Interruption of Vascular Thrombus Formation and Vascular Lesion Formation by Dietary n-3 Fatty Acids in Fish Oil in Nonhuman Primates

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Background. Because of discrepant claims regarding the relative biological effects of n-3 fatty acids (n-3FAs), we have concurrently measured the effects of dietary n-3FAs on blood and vascular lipid composition, hemostatic function, blood thrombotic responses, vascular thrombus formation, and vascular lesion formation in baboons.

Methods and Results. Dietary n-3FAs displaced n-6FAs in plasma, platelets, blood vessels, and corresponding urinary eicosanoid metabolites (p<0.01 in all cases) within weeks after initiation of a semipurified diet containing 1 g/kg per day n-3FA-ethyl ester concentrate (composed of two thirds eicosapentanoic acid and one third docosahexanoic acid). Coincidentally, platelet hemostatic function became minimally impaired (template bleeding times prolonged from 4.3±0.5 minutes to 7.6±1.3 minutes, p=0.039); concentrations of collagen producing half-maximal platelet aggregation increased (from 6.4±2.1 to 8.5±2.5 μg/mL, p=0.045); and tissue factor expression by endotoxin-stimulated blood monocytes fell (from 6.5±1.2 to 1.7±0.14 mU/10⁶ cells, p<0.005). Dietary n-3FAs decreased deposition of platelets onto thrombogenic segments of Dacron vascular graft incorporated into chronic exteriorized femoral arteriovenous (AV) shunts, a thrombotic process resistant to the effects of both aspirin and heparin (10¹⁴In-labeled platelet deposition decreased from 14.1±1.4×10⁹ platelets/5-cm segment at 40–60 minutes with occlusion to 7.5±0.8×10⁹ platelets/5-cm segment without occlusion; p<0.001). Platelet deposition onto segments of endarterectomized homologous normal aorta in the AV shunts of n-3FA-treated animals was similarly reduced (from 4.4±0.9 to 1.8±0.4×10⁹ platelets; p<0.01). Dietary n-3FAs interrupted vascular thrombus formation at sites of surgical carotid endarterectomy (platelet deposition, 1.5±0.4 versus 4.4±1.0×10⁹ platelets in untreated controls; p<0.001). Moreover, endarterectomized aortic segments (EAs) from n-3FA-treated donors exhibited little capacity to induce thrombus formation when tested in the AV shunts of control recipient animals (0.24±0.10 versus 4.4±0.90×10⁹ platelets). However, in the converse crossover experiments, EAs from control animals actively accumulated platelets when studied in the AV shunts of n-3FA-treated animals (1.8±0.4×10⁹ platelets; p<0.01 versus n-3FA-treated EAs in shunts of normal animals). Dietary n-3FAs also abolished vascular lesion formation at sites of carotid endarterectomy 6 weeks after surgery (cross-sectional area of neointima 0.048±0.031 mm² compared with 0.428±0.104 mm² in control arteries; p=0.010).

Conclusions. In nonhuman primates, dietary n-3FAs in high doses eliminate both vascular thrombus formation and vascular lesion formation after mechanical vascular injury while largely sparing hemostatic function and modestly reducing blood thrombotic responses. These effects are attributed to selective n-3FA-dependent alterations in cellular membrane functions. (Circulation 1993;87:1017–1029)

Key Words • thrombosis • endarterectomy • restenosis • fish oils • antithrombotic therapy

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evidence arising from epidemiological,1–6 biochemical,1,6–13 experimental-animal,14–19 and clinical studies20–27 indicates that dietary n-3 fatty acids (n-3FAs) may benefit individuals with estab-
tered cell membrane composition, 3) decreased blood viscosity, 4) impaired platelet hemostatic function, and 5) production of characteristic n-3FA urinary eicosanoid metabolic products. Although the reduction in thrombotic vascular events has been attributed to the metabolic results of substituting n-3FAs for arachidonic acid and the resultant generation of eicosanoid products capable of modifying platelet and vascular functions, dietary n-3FAs also reduce experimental vascular lesion formation (VLF) in dogs, swine, and nonhuman primates, and increased fish consumption is associated with decreased mortality from coronary artery disease. Yet, the effects of dietary n-3FAs on restenosis in patients undergoing coronary angioplasty have been inconclusive.

To determine quantitatively the consequences of administering n-3FAs on vascular thrombus formation (VTF) and VLF, we have measured directly in nonhuman primates the relative effects of dietary n-3FAs on lipid cellular composition, platelet hemostatic function, thrombotic responses of circulating blood, vascular thrombogenicity, and intimal proliferative lesion formation induced by mechanical damage. In these studies, the baboon was used as the experimental model because this species closely resembles humans with respect to its 1) molecular hemostatic activation and inhibition pathways involving platelets, coagulation, and fibrinolysis; 2) vascular structure and function; 3) protein antigenic properties permitting common use of immunologic probes and assays; 4) lipid composition and metabolic pathways; and 5) size, logistic application of imaging protocols, ease of frequent blood sampling, and acceptance of long-term exteriorized arteriovenous (AV) access shunts. In addition, well-characterized models of clinically relevant VTF and VLF are established in this species.

Methods

Animals Studied

Twenty normal male baboons (Papio anubis) weighing 8–12 kg were studied. Eight animals received dietary n-3FAs (see below), and 12 animals served as controls for the studies of VLF. The animals had been dewormed and observed to be disease free for 3 months before use. All procedures were previously reviewed and approved by the Institutional Animal Care and Use Committee of Emory University and were in compliance with procedures and methods outlined by the National Institutes of Health (NIH) (Guide for the Care and Use of Laboratory Animals, National Institutes of Health, Bethesda, Md., NIH Publication No. 86-23) as well as the Animal Welfare Act and related university policies. Initially, chronic femoral AV exteriorized access shunts were surgically placed in the animals allocated to receive n-3FAs under halothane anesthesia after induction by ketamine (10 mg/kg i.m.) and valium (0.5 mg/kg i.v.). For subsequent short-term immobilization for experimental procedures after surgery, we used ketamine hydrochloride (5–20 mg/kg i.m.). The shunt system provided predictable and durable blood access, thereby permitting each animal to serve as its own control for the studies assessing the changes in blood and thrombotic responses (see below).

Experimental Design

The overall design of the experiments is depicted in Figure 1. Eight animals initially received the control olive oil–supplemented diet (Table 1) for 8 weeks, followed by the n-3FA-supplemented diet (Table 1) for 16–22 weeks (after 16 weeks of n-3FA treatment, carotid endarterectomy was performed, and sites were harvested after 3 days in two animals and after 6 weeks in six animals). In the 12 VLF control animals, a diet not containing n-3FAs was administered throughout the 30-week study. At 4-week intervals, assessment was made of 1) lipid composition of plasma and platelets, 2) platelet hemostatic function, 3) thrombotic responses of blood to thrombogenic segments in the exteriorized shunts, and 4) plasma and urinary eicosanoid products.

After 8 weeks of control diet and before initiation of dietary n-3FA therapy in eight animals, surgical endarterectomies were performed and control (basal) endarterectomy thrombosis was measured on the left carotid arteries (see below). In addition, segments of the left superficial femoral arteries were harvested for analysis of baseline lipid composition.

After 12 weeks of dietary n-3FAs, the right superficial femoral artery was obtained for lipid analysis, in addition to the regular every-4-week assessment of lipid composition of plasma and platelets, platelet hemostatic function, thrombotic responses of blood to thrombogenic segments in the exteriorized shunts, and plasma and urinary eicosanoid products (Figure 1).

After 16 weeks of dietary n-3FAs, endarterectomies were performed on the right carotid arteries and consequent endarterectomy thrombosis was measured (see below). Also, thrombus formation on segments of endarterectomized normal aorta in the AV shunts of animals treated with n-3FA for 12–16 weeks was determined (see below). In two animals receiving dietary n-3FAs, the right carotid artery was harvested under anesthesia 3 days after surgical endarterectomy, thereby providing endarterectomy sites for the 3-day VLF studies (see below). After an additional 6 weeks of dietary n-3FAs in the six remaining animals, the right superficial femoral arteries were obtained under anesthesia for lipid analyses, and the aortas were harvested for use in assessing the thrombotic responses of endarterectomized aortic segments in the AV shunts of normal animals. Subsequently, but during the same procedure, in situ pressure-perfusion fixation with 4% paraformaldehyde was carried out for 30 minutes by the procedure described previously, followed by removal of the right and left common carotid endarterectomy sites. The paraformaldehyde-fixed specimens of carotid arteries were prepared as two 5-mm rings allocated to paraffin section preparations for morphometric analysis of intimal lesion formation and glutaraldehyde postfixed material for epon sections used in ultrastructural morphological evaluation. This design allowed animals to serve as their own controls with respect to 1) blood cell counts; 2) plasma, platelet, and arterial lipid composition; 3) platelet function testing; 4) monocyte expression of tissue factor; 5) acute thrombus formation on segments of endarterectomized aorta and Dacron vascular graft in exteriorized shunts; and 6) thrombus...
formation at carotid endarterectomy sites. The twelve additional animals served as separate controls in evaluating vascular lesion formation 6 weeks after carotid endarterectomy.

**Diet Administered**

The control semipurified diet (Table 1) containing olive oil (1 g/kg per day) was administered to the eight study animals for the initial 8 weeks and to the 12 VLF control animals throughout 30 weeks (Figure 1). The use of a defined diet guaranteed that the animal’s intake was calorically balanced and equivalently distributed with respect to carbohydrate, protein, and fat and precluded uncontrollable variations in dietary formulation to which commercial diets are prone. The olive oil was composed of 72% monounsaturated fatty acid as n-9 C\(_{18:1}\) and 12% as n-6 C\(_{18:2}\), 12.6% polysaturated, and 0.6% content as n-3 C\(_{18:3}\).

After 8 weeks of control diet, n-3FAs (1 g/kg per day) were substituted for olive oil during the subsequent 16–22 weeks in eight animals. n-3FAs were provided as n-3 ethyl ester concentrate prepared from vacuum-deodorized menhaden oil by use of transesterification, urea adduction, and short-path distillation through the Fish Oil Test Material Program.\(^4\) The concentrate contained approximately 80% n-3FA ethyl ester (44% EPA, 24% DHA, 10–12% other n-3FA ethyl esters, and 3% C\(_{18}\) other than n-3), 6% C\(_{18}\) FAs, and the remainder as other esters. The n-3FA concentrate contained 0.2 mg/g tertiary butyl hydroquinone and 2 mg/g tocopherol as antioxidants.\(^4\) It also contained 2 mg/g cholesterol. The dietary content of cholesterol was maintained at low levels to assess specifically the effects of n-3FAs on vascular processes in the absence of confounding effects arising from other lipid variables (Table 1).

The diet was prepared by the Oregon Regional Primate Research Center (Beaverton, Ore.) as granular coarse cakes containing the n-3FAs and added antioxidants (see above). To further minimize the possibility of fatty acid oxidation, the diet was prepared every 2 weeks, immediately frozen in plastic bags, shipped on dry ice, and stored at -20°C until used. The daily dietary allocation was 3–4% of body weight for each animal, and the amount eaten was recorded. Animal weights were also monitored regularly to document normal growth patterns.

**Measurements of Plasma, Platelet, and Arterial Lipid Composition**

Plasma lipoproteins were separated by ultracentrifugation and heparin-MnCl\(_2\) precipitation.\(^4\) The cholesterol and triglyceride contents in the plasma and in lipoprotein fractions were determined every 4 weeks by procedures previously described.\(^4\)

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**Figure 1. Experimental Design**

<table>
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<th>Time (weeks)</th>
<th>Experimental Group (n = 8)</th>
<th>Procedures</th>
<th>VLF Control Group (n = 12)</th>
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<td>n-3 FA Diet</td>
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**Figure 1.** Diagram showing experimental design. VLF, vascular lesion formation; n-3 FA, n-3 fatty acid; L, left; R, right; EA, endarterectomy.
Ingredients
1.20
bic
5H2O, 0.0299; sodium
10.910; 10.910;
18 18
Sugar
n-3
Vitamin
Carbohydrate
18.7
18.7
Protein
41.2
41.2
Fat
40.1
40.1

Tertiary butyl hydroquinone (0.2 mg/g) and tocopherol (2 mg/g) were added as antioxidants to both n-3FAs and control olive oil.

*Ingredients (g/kg) with added choline chloride, 250 g/kg: dextrose, 652.05; vitamin A, 1.25; vitamin E acetate, 9.975; ascorbic acid, 24.975; inositol, 50.02; menadione sodium bisulfite, 6.0; niacin, 2.45; riboflavin, 0.5; thiamine, 0.5; pyridoxine, 0.525; calcium pantothenate, 1.5; biotin, 0.01; folic acid, 0.05; vitamin B12, 1%; mannitol, 0.15 (Monkey DWP product, Bio-Serv, Inc., Frenchtown, N.J.).

†Composition (%): calcium carbonate, 29.974; potassium phosphosphate dibasic, 32.220; calcium phosphate monobasic, 7.493; magnesium sulfate · 7H2O, 10.910; sodium chloride, 16.735; ferric citrate (16–17% Fe), 2.747; potassium iodide, 0.0799; manganese sulfate · H2O, 0.499; copper sulfate · 5H2O, 0.0299; zinc chloride, 0.0249 (Hegsted salt mixture, U.S. Biochemical Corp., Cleveland, Ohio). Selenium content (by analysis): 6 µg/100 g of similar specific purity determination.

‡Ergocalciferol, 8,000 IU/mL (Drisdol, Winthrop-Breon Laboratories, New York).

For platelet lipid determinations, platelet-rich plasma (PRP) was prepared from EDTA-anticoagulated blood, platelets were pelleted at 7,000g for 20 minutes, and the lipids were extracted by the procedure described previously. For lipids were separated into individual lipid classes by addition of recovery standard [4-14C]cholesterol. Methyl heptadecanoate was then added to the phospholipid band as a recovery marker and an internal standard for gas–liquid chromatography. Cholesterol was quantified as previously described. Fatty acids were measured with an HP 5830A chromatograph (Hewlett-Packard, Avondale, Pa.) with an SP2330 column (Supelco, Inc., Bellefonte, Pa.) at 180°C for 10 minutes, subsequently rising at 5°C/min to 200°C for 20 minutes; injection port, 250°C; N2 flow, 44 mL/min. Both the relative proportions of fatty acids in platelet phospholipids and the absolute amount of each acid were determined by including internal standards.

Arterial tissue was immediately washed with physiological saline and blotted dry. Each sample was minced, weighed, freeze-dried, reweighed, and then extracted by grinding with a mortar and pestle in chloroform–methanol. Aliquots of the extracts were plated on silica gel G thin-layer chromatography (TLC) plates after [4-14C]cholesterol and cholesterol [14C]stearate were added as internal standards. The plates were developed in hexane:chloroform:ether:acetic acid 80:10:10:1. After the plates were sprayed with rhodamine G, the lipid bands were visualized with ultraviolet light. The free cholesterol and cholesteryl ester bands were removed and extracted with ether. Cholesteryl ester was saponified with alcoholic KOH and the cholesterol was extracted with hexane. The cholesteryl ester fatty acids were recovered by acidifying the aqueous phase and reextracting with hexane. Cholesterol content was determined by gas–liquid chromatography (Hewlett-Packard 7610A) on a 3.8% SE-30 glass column. Cholesterol and stigmastanol were used as internal standards as previously described. The fatty acids were methylated with boron trifluoride-methanol, and relative concentrations were determined by gas–liquid chromatography (Hewlett-Packard 5830A) as for platelets above. Because the peaks of three pairs of fatty acids overlap in this system, i.e., C16:3(n-3) and C16:1(n-9); C20:4(n-6) and C22:1(n-11); and C22:4(n-6) and C24:1(n-9), separation of these mono- and polyunsaturated fatty acids was achieved by TLC of the fatty acid methyl esters on silver nitrate plates as described previously. After the samples were extracted with hexane, they were analyzed by gas–liquid chromatography as described above.

Measurements of Urinary Eicosanoid Products
Free-flowing urine was obtained by suprapubic expression. Urinary eicosanoid metabolites were measured with stable isotope dilution assays in which capillary gas chromatography–negative chemical ionization mass spectrometry was used as previously described. The concentrations of excreted 2,3-dinor-6-keto-prostaglandin F1a and 2,3-dinor-thromboxane B2, major urinary metabolites of dienoic prostacyclin and thromboxane derived from arachidonic acid, respectively, were determined by adding known amounts of internal standards to 3–5 mL of urine.

Purification was performed by sequential column chromatography and TLC. The purified compounds were derivatized to their methoxime, pentafluorobenzyl ester, and trimethylsilyl ethers and were analyzed by selected-ion monitoring, at mass-to-charge ratio (m/z) 586 for the endogenous compounds and m/z 590 for the tetradegerated standards. To quantify the trienoic metabolites of prostaglandin I2 and thromboxane B2, derived from eicosapentaenoic acid, monitoring was performed at two mass units lower (for the extra double bond) to detect a peak with a shape identical to and with a slightly longer retention time than the shape and retention time of the analogous dienoic metabolites.

Measurements of Hemostatic Function
Platelet counts, hematocrit determinations, and white blood cell counts were performed weekly on whole blood collected in 2 mg/mL disodium EDTA with a J.T. Baker (Allentown, Pa.) model 810 whole blood analyzer. Bleeding time measurements were performed on the shaved volar surface of the forearm by the standard template method as previously described for studies in baboons. Bleeding time measurements were performed in duplicate and averaged. Platelet aggregation was measured with a Chrono-Log aggregometer (Havertown, Pa.) by recording the increase
in light transmission through a stirred suspension of PRP maintained at 37°C. PRP and platelet-poor plasmas were prepared as previously described.53,54 In all cases, the platelet count in the PRP was adjusted to 200,000 platelets/μL ADP (Sigma Chemical Co., St. Louis, Mo.) and collagen (Hormon, Munich, Germany) were added at doses that spanned the range of responsiveness. The results were plotted and expressed as the concentration of agonist that induced half-maximal aggregation.54

Serum thromboxane B2 levels were determined by radioimmunoassay on serum prepared by adding 5 IU thrombin/mL to fresh whole blood incubated at 37°C for 30 minutes as described previously.54

Tissue factor activity was measured on mononuclear cells in a purified system. The cells were prepared by centrifugation density gradient and stimulated by endotoxin in vitro as described previously.13,55 In brief, blood was drawn into plastic syringes and immediately transferred into a polycarbonate tube containing heparin at a final concentration of 10 units/mL. Lipopolysaccharide (E. coli 026:B6, Difco Laboratories, Detroit, Mich.) (2 ng) was added to 1 mL of heparin-anticoagulated whole blood and incubated in a temperature-regulated rotary incubator at 180 rpm for 2 hours. Then 0.1 ml 2% EDTA, pH 7.4, was added to stop the activation reaction. Monocytes were isolated from the incubated blood and applied on top of 1.5 mL Lymphopaque (Nycome, Oslo, Norway) and centrifuged and handled as previously described.13 Tissue factor activity was quantified by incubating the lysed monocyte test samples with factor VII and factor X as reported.15,55 Generated factor Xa was then measured, and the activity was related to a crude standard human tissue factor thromboplastin preparation that gave a clotting time of 16 seconds in a prothrombin-time coagulation system. Anti–tissue factor neutralizing antibodies completely inactivated the production of tissue factor activity.

Chronic Exteriorized AV Shunt Models of Arterial Thrombosis

Surgically implanted exteriorized femoral AV “Scribner-type” access shunts were used for interposition of thrombogenic segments.37–39,52–54,56 The permanent shunt system consisted of two 5-cm lengths of silicone rubber tubing (3.0-mm i.d.) (Silastic, Dow Corning Corp., Midland, Mich.) connected to establish blood flow by 2-cm-long blunt-edged Teflon tubing (2.8-mm i.d.). AV shunts of this design do not detectably shorten platelet survival times or fibrinogen removal rates and do not produce measurable activation of platelets or fibrinogen.56

To model acute thrombotic responses of native blood under conditions of well-controlled flow and geometry, thrombus formation was induced by thrombogenic segments incorporated as extension pieces into the chronic AV shunts.38,39,57 Blood flows were maintained at 100–150 mL/min for these studies. Thrombogenic segments were interposed between the arms of the permanent shunt system of awake animals for 1 hour while the extent of acute thrombus formation was measured by 1) scintillation camera imaging of deposited autologous 111In-labeled platelets; 2) segment patency assessed by Doppler flow analysis through the shunt using a C-clamp-type flow probe interfaced with a Transonic T206 Blood Flow Analyzer (Transonic Corp., Ithaca, N.Y.); and 3) blood tests of thrombosis, including plasma levels of platelet factor 4 (PF4), β-thromboglobulin (βTG), fibrinopeptide A (FPA), thrombin–antithrombin complex (TAT), and D-dimer.37–41,52–57

Two different thrombogenic segments were studied: endarterectomized homologous arteries38 and Dacron vascular grafts.57 These thrombogenic surfaces were selected for study because of their clinical relevance and because they induced thrombus that was resistant to the conventional antithrombotic agents aspirin and heparin.52,54 Endarterectomized aortic segments were prepared from homologous baboon aorta as previously described.38–41 A central circumferential 1-cm area of intima was removed, extending to the internal elastic lamina. Flanged Teflon (Small Parts Inc., Miami, Fla.), 4-mm i.d., was used to cannulate each end of the aortic segment, and each endarterectomized aortic segment was encased by a shrinkable Teflon sleeve to constrain the vascular segment in 4-mm-i.d. cylindrical configuration with a smooth transition from tubing to vessel suitable for incorporation into the chronic silicone rubber AV shunts. Nonendarterectomized aortic control segments were handled similarly and were shown to be nonthrombogenic.38 Segments of Dacron vascular graft (5-cm-long uncrimped Dacron graft, 4-mm i.d., obtained from C.R. Bard, Inc., Billerica, Mass.) were rendered impervious to blood leakage by external wrapping in Parafilm (American Can Co., New York) and 5.3-mm-i.d. “heat shrink” Teflon tubing.57 Butt joints were constructed that ensured smooth transition suitable for incorporation into the AV shunts.39,52,53

Platelet deposition was measured in real time by gamma camera imaging of 111In-labeled platelets. Autologous baboon platelets were labeled with 1 mCi 111In oxine as previously described57 and reinfused at least 1 hour before imaging, at which time 111In-labeled platelets were functionally normal.58 The imaging routines and isotopic detection protocols for these shunt studies were performed by use of procedures that have been reported previously.38–41

Blood tests of thrombosis in vivo were determined in plasma-obtained baseline before the incorporation of thrombogenic segments into the AV shunt and after the thrombogenic segments were exposed to arterial rates of blood flow for 1 hour.37 Plasma levels of PF4, βTG, FPA, TAT, and D-dimer were determined by radioimmunoassay on blood samples collected and processed as previously described.39,52–57

The concentration of fibrinogen in plasma was estimated spectrophotometrically by a modification of Jacobsson’s method.59

Carotid Endarterectomy Thrombosis Model

Carotid endarterectomy was performed through a midline neck incision by a previously described technique.40,41 Endarterectomy was chosen as the vascular injury model because of its clinical relevance and because endarterectomy of normal vessels, as opposed to balloon angioplasty, induced reproducible deposition of platelet-dependent thrombus formation that was readily quantified by scintillation camera imaging in vivo. The endarterectomy involved mechanical removal of the normal intima and a partial thickness of media for a measured distance of 1 cm by use of forceps and a
surgical microscope (magnification, ×32; Zeiss operating microscope, Germany).

Scintillation camera images were taken at 30, 60, and 90 minutes and at 24 and 48 hours after flow was reestablished in the endarterectomized artery. To localize the endarterectomy site precisely with respect to the gamma camera imaging, a ⁵⁷Co radioisotope source (approximately 5 μCi) was sealed in a 1.0-cm length of 0.6-mm-i.d. polyethylene tubing (PE-50, Clay Adams Inc., New York). The ⁵⁷Co radioisotope source was sutured to the adventitia of the underlying endarterectomy site with 7-0 polypropylene sutures. The endarterectomy region of interest was defined by localizing the lower-energy ⁷⁷Co 1-cm source. Subsequently, the higher-energy ¹¹¹In-labeled platelet activity was measured in the same region of interest. Through this colocalization technique, the endarterectomy site was positively identified despite the elimination of detectable ¹¹¹In-labeled platelet activity after treatment, particularly when there was the possibility that blood containing ¹¹¹In-labeled platelets could accumulate excessively within the endarterectomy surgical site. To measure the attenuation of ¹¹¹In-labeled platelet radioactivity produced by overlying soft tissue, an ¹¹¹In standard (approximately 5 μCi ¹¹¹In sealed within 0.6-mm-i.d. polyethylene tubing) was also implanted adjacent to the carotid artery.

Gamma camera images of both the normal and operated carotid arteries were acquired with a Searle PHO/Gamma V scintillation camera (Siemens Medical System, Iselin, N.J.) and stored and analyzed on a Medical Data System A³ Computer (Medtronics, Ann Arbor, Mich.). A medium-energy collimator was placed close to the animal. Total deposited ¹¹¹In-labeled platelet activity was determined at the endarterectomy site by placing a region of interest representing 0.5×1.0 cm on the coordinates previously identified by the ⁷⁷Co standard. Measurements of ¹¹¹In activity at the endarterectomy site were corrected for background activity by subtracting the ¹¹¹In activity in the contralateral, undiseased carotid artery using a region of interest of identical size at the appropriate location, thereby yielding deposited radioactivity only. The ¹¹¹In activity at the site of endarterectomy was then corrected for tissue attenuation as described above. Activity of a 5-mL whole blood standard was also determined. The activity of the blood standard was also corrected for the small fraction of circulating nonplatelet radioactivity to give platelet-associated ¹¹¹In activity per 1 mL of whole blood. The total platelet deposition, including both labeled and unlabeled platelets, was calculated by dividing the endarterectomy-deposited counts per minute by circulating-platelet counts per minute (blood standard) and multiplying by the circulating platelet count (platelets per 1 mL of whole blood) as measured in the blood standard sample.

Because circulating ¹¹¹In-labeled platelet activity is cleared continuously through normal physiological mechanisms, platelet accumulation after the acute 30–90-minute time period was expressed as the ratio of the ¹¹¹In-labeled platelet activity at the endarterectomy site to the ¹¹¹In-labeled platelet activity in blood. This measurement was independent of the size of the animal, the amount of isotope injected, or the extent to which the isotope might have decayed. Radioactivity values in these calculations refer to platelet activity only, with blood and standard values corrected for the small fraction of nonplatelet ¹¹¹In activity.

Duplex Ultrasound Analysis

Duplex ultrasound analysis, combining both high-resolution B-mode imaging and Doppler spectral flow analysis, was carried out to determine patency of the operated carotid artery noninvasively after the 90-minute gamma camera image and at 24 and 48 hours after surgery with an ATL-MK600 duplex scanner (Advanced Technology Laboratories, Inc., Seattle, Wash.). This technique has been shown to be an accurate method for verifying patency and occlusion in manipulated vessels in baboons.

Morphological Evaluation of VLF

Under anesthesia and after in vivo pressure-perfusion fixation at 100 mm Hg with 4% paraformaldehyde (see above), both carotid arteries were obtained en bloc 6 weeks (range, 26–77 days) after completion of the right carotid endarterectomy, placed directly in fixative, cut into rings, and processed as paraffin sections for morphometry. For morphometric analysis, sections embedded in paraffin and stained for connective tissue components (collagen, elastin) and with conventional hematoxylin and eosin were evaluated with a Zeiss Photomicroscope coupled with image analysis system (Thomas Optical Measurement Systems, Columbus, Ga.) consisting of high-resolution (580 lines) CCD microscope camera coupled to a high-resolution (700 lines) monitor, an IBM 386-chip, 80-MB computer with high-resolution graphics digitizable for image acquisition and storage. Quantitative image analysis was performed with a morphometric software driver (Optimas, Bioscan, Inc., Edmonds, Wash.). Arterial cross sections were analyzed with respect to total area of neointimal proliferative lesion and corresponding area of arterial media.

Statistical Analyses

Comparisons between groups were made with Student's t test (two-tailed) for paired and unpaired data, unless the data were not randomly distributed, in which case nonparametric methods of analysis were used to compare the groups. Interanimal differences produced significant variability in modeling response data, a factor that supported the utility of the crossover design whereby animals served as their own controls.

Results

The control and n-3FA–supplemented diets were accepted by the animals, who consumed about 90% of what was offered and showed appropriate weight gain during the period of study. Dietary n-3FAs produced no significant changes in hematocrits (36±1% versus the control value of 36±1%; p>0.5) or leukocyte counts (7,000±620 versus 8,600±1,400 in control animals; p=0.29). No systemic functional adverse effects were noted throughout the period of study (Figure 1).

Metabolic Effects of Dietary n-3FAs

Lipid analyses were performed on plasma and platelets at 4, 8, and 12 weeks after n-3FA treatment was initiated (Table 2). By the fourth week, the diet-induced changes were largely complete (data not shown). At 12
TABLE 2. Incorporation of n-3 Fatty Acids Into Plasma, Platelets, and Artery Wall by 12 Weeks of Dietary Supplementation

<table>
<thead>
<tr>
<th>Fatty acid content (%)</th>
<th>Baseline</th>
<th>n-3FA treated</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sat</td>
<td>29.0±2.5</td>
<td>28.4±3.0</td>
<td>NS</td>
</tr>
<tr>
<td>Mono</td>
<td>35.3±6.4</td>
<td>20.9±13.0</td>
<td>0.0008</td>
</tr>
<tr>
<td>n-6</td>
<td>30.5±2.5</td>
<td>21.1±2.4</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>EPA</td>
<td>0.2±0.1</td>
<td>17.2±5.6</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>DHA</td>
<td>1.6±0.5</td>
<td>5.8±18</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>n-3</td>
<td>2.6±0.6</td>
<td>27.2±8.4</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Platelets</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sat</td>
<td>40.1±2.8</td>
<td>42.2±3.0</td>
<td>NS</td>
</tr>
<tr>
<td>Mono</td>
<td>29.0±2.2</td>
<td>20.6±3.6</td>
<td>0.017</td>
</tr>
<tr>
<td>n-6</td>
<td>28.1±2.1</td>
<td>20.0±2.7</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>EPA</td>
<td>0.4±0.2</td>
<td>11.1±2.7</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>DHA</td>
<td>0.7±0.2</td>
<td>2.0±0.7</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>n-3</td>
<td>1.7±3.1</td>
<td>15.0±3.1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Femoral artery</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sat</td>
<td>42.2±4.1</td>
<td>40.2±9.5</td>
<td>NS</td>
</tr>
<tr>
<td>Mono</td>
<td>34.2±6.0</td>
<td>33.7±11.7</td>
<td>NS</td>
</tr>
<tr>
<td>n-6</td>
<td>18.5±5.1</td>
<td>18.1±8.5</td>
<td>NS</td>
</tr>
<tr>
<td>EPA</td>
<td>0.1±0.1</td>
<td>1.2±0.8</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>DHA</td>
<td>1.9±1.2</td>
<td>2.7±1.8</td>
<td>NS</td>
</tr>
<tr>
<td>n-3</td>
<td>3.3±1.9</td>
<td>6.0±3.1</td>
<td>0.016</td>
</tr>
</tbody>
</table>

n-3FA, n-3 fatty acids; Sat, saturated; Mono, monounsaturated; n-6, n-6 fatty acids; EPA, eicosapentaenoic acid (20:5 n-3); DHA, docosahexaenoic acid (22:6 n-3).

weeks, the n-3FA content of plasma and platelets had increased 10-fold, with reciprocal decreases in the contents of n-6FAs. Similarly, after 12 weeks of dietary n-3FAs, the contents of n-3FAs were increased and n-6FAs reciprocally decreased in segments of femoral artery (Table 2). At 12 weeks, the mean plasma triglyceride level was 75±35 mg/dL, compared with the mean value obtained during the control diet of 61±31 mg/dL (p=0.4 by paired two-tailed t test), and the total mean plasma cholesterol concentration was 71±52 mg/dL compared with the results obtained during the control diet of 129±23 mg/dL (p=0.01). The mean values for plasma very low density lipoprotein (VLDL), low density lipoprotein (LDL), and HDL cholesterol compared with the control values (mg/dL) and corresponding p calculations were 15±6.9 versus 12±6.0 (p=0.4) for VLDL, 41±39 versus 58±17 (p=0.3) for LDL, and 15±4 versus 49±6 (p=0.0001) for HDL. Thus, dietary n-3FAs profoundly reduced the plasma concentrations of HDL cholesterol without changing other cholesterol fractions or triglyceride levels significantly.

Dietary n-3FAs produced the expected modifications in urinary eicosanoid metabolites (Table 3). The excreted concentrations of major dienioic metabolites of prostacyclin (2,3-dinor-6-keto prosta glandin F1α) and thromboxane (2,3-dinor thromboxane B2) fell to <33% of basal levels, and corresponding amounts of n-3FA metabolites derived from prostaglandin I2 and thromboxane B2 appeared in the urine (Table 3).

**Effects of Dietary n-3FAs on Homostasis**

Dietary n-3FAs did not significantly modify either platelet counts (341±33 versus 269±25 x103 platelets/mL in control and treated animals; p=0.096) or fibrinogen concentrations (2.9±0.24 mg/mL in control animals and 3.4±0.40 mg/mL in the treated group, respectively; p>0.1). Overall platelet hemostatic function was detectably impaired (bleeding time measurements were prolonged from 4.3±0.5 to 7.6±1.2 minutes; p=0.039). Platelet responsiveness to collagen-induced aggregation was also minimally decreased (Table 4). After 12 weeks of n-3FA treatment, the concentration of collagen required to induce half maximal platelet aggregation was increased from 6.4±2.1 to 8.5±2.5 μg/mL (p=0.045). However, there was no significant change in the responsiveness of platelets to ADP-induced aggregation by dietary n-3FAs (Table 4), and the levels of plasma βTG, PF4, and FPA were not reduced by dietary n-3FAs compared with the period of control diet (4.8±0.7 versus 2.0±0.5 ng/mL, 1.5±0.2 versus 1.1±0.2 ng/mL, and 6.4±1.6 versus 4.1±1.0 pmol/L for PF4, βTG, and FPA, respectively; p>0.20 in all cases).

As anticipated, serum thromboxane B2 levels were reduced by dietary n-3FAs (from 148±38 ng/109 platelets to 41±16 ng/109 platelets; p=0.005).

The expression of tissue factor activity by monocytes stimulated with endotoxin was substantially reduced by dietary n-3FAs. Monocytes obtained before and 12 weeks after n-3FA treatment was initiated were prepared by density gradients, stimulated with endotoxin in vitro, and assayed for the production of tissue factor activity by use of a purified system. Monocytes obtained from animals during dietary n-3FA administration exhibited little capacity to express tissue factor activity (1.7±0.14 mU/10⁶ cells) compared with baseline values (6.5±1.2 mU/10⁶ cells; p<0.005).

**Effects of Dietary n-3FAs on Thrombotic Responses of Blood**

The thrombotic responses of blood to segments of endarterectomized homologous aorta and Dacron vas-

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**TABLE 3. Effects of Dietary n-3 Fatty Acids on Urinary Eicosanoid Metabolites**

<table>
<thead>
<tr>
<th>Time</th>
<th>Thromboxane B2</th>
<th>Thromboxane B3</th>
<th>Prostaglandin I2</th>
<th>Prostaglandin I3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Base</td>
<td>8.52±4.27</td>
<td>0.09±0.36</td>
<td>1.05±0.43</td>
<td>0.02±0.01</td>
</tr>
<tr>
<td>4 Weeks</td>
<td>5.07±3.92</td>
<td>1.41±0.84*</td>
<td>0.45±0.21*</td>
<td>0.12±0.02*</td>
</tr>
<tr>
<td>8 Weeks</td>
<td>2.92±0.90†</td>
<td>1.81±1.18</td>
<td>0.32±0.34</td>
<td>0.09±0.02†</td>
</tr>
<tr>
<td>12 Weeks</td>
<td>2.47±1.73‡</td>
<td>1.38±0.42‡</td>
<td>0.37±0.22‡</td>
<td>0.05±0.04‡</td>
</tr>
</tbody>
</table>

*p<0.001; †p<0.01; ‡p<0.05.
circular graft under arterial flow conditions were measured in exteriorized AV shunts before and periodically after dietary n-3FAs were initiated (Figure 1). In each case, the deposition of platelets onto thrombogenic segments was measured by $^{111}$In-labeled platelet imaging. In control studies, the deposition of $^{111}$In-labeled platelets onto the Dacron vascular graft segments was rapid, reaching a plateau of $14.1 \pm 1.4 \times 10^9$ platelets for the 5-cm segment of graft with occlusion by about 60 minutes (Figure 2). Dietary n-3FAs decreased platelet deposition significantly compared with pretreatment values without occlusion (by 60 minutes) ($7.5 \pm 0.8 \times 10^9$ platelets/5-cm segment of graft; $p<0.001$). When endarterectomized aortic segments (EASs) were prepared from normal homologous donors and incorporated into chronic femoral AV shunts of n-3FA-treated animals (Figure 3), platelet deposition was similarly reduced (from $4.2 \pm 0.9$ to $1.8 \pm 0.4 \times 10^9$ platelets/cm for control animals compared with the results in animals receiving dietary n-3FAs, respectively; $p<0.01$).

Effects of Dietary n-3FAs on Vascular Thrombus Formation

To determine the effect of n-3FAs on VTG, the deposition of platelets was measured at sites of carotid endarterectomy by gamma camera imaging of $^{111}$In-labeled platelets acutely for 90 minutes and at 24 and 48 hours after the surgical procedures. Platelets accumulated rapidly in control studies, reaching a plateau of $4.4 \pm 1.0 \times 10^9$ platelets/cm (Figure 4). By contrast, dietary n-3FAs interrupted platelet deposition at sites of surgical carotid endarterectomy (platelet deposition, $1.5 \pm 0.4 \times 10^9$ platelets/cm; $p<0.001$ compared with untreated controls).

To determine the relative importance of altered thrombotic blood responses versus modified vascular thrombogenicity in achieving this reduction in vascular thrombosis, measurements of platelet deposition were compared for EASs derived from control versus n-3FA-treated donors in the chronic AV shunts of n-3FA-treated versus untreated control animals in crossover experiments. In control studies, platelets accumulated rapidly on normal EASs in chronic AV shunts of normal animals, reaching a plateau of $4.4 \pm 0.90 \times 10^9$ platelets/cm after 60 minutes, and no significant platelet deposition occurred on segments of normal nonendarterectomized homologous aorta placed in the AV shunts of normal control animals, consistent with previous

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**Figure 2.** Graph showing reduction in $^{111}$In-labeled platelet deposition on vascular graft segments incorporated into exteriorized arteriovenous shunts in n-3 fatty acid (n-3FA)–treated baboons. Results obtained in the same animals (n=8) before n-3FA administration (closed circles) are compared with findings after 12 weeks of n-3FAs (open triangles).

**Figure 3.** Graph showing impaired thrombogenicity of damaged arterial wall by dietary n-3 fatty acids (n-3FA) in baboons. Endarterectomy (EA) thrombus formation on homologous aortic segments is measured in chronic exteriorized arteriovenous shunts using $^{111}$In-labeled platelet imaging analysis. Platelet accumulation at EA sites of normal aortic segments is rapid, reaching a plateau by 60 minutes (closed circles). Platelet deposition at EA sites of normal aortic segments in the shunts of n-3FA–treated animals is significantly reduced (open triangles). However, platelet accumulation at EA sites of aortic segments obtained from n-3FA–treated animals placed in the shunts of normal animals is abolished (closed triangles).

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**Table 4. Effects on Hemostatic Function of Dietary n-3 Fatty Acids for 12 Weeks**

<table>
<thead>
<tr>
<th>Measurements</th>
<th>Baseline</th>
<th>Treated</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet count ($\times 10^9/\mu L$)</td>
<td>341±33</td>
<td>269±25</td>
<td>0.096</td>
</tr>
<tr>
<td>Bleeding time (minutes)</td>
<td>4.3±0.5</td>
<td>7.6±1.2</td>
<td>0.039</td>
</tr>
<tr>
<td>Serum thromboxane B2 (ng/10^9 platelets)</td>
<td>148±38</td>
<td>41±16</td>
<td>0.005</td>
</tr>
<tr>
<td>Platelet aggregation (AC50) *</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ADP (µM)</td>
<td>5.4±0.7</td>
<td>4.8±0.9</td>
<td>&gt;0.5</td>
</tr>
<tr>
<td>Collagen (µg/mL)</td>
<td>6.4±2.1</td>
<td>8.5±2.5</td>
<td>0.045</td>
</tr>
</tbody>
</table>

*AC50 refers to the agonist concentration inducing half-maximal aggregation.*
findings. As reported above, platelet deposition onto normal homologous EASs in AV shunts of n-3FA-treated animals was modestly reduced (1.8±0.4×10⁶ platelets/cm; p<0.01) at the end of 60 minutes (Figure 3). By contrast, platelet accumulation was eliminated on EASs obtained from four n-3FA-treated donors and placed in the shunts of normal ¹¹¹In-labeled platelet recipient animals (0.24±0.08×10⁶ platelets/cm; p<10⁻⁵; Figure 3).

**Effects of Dietary n-3FAs on Vascular Lesion Formation**

In untreated control animals, vascular intimal proliferative lesions formed at sites of carotid endarterectomy (Figures 5 and 6; Table 5). By morphometric analysis of tissue sections prepared from carotid endarterectomy specimens obtained 46±5 days after surgery, the cross-sectional area of neointimal lesions in 12 untreated control animals measured 0.428±0.104 mm², with 1.39±0.23 mm² of media remaining after the endarterectomy procedures (Table 5). By contrast, VLF was essentially abolished at sites of carotid endarterectomy 46±9 days after surgery in animals receiving dietary n-3FAs (0.048±0.031 mm² neointimal area, p=0.010, with 1.27±0.36 mm² of remaining media; Table 5).

**Discussion**

This study demonstrates that dietary n-3FAs 1) are rapidly incorporated into blood and vascular tissues, 2) modestly reduce the thrombotic responses of flowing blood to thrombogenic surfaces, and 3) largely eliminate both the thrombotic and proliferative responses of endarterectomized carotid arteries while producing trivial effects on platelet hemostatic function and no other notable adverse effects when administered in high doses to baboons. Dietary n-3FAs are postulated to produce these striking biological effects by disordering cellular membrane functions that are critical for both VTF and VLF at sites of arterial mechanical injury but largely irrelevant in hemostatic mechanisms.

The hemostatic apparatus involves the formation of provisional hemostatic plugs by platelets adhering to exposed subendothelial adhesive proteins and subsequent stabilization by fibrin generated through a set of tightly controlled sequential catalytic events leading to activation of platelets by thrombin and cleavage of

**Figure 4.** Graphs showing interruption of carotid endarterectomy thrombus formation by dietary n-3 fatty acids (n-3FAs) in baboons. ¹¹¹In-labeled platelet deposition at sites of surgical carotid endarterectomy is measured by gamma camera imaging. Baseline results obtained before dietary n-3FA therapy was initiated are shown by the open circles. The findings after 12 weeks of n-3FA administration are shown by the closed circles (n=8).

**Figure 5.** Photomicrographs showing effect of dietary n-3FAs on carotid endarterectomy lesion formation in baboons. Representative low-power photomicrographs of carotid artery histological cross sections prepared from normal control animals (panel A), untreated animals 46 days after endarterectomy (panel B), and animals 46 days after endarterectomy that were treated with dietary n-3FAs (panel C). Original magnification, ×25.
circulating fibrinogen to fibrin. The minimal impairment in platelet function observed in the present study (Table 4) is consistent with previous reports in that the effects are attributable to decreased spontaneous thromboxane A2 generation by aspirin resulting from its covalent inactivation of platelet cyclooxygenase. However, therapy with dietary n-3FAs differs from treatment with aspirin in that n-3FAs largely fail to affect overall vascular prostaglandin activities despite reciprocal effects on prostaglandin I2 and prostaglandin I3 production, because both prostaglandin I2 and prostaglandin I3 exhibit antiaggregatory and vasodilatory activities, whereas aspirin blocks the generation of thromboxane by platelets and prostaglandins by vessels.

Under arterial flow conditions, dietary n-3FAs inhibit the thrombotic responses of blood to thrombogenic surfaces, including Dacron vascular grafts and surgical endarterectomies (Figures 2 and 3). Since aspirin fails to affect these platelet-dependent thrombotic processes, it follows that dietary n-3FAs must affect thrombotic blood responses in addition to the reduction in thromboxane A2 generation by activated platelets. Several membrane functions of other blood cells are known to be altered by dietary n-3FAs that may compromise the blood's thrombotic responses. First, n-3FAs increase erythrocyte deformability, which may limit platelet recruitment by reducing 1) erythrocyte-dependent platelet diffusion and 2) the release of proaggregatory ADP from erythrocytes under shear stress. Second, n-3FAs alter leukocyte responses that may decrease the accumulation of platelets on thrombogenic surfaces by impairing leukocyte expression of proaggregatory adhesion molecules interacting with platelets or their production of prothrombotic factors such as tissue factor. Indeed, the present study demonstrates that dietary n-3FAs greatly inhibit the capacity of blood monocytes to express tissue factor activity after stimulation by endotoxin (see “Results”). Third, surface-dependent platelet adhesion reactions are impaired by n-3FA–dependent modifications that may lead to decreased recruitment of ambient platelets onto these thrombogenic surfaces.

Vascular thrombosis at endarterectomy sites is interrupted by dietary n-3FAs (Figures 3 and 4). Thrombin plays a central role in this complex platelet-dependent process, as shown by the capacity of direct antithrombins, such as hirudin and synthetic antithrombin peptides, to abolish VTF. VTF involves the localized

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**Table 5. Carotid Endarterectomy Vascular Lesion Formation**

<table>
<thead>
<tr>
<th>Endarterectomy site</th>
<th>Area of neointima (mm²)</th>
<th>Area of media (mm²)</th>
<th>Neointima/media (×100)</th>
<th>Uninjured site Area of media (mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control animals</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 Days (n=4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>46 Days (n=12)</td>
<td>0.428±0.104</td>
<td>1.39±0.23</td>
<td>31±23</td>
<td>1.47±0.22</td>
</tr>
<tr>
<td>n-3FA treated</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 Days (n=4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>46 Days (n=6)</td>
<td>0.048±0.031</td>
<td>1.27±0.36</td>
<td>4±3</td>
<td>1.47±0.21</td>
</tr>
</tbody>
</table>
surface-mediated interactions among vascular constituents, platelets (and to a lesser extent erythrocytes and leukocytes), and plasma coagulation factors.\textsuperscript{40-42,65,66} These reactions occur on negatively charged phospholipid surfaces, including subendothelial connective tissue elements, platelet and vascular cell membranes, and tissue factor released from damaged vascular tissues and monocytes/macrophages.\textsuperscript{65,66,76-78} Mechanical vascular injury initiates thrombin generation by inducing tissue factor–activated factor VII complex (TF:fVIIa)–dependent activation of factor X on phospholipid surfaces in the presence of the cofactor-activated factor V and ionized calcium to form the prothrombinase complex that cleaves prothrombin to form thrombin.\textsuperscript{65,66,76-78} Thrombin generation is greatly augmented through an amplification loop that activates factor X to factor Xa on phospholipid surfaces by the proteolytic complex composed of thrombin-activated factor VIII cofactor activity and TF:fVIIa-activated factor IX.\textsuperscript{76-78} Thrombin recruits ambient platelets into the forming thrombus by cleaving an extracellular amino-terminal extension of the seven-transmembrane thrombin receptors on platelets\textsuperscript{76} with receptor signaling initiated by the tethered neoamino-terminus.\textsuperscript{80} Thrombin also binds tightly with elements of already formed thrombus\textsuperscript{41,81,82} in a proteolytically competent configuration capable of activating platelets and cleaving fibrinogen\textsuperscript{41,81} but inaccessible to the inhibitory effects of heparin complexed to plasma antithrombin.\textsuperscript{41,81,82} Membrane phospholipids of platelets and vascular wall cells incorporate dietary n-3FAs (Table 2) that affect their physicochemical properties,\textsuperscript{8,29-32,71,72} particularly with respect to the complex membrane changes underlying the enhanced conversion of coagulant zymogens to their respective serine protease complexes required for thrombin generation.\textsuperscript{76-78} We postulate that dietary n-3FAs prevent the formation of vascular thrombosis at sites of mechanical vascular injury by inhibiting the capacity of the exposed subendothelial constituents to initiate and sustain the membrane-dependent generation and binding of thrombin. The mechanisms whereby dietary n-3FAs abolish vascular lesion formation are not clear. The proliferative smooth muscle cell (SMC) response induced by mechanical vascular injury is a variable multifactorial process that is postulated to involve the luminal migration of medial SMCs, stimulated by platelet-derived growth factor (PDGF) arising from platelets accumulating at the site of injury, and their luminal proliferation, mediated initially by fibroblast growth factor (FGF) (derived from damaged vascular wall cells) and subsequently sustained by PDGF (derived from stimulated vascular wall and inflammatory cells).\textsuperscript{61,83-88} This hypothesis is supported by the observations that 1) the initial mitogenic response is inhibited by infusing antibodies against FGF,\textsuperscript{62} 2) subsequent intimal SMC accumulation is decreased by infusing antibodies against PDGF,\textsuperscript{84} and 3) mechanical vascular injury upregulates PDGF and downregulates PDGF receptor expression by medial and intimal SMCs and macrophages and by adjacent endothelial cells.\textsuperscript{88} However, it now appears that thrombin may also be important in the intimal proliferative responses that constitute VLF.\textsuperscript{89-93} Among the many factors present at the site of vascular injury, only thrombin reproduces in cultured medial SMCs the pattern of modulated gene expression observed in vivo after balloon catheter vascular injury.\textsuperscript{88,89} Moreover, infusing the antithrombin peptide D-FPRCH$_2$Cl for 6 hours after balloon catheter arterial injury prevents the expression of PDGF mRNA by damaged medial SMCs in nonhuman primates.\textsuperscript{89} Since macrophages normally accumulate in the human atheroma and express tissue factor, they provide a potential continuing source of thrombin generation capable of producing progressive proliferative lesions.\textsuperscript{62} Finally, it has recently been shown that the inhibition of thrombin activity for 3 days by continuously infused hirudin greatly decreases (by >80\%) the early mitogenic response of balloon-injured arterial SMCs.\textsuperscript{93}

Dietary n-3FAs may modulate several of the putative mechanisms contributing to VLF, although their relative importance and susceptibility to n-3FA modification are not presently known. For example, dietary n-3FAs may decrease the production of PDGF at sites of vascular injury, since n-3FAs reduce the expression of PDGF by cultured endothelial cells.\textsuperscript{10} Furthermore, because n-3FAs block tissue factor expression by blood monocytes stimulated by endotoxin and abolish the accumulation of macrophages and production of tissue factor at sites of vascular injury, it is reasonable to postulate that the interruption of VLF by dietary n-3FAs may reflect the inhibition of thrombin-dependent proliferation in VLF. Thus, n-3FA interruption of thrombin generation may be responsible for eliminating both VTF and VLF. Contrary to predictions based on clinical correlations, the interruption of VLF by dietary n-3FA occurs despite the striking drop in HDL cholesterol (from control values of 59±6 to 15±4 mg/dL after 12 weeks of dietary n-3FAs; p=0.0001).

The dose of n-3FAs administered in the present study is fivefold to 10-fold greater than that used in reported clinical trials.\textsuperscript{20-28} Presumably, this difference in dosage explains the contrast between the striking effects reported in the present study and the inconclusive results obtained in the clinical trials,\textsuperscript{20-28} although no dose–response data are presently available in either humans or nonhuman primates. It will be important to determine whether larger doses in human studies are accompanied by enhanced beneficial effects. It will also be important to establish the relative roles of EPA versus DHA in mediating n-3FA–dependent outcomes.

We conclude that dietary n-3FAs produce profound effects on both VTF and VLF, with lesser effects on blood thrombotic responses, while largely sparing hemostasis in baboons. The safe antithrombotic and antiproliferative profile for this dose of dietary n-3FAs has important implications in the design of therapeutic trials in patients with symptomatic atherosclerotic vascular disease.

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