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

Rajbabu 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-HT<sub>2</sub> receptor mRNA. This anti-mitogenic 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 α<sub>3</sub> polyunsaturated fatty acids (fish oils).<sup>1,2</sup> Clinical trials involving survivors of acute myocardial infarction have demonstrated a reduction in subsequent coronary events through an increase in the consumption of fish<sup>3</sup> or ω-3 fatty acids, a precursor of ω-3 fatty acids derived from vegetable sources.<sup>4</sup> However, the exact mechanism(s) of action of these complex heterogeneous compounds remain incompletely characterized. Among the diverse biological effects of ω-3 fatty acids, favorable alteration of lipoprotein levels, changes in eicosanoid metabolism, and inhibition of platelet aggregation have been implicated in the prevention of atherosclerosis.<sup>5–8</sup> Dietary ω-3 fatty acids have also been shown to reduce experimental vascular lesion formation in dogs,<sup>9,10</sup> swine,<sup>11,12</sup> rabbits,<sup>13,14</sup> and nonhuman primates.<sup>15,16</sup> Yet the results of dietary ω-3 fatty acids on restenosis in patients undergoing coronary angioplasty have been inconclusive.<sup>17,18</sup>

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<sup>19–21</sup> and nonpeptide vasoactive compounds like serotonin (5HT), thromboxane A<sub>2</sub>, (TXA<sub>2</sub>), norepinephrine, histamine, bradykinin, and platelet activating factor.<sup>22,23</sup> Recent studies from our laboratory and those of others indicate that some of these vasoactive compounds like 5HT and TXA<sub>2</sub> are also mitogens to vascular SMC in culture,<sup>22,24–26</sup> 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), ω-6-linolenic acid ethyl ester (9, 12, 15-octadecatetraenoic acid, ω-6-LA; 18:3n-3), ω-6-linolenic acid ethyl ester (9, 12-octadecatetraenoic acid, ω-6-LA, 18:3n-6), oleic acid ethyl ester (cis-9-octadecenoic acid, OA, 18:1n-9), and lysyrgic acid diethylamide (LSD) were obtained from Sigma Chemical Co; DMEM and fetal bovine serum (FBS) were obtained from Whittaker Bioproducts, Walkersville, Md; <sup>3</sup>H-thymidine (20 Ci/mole) and <sup>3</sup>H-LSD (N-methyl-[3H]-lysylgic 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.<sup>24</sup> 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 cm<sup>2</sup> 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% CO<sub>2</sub> (vol/vol) at 37°C. After 1 to 2 weeks, the tissue blocks were removed and the migrated SMC were cultured. Following isolation, the
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 mM/L EDTA in Ca"+ Mg"+-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.

\[ \text{3} \text{H]-Thymidine Incorporation} \]

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 \( \approx \)72 hours. After 72 hours, the growth medium was replaced with 2 mL DMEM containing 10% FBS and incubated for \( \approx \)72 hours for arresting cell growth and synchronization. After growth arrest, the medium was replaced with 2 mL DMEM containing 1% FBS and 100 μM/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 the medium. After 20 hours of incubation, 1 μCi of [\( \text{3} \text{H} \text{-thymidine} \) was 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 [\( \text{3} \text{H} \text{-thymidine} \) 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.

\[ \text{Isolation of pol(A) RNA and Northern Blot Analysis} \]

SMCs from 2 or 3 passage were seeded into 150-mm tissue culture plates in DMEM containing 10% FBS and allowed to proliferate for \( \approx \)72 hours. The growth medium was replaced with DMEM containing 10% FBS, and allowed to proliferate for \( \approx \)72 hours. After 72 hours, the growth medium was replaced with 2 mL DMEM containing 1% FBS and 100 μM/L pargyline (control), or 1 μM/L EPA or 1 μM/L DHA with or without 5HT. After 24 hours of incubation, the cells were rinsed twice with phosphate-buffered saline (PBS, 160 mM/L NaCl, 1.5 mM/L KCl, 8 mM/L Na, 8 mM/L HPO4), and scraped into ice-cold homogenizing buffer (50 mM/L Tris-HCl, 0.5 mM/L Nao EDTA, 10 mM/L MgSO4, pH 7.4). 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 recentrifuged at 30,000g for 15 minutes; the supernatant was decanted and pellets stored at \(-45°C \) until use. Saturation analysis for 5HT receptors was performed with 0.3 to 20 μM/L [\( \text{3} \text{H} \text{-LSD} \) at \( \text{3} \text{H} \text{-thymidine incorporation} \) was assayed in the presence of unlabeled ligand. Assays were performed with \( \approx \)120 μg 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 mM/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 mM/L Tris-HCl (pH 7.0) and radioactivity that remained bound to the filters was measured using a liquid scintillation counter.

\[ \text{Statistical Analyses} \]

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 \( P \leq 0.05 \). In each figure, mean values ±SD are shown. Radioligand binding results were analyzed by standard linear regression methods.

\[ \text{Results} \]

\text{Effect of 5HT on [\( \text{3} \text{H} \text{-thymidine} \) 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 [\( \text{3} \text{H} \text{-thymidine} \) incorporation in the presence of 5HT. 5HT at an added concentration >10 μM/L induced an increase in [\( \text{3} \text{H} \text{-thymidine} \) incorporation. At 150 μM/L of added 5HT, there was \approx 4-fold increase in the amount of [\( \text{3} \text{H} \text{-thymidine} \) 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 [\( \text{3} \text{H} \text{-thymidine} \) incorporated.

\[ \text{Effect of Fatty Acids on [\( \text{3} \text{H} \text{-thymidine} \) Incorporation by SMCs} \]

Next, the effect of \( \omega \) fatty acids EPA and DHA or the effect of non-\( \omega \) fatty acids AA, α-LA, γ-LA, and OA (as control fatty acids) on [\( \text{3} \text{H} \text{-thymidine} \) 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 [\( \text{3} \text{H} \text{-thymidine} \) incorporation (Figure 2). In contrast, when growth-arrested SMC were incubated with AA, up to an added concentration of 1.67 μM/L, AA did not have a significant effect on [\( \text{3} \text{H} \text{-thymidine} \) incorporation. However, at higher concentrations, AA significantly induced [\( \text{3} \text{H} \text{-thymidine} \) incorporation, resulting in approximately 3-fold increase to \approx 16.7 μM/L (Figure 2). When growth-
arrested SMC were incubated with EPA or DHA, EPA up to a concentration of 3.3 \( \mu \text{mol/L} \) and DHA up to a concentration of 7.5 \( \mu \text{mol/L} \), did not have any significant effect on the \(^{3}\text{H}\)-thymidine incorporation (Figure 2). With concentrations higher than these values, there was a gradual decrease in the amount of \(^{3}\text{H}\)-thymidine incorporated into the DNA of SMC (Figure 2). At concentrations >75 \( \mu \text{mol/L} \), both EPA and DHA appeared to be cytotoxic to SMC (data not shown).

**Effect of \( \omega3 \) Fatty Acids on Serotonin-Induced \(^{3}\text{H}\)-Thymidine Incorporation by SMCs**

Growth-arrested SMC were incubated with different concentrations of EPA or DHA (0.1665 to 1.665 \( \mu \text{mol/L} \) along with

mitogenic concentrations of 5HT, 50 to 200 \( \mu \text{mol/L} \)). EPA at a concentration of 0.33 \( \mu \text{mol/L} \) or greater (Figure 3A) and DHA at a concentration of 1.67 \( \mu \text{mol/L} \) or greater (Figure 3B), completely blocked the 5HT induced \(^{3}\text{H}\)-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, \( \alpha \)-LA, \( \gamma \)-LA, or OA along with mitogenic concentrations of 5HT, AA, \( \gamma \)-LA, \( \alpha \)-LA, or OA did not inhibit 5HT-induced \(^{3}\text{H}\)-thymidine incorporation (Figure 4). These results suggest that the growth inhibitory effects of fatty acids on SMC appears to be specific for the \( \omega3 \) 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 \(^{3}\text{H}\)-thymidine incorporation (Figure 5). With higher concentrations of \( \omega3 \) fatty acids (0.22 \( \mu \text{mol/L} \) EPA + 0.11 \( \mu \text{mol/L} \) DHA), mitogenic effect of 5HT was completely abolished (Figure 5). These results suggest

![Figure 1](http://atvb.ahajournals.org/)

**Figure 1.** Concentration-dependent stimulation of aortic SMC proliferation by serotonin. \(^{3}\text{H}\)-thymidine incorporation into DNA was measured in growth-arrested aortic SMC stimulated by varying concentrations of serotonin (5HT) in 1% FBS in the presence of 100 \( \mu \text{mol/L} \) pargyline, as described in Methods. One hundred percent equals the baseline value of \(^{3}\text{H}\)-thymidine uptake; 100% = 17551 ± 487 cpm/10\(^6\) cells. Experiments were performed with 2 different batches of cells and each batch was tested in triplicate. Results are mean ± SD.

![Figure 2](http://atvb.ahajournals.org/)

**Figure 2.** Effect of fatty acids on SMC growth. Varying concentrations of eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA), arachidonic acid (AA), oleic acid (OA), \( \alpha \)-linolenic acid (\( \alpha \)-LA), or \( \gamma \)-linolenic acid (\( \gamma \)-LA) in 1% FBS and 100 \( \mu \text{mol/L} \) pargyline containing DMEM were added to growth-arrested aortic SMC and the amount of \(^{3}\text{H}\)-thymidine incorporated into the DNA measured as described in Methods. Baseline value of \(^{3}\text{H}\)-thymidine uptake is 100; 100% = 16430 ± 590 cpm/10\(^6\) cells. Experiments were performed with 2 different batches of cells and each batch was tested in triplicate. Results are mean ± SD.

![Figure 3](http://atvb.ahajournals.org/)

**Figure 3.** Interaction between serotonin and eicosapentaenoic acid (A) and docosahexaenoic acid (B) in modulating SMC growth. Growth-arrested aortic SMC were incubated with indicated concentrations of serotonin (5HT) and eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) in 1% FBS and 100 \( \mu \text{mol/L} \) pargyline containing DMEM; the amount of \(^{3}\text{H}\)-thymidine incorporated was determined as described in Methods. Baseline value of \(^{3}\text{H}\)-thymidine uptake is 100. 100% = 16942 ± 467 cpm/10\(^6\) cells for A, 100% = 1712 ± 560 CPM/10\(^6\) cells for B. Experiments were performed with 2 different batches of cells and each batch was tested in triplicate. Results are mean ± SD. *P < 0.05, **P < 0.01.
that when EPA and DHA are present together they can act synergistically in reversing the proliferative effect of 5HT on SMC.

**Effect of \( \omega-3 \) Fatty Acids on 5-HT \(_2\) Receptor mRNA Levels**

It has been shown that in vascular SMC, 5HT mediates its effects via the 5HT\(_2\) receptors. Therefore, we examined whether the 5HT-induced mitogenic effect and its reversal by \( \omega-3 \) fatty acids was due to an alteration in the regulation of mRNA levels for 5HT\(_2\) receptors. Growth-arrested SMC were incubated with medium alone (control) or with the same medium containing 5HT (50 \( \mu \)mol/L), EPA (1 \( \mu \)mol/L), or DHA (1 \( \mu \)mol/L) alone or EPA or DHA (1 \( \mu \)mol/L) with 5HT (50 \( \mu \)mol/L) for 24 hours. mRNA levels for 5HT\(_2\) were determined as described in Methods. Incubation of SMC with 5HT resulted in \( \approx 100\% \) increase in the 5HT\(_2\) receptor mRNA levels as compared with the controls (Figure 6). Although EPA and DHA did not have a significant effect on 5HT\(_2\) receptor mRNA levels, when SMC were stimulated with 5HT in the presence of EPA, the 5HT-induced increase in mRNA for 5HT\(_2\) receptor was significantly blunted. Similarly, in the presence of DHA, 5HT-induced increase in 5HT\(_2\) mRNA levels was limited to \( \approx 35\% \) (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 5HT\(_2\) receptors in vascular SMC.

**Effect of \( \omega-3 \) Fatty Acids on 5HT\(_2\) Receptor Number**

We also examined the effect of \( \omega-3 \) fatty on 5HT\(_2\) receptor numbers. Growth-arrested SMC were incubated with medium alone (control) or the same medium containing 5HT (50 \( \mu \)mol/L), EPA (1 \( \mu \)mol/L), or DHA (1 \( \mu \)mol/L) alone or EPA or DHA (1 \( \mu \)mol/L) with 5HT (50 \( \mu \)mol/L) for 24 hours. Scatchard analysis revealed that despite the alteration in regulation of 5HT\(_2\) mRNA levels by 5HT and EPA or DHA, there were no significant differences in the steady state levels of 5HT\(_2\) 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.\(^{22,23}\) Recent studies from our group and others have indicated that nonpeptide growth factors like 5HT, TXA\(_2\), and ADP can stimulate vascular SMC to proliferate.\(^{22,24–26}\) These nonpeptide growth factors have been shown to act as amplification factors to known peptide growth factors like PDGF and EGF.\(^{24,25,26}\) and also act...
synergistically among themselves to stimulate SMC proliferation.\textsuperscript{24} 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 number of epidemiological and clinical studies.\textsuperscript{27,28} Diets rich in fish oils have been shown to reduce vascular lesion formation following endarterectomy\textsuperscript{16} or intimal hyperplasia in autologous vein grafts.\textsuperscript{9,10} Depending on the regimen used, fish oil supplementation has also been shown to reduce restenosis following angioplasty.\textsuperscript{17,18} All these beneficial effects of fish oils have been ascribed to the \(\omega_3\) fatty acids EPA and DHA present in the fish oils. Despite the epidemiological and clinical evidence to suggest that EPA and DHA may reduce SMC proliferation, there are very few studies to indicate this at the cellular level. This is the first study to demonstrate that EPA and DHA inhibit 5HT-induced SMC proliferation at concentrations that did not induce free radical formation. Even at very low concentrations (0.3 \textmu mol/L), both EPA and DHA inhibited 5HT-induced \([H]-\text{thymidine incorporation by 60}\%\) to 70\% and completely abolished the effect at a concentration of 1.6 \textmu mol/L EPA or DHA. At lower concentrations, EPA was more potent than DHA in inhibiting the 5HT-induced 3\textsuperscript{[H]}-thymidine incorporation. The inhibitory effect on 5HT-induced 3\textsuperscript{[H]}-thymidine incorporation was specific for \(\omega_3\) fatty acids EPA and DHA only, because \(\alpha\)-LA (n-3) AA, \(\gamma\)-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.
In vascular SMC, 5HT mediates its effects via the 5HT$_2$ receptor.\textsuperscript{35} Incubation of SMC with 5HT resulted in an upregulation of 5HT$_2$ receptor mRNA levels. Ligand-induced upregulation of 5HT$_2$ receptor mRNA has been demonstrated in myometrial SMC.\textsuperscript{36} Ligand-mediated upregulation of receptor mRNA levels has also been reported for EGF and interleukin-2.\textsuperscript{37,38} Incubation of SMC with EPA or DHA downregulated the 5HT$_2$ receptor mRNA. Similarly, when SMC were incubated with EPA or DHA along with 5HT, there was a partial decrease in 5HT-induced 5HT$_2$ receptor mRNA levels, indicating that EPA and DHA may inhibit the 5HT-induced SMC proliferation by downregulating the 5HT$_2$ receptor mRNA levels. Surprisingly, the changes in the 5HT$_2$ receptor mRNA levels brought about by 5HT and EPA or DHA were not followed by a corresponding change in the 5HT$_2$ receptor affinity or numbers. Fitzgerald et al.\textsuperscript{39} have reported that in uterine SMC, 5HT and its analogues upregulated the 5HT$_2$ receptor mRNA levels and antagonists downregulated the 5HT$_2$ receptor mRNA levels. However, in both instances they did not observe a significant difference in the affinity for the ligand or 5HT$_2$ receptor numbers.\textsuperscript{36} Changes in the levels of mRNA without analogous changes in the corresponding protein has also been shown for other receptors, like EGF and interleukin-2.\textsuperscript{37,38} and in some instances this was found to be the result of specific processes regulating receptor turnover.\textsuperscript{39} Moreover, the disparity between mRNA levels and receptor densities could be the result of mechanisms regulating distinct steps of protein synthesis like transcription and translation.\textsuperscript{40} Several studies, including the classic experiments of Davis et al.\textsuperscript{15} and Weiner et al.\textsuperscript{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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