Vascular smooth muscle cells preloaded with eicosapentaenoic acid and docosahexaenoic acid fail to respond to serotonin stimulation

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Received 14 July 1999; received in revised form 16 November 1999; accepted 19 January 2000

Abstract

Epidemiological, animal and clinical studies indicate that n-3 fatty acids may benefit individuals with known history of cardiovascular disease or at risk of developing it. Though there is indirect evidence to suggest that the beneficial effects of n-3 fatty acids may be because of their ability to inhibit smooth muscle cell (SMC) proliferation, there are no studies that have examined this hypothesis. In this study, the mitogenic effect of serotonin (5HT) and platelet derived growth factor (PDGF), known mitogens for vascular SMC, on aortic SMCs preloaded with eicosapentaenoic acid (EPA) and/or docosahexaenoic acid (DHA) is examined. 5HT and PDGF could only partially stimulate proliferation of SMC that were preloaded with EPA or DHA as compared to the control cells. γ-Linolenic acid (LA) and oleic acid (OA) did not block the 5HT or PDGF induced 3[H]thymidine incorporation suggesting that the anti-proliferative effect was specific to n-3 fatty acids only. Further, when EPA and DHA were combined in the ratio they are present in fish oils, there was a synergistic interaction in inhibiting the proliferation of SMC. Further, SMC grown in the presence of EPA or DHA, when stimulated with 5HT, failed to show an increase in 5HT2 receptor mRNA. One of the potential mechanism by which fish oils may prevent the development of atherosclerosis or restenosis could be inhibition of the mitogen induced SMC proliferation. Combination of EPA with DHA is likely to be more beneficial. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Eicosapentaenoic acid; Docosahexaenoic acid; Vascular smooth muscle cells; Atherosclerosis; Restenosis

1. Introduction

Evidence from epidemiological [1–4], biochemical [5–8], animal [9–16] and clinical studies [17–19] indicate that dietary n-3 fatty acids may benefit individuals with established atherosclerosis or at risk of developing it, including patients undergoing interventional procedures for symptomatic coronary artery disease. Populations consuming traditional diets rich in n-3 fatty acids, or western diets supplemented with n-3 fatty acids, primarily C20: 5n-3, eicosapentaenoic acid (EPA) and C22:6 n-3 docosahexaenoic acid (DHA) exhibit characteristic biochemical and functional changes including decreased serum triglycerides, variable decrease in total serum cholesterol and an increase in high density lipoprotein cholesterol [5,6], altered cell membrane composition [8], decreased blood viscosity [7] and impaired platelet Hemostatic functions [7,8]. Although the reduction in thrombotic vascular events has been attributed to the metabolic effects of substituting n-3 fatty acids for arachidonic acid and the generation of eicosanoid products that modify platelet and vascular functions, animal experiments and clinical data provide only indirect evidence in this regard. Dietary n-3 fatty acids reduce experimental vascular lesion formation in dogs [10,20], swine [11,12], rabbits [13,14] and non-human primates [15,16], and increased fish consumption has been associated with decreased mortality from coronary artery disease [17,19–21]. However, the effects of dietary n-3 fatty acids on restenosis in patients undergoing coronary angioplasty has been inconclusive [22,23]. Restenosis following angioplasty involves migration and proliferation of vascular smooth muscle cells (SMC) probably in response to mitogens released from: (a) aggregating platelets [24,25]; (b) monocyte that are

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derived from macrophages accumulating at the injured site [26], and from endothelial cells subjected to injury [27,28]. In vitro cell culture and in vivo animal studies have indicated that the vasoactive compounds like serotonin (5HT) and thromboxane A₂ released from the aggregating platelets may play a major role in the development of neointima [29–32]. These compounds have been shown not only to act as mitogens by themselves but also act as amplification factors for known peptide growth factors as platelet derived growth factor (PDGF) and fibroblast growth factor (FGF) [24,32]. Recently, it has been reported that 5HT and thromboxane A₂ can also act synergistically among themselves in inducing SMC proliferation [31]. The objective of the present study is to find out whether preloading of SMC with EPA and/or docosahexaenoic acid (DHA) (n-3 fatty acids present in fish oils) makes them less responsive to mitogens that are known to play an important role in the development of atherosclerosis and restenosis. Results of this study indicate that SMC grown in the presence of (EPA) and/or (DHA) are non-responsive to 5HT and PDGF stimulation. This suggests that one of the mechanisms by which fish oils may reduce the neointima formation is by inhibiting the SMC responsiveness to mitogenic factors released by aggregating platelets.

2. Materials and methods

2.1. Materials

5HT (as creatine sulphate), ethylene diamine tetraacetic acid (EDTA), pargyline, Hank’s balanced salts (HBSS), γ-linolenic acid (6,9,12-octadecatrienoic acid, LA), oleic acid (cis-9 octadecenoic acid, OA) and lysergic acid diethylamide (LSD) were obtained from Sigma, St. Louis, MO; Dulbecco’s modified Eagles medium (DMEM) and fetal bovine serum (FBS) were obtained from Whittaker Bioproducts, Walkersville, MD; Dulbecco’s modified Eagles medium (DMEM) and fetal bovine serum (FBS) were obtained from Amersham Life Science, Arlington Heights, IL. [3H]thymidine (20 Ci/mol) and [3H]LSD (N-methyl-[3H]lysergic acid diethylamide from New England Nuclear, Boston, MA). Other reagents were purchased from local vendors. EPA and DHA were provided by the United States Department of Commence, National Oceanic and Atmospheric Administration, Charleston, SC.

2.2. Isolation, culture and characterization of canine primary aortic SMC

Canine primary aortic SMCs were isolated using the explant method described by Pakala et al. [31]. Briefly the intima was first peeled off from the aorta and then the media carefully stripped away from the adventitia and placed in a petridish containing warmed DMEM (37°C). The medial layer was cut into approximately 1 mm squares, which were transferred into a 25 cm² tissue culture flask and barely covered with DMEM supplemented with 20% FBS. The blocks of tissue were cultured in a humidified atmosphere of 95% air and 5% CO₂ (vol/vol) at 37°C. After 1–2 weeks, the tissue blocks were removed and the migrated SMCs were cultured. Following isolation, the identity of the SMC was confirmed by morphological examination and by staining for α-actin.

Subcultures of SMC done once they became confluent, media from the plates aspirated and the cells washed with 10 ml of phosphate buffered saline (PBS). Then 2–3 ml of trypsin–EDTA (0.05% trypsin, 0.53 mM EDTA in Ca²⁺, Mg²⁺ free HBSS) were added to the cells and incubated at room temperature for 2–3 min. The action of trypsin was stopped by the addition of 7–8 ml of DMEM containing 10% FBS. The cells were collected by centrifugation at 150 × g for 10 min. After removing the supernatant, the pellets cells were dispersed in 10 ml of DMEM containing 10% FBS and fresh cultures were initiated from these cells.

2.3. [3H]Thymidine incorporation

SMCs from passages 2 or 3 were seeded into 35 mm diameter plates at a density of 65,000–75,000 cells/plate in DMEM containing 10% FBS and allowed to proliferate for 72 h. After 72 h, the growth medium was replaced with 2 ml DMEM containing 0.1% FBS and incubated for an additional 72 h for growth arrest and synchronization. After growth arrest cells were stimulated with serum free medium containing 500 μg/ml bovine serum albumin (BSA), 10 μg/ml bovine insulin, 25 ng/ml selenium, 0.2 nM ascorbate and 100 mM pargyline or the same medium with indicated concentrations of 5HT or PDGF. After 20 h of incubation with 5HT or PDGF, 1 μCi of [3H]thymidine was added to each plate and then incubated for an additional 4 h. Twenty-four hours after addition of compounds, the medium was removed and the plates washed three times with ice cold PBS. Then 6% trichloroacetic acid was added to the cells and the acid insoluble thymidine collected on to glass fiber filter. The filters were washed with 100% ethanol, air dried and the amount of [3H]thymidine incorporated into the DNA was determined, using a liquid scintillation counter. In another set of experiment SMC were seeded in DMEM containing 10% FBS and different concentrations of EPA, DHA, OA or LA, and allowed to proliferate for approximately 72 h. After 72 h, the growth medium was replaced with 2 ml DMEM containing 0.1% FBS and the same concentrations of fatty acid in which they were grown previously and incubated for an additional 72 h for growth arresting and synchronization. After
growth arrest, the cells were stimulated with 1% FBS containing medium and the amount of [H]thymidine incorporated into the DNA determined as described earlier. In a third set of experiment SMC were growth arrested and synchronized in non-cytotoxic concentrations of EPA, DHA, OA or LA and then stimulated with mitogenic concentrations of 5HT or PDGF in serum free medium and the amount of [H]thymidine incorporated into the DNA measured as described earlier. The cells were counted (Coulter counter) on the day of seeding and before changing to 0.1% FBS containing medium to ensure that they were growing normally, and finally on day 6 to determine that the cells were growth arrested. All experiments were performed in triplicate and each experiment was repeated minimum 3 times.

2.4. Determination of SMC number

After preloading the SMC with fatty acids as described in the [H]thymidine incorporation section, cells were stimulated with either 5HT or PDGF. Twenty-four hours after stimulation, 0.4 ml of 2% (w/v) crude pancreatic trypsin in Dulbecco’s PBS containing 152 mM EDTA was added to each dish. The dishes were incubated at room temperature for 2 min before addition of 0.8 ml of horse serum. The contents of each dish were diluted to 20 ml with isotone II (Coulter Electronics) and the cell number determined by using a Coulter counter. Triplicate counts were taken for each plate and quadruplicate plates were used for each determination.

2.5. Isolation of poly(A+) RNA and Northern blot analysis

After preloading SMC with EPA and DHA as described earlier cells were stimulated with serum free medium alone or with serum free medium containing 100 μM 5HT. Twenty-four hours after stimulation cells were rinsed twice with PBS, and mRNA isolated using the ‘on track’ mRNA isolation kit (Biotex Laboratories, Houston, TX) and 5HT2 receptor mRNA quantified Poly(A+) RNA was denatured with formaldehyde and formamide and size-fractionated on a 0.66 M formaldehyde/1.3% agarose gel for 304 h at 80 AV RNA transferred overnight onto a magnagraph nylon transfer membrane (MSI, Westboro, MA) by electroblotting in 10 × SSC (1 × SSC contains 0.15 M NaCl, 0.015 M trisodium citrate). The RNA was cross-linked to the membrane at 120 000 μJ using UV cross linker (Hoeffer Scientific Instruments, San Francisco, CA) and prehybridized in 50% deionized formamide, 4 × SSC, 20 mM Tris Hel (PH 8.0), 1 × Denhardt’s solution (0.02% ficoll, 0.02% polyvinyl pyrrolidine, 0.02% BSA), 0.1% sodium dodecyl sulfate (SDS), 200 mg/ml denatured salmon sperm DNA and 10% dextran sulfate for 20 min. 35S-labeled cRNA probe was synthesized using the full-length 5HT2 receptor coding region (provided by Dr Julies) and the ‘MAXIscript’ kit (Ambion, Austin, TX). Approximately 3 ng/ml of probe was added to the pre-hybridization solution and hybridization was performed overnight at 42°C. Filters were washed 3 times for 20 min with 0.1 × SSC containing 0.1% SDS at room temperature and 3 times for 20 min with 0.1 × SSC 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 random primed GAPDH. The relative quantity of 5HT2 mRNA in each sample was analyzed by densitometry using OPTIMIUS program on a Toshiba Image analyzer and corrected for loading conditions by the quantity of mRNA for GAPDH. The concentrations of mRNA are expressed as densitometric units.

2.6. Radioligand binding studies

After preloading the cells with EPA and DHA and stimulating with 5HT as described earlier, cell membranes isolated and radioligand binding studies were performed. Briefly the medium was removed and cell layers rinsed twice with PBS and scrapped into ice cold homogenizing buffer (50 mM Tris–Hcl, 0.5 mM Na2 EDTA, 10 mM Mg 504, PH 7.4). Cells were homogenized and membranes pelleted at 30 000 × g for 15 min. Pellets were resuspended in homogenizing buffer, incubated at 37°C for 15 min. and re-centrifuged at 30 000 × g for 15 min. Supernatant decanted, and pellets stored at −45°C until use. Saturation analysis for 5HT2 receptors was performed with 3[H]LSD (0.3125–20 nM). Non-specific binding was determined in the presence of 100-fold excess of unlabeled ligand. Assays were performed with approximately 120 mg of cell membrane protein in a final volume of 1 ml. The assay buffer was identical to the homogenizing buffer described above except that it contained 10 mM pargyline and 0.1% ascorbic acid. Samples were incubated for 30 min at 25°C and filtered through glass fiber filters which were presoaked in 0.1% poly-ethelene for 30 min. Filters were washed rapidly with 10 ml of ice cold 50 mM Tris–HCl (PH 7.0) and radioactivity that remained bound to the filters was measured using liquid scintillation counter.

2.7. Statistical analyses

Data were analyzed by one way analysis of variance (ANOVA) for each interaction. When a statistically significant difference was obtained further analysis was conducted using Scheffe’s post-hoc tests. For all comparisons statistical significance was assumed as P < 0.05. In each figure and table, mean values ± S.D. are
shown. Radioligand binding results were analyzed by standard linear regression analysis.

3. Results

3.1. Effect of 5HT and PDGF on $^3$[H]thymidine incorporation by SMC

To find out the mitogenic concentration of 5HT and PDGF growth arrested and synchronized SMC were stimulated with 5HT or PDGF in serum free medium and the amount of $^3$[H]thymidine incorporated into the DNA determined as described in Section 2. The effect of 5HT and PDGF on SMC proliferation was determined on growth arrested SMC. 5HT at an added concentration of 100 nM induced $^3$[H]thymidine incorporation into SMC and the effect was maximal at a concentration of 100 μM of 5HT. At concentrations greater then 200 μM of 5HT, there was a decrease in $^3$[H]thymidine incorporation, suggesting that higher concentrations of 5HT may be cytotoxic to SMC (Fig. 1). Exposure of SMC to PDGF was similarly associated with enhanced DNA synthesis (Fig. 1B). SMCs responded to PDGF with concentration dependent increase in $^3$[H]thymidine incorporation into DNA. Stimulation of $^3$[H]thymidine incorporation was maximal at PDGF concentrations above 20 ng/ml.

3.2. Effect of n-3 fatty acid pretreatment on $^3$[H]thymidine incorporation by SMC

To find out the effect of preloading of SMC with EPA, DHA, OA or LA on SMC proliferation, SMC were preloaded and synchronized in the presence of different concentrations of fatty acids. After synchronization SMC were stimulated with 1% FBS containing DMEM and the amount of $^3$[H]thymidine incorporated into the DNA determined as described in Section 2. When SMC were preloaded with different concentrations of EPA or DHA, up to a concentration of 7.5 μM of EPA or DHA there was no significant effect on $^3$[H]thymidine incorporation by the SMC (Fig. 2). However, when SMC were grown in concentrations of EPA or DHA higher than 7.5 μM, there was a gradual decrease in the amount of $^3$[H]thymidine incorporation up to a concentration of 30 μM of EPA or DHA (Fig. 2). Concentrations higher than 30 μM EPA or DHA completely inhibited $^3$[H]thymidine incorporation by SMC. These results indicate that higher concentrations of EPA and DHA may be cytotoxic to SMC. To determine whether this response was specific to n-3 fatty acids or is it common to all classes of fatty acids, SMC were preincubated with either LA (n-6) or OA (n-9) in the same concentration range (0–100 μM) and the amount of $^3$[H]thymidine incorporated into the DNA measured. The results indicate that when SMCs were incubated with LA the $^3$[H]thymidine incorpora-

Fig. 1. Graph showing concentration dependent stimulation of aortic smooth muscle cell (SMC) by serotonin (5HT) (A) and platelet derived growth factor (PDGF) (B). $^3$[H]Thymidine incorporation into DNA was determined in synchronized cells stimulated by various concentrations of 5HT and PDGF in serum a free medium in the presence of 100 μM pargyline, as described in Section 2. One hundred percent equals the baseline value of $^3$[H]thymidine uptake. 100% = 8130 ± 210 CPM/10⁶ cells for A, 100% = 7642 ± 330 CPM/10⁶ cells for B. The experiments were performed with two different batches of cells and each batch was tested in triplicate. Results are mean ± S.D., * P < 0.05, ** P < 0.01.
Fig. 2. Effect of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), oleic acid (OA) and γ-linolenic acid (LA) on smooth muscle cell (SMC) proliferation. Aortic SMC were grown in the presence of different concentrations of EPA, DHA, LA or OA in 10% fetal bovine serum (FBS) containing medium for 72 h, and then with the same concentrations of fatty acids in 0.1% containing medium for 72 h and then stimulated with serum free medium containing 100 mM pargyline. The amount of 3[H]thymidine incorporated into DNA was measured as described in Section 2. One hundred percent equals the baseline value of 3[H]thymidine uptake. 100% = 7960 ± 300 CPM/10⁶ cells. The experiments were performed with two different batches of cells and each batch was tested in triplicate. Results are mean ± S.D., *P < 0.05, **P < 0.01.

3.3. Effect of 5HT and PDGF on 3[H]thymidine incorporation by SMC grown in the presence of n-3 fatty acids

To determine the effect of EPA and DHA on 5HT and PDGF induced SMC proliferation, primary SMC were grown in the presence of different concentrations of EPA or DHA (0–3.33 μM) in 10% FBS containing medium for 72 h. Only the non-inhibitory and non-cytotoxic concentrations of EPA and DHA were selected to make sure that the observed effects of EPA and DHA on 5HT and PDGF induced SMC proliferation were not due to the inhibitory or cytotoxic effect of EPA and DHA. As controls, another group of cells was grown in the presence of same concentrations (0–3.33 μM) of LA or OA. Following this, the cells were growth arrested for 72 h in the same concentrations of fatty acids. Then SMC were preloaded with 50, 100 or 200 μM of 5HT or 10, 20 or 30 ng/ml PDGF. The results indicated that when SMC preloaded with 3.33 μM EPA or DHA was stimulated with 5HT, even the highest concentration of 5HT tested failed to induce significant 3[H]thymidine incorporation (Fig. 3A,B). However, when the same cells were stimulated with PDGF, the amount of 3[H]thymidine incorporated into the DNA of EPA treated cell was decreased by 40% (Fig. 3C) and DHA treated cells by 30% (Fig. 3D) as compared to the control cells. In contrast, preloading of SMC with LA or OA did not inhibit their proliferative response either to 5HT or to PDGF (Fig. 4). In fact, even modest concentrations of 5HT (50 μM) could stimulate 3[H]thymidine incorporation in SMC grown in 3.33 μM of LA or OA (Fig. 4B). These results suggest that preloading of SMC with either EPA or DHA makes them non-responsive to 5HT and partially responsive to PDGF and this effect was specific for only n-3 fatty acids not shared by n-6 or n-9 fatty acids.

Next the combined effect of EPA and DHA on SMC in preventing 5HT or PDGF induced SMC proliferation is determined. Since EPA and DHA are present approximately in 2.1 ratio in fish oils, SMC were preloaded with: (a) 0.11 or 0.22 μM EPA; or (b) 0.055 or 0.11 μM DHA alone; or (c) with 0.11 μM EPA and 0.055 μM DHA (0.165 nM of total n-3 fatty acids); or (d) with 0.22 μM EPA and 0.11 μM DHA (0.33 μM of total n-3 fatty acids) and then stimulated with 5HT or PDGF. The results indicate that EPA or DHA at the concentrations tested did not prevent 5HT or PDGF induced SMC proliferation (Fig. 5). Further, EPA or DHA by themselves, at a concentration of 0.33 μM alone produced only a modest inhibition of SMC proliferation (Fig. 3A for EPA and Fig. 3B for DHA). However, when EPA and DHA were added together at a total concentration of 0.33 μM of n-3 fatty acids (0.22 μM EPA and 0.11 μM DHA) 5HT induced SMC proliferation was completely inhibited (Fig. 5A) and PDGF induced SMC proliferation was partially inhibited (Fig. 5B). These results indicate that EPA and DHA may act synergistically in inhibiting the SMC proliferative response to 5HT and PDGF.

3.4. Effect of n-3 fatty acids on SMC cell number

To determine whether the inhibition of DNA synthesis results in decreased cell number, SMCs were preloaded with EPA or DHA or EPA and DHA together were stimulated with 5HT or PDGF and the number of cells counted. The results show that the inhibition of DNA synthesis by EPA and DHA preloading results in decreased cell number (Fig. 6). Stimulation of control cells with 100 μM 5HT resulted in an increase of 103 500 ± 6750 cells over the control, whereas when 3.2, 3 μM EPA or DHA or 0.33 μM EPA + DHA preloaded cells were stimulated with the same concentration of 5HT the cell numbers were increased only by 11 250 ± 1050, 16 750 ± 2100, 21 500 ± 3100 cells respectively (Fig. 6A). Similarly with PDGF stimulated cells, the cell numbers were increased by 52 500 ± 5670, 56 900 ± 6070, 56 250 ± 5750 cells respectively as compared to an increase of 756 500 ± 12 500 cells in the control (Fig. 6B).
3.5. Effect of n-3 fatty acids on 5HT<sub>2</sub> receptor mRNA levels

Next the mechanism by which EPA and DHA may inhibit the proliferative response of SMC to 5HT is determined. In vascular cells 5HT mediate its proliferative effect via the 5HT<sub>2</sub> receptor and 5HT<sub>2</sub> receptor antagonists can block the 5HT induced [H]thymidine incorporation [30,31]. Therefore, the effect of 5HT alone, or n-3 fatty acids alone, or the effect of n-3 fatty acids with 5HT on 5HT<sub>2</sub> receptor mRNA level was determined. Stimulation of SMC with 5HT induced an ≈ 55% increase in the 5HT<sub>2</sub> receptor mRNA levels when compared to the unstimulated cells control. When SMC preloaded with EPA or DHA were stimulated with 5HT, there was an 11 or a 22% increase respectively of the 5HT<sub>2</sub> receptor mRNA levels as compared to controls (≈ 55% increase) (Fig. 7). These results suggest that EPA or DHA may attenuate the SMC proliferative response to 5HT by down regulating the mRNA levels for 5HT<sub>2</sub> receptor in SMC.

3.6. Effect of n-3 fatty acids on 5HT<sub>2</sub> receptor number

To determine whether the changes in 5HT<sub>2</sub> mRNA levels induced by EPA or DHA, translates to an alteration in 5HT<sub>2</sub> receptor number, Scatchard analyses were performed using [H]LSD as a 5HT<sub>2</sub> receptor ligand. The results suggest, that although EPA and DHA attenuate the 5HT induce increase in mRNA levels for 5HT<sub>2</sub> receptor, there was no significant changes in 5HT<sub>2</sub> receptor density (data not presented).

4. Discussion

Platelets aggregate in response to arterial injury releasing vasoactive compounds and peptide growth factors [33,34]. Some of these factors have been shown to stimulate the proliferation of SMC [25,31,32,35,36], indicating that factors released from aggregating platelets may play an important role in the development of restenosis. Antiplatelet agents have been shown to decrease intimal hyperplasia and to improve long
term patency of autologous vein grafts [37,38]. Fish oil feeding has been shown to decrease platelet counts, prolong bleeding time and reduce platelet aggregation [5,7]. These effects of fish oils on platelet metabolism resulted in studies to examine the effect of fish oils on intimal hyperplasia and atherosclerosis [9,11,15,16,20]. Although these studies indicate that fish oils may have an inhibitory effect on SMC proliferation, efforts to understand the mechanisms of action of n-3 fatty acids have focused largely on their effects on eicosanoid production [39,40]. Further, an ever-growing number of other effects, potentially linked to the inhibition of atherogenesis or restenosis appear also to be eicosanoid independent, which include decreased production of tissue factor and cytokines [41,42], inhibition of production of growth factor like mitogens [43,44] and attenuation of expression of endothelial cell adhesion molecules [45]. In general, polyunsaturated fatty acids (PUFA) have been reported to inhibit proliferation of vascular SMC [46,47], but the difference in efficacy between different PUFAs and the underlying mechanism involved have not yet been fully characterized. In this study, the effects of fish oil derived n-3 PUFAs EPA and DHA are examined. OA (n-9 monounsaturated fatty acid) and LA (n-6 PUFA) were used as control fatty acids. In some studies arachidonic acid, has been used as a control, but since arachidonic acid and it is metabolites, like prostaglandins can directly influence SMC proliferation [48], LA might be a better alternative for n-6 PUFA because it is not converted to prostaglandins. In this study at low concentrations none of the fatty acids tested had any effect on SMC proliferation.

This study demonstrates for the first time, that SMC preloaded with non-cytotoxic concentrations of EPA or DHA, fail to proliferate when stimulated with 5HT and PDGF. In contrast, OA or LA were not effective, suggesting that only preincubation with n-3 fatty acids makes the SMC non-responsive to the mitogenic effect of 5HT and PDGF. In this study, it is also demonstrated that when EPA and DHA were combined approximately in the ratio, present in fish oils, they act synergistically in inhibiting the SMC proliferative response. Shiina et al. [49] have shown that EPA and
DHA at a concentration of 20 μM and above inhibited SMC proliferation by 30 and 20%, respectively. They also demonstrated that incubating the cells with free radical scavenger butylated hydroxytoluene (BHT) reversed this inhibitory effect, which indicates that the concentrations of EPA and DHA used by Shiina et al. may have produced free radicals that are known to be cytotoxic at higher concentration [50]. In the present study, also when preloaded with 30 μM and higher concentrations of EPA or DHA or LA or 250 μM and higher concentration of OA, SMC failed to grow at a normal growth rate when transferred to normal growth medium (data not presented). However cells preloaded with 7.5 μM and lower concentrations of EPA or DHA or LA or OA continued to grow at normal rate (data not presented) indicating that the concentrations of fatty acids used in this study are not cytotoxic. Addition of BHT to the culture medium did not alter the blocking effect of EPA and DHA (data not presented) indicating that the blocking effects observed in this study were not due to the formation of cytotoxic concentrations of free radicals.

In vascular SMC 5HT has been shown to exert its proliferative effect via the 5HT2 receptors [51]. In this study stimulation of SMC with 5HT, resulted in upregulation of the 5HT2 mRNA. Ligand induced upregulation of mRNA levels has been reported for epidermal growth factor and interleukin-2 [52,53]. When EPA and DHA preloaded SMC was stimulated with 5HT, it failed to upregulate the 5HT2 receptor mRNA levels to
the same level as in the control cells, suggesting that receptor down regulation may be one of the mechanism by which EPA and DHA prevent 5HT induced proliferation of the SMC. Even though there is a decrease in the amount of mRNA for 5HT2 receptors in EPA or DHA preloaded SMC, the receptor affinity or the number was not significantly different from the control SMC. Fitzgerald et al. have reported that in uterine 5HT and its analogues unregulated and antagonists down regulated the 5HT2 receptor mRNA levels without any significant difference in the affinity between the ligand and receptor number [54]. Changes in the levels of mRNA without analogous changes in corresponding protein levels have been shown for epidermal growth factor and interleukin-2 also [52,53]. In some instances this was found to be the result of specific processes regulating receptor turnover [55,56]. 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 [56].

On the basis of the present knowledge growth factors can be divided into two classes based on their transmembrane signaling: (1) the ones act through receptor tyrosine kinases (PDGF, FGF epidermal growth factor insulin like growth factor) [57]; (2) The ones act through G-protein coupled receptors (bomberin, bradykinin, vasopressin, thrombin, 5HT and thromboxane A2 [31,58–60]. In the present study, it has been demonstrated that upregulation of 5HT2 mRNA is blocked by n-3 fatty acid pretreatment. Kiminski et al. [44] have already shown that n-3 fatty acids down regulates PDGF mRNA. Thus the present results and that of others suggest that n-3 fatty acids block the mitogenic effect of both the classes of growth factors. The partial blocking effect on PDGF induced stimulation maybe because of the fact that growth factors which act through tyrosine kinase pathway (‘classical’ growth factors) are very strong mitogens as compared to the mitogens which act through G-protein coupled receptors (hormones).

The effective concentrations of EPA and DHA in the present study are well below the concentrations normally used in in vitro studies Wallace et al. [61] and Tremoli et al. [62] have shown that in healthy humans even low concentration of fish oil supplementation (2–3 g/day as compared to 5.4–20 g/day) has significantly reduced cytokine and tissue factor production. Results from this study and that of Wallace et al. [61] and Tremoli et al. [62] indicate that the beneficial effects of fishoils could be ascertained even lower concentrations. In fact, using lower concentrations of EPA and DHA may be more beneficial, because presence of high concentrations of EPA and DHA in the membrane phospholipids makes them more prone to lipid peroxidation [63–65] and free radicals generated as a result of increased lipid peroxidation are known to be cytotoxic [50,66,67].

Although earlier studies have indicated that the decreased rate of intimal hyperplasia [9,20] and atherosclerosis [15,16] in fish oil fed animals may be due to the decreased production of SMC growth factors by fish oil treated endothelial cells [43] and monocytes [44], a specific effect of EPA and DHA on vascular SMC proliferation was demonstrated that suggests additional mechanistic beneficial effects of n-3 fatty acid rich diets in reducing cardiovascular events.

Acknowledgements

The authors would like to thank Dr David Julius,
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