Eicosapentaenoic Acid and Docosahexaenoic Acid Block Serotonin-Induced Smooth Muscle Cell Proliferation

Rajabu Pakala, Rajashree Pakala, Wen Lu Sheng, Claude R. Benedict

Abstract—Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) present in fish oils have been ascribed as having significant antithrombotic and antiatherosclerotic effects. Vascular smooth muscle cell (SMC) proliferation plays an important role in the pathogenesis of atherosclerosis and restenosis. Recent studies have indicated that serotonin at concentrations present at sites of vascular injury stimulates SMC proliferation and may contribute to the restenotic process. In the present study we demonstrate that among the fatty acids tested, only EPA and DHA could block the mitogenic effect of serotonin on vascular SMC. Further, when added together these fatty acids act synergistically in blocking the mitogenic effect of serotonin. EPA and DHA blocked the 5HT-induced increase in the 5-HT2 receptor mRNA. This antimitogenic effect of EPA and DHA may partially explain some of the beneficial effects of fish oils.

Key Words: smooth muscle cell ■ eicosapentaenoic acid ■ docosahexaenoic acid ■ serotonin ■ restenosis

Coronary events appear to be less frequent among populations consuming large amounts of ω3 polyunsaturated fatty acids (fish oils).1,2 Clinical trials involving survivors of acute myocardial infarction have demonstrated a reduction in subsequent coronary events through an increase in the consumption of fish3 or ω3 fatty acids, a precursor of ω3 fatty acids derived from vegetable sources.4 However, the exact mechanism(s) of action of these complex heterogeneous compounds remain incompletely characterized. Among the diverse biological effects of ω3 fatty acids, favorable alterations of lipoprotein levels, changes in eicosanoid metabolism, and inhibition of platelet aggregation have been implicated in the prevention of atherosclerosis.5–8 Dietary ω3 fatty acids have also been shown to reduce experimental vascular lesion formation in dogs,9,10 swine,11,12 rabbits,13,14 and nonhuman primates.15,16 Yet the results of dietary ω3 fatty acids on restenosis in patients undergoing coronary angioplasty have been inconclusive.17,18

Restenosis after coronary angioplasty involves intimal proliferation of vascular smooth muscle cell (SMC), probably in response to mitogens released from aggregating platelets as well as from monocyte-derived macrophages that accumulate at the site of vascular injury. Platelets contain peptide growth factors like platelet derived growth factor (PDGF), epidermal growth factor (EGF), and transforming growth factor-β,19–21 and nonpeptide vasoactive compounds like serotonin (5HT), thromboxane A2 (TXA2), norepinephrine, histamine, bradykinin, and platelet activating factor.22,23 Recent studies from our laboratory and those of others indicate that some of these vasoactive compounds like 5HT and TXA2 are also mitogens to vascular SMC in culture,22–24,26 which suggests that vasoactive compounds may play an important role in the development of neointima. In the present study we investigated the putative mechanism by which eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), the active ingredients of fish oils, may inhibit the 5HT-induced vascular SMC proliferation.

Materials and Methods

Serotonin (as creatine sulfate), EDTA, pargyline, Hanks’ balanced salts (HBSS) Arachidonic acid ethyl ester (5, 8, 11, 14-eicosatetraenoic acid, AA, 20:4n-6), α-linolenic acid ethyl ester (9, 12, 15-octadecatrienoic acid, α-LA; 18:3n-3), γ-linolenic acid ethyl ester (6, 9, 12-octadecatrienoic acid, γ-LA; 18:3n-6), oleic acid ethyl ester (cis-9-octadecenoic acid, OA, 18:1n-9), and lysergic acid diethylamide (LSD) were obtained from Sigma Chemical Co; DMEM and fetal bovine serum (FBS) were obtained from Whittaker Bioproducts, Walkersville, Md; [3H]-thymidine (20 Ci/mole) and [3H]-LSD (N-methyl-[3H]-lysergic acid diethylamide), from New England Nuclear Corp, Boston, Mass. Other reagents were purchased from local vendors. EPA ethyl ester and DHA ethyl ester were provided by the United States Department of Commerce, National Oceanic and Atmospheric Administration, Charleston, SC.

Isolation, Culture, and Characterization of Primary Aortic SMCs

Primary canine aortic SMC were isolated using the explant method as described by Pakala et al.24 Briefly, the intima was first peeled off from the aorta and then the media carefully stripped away from the adventitia and placed in a petri dish containing warmed DMEM (37°C). The medial layer was cut into ∼1-mm squares, which were transferred into a 25 cm² tissue culture flask and barely covered with DMEM supplemented with 10% FBS. The blocks of tissue were cultured in a humidified atmosphere of 95% air and 5% CO₂ (vol/vol) at 37°C. After 1 to 2 weeks, the tissue blocks were removed and the migrated SMC were cultured. Following isolation, the

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identity of the SMC was confirmed by morphological examination and by staining for α-actin.

Subcultures of SMC were done once they became confluent, media from the plates was aspirated and the cells washed with 10 mL of phosphate buffered saline. Then, 2 to 3 mL of trypsin EDTA (0.05% trypsin, 0.53 mMol/L EDTA in CaCl2, MgCl2-free HBSS) was added to the cells and incubated at room temperature for 2 to 3 minutes. The action of trypsin was stopped by the addition of 7 to 8 mL of DMEM containing 10% FBS. The cells were collected by centrifugation at 150g for 10 minutes. After removing the supernatant, the pelleted cells were dispersed in 10 mL of DMEM containing 10% FBS and fresh cultures were initiated from these cells.

3[H]-Thymidine Incorporation
SMCs from passages 2 or 3 were seeded into 35-mm plates at a density of 65 000 to 75 000 cells/plate in DMEM containing 10% FBS, and allowed to proliferate for ∼72 hours. After 72 hours, the growth medium was replaced with 2 mL DMEM containing 1% FBS and incubated for ∼72 hours for arresting cell growth and synchronization. After growth arrest, the medium was replaced with 2 mL DMEM containing 1% FBS and 100 mMol/L pargyline. Where indicated, serotonin with pargyline and with or without the respective fatty acid (EPA, DHA, OA, α-LA, γ-LA) were added to each plate and then incubated for an additional 4 hours (24 hours after addition of compounds); the medium was removed and the plates washed 3 times with ice-cold phosphate-buffered saline. Then, 6% trichloroacetic acid was added to the cells and the acid insoluble thymidine collected on a glass fiber filter. The filters were washed with 100% ethanol, air dried, and [3H]-thymidine was quantified using a liquid scintillation counter. The cells were counted (Coulter counter) on the day of seeding, before changing to 0.1% FBS containing medium to ensure that they were growing, and finally on day 6 to determine that the cells were growth-arrested. All experiments were performed in triplicate.

Isolation of poly(A) RNA and Northern Blot Analysis
SMCs from 2 or 3 passage were seeded into 150-mm tissue culture plates in DMEM containing 10% FBS and allowed to proliferate for ∼72 hours. The growth medium was replaced with DMEM containing 0.1% FBS and incubated for ∼72 hours for arresting cell growth and synchronization. After growth arrest, cells were stimulated with DMEM containing 1% FBS and 100 mMol/L pargyline (control), or 1 μMol/L EPA or 1 μMol/L DHA with or without 5HT. After 24 hours of incubation, cells were rinsed twice with phosphate-buffered saline (PBS, 160 mMol/L NaCl, 1.5 mMol/L K2HPO4, 8 mMol/L Na2HPO4). Poly(A) RNA was isolated from SMC using the Oligotex mRNA isolation kit (Biotex Laboratories). Poly(A) RNA was denatured with formaldehyde and formamide and size-fractionated on a 0.66 mol/L formaldehyde/1.3% agarose gel for 3 to 4 hours at 80 V. RNA was transferred overnight onto a magnagraph nylon transfer membrane (MSD) by electro-blotting in 10×SSC (1×SSC contains 0.15 mol/L NaCl, 0.015 mol/L trisodium citrate). The RNA was cross-linked to the membrane at 120 000 μl using UV cross linker (Hoeffer Scientific Instruments) and prehybridized in 50% deionized formamide, 4ΧSSC, 20 mMol/L Tris HCl (pH 8.0), 1ΧDenhardt’s solution (0.02% ficoll, 0.02% polyvinyl pyrrolidine, 0.02% bovine serum albumin), 0.1% sodium dodecyl sulfate, 200 mg/mL denatured salmon sperm DNA, and 10% dextran sulfate for 30 minutes. 32P-labeled cRNA probe was synthesized using the full-length 5-HT2 receptor coding (obtained by Dr Julies) region by the MAXIscript kit (Ambion). Approximately 3 mg/mL of probe was added to the prehybridization solution, and hybridization was performed overnight at 42°C. Filters were washed 3 times for 20 minutes with 2ΧSSC containing 0.1% SDS at room temperature and 3 times for 20 minutes with 0.1ΧSSC containing 0.1% SDS at 60°C. The filters were then exposed to x-ray film at −80°C. For semi-quantification, filters were stripped of 5HT2 probe and reprobed with GAPDH. The relative quantity of 5HT2 mRNA in each sample was analyzed by densitometry using OPTIMUS program on a Toshiba Image analyzer and corrected for loading conditions by the quantity of rRNA for GAPDH. Results were expressed as densitometric units.

Scatchard Analysis for 5-HT2 Receptor Binding Studies
Primary SMC from 2 or 3 passage were seeded into 150-mm tissue culture plates in DMEM containing 10% FBS and allowed to proliferate for ∼72 hours. The growth medium was replaced with DMEM containing 0.1% FBS and cultured for another 72 hours for arresting cell growth and synchronization. After growth arrest, cells were stimulated with DMEM containing 1% FBS and 100 μMol/L pargyline (control), or 1 μMol/L EPA or 1 μMol/L DHA with or without 5HT. After 24 hours of incubation, SMC were rinsed twice with phosphate-buffered saline (PBS, 160 mMol/L NaCl, 1.5 mMol/L K2HPO4, 8 mMol/L Na2HPO4) and scraped into ice-cold homogenizing buffer (50 mMol/L Tris-HCl, 0.5 mMol/L Na2EDTA, 10 mMol/L MgSO4, pH7.4). Cells were homogenized and membranes pelleted at 30 000g for 15 minutes. Pellets were resuspended in homogenizing buffer, incubated at 37°C for 15 minutes, and centrifuged at 30 000g for 15 minutes; the supernatant was decanted and pellets stored at −45°C until use. Saturation analysis for 5-HT2 receptors was performed with 0.3 to 20 mMol/L [3H]-LSD. Nonspecific binding was determined in the presence of 100-fold excess concentration of unlabeled ligand. Assays were performed with ∼120 mg of cell membrane protein/assay (determined by Bradford method) in a final volume of 1 mL. The assay buffer was identical to homogenizing buffer described above, except that it contained 10 mMol/L pargyline and 0.1% ascorbic acid. Samples were incubated for 30 minutes at 25°C and filtered through glass fiber filters which had been presoaked in 0.1% polyethyleneimine for 30 minutes. Filters were washed rapidly with 10 mL of ice-cold 50 mMol/L Tris-HCl (pH7.0) and radioactivity that remained bound to the filters was measured using a liquid scintillation counter.

Statistical Analyses
Data were analyzed by 1-way ANOVA for each interaction. When a statistically significant difference was observed, further analysis were conducted using Scheffe’s post-hoc tests. For all comparisons, statistical significance was assumed as P<0.05. In each figure, mean values ±SD are shown. Radioligand binding results were analyzed by standard linear regression methods.

Results
Effect of 5HT on [3H]-Thymidine Incorporation by SMCs
The effect of increasing concentrations of 5HT on thymidine incorporation into the DNA of quiescent SMC was assessed. Figure 1 illustrates the increase in SMC proliferation as measured by [3H]-thymidine incorporation in the presence of 5HT. 5HT at an added concentration >10 μMol/L induced an increase in [3H]-thymidine incorporation. At 150 μMol/L of added 5HT, there was ∼4-fold increase in the amount of [3H]-thymidine incorporated into the DNA of SMC when compared with controls incubated with media alone and without fatty acids (P<0.01). However, at higher concentrations of added 5HT, there was a decrease in the amount of [3H]-thymidine incorporated.

Effect of Fatty Acids on [3H]-Thymidine Incorporation by SMCs
Next, the effect of ω3 fatty acids EPA and DHA or the effect of non-ω3 fatty acids AA, α-LA, γ-LA, and OA (as control fatty acids) on [3H]-thymidine incorporation into the DNA of growth-arrested SMC was measured. Incubation of SMC with α-LA, γ-LA, or OA at concentrations tested did not have any significant effect on [3H]-thymidine incorporation (Figure 2). In contrast, when growth-arrested SMC were incubated with AA, up to an added concentration of 1.67 μMol/L, AA did not have a significant effect on [3H]-thymidine incorporation. However, at higher concentrations, AA significantly induced [3H]-thymidine incorporation, resulting in approximately 3-fold increase to ∼16.7 μMol/L (Figure 2). When growth-
arrested SMC were incubated with EPA or DHA, EPA up to a concentration of 3.3 μmol/L and DHA up to a concentration of 7.5 μmol/L, did not have any significant effect on the 3[H]-thymidine incorporation (Figure 2). With concentrations higher than these values, there was a gradual decrease in the amount of [H]-thymidine incorporated into the DNA of SMC (Figure 2). At concentrations >75 μmol/L, both EPA and DHA appeared to be cytotoxic to SMC (data not shown).

Effect of ω3 Fatty Acids on Serotonin-Induced [H]-Thymidine Incorporation by SMCs

Growth-arrested SMC were incubated with different concentrations of EPA or DHA (0.1665 to 1.665 μmol/L along with mitogenic concentrations of 5HT, 50 to 200 μmol/L). EPA at a concentration of 0.33 μmol/L or greater (Figure 3A) and DHA at a concentration of 1.67 μmol/L or greater (Figure 3B), completely blocked the 5HT induced 3[H]-thymidine incorporation. These results suggest that EPA and DHA can block the proliferative effect of 5HT. In contrast, when SMC were incubated with the same concentrations of AA, α-LA, γ-LA, or OA along with mitogenic concentrations of 5HT, AA, γ-LA, α-LA, or OA did not inhibit 5HT-induced [H]-thymidine incorporation (Figure 4). These results suggest that the growth inhibitory effects of fatty acids on SMC appears to be specific for the ω3 class of fatty acids only.

Because both EPA and DHA are present in a 2:1 ratio in fish oils, we also examined the combined effect of EPA and DHA (at a ratio of 2:1) on 5HT-induced [H]-thymidine incorporation. Growth-arrested SMC were incubated with 0.11 or 0.22 μmol/L EPA or 0.055 or 0.11 μmol/L DHA along with mitogenic concentrations of 5HT (100 μmol/L to 200 μmol/L). The above concentrations of EPA or DHA did not inhibit the mitogenic effect of 5HT. However, when SMC were incubated with both EPA (0.11 μmol/L) and DHA (0.055 μmol/L) (total ω3 fatty acid concentration 0.165 μmol/L) and mitogenic concentrations of 5HT, the combination (0.165 μmol/L of EPA+DHA) could significantly inhibit the proliferative effects of 5HT (P<0.01) (Figure 5). With higher concentrations of ω3 fatty acids (0.22 μmol/L EPA+0.11 μmol/L DHA), mitogenic effect of 5HT was completely abolished (Figure 5). These results suggest...
that when EPA and DHA are present together they can act synergistically in reversing the proliferative effect of 5HT on SMC.

**Effect of ω3 Fatty Acids on 5-HT; Receptor mRNA Levels**

It has been shown that in vascular SMC, 5HT mediates its effects via the 5HT₂ receptors. Therefore, we examined whether the 5HT-induced mitogenic effect and its reversal by ω3 fatty acids was due to an alteration in the regulation of mRNA levels for 5HT₂ receptors. Growth-arrested SMC were incubated with medium alone (control) or with the same medium containing 5HT (50 μmol/L), EPA (1 μmol/L), or DHA (1 μmol/L) alone or EPA or DHA (1 μmol/L) with 5HT (50 μmol/L) for 24 hours. mRNA levels for 5HT₂ were determined as described in Methods. Incubation of SMC with 5HT resulted in a 100% increase in the 5HT₂ receptor mRNA levels as compared with the controls (Figure 6). Although EPA and DHA did not have a significant effect on 5HT₂ receptor mRNA levels, when SMC were stimulated with 5HT in the presence of EPA, the 5HT-induced increase in mRNA for 5HT₂ receptor was significantly blunted. Similarly, in the presence of DHA, 5HT-induced increase in 5HT₂ mRNA levels was limited to ~35% (Figure 6). These results indicate that one of the mechanism for the effect of EPA and DHA could be to produce a decrease in mRNA for 5HT₂ receptors in vascular SMC.

**Effect of ω3 Fatty Acids on 5HT₂ Receptor Number**

We also examined the effect of ω3 fatty on 5HT₂ receptor numbers. Growth-arrested SMC were incubated with medium alone (control) or the same medium containing 5HT (50 μmol/L), EPA (1 μmol/L), or DHA (1 μmol/L) alone or EPA or DHA with 5HT for 24 hours. Scatchard analysis revealed that despite the alteration in regulation of 5HT₂ mRNA levels by 5HT and EPA or DHA, there were no significant differences in the steady state levels of 5HT₂ receptors (Figure 7).

**Discussion**

Platelets aggregate in areas of endothelial dysfunction (atherosclerosis) or at sites of vascular injury such as those following percutaneous transluminal angioplasty. These aggregating platelets release peptide growth factors like PDGF from alpha granules and nonpeptide growth factors like 5HT and adenosine diphosphate (ADP) from dense granules. Recent studies from our group and others have indicated that nonpeptide growth factors like 5HT, TXA₂, and ADP can stimulate vascular SMC to proliferate. These nonpeptide growth factors have been shown to act as amplification factors to known peptide growth factors like PDGF and EGF and also act...
synergistically among themselves to stimulate SMC proliferation. SMC migration and proliferation contributes to the intimal hyperplasia of the arterial wall, which is the main pathological feature underlying the development of the fibrofatty atherosclerotic lesions and neointima that develops following percutaneous transluminal angioplasty. Fish oils exert antiatherosclerotic effects as demonstrated in a number of epidemiological and clinical studies. Diets rich in fish oils have been shown to reduce vascular lesion formation following endarterectomy or intimal hyperplasia in autologous vein grafts. Depending on the regimen used, fish oil supplementation has also been shown to reduce restenosis following angioplasty. Several investigators have reported that polyunsaturated fatty acids like EPA and DHA generally inhibit the proliferation of cells in culture mainly through the formation of free radicals. Although free radicals are known to be cytotoxic to different types of cells, recent studies have indicated that these molecules at low concentrations can also promote SMC proliferation. In this study, we demonstrate that 5HT-induced \(^{3}\)H-thymidine incorporation was dose-dependently inhibited by both EPA and DHA at concentrations that did not induce free radical formation. Even at very low concentrations (0.3 \(\mu\)mol/L), both EPA and DHA inhibited 5HT-induced \(^{3}\)H-thymidine incorporation by 60% to 70% and completely abolished the effect at a concentration of 1.6 \(\mu\)mol/L EPA or DHA. At lower concentrations, EPA was more potent than DHA in inhibiting the 5HT-induced \(^{3}\)H-thymidine incorporation. The inhibitory effect on 5HT-induced \(^{3}\)H-thymidine incorporation was specific for \(\omega_3\) fatty acids EPA and DHA only, because \(\omega_6\)-LA (n-3) AA, \(\omega_6\)-LA (n-6), or OA (n-9) failed to block the 5HT-induced proliferative effect. The inhibitory effect of EPA and DHA was not due to cytotoxicity, as cells were morphologically intact and resumed normal growth after transferring to serum containing medium without \(\omega_3\) fatty acids (data not shown).

The concentrations of EPA and DHA used by Shiina et al may have produced cytotoxic concentrations of oxidized species, thus resulting in inhibition of vascular SMC proliferation. Several investigators have reported that polyunsaturated fatty acids like EPA and DHA generally inhibit the proliferation of cells in culture mainly through the formation of free radicals. Although free radicals are known to be cytotoxic to different types of cells, recent studies have indicated that these molecules at low concentrations can also promote SMC proliferation. In this study, we demonstrate that 5HT-induced \(^{3}\)H-thymidine incorporation was dose-dependently inhibited by both EPA and DHA at concentrations that did not induce free radical formation. Even at very low concentrations (0.3 \(\mu\)mol/L), both EPA and DHA inhibited 5HT-induced \(^{3}\)H-thymidine incorporation by 60% to 70% and completely abolished the effect at a concentration of 1.6 \(\mu\)mol/L EPA or DHA. At lower concentrations, EPA was more potent than DHA in inhibiting the 5HT-induced \(^{3}\)H-thymidine incorporation. The inhibitory effect on 5HT-induced \(^{3}\)H-thymidine incorporation was specific for \(\omega_3\) fatty acids EPA and DHA only, because \(\omega_6\)-LA (n-3) AA, \(\omega_6\)-LA (n-6), or OA (n-9) failed to block the 5HT-induced proliferative effect. The inhibitory effect of EPA and DHA was not due to cytotoxicity, as cells were morphologically intact and resumed normal growth after transferring to serum containing medium without \(\omega_3\) fatty acids (data not shown).

Further, because of the synergistic interaction between EPA and DHA when used in combination, very low concentration of EPA and DHA are necessary to demonstrate the same beneficial effects.

Figure 6. Northern blot analysis for 5-HT2 receptor in SMC. mRNA was isolated from growth-arrested aortic SMC incubated with 1% FBS and 100 \(\mu\)mol/L pargyline containing medium (control) or the same medium containing 5HT, EPA, or DHA alone, or EPA+5HT or DHA+5HT and used for northern blot analysis. A, Expression of mRNA for the 5HT2 receptor and panel; B, mRNA for GAPDH; and C, densitometric ratio of the 5-HT2 receptor mRNA normalized to loading conditions with mRNA for GAPDH.

Figure 7. Scatchard analysis of 5-HT2 receptor number in aortic SMC. Cell membranes were isolated from growth-arrested aortic SMC incubated with 1% FBS and 100 \(\mu\)mol/L pargyline containing medium (control) or the same medium with 5HT, EPA, or DHA alone, or EPA+5HT or DHA+5HT and used for Scatchard analysis. \(^{3}\)H-LSD was used as radioligand for 5-HT2 receptors. Nonspecific binding was determined in the presence of 100-fold excess of unlabeled LSD.
In vascular SMC, 5HT mediates its effects via the 5HT₂ receptor.³⁵ Incubation of SMC with 5HT resulted in an upregulation of 5HT₂ receptor mRNA levels. Ligand-induced upregulation of 5HT₂ receptor RNA has been demonstrated in myometrial SMC.³⁶ Ligand-mediated upregulation of receptor mRNA levels has also been reported for EGF and interleukin-2.³⁷,³⁸ Incubation of SMC with EPA or DHA downregulated the 5HT₂ receptor mRNA. Similarly, when SMC were incubated with EPA or DHA along with 5HT, there was a partial decrease in 5HT-induced 5HT₂ receptor mRNA levels, indicating that EPA and DHA may inhibit the 5HT-induced SMC proliferation by downregulating the 5HT₂ receptor mRNA levels. Surprisingly, the changes in the 5HT₂ receptor mRNA levels brought about by 5HT and EPA or DHA were not followed by a corresponding change in the 5HT₂ receptor affinity or numbers. Fitzgerald et al.³⁶ have reported that in uterine SMC, 5HT and its analogues upregulated the 5HT₂ receptor mRNA levels and antagonists downregulated the 5HT₂ receptor mRNA levels. However, in both instances they did not observe a significant difference in the affinity for the ligand or 5HT₂ receptor numbers.³⁶ Changes in the levels of mRNA without analogous changes in the corresponding protein has also been shown for other receptors, like transcription and translation.³⁰ Several studies, including the classic experiments of Davis et al.¹⁵ and Weiner et al.¹¹ have shown that fish oil feeding inhibits the development of atherosclerosis. The present study may explain the mechanism by which fish oils exert their beneficial effects.

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References


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