at concentrations that inhibit tumor cell growth, (n-3) PUFA exert little or no cytotoxic effects on normal breast cells (3,4,41). Thus, with further study, (n-3) PUFA may hold promise as nontoxic adjuvants to standard cancer therapies. The results of our study demonstrate for the first time, to our knowledge, that (n-3) PUFA have significant effects on the FA composition as well as the phospholipid, cholesterol, ceramides, and DAG content of membrane rafts in human breast cancer cells. These lipid alterations reduced the levels of EGFR in rafts and increased whole cell levels of phosphorylated EGFR, which was associated with apoptotic signaling and growth inhibition, rather than growth promotion. Our results describe a novel membrane mechanism by which (n-3) PUFA may inhibit the growth of breast tumors.

Acknowledgment

We thank Jay Dewald for measuring ceramide and DAG concentrations.

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