n-6 and n-3 polyunsaturated fatty acids differentially modulate oncogenic Ras activation in colonocytes

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Collett, Esther D., Laurie A. Davidson, Yang-Yi Fan, Joanne R. Lupton, and Robert S. Chapkin. n-6 and n-3 polyunsaturated fatty acids differentially modulate oncogenic Ras activation in colonocytes. Am J Physiol Cell Physiol 280: C1066–C1075, 2001.—Ras proteins are critical regulators of cell function, including growth, differentiation, and apoptosis, with membrane localization of the protein being a prerequisite for malignant transformation. We have recently demonstrated that feeding fish oil, compared with corn oil, decreases colonic Ras membrane localization and reduces tumor formation in rats injected with a colon carcinogen. Because the biological activity of Ras is regulated by post-translational lipid attachment and its interaction with stimulatory lipids, we investigated whether docosahexaenoic acid (DHA), found in fish oil, compared with linoleic acid (LA), found in corn oil, alters Ras posttranslational processing, activation, and effector protein function in young adult mouse colon cells overexpressing H-ras (YAMC-ras). We show here that the major n-3 polyunsaturated fatty acid (PUFA) constituent of fish oil, DHA, compared with LA (an n-6 PUFA), reduces Ras localization to the plasma membrane without affecting posttranslational lipidation and lowers GTP binding and downstream p42/44erk-dependent signaling. In view of the central role of oncogenic Ras in the development of colon cancer, the finding that n-3 and n-6 PUFA differentially modulate Ras activation may partly explain why dietary fish oil protects against colon cancer development.

docosahexaenoic acid; linoleic acid; colon cancer; fish oil

COLORECTAL CANCER continues to pose a serious health problem in the United States, accounting for an estimated 129,400 new cases diagnosed this year, with ~155 deaths each day (34). Over a lifetime, a person has 1 chance in 18 of developing invasive colorectal cancer (34). Colorectal cancer evolves from a multistep process (20) and is a disease strongly influenced by environmental factors, with diet being one of the most important modifying agents (43). Among dietary factors, there are strong epidemiological, clinical, and experimental data indicating a protective effect of fish oil-derived n-3 polyunsaturated fatty acids (PUFA) on colon cancer (2, 10, 35, 43). In general, dietary lipids rich in linoleic acid (LA; 18:2n-6), found in a variety of vegetable oils, enhance the development of colon tumors (8, 17, 46, 47), whereas n-3 PUFA-enriched diets, containing docosahexaenoic acid (DHA; 22:6n-3), reduce colon cancer incidence (10, 17, 43, 45, 48). This protective effect is exerted at both the initiation and postinitiation stages of carcinogenesis (11, 29, 45). Recently, we have shown that the balance between colonic epithelial cell proliferation and apoptosis can be favorably modulated by dietary n-3 PUFA, conferring resistance to toxic carcinogenic agents (11, 29). However, the underlying molecular mechanism(s) by which distinct classes of dietary PUFA (n-6 vs. n-3) exert their effects is not known.

Ras genes code for 21-kDa guanine nucleotide binding proteins that play an important role in colonic epithelial cell growth, differentiation, and tumor formation (38, 59). Ras proteins (H-Ras, K-Ras and N-Ras) differ only in the carboxy-terminal region of the protein, bind guanine nucleotides (GTP and GDP) with high affinity, and possess intrinsic GTPase activity. Biological Ras activity is determined by the bound nucleotide (38, 51), which in turn is regulated by the membrane lipid microenvironment, specifically posttranslational lipid attachment (isoprenylation and palmitoylation) (23, 25) and the interaction with stimulatory lipids (53). Ras proteins are synthesized in the cytosol on ribosomes and have a half-life of 24 h (13). After synthesis, Ras proceeds through a series of posttranslational processing steps at the COOH terminus that increase protein affinity for the plasma membrane. Specifically, the H-Ras and N-Ras isoforms are transported to the plasma membrane through the Golgi, while K-Ras is transported through the cytosol after cleavage and methylation (3, 13, 38). The palmitoylation of H-Ras targets the protein to the plasma membrane in flask-shaped invagination domains rich in cholesterol and sphingolipids referred to as caveolae (39, 55). Importantly, we have recently demonstrated that dietary fish oil reduces colonic Ras membrane localization in carcinogen-treated rats (16). Because Ras must be localized to the plasma membrane to be biologically active, and its constitutive activation di-

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rectly drives early colonic tumor development (52, 59), we have determined the action of DHA (antitumorigenic) vs. LA (protumorigenic) on Ras posttranslational processing, activation, and signal transduction in colonocytes.

MATERIALS AND METHODS

Materials. RPMI 1640 and Hanks’ balanced salt solution were from Mediatech (Herndon, VA). Fetal bovine serum (FBS) was from Hyclone (Logan, UT). Insulin/transferrin/selenium (ITS) was obtained from Collaborative Biomedical Products (Bedford, MA). Glutamax and recombinant mouse interferon-γ (IFN-γ) were from Gibco-BRL (Grand Island, NY). Fatty acid-free bovine serum albumin (BSA) and 1% penicillin-streptomycin were from Sigma (St. Louis, MO). Fluorescein diacetate and propidium iodide were obtained from Millipore (Burlington, MA). Bicinchoninic acid protein assay and SuperSignal chemiluminescent detection reagents were from Pierce (Rockford, IL). Peroxi-
dase-conjugated secondary antibodies were from Kirkegaard & Perry (Gaithersburg, MD). Mycoplasma PCR-based ELISA was purchased from Roche Molecular Biochemical (Indianapolis, IN). Bromodeoxyuridine (BrdU) labeling kit was from Molecular Probes (Eugene, OR). Prepoured silica gel 60 thin-layer chromatography (TLC) plates were from Merck (Darmstadt, Germany). All other reagents were obtained from Sigma.

Cell culture. Malignant transformed young adult mouse colon (YAMC)-Ras cells overexpressing v-H-Ras were kindly donated by Dr. Robert Whitehead (Ludwig Institute for Cancer Research, Melbourne, Australia) (15). Cells (passages 16–20) were maintained in RPMI 1640 supplemented with 5% FBS, 1% glutamax-1, 1% ITS, and 1% penicillin-streptomycin at 33°C. The medium was supplemented with 5,000 U/ml of mouse recombinant IFN-γ because the temperature-sensitive mutant SV40 large T antigen gene (tsA58) is under an IFN-γ-inducible promoter, mouse H-2Kb class I gene (60). For fatty acid treatment, subconfluent cells were treated for 72 h with 0–100 μM DHA or LA complexed to fatty acid-free BSA (27). Cells treated with BSA plus medium or medium alone were used as controls for each experiment. All cultures were mycoplasma free, as determined by a PCR-based ELISA.

Cell proliferation. Cells were plated at an initial density of 20,000 cells per 35-mm dish. After 24 h, dishes were treated for 72 h with 50 μM DHA or LA, with medium replenished every 24 h. Medium alone was used as a negative control. After incubation at 33°C, adherent cells were trypsinized and counted with a hemacytometer. For quantitation of DNA synthesis, 1–2 × 10⁶ cells were pulsed with BrdU (1:2,000 dilution, 0.3 μg/ml) 3 h before harvest and then processed according to kit instructions.

Cell viability. Cells were plated at 20,000 cells per 35-mm dish, and the effect of fatty acid treatment on viability was assessed with fluorescein diacetate/propidium iodide (31). After incubation with 50 μM DHA or LA for 72 h, cultures were rinsed with phosphate-buffered saline (PBS) and stained. A minimum of 100 adherent cells were counted and scored as red (nonviable) or green (viable) with the use of a Nikon Eclipse TE300 fluorescence inverted microscope equipped with a Prinvention Instruments Micromax cooled digital camera chip and a Metamorph imaging workstation.

As a positive control, YAMC cells (parental cell line) were incubated at a nonpermissive temperature (39°C) overnight. Data were expressed as percent viability (100 × [no. of green cells/no. of total cells]).

Caspase 3 activity. Caspase 3 activity was determined with the EnzChek caspase 3 assay kit. Floating and adherent cells were harvested, and supernatants were utilized for the activity assay. Fluorescence was measured at a ratio of 496 to 520 nm 30, 45, 60, and 90 min after substrate addition. Data were transformed with a log function before statistical analysis. In addition, a morphological assessment of apoptosis was performed by acridine orange staining of floating cells as previously described (5).

Measurement of membrane phospholipid fatty acid composition. Cell lipids were extracted with chloroform-methanol (2:1 vol/vol), and individual phospholipid classes were separated by TLC with chloroform-methanol-acetic acid-water (50:37.5:3.5:2 vol/vol/vol/vol) as previously described (12). After transesterification, fatty acid methyl esters from the diposphatidylglycerol, glycerophosphocholine, and glycerophosphocholeolamine phospholipids were quantitated by capillary gas chromatography (1).

Subcellular fractionation. Cell extracts were prepared as previously described (16). Briefly, after fatty acid incubation, cells were grown to near confluence in T-75 or T-175 flasks, trypsinized, pelleted at 200 g, and washed three times with PBS. Subsequently, the cell pellet was passed through a 27-gauge needle three times with 500 μl of homogenizing buffer [50 mM Tris-HCl, pH 7.2, 250 mM sucrose, 2 mM EDTA, 1 mM EGTA, 50 μM sodium fluoride, 100 mM ortho-
vandate, 25 μg/ml each of leupeptin, pepstatin, and aproti-
nin, and 150 μM 4-(2-aminoethyl)benzenesulfonyl fluoride]. Samples were centrifuged at 100,000 g for 30 min at 4°C, and the supernatant was taken as the cytosolic extract and frozen in aliquots at −80°C until use. The pellet was further ex-
tacted with the above buffer supplemented with Triton X-100 at a final concentration of 1% and then incubated for 30 min on ice. Insoluble material was removed by a second round of centrifugation at 100,000 g for 30 min at 4°C. The supernatant was saved as the total membrane extract. In addition, total cell lysates were prepared with the use of the Triton X-100-containing buffer, incubated on ice for 30 min, and centrifuged at 14,000 g for 20 min. The supernatant was designated the total cell extract. Cellular protein was quanti-
fied with the Coomassie Plus assay (Pierce).

Immunoblotting. Total cellular, membrane, or cytosolic extracts were treated with SDS sample buffer and subjected to polyacrylamide gel electrophoresis in 4–20% precast mini gels (16). After electrophoresis, proteins were electrobotted onto a PVDF membrane with the use of a Hoefer Mighty Small Transphor Unit (Pharmacia, Piscataway, NJ) at 400 mA for 1.5 h. After transfer, the membrane was processed according to the method of Davidson et al. (16), including blocking of the membrane in 4% nonfat dry milk and 0.1% Tween 20 in PBS at room temperature for 1 h with shaking, followed by incubation with shaking overnight at 4°C with primary antibody [pan-Ras Ab-3 (Clone 10; Oncogene Science), anti-HMG-CoA reductase (CRL 18811) isolated from the A9 hybridoma cell line (ATCC, Rockville, MD), or anti-
SV40 (Oncogene Science) diluted in PBS containing 4% milk and 0.1% Tween 20. Membranes were washed with PBS containing 0.1% Tween 20 and incubated with secondary antibody (peroxidase-conjugated goat anti-mouse IgG; Kirkegaard & Perry) per manufacturer’s instructions. Recombinant H-Ras standard (0.75 ng) from Panvera (Madison, WI) was used as a marker and resulted in a 21-kDa band. In addition, direct molecular weight determination of Ras pro-

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teins was performed with the use of positive-ion MALDI-TOF-MS on a PerSeptive Biosystems Voyager Elite XL system (Framingham, MA) equipped with delayed extraction and a LSI nitrogen laser operating at 337 nm (4). For HMG-CoA reductase analysis, mouse brain extract was used as the positive control. For quantitation, linearity of detection was validated over a range of sample protein, and blots were scanned and quantitated with a Fluor-S Max Multilimage System (Bio-Rad, Hercules, CA). Band intensities were reported as intensity multiplied by band area.

Confocal immunofluorescence. Cells were plated into four-well pretreated (overnight with 20% FBS in culture medium) chamber glass slides (catalog no. 136420; Lab-Tek) at a density of 2,000 or 3,500 cells per well. After 48–72 hr, cell monolayers were rinsed with PBS and fixed with 2% paraformaldehyde-PBS for 20 min at room temperature (61). Cells were permeabilized and blocked in PBS containing 0.02% saponin and 5% normal rabbit serum (SNRS-PBS) for 30 min at room temperature and were subsequently probed with 20 μg/ml Ras antibody Y13-238 (Oncogene Science) in SNRS-PBS for 1 h at room temperature. Slides were washed with PBS containing 0.02% saponin and then incubated with FITC-conjugated rabbit anti-rat IgG (20 μg/ml; Dako, Carpinteria, CA) in the dark for 30 min at room temperature, followed by 10 min of treatment with 100 nM 4’-6-diamidino-2-phenylindole dihydrochloride (Roche Molecular Biochemical) to label nuclei. Finally, cells were washed for 30 min in PBS containing 0.02% saponin and mounted in ProLong anti-fade mounting medium (Molecular Probes). Slides were viewed on an Ultima confocal microscope (Meridian Instrument, Okemos, MI).

Farnesyl protein transferase assay. Farnesyl protein transferase activity was assayed as the ability of cytosolic protein extracts to catalyze the prenylation of Ras (16). Cytosolic extracts (40 μg protein) from YAMC-Ras cells were incubated for 1 h with 5 μg of recombinant H-Ras (Panvera) and 1 μCi of [3H]farnesylpyrophosphate (15.0 Ci/mmol; NEN, Boston, MA) in a buffer containing 50 mM Tris·HCl, pH 7.5, 20 mM MgCl2, 144 μM [32P]orthophosphoric acid (285 Ci/mg; NEN) for 4 h in a low-phosphate medium (23, 44). After incubation, cells were harvested in a lysis buffer containing 25 mM Tris·HCl, pH 7.5, 20 mM MgCl2, 144 μM 4-(2-aminoethyl)-benzenesulfonyl fluoride, and 1% aprotinin. Samples were centrifuged at 100,000 g for 30 min, and the supernatant was taken as the cytosolic extract. For preparation of the membrane fraction, the pellet was resuspended in the above buffer containing Triton X-100 at a final concentration of 1%, incubated for 30 min on ice, and centrifuged at 100,000 g for 30 min. The supernatant was saved as the membrane extract. Membrane and cytosolic extracts were immunoprecipitated with pan-Ras antibody as described in Ras palmitoylation; immunoprecipitates bound to protein A/G beads were washed with buffer containing 50 mM Tris·HCl, pH 7.5, 0.2% Triton X-100, 5 mM MgCl2, 500 mM NaCl, and 0.005% SDS (16). Ras immunoprecipitate was resuspended in 2 mM EDTA, 2 mM DTT, 0.2% SDS, 10 mM GTP, and 10 mM GDP, as adapted from the procedure of Downward et al. (18), and incubated for 80 min at 68°C to elute bound nucleotides. The GTP and GDP from membrane or cytosolic fractions were resolved by TLC on polyethyleneimine-cellulose plates (Merck), developed with 2.0 M ammonium formate in 1.0 M HCl as solvent (40), and detected with a Packard InstantImager scanner. The percentage of GTP-associated Ras in membrane or cytosolic fractions was calculated as [dpm GTP/1.5(dpm GTP + dpm GDP)] × 100 (50).

Ras-dependent signal transduction. To assess the effect of fatty acid treatment on Ras downstream signaling, cells were incubated with 50 μM LA, DHA, or no fatty acid under standard culture conditions for 72 h, followed by serum deprivation (0.1% FBS) for 18 h. Select cultures were subsequently stimulated with 1 μg/ml lipopolysaccharide (Sigma) for 10 min, and total cell lysates were prepared. Activated p42 and p44 extracellular signal-regulated kinase (ERK) levels were determined by immunoblot analysis with phospespecific p44/42 ERK antibody, which detects ERK1 and ERK2 only when catalytically activated by phosphorylation. Statistical analysis. Effects of fatty acid treatment on Ras downstream signaling, cells were incubated with 50 μM LA, DHA, or no fatty acid under standard culture conditions for 72 h, followed by serum deprivation (0.1% FBS) for 18 h. Select cultures were subsequently stimulated with 1 μg/ml lipopolysaccharide (Sigma) for 10 min, and total cell lysates were prepared. Activated p42 and p44 extracellular signal-regulated kinase (ERK) levels were determined by immunoblot analysis with phosphospecific p44/42 ERK antibody, which detects ERK1 and ERK2 only when catalytically activated by phosphorylation at Thr-202 and Tyr-204 (Cell Signaling Technology, Beverly, MA), and p44/42 ERK antibody, which detects total ERK1 and ERK2 (16). Phosphorylated ERK2 control protein was used as a standard for ERK blots and yielded a 42-kDa band. Nonphosphorylated ERK2 was used as a negative control to assess the specificity of the antibody for the phosphorylated form only. In addition, activated Akt was determined by immunoblot with a phospho-Akt (Ser-473) antibody, which detects Akt1 when phosphorylated at Ser-473 and Akt2 and Akt3 when phosphorylated at equivalent sites (Cell Signaling Technology). Expression levels of total Akt were also quantitated with the use of phosphorylation state-independent Akt antibody (Cell Signaling Technology).

RESULTS

Functional effects of fatty acids on colonocytes in vivo. We have previously demonstrated that the YAMC cell line is a useful model system to define the molec-
ular mechanisms by which dietary constituents modulate colonocyte growth, differentiation, and apoptosis (5, 19). The YAMC-Ras cell line overexpresses H-Ras approximately fourfold compared with the parental cell line (data not shown) and forms visible tumors (15). Using this model, we first investigated the effects of fatty acids known to promote (LA, 18:2n-6) and prevent (DHA, 22:6n-3) colon cancer in experimental animal models (11, 29, 48, 49). After a 72-h incubation, a significant reduction (P < 0.05) in cell number was observed in 50 μM DHA-treated cultures compared with LA-treated and control cells at subconfluent densities (Fig. 1). This dose is considered physiologically relevant because it lies well within the range of blood levels in human subjects supplemented with DHA (14).

Fatty acid treatment had no effect on adherent cell viability (>98%) as assessed by propidium iodide exclusion. To examine whether growth arrest by DHA treatment was associated with changes in cell proliferation and/or apoptosis, we determined BrdU uptake and caspase 3 activity. DHA treatment significantly reduced DNA synthesis compared with LA treatment and control (Fig. 2A). In contrast, fatty acid treatment had no effect on the level of apoptosis in adherent and floating populations (Fig. 2B). The effects of DHA on cell proliferation were not the result of alterations in the SV40 gene product because the expression of SV40 large T antigen was not affected (data not shown).

**Incorporation of fatty acids into membrane phospholipids.** The exogenous fatty acids were differentially incorporated into membrane phospholipids (Tables 1–3). DHA was primarily incorporated into the glycerocephosphoethanolamine (EtnGro) and cardiolipin (Ptd2Gro) classes. A significant (P < 0.05) reduction in arachidonic acid (AA; 20:4n-6) levels was observed in EtnGro, Ptd2Gro, and glycerophosphocholine (ChoGro) compared with LA-treated and untreated cells. Interestingly, compared with untreated cells, DHA-treated cells exhibited an elevation in the mole-percent level of LA. In contrast, LA treatment was associated with an elevation in the weight-percent level of LA in Ptd2Gro and ChoGro and of its metabolic elongation-desaturation product, AA, in EtnGro compared with DHA-treated and untreated cells.

**n-6 and n-3 PUFA differentially modulate Ras membrane localization.** There were no significant effects of 50 μM fatty acid treatment on total Ras protein expression (Fig. 3A). Similar to previous reports (13), five- to eightfold more Ras resided at the membrane compared with the cytosol (Fig. 3B). Interestingly, the Ras membrane-to-cytosol ratio was 41% higher in LA- vs. DHA-treated cells (Fig. 3B). When the effects of fatty acid dose were examined, significant differences (P < 0.05) between LA and DHA treatments were observed at both the 25 and 50 μM level (Fig. 4). To corroborate protein identification by SDS-PAGE, direct molecular weight determination of the Ras protein was per-
formed with the use of positive-ion MALDI-TOF-MS and was found to be 21,296.8 daltons, consistent with published data (59). Because Ras must be localized to the inner surface of the plasma membrane to be biologically active (3, 61), we examined its intracellular localization using confocal immunofluorescence. As expected, Ras was largely targeted to the plasma membrane (data not shown). Together, these data indicate that fatty acid treatment can modulate Ras trafficking to the plasma membrane.

**Fatty acid treatment does not affect Ras posttranslational processing.** After synthesis, Ras proceeds through a series of posttranslational steps at the COOH terminus that increase its affinity for the plasma membrane. The first of these posttranslational modifications is the addition of a 15-carbon farnesyl lipid at Cys-186. Therefore, we determined whether the lower Ras membrane-to-cytosol ratio in DHA- vs. LA-treated cells was due to an effect on farnesylation. As shown in Fig. 5, no effect on HMG CoA reductase expression was observed.

### Table 1. Cardiolipin fatty acid composition

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<th>Fatty Acid</th>
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<td>UT</td>
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<tr>
<td>16:0</td>
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<td>18:0</td>
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<td>18:1(n-9)</td>
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<td>18:2(n-6)</td>
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<tr>
<td>20:4(n-6)</td>
<td>17.39 ± 2.39*</td>
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<td>20:5(n-3)</td>
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<tr>
<td>22:6(n-3)</td>
<td>1.99 ± 0.58*</td>
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Young adult mouse colon (YAMC)-Ras cells were incubated with 50 μM linoleic acid (LA) or docosahexaenoic acid (DHA) or medium alone (untreated, UT) for 72 h. Fresh media containing fatty acid were given daily. Total lipids from the cells were extracted. Isolated cardiolipin, glycerophosphocholine, and glycerophosphoethanolamine fractions were transesterified, and the resultant fatty acid methyl esters were analyzed by gas chromatography. Values represent means ± SE from 5 separate experiments. Bars with different symbols represent treatments that are significantly different (P < 0.05).

### Table 2. Glycerophosphocholine fatty acid composition

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<tr>
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<td>18:1(n-9)</td>
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<td>18:2(n-6)</td>
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<td>20:4(n-6)</td>
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See Table 1 legend for details. Values represent means ± SE (n = 3–4) of glycerophosphocholine compositions from 3 separate experiments. Values with different symbols (*, †, ‡) are significantly different (P < 0.05).

### Table 3. Glycerophosphoethanolamine fatty acid composition

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<td>20:5(n-3)</td>
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<tr>
<td>22:6(n-3)</td>
<td>1.84 ± 1.54*</td>
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See Table 1 legend for details. Values represent means ± SE (n = 5) of glycerophosphoethanolamine compositions from 3 separate experiments. Values with different symbols (*, †) are significantly different (P < 0.05).
expression, which produces the farnesylpyrophosphate precursor mevalonate, was observed after the fatty acid treatment. Similarly, farnesyltransferase activity was not altered (Fig. 6). Interestingly, although isoprenylation of Ras is required for cell-transforming activity, it is posttranslational acylation (palmitoylation of Cys-181 and/or Cys-184) that directly regulates the amount of protein in the membrane (25, 26). However, fatty acid treatment had no effect on the relative ratio of [3H]palmitate- to [35S]methionine-labeled Ras, an index of palmitoyl transferase activity in vivo (Fig. 7). Although farnesylation activity and HMG-CoA reductase expression are indirect markers of farnesylation status, without isoprenoid attachment to Cys-186 the H-Ras protein remains soluble, and the adjacent Cys-181 and Cys-184 do not become palmitoylated (3, 7, 37). Therefore, any perturbation in farnesylation would negatively impact palmitoylation, the terminal processing step. Because fatty acid treatment had no effect on Ras palmitoylation status, we conclude that post-

Fig. 4. Dose effect of fatty acid treatment on Ras membrane-to-cytosol ratio. Cells were incubated with fatty acids (0–100 μM) as described in Fig. 3. ● LA (18:2n-6)-treated cells; ○ DHA (22:6n-3)-treated cells. Results are expressed as percentages of untreated control (n = 3). *Significant difference (P < 0.05) between LA and DHA treatment at 25 and 50 μM levels.

Fig. 5. Colonocyte HMG CoA reductase expression. Cells were treated with 50 μM LA or DHA or with medium alone for 72 h. Membrane (100,000 g pellet) extracts (20 μg of protein) were immunoblotted as described in MATERIALS AND METHODS. Expression was quantified as band intensity (optical density) multiplied by band area. Data are mean (±SE) percentages of untreated control from 3 separate experiments (n = 5–11). There were no significant differences among treatment groups (P > 0.05).

Fig. 6. Farnesyl protein transferase activity 72 h after fatty acid treatment. Cells were treated with 50 μM LA or DHA or with medium alone for 72 h. Cytosolic (100,000 g supernatant) extracts (40 μg) were incubated for 1 h at 37°C with [3H]farnesylpyrophosphate (0.5 μCi/μl) and 5 μg of recombinant H-Ras. Enzyme activity was assayed as counts (dpm) of [3H]farnesylpyrophosphate transferred per 40 μg of protein per hour. Data are means ± SE from 3 separate experiments (n = 5–6). No significant differences among treatment groups were noted (P > 0.05).

Fig. 7. Lack of an effect of fatty acid treatment on the ratio of [3H]palmitate- to [35S]methionine-labeled Ras, an index of palmitoyl transferase activity. Cells were treated for 72 h with 50 μM DHA or LA or with medium alone. During the final 24 h of fatty acid treatment, cells were metabolically labeled overnight (16 h) with [35S]methionine (250 μCi/ml) in a low-methionine medium, followed by a 4-h incubation with [3H]palmitic acid (165 μCi/ml). After fatty acid incubation, cells were harvested and Ras immunoprecipitated with pan-Ras antibody. Immunoprecipitates were subjected to SDS-PAGE, and the 21-kDa band was excised and dual counted for 3H and 35S as described in MATERIALS AND METHODS. Counts (dpm) from 35S-labeled Ras were used as an internal standard in each experiment to normalize the palmitoylation dpm values. Data are means ± SE from 3 separate experiments (n = 7–8). There were no significant differences among treatment groups (P > 0.05).
translational processing was not significantly influenced by fatty acid treatment.

**DHA suppresses Ras activation.** Because localization of Ras to the plasma membrane and its subsequent binding to GTP are required steps for activation and downstream signaling, the percentage of Ras bound to GTP in membrane and cytosol fractions was assessed. A significant reduction ($P < 0.05$) was observed in the percentage of GTP bound Ras in the membrane fraction of DHA-treated cells compared with LA-treated and untreated cells (Fig. 8A). In contrast, no significant differences were observed in the percentage of GTP bound Ras in the cytosolic fraction (Fig. 8B). As expected, the majority (89%) of GTP bound Ras was localized at the membrane (Fig. 8C).

Ras activation can lead to the sequential stimulation of ERK; therefore, expression of activated ERK was quantified by using a phosphorylated ERK-specific antibody. Because it is difficult to quantify the activation of many signaling molecules under “nonstimulated/basal” growth conditions, we attempted to highlight the differential induction of ERKs using a potent inflammatory stimuli, i.e., lipopolysaccharide (36, 42). The level of activated p42 and p44 ERK was 63% lower in DHA-treated cells compared with LA-treated cells (Fig. 9). In addition, there was no difference in total ERK expression (phosphorylated plus nonphosphorylated) in any of the treatments (data not shown). We next examined the ability of DHA to modulate the activation of a second Ras effector, i.e., the phosphatidylinositol (PI) 3-kinase/Akt signaling pathway. Data from these experiments indicated no effect of fatty acid treatment on either the level of activated Akt or total Akt, i.e., phosphorylated and nonphosphorylated (data not shown). These data suggest that PUFA modulate the ERK signaling pathway.

**Fig. 8.** DHA incubation suppresses membrane Ras GTP-binding. Fatty acid-treated cells (50 μM for 72 h) were incubated with 325 μCi/ml [32P]orthophosphoric acid in phosphate-free medium for 4 h. After incubation, cells were harvested and fractionated into 100,000 g membrane and cytosolic compartments as described in MATERIALS AND METHODS. Ras immunoprecipitates were subjected to thin-layer chromatography to separate nucleotide classes. Radiolabel was quantitated with an InstantImager scanner to determine the percent GTP vs. percent GDP-bound Ras. A: membrane Ras GTP levels ($n = 9$). B: cytosolic Ras GTP levels ($n = 9$). C: total GTP-bound Ras levels, membrane vs. cytosol ($n = 27$). Data are means ± SE from 3 separate experiments. Bars with different symbols represent treatments that are significantly different ($P < 0.05$).

**Fig. 9.** DHA incubation suppresses lipopolysaccharide-induced extracellular signal-regulated kinase (ERK)1/ERK2 activation. After fatty acid incubation (50 μM) for 72 h, cultures were serum deprived for 18 h and stimulated with lipopolysaccharide (1 μg/ml) for 10 min. Cell lysates were prepared, and activated p42/44 ERK levels were determined by immunoblotting with phosphospecific ERK antibody as described in MATERIALS AND METHODS. Data are means ± SE from 2 separate experiments ($n = 3–5$). Bars with different symbols represent treatments that are significantly different ($P < 0.05$).
DISCUSSION

At present, the molecular basis for the protective effect of fish oil on the incidence of colon cancer is a complete black box. In a previous study, we demonstrated that dietary fish oil modulates Ras intracellular localization by reducing its levels at the plasma membrane in colonocytes isolated from rats injected with carcinogen (16). This was associated with a suppression of colonic tumor development (10, 11). In the present study, we conducted a series of complementary experiments to investigate the mechanism(s) by which fish oil decreases Ras membrane localization. Specifically, we examined the biological properties of DHA (antitumorigenic), the major n-3 PUFA found in fish oil, compared with those of LA (protumorigenic), the major n-6 PUFA found in corn oil, in a mouse malignant transformed colonic cell line that overexpresses oncogenic H-Ras (YAMC-Ras). Because posttranslational processing is essential for Ras membrane anchoring, we examined whether purified fatty acids found in fish oil and corn oil affect protein farnesylation and/or palmitoylation. In addition, for the purpose of contrasting PUFA treatments, a third “control” group was supplemented with fatty acid-free BSA only. It is important to note, as a caveat to this approach, that uncomplexed BSA can serve as a fatty acid sink and therefore also can exert a biological effect distinct from fatty acid supplementation (54). Our data demonstrate that although the Ras membrane-to-cytosol ratio, an indication of activation state, was lower in DHA-treated cells (4.9) than in LA-treated cells (8.3), Ras farnesylation and palmitoylation were not affected. These data are consistent with our rat colon data (16) and indicate that long-chain PUFA do not modulate Ras posttranslational processing. Although the mechanism(s) responsible for these observations has not been elucidated, it is likely that distinct classes (n-3 vs. n-6) of PUFA can alter membrane structure, thereby influencing the interaction of Ras proteins with specific domains within the plasma membrane (58). Interestingly, compared with the untreated control, it appears as though LA treatment actually increases Ras membrane association. However, this type of contrast is difficult to interpret because, in vivo, there is no comparable control diet.

Ras activation is dependent on its binding to GTP (30). Ras is predominantly in the GDP-bound state, and upon interaction with the Grb2-SOS complex at the plasma membrane, GTP is exchanged for GDP (21, 50). Subsequently, it is cycled back to the GDP state by GTPase-activating protein (GAP) activity (56). Select fatty acids may affect the GTP/GDP binary switch by modulating GAP activity and function, which regulates hydrolysis of GTP to GDP and, hence, the Ras activation state (6, 21, 22). Interestingly, investigators have shown that AA is capable of inhibiting GAP activity (53, 62). Therefore, we further investigated the effect of DHA and LA (an AA precursor) on the fatty acid composition of membrane phospholipid classes. It is evident that DHA treatment enriches membrane phospholipid levels of DHA and eicosapentaenoic acid (EPA) and reduces AA, indicating that exogenous PUFA are incorporated into membrane lipids. This is consistent with previously published data where fish oil was fed to rats and was shown to decrease the AA content of colonic epithelial cell total phospholipids (35). The antagonism of AA by DHA is noteworthy in view of the documented inhibitory effect of AA on Ras GAPs (28, 53, 57). Because GAPs deactivate Ras, the anticipated outcome of a suppression of membrane AA content would be a decrease in the levels of GTP-bound (active) Ras in colonocytes. This is precisely what was observed in cells treated with DHA.

It is well established that in the active, GTP-bound state, Ras can activate the mitogen-activated protein kinase/ERK kinase pathway (9). Activated ERK kinases can translocate to the nucleus and regulate the activity of transcription factors such as Elk-1 (40). Membrane GTP-bound Ras additionally interacts with and activates the heterodimer enzyme PI 3-kinase (41). Increased PI 3-kinase activity results in the activation of Akt, a serine/threonine kinase (32, 41) that promotes cell survival (33). To further examine the influence of DHA on Ras activation and effector protein function, p42/44ERK activation was determined by phosphospecific immunoblotting after lipopolysaccharide stimulation. ERK activation was reduced by 63% in DHA- vs. LA-treated cells. Interestingly, DHA treatment did not block Akt activation, which is consistent with a lack of effect on the level of apoptosis. Collectively, these data indicate that DHA disrupts Ras-dependent signal transduction by suppressing plasma membrane localization, GTP binding, and p42/44ERK activation. This may partly explain why dietary fish oil protects against colon cancer development.

In conclusion, we have demonstrated that DHA (isolated from fish oil), when compared with LA (isolated from corn oil), lowers the activation of the Ras oncogene by 1) reducing Ras localization to the plasma membrane without affecting posttranslational processing, 2) suppressing levels of GTP-bound (activated) Ras at the plasma membrane, and 3) partially blocking downstream signal transduction. These observations are relevant because they corroborate previously published in vivo findings (16). In these studies, we demonstrated that dietary fish oil compared with corn oil downmodulates Ras intracellular localization by reducing its levels at the plasma membrane in colonocytes isolated from rats injected with azoxymethane (a colon carcinogen). This was associated with a suppression of colonic tumor development (10) and altered crypt cytokinetics (29). Because the in vivo vs. in vitro effects of DHA and LA on Ras intracellular localization are comparable, we propose that YAMC-Ras cells are a suitable model system to probe unresolved mechanistic questions. Further experiments with the use of quantitative immunofluorescence are required to determine the effect of PUFA classes on the precise intracellular localization of specific isoforms of Ras. In addition, whether other prenylated G proteins such as Rho...
GTPases are modulated by select PUFA merits further investigation.

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REFERENCES


