Dietary n-3 Fatty Acid Effects on Neutrophil Lipid Composition and Mediator Production

Influence of Duration and Dosage

Floyd H. Chilton,*‡ Manish Patel,* Alfred N. Fonteh,* Walter C. Hubbard,§ and Massimo Triggiani§

**Department of Medicine and ‡Biochemistry, Bowman Gray School of Medicine of Wake Forest University, Winston-Salem, North Carolina 27157; and §Department of Medicine, Division of Clinical Immunology, The Johns Hopkins University School of Medicine Asthma and Allergy Center, Baltimore, Maryland 21224*

Abstract

Healthy volunteers supplemented their usual Western diets with Promega fish oil supplement (eicosapentaenoic acid [EPA], 0.28 g; docosahexaenoic acid [DCHA], 0.12 g; other n-3 fatty acids 0.10 g per capsule) using three protocols. Initial experiments (protocol 1 and 2) investigated the kinetics of incorporation of n-3 fatty acids into serum and neutrophil lipids after 10 capsules/d of Promega. EPA was rapidly detected in both serum and neutrophil lipids; the arachidonic acid (AA) to EPA ratio in neutrophil phospholipids showed a maximal reduction of 49:1 to 8:1 within 1 wk of beginning supplementation. EPA was preferentially incorporated into phosphatidylethanolamine and phosphatidylcholine but not phosphatidylinositol. Long-term supplementation for up to 7 wk did not influence the AA/EPA ratio or the distribution of EPA among neutrophil phospholipids in a manner that was not observed after the first week. Neutrophils produced similar quantities of platelet-activating factor and slightly lower quantities of leukotriene B_4 during long-term supplementation when compared with presupplementation values. Experiments examining the influence of Promega dosage indicated that the AA/EPA ratio in neutrophil lipids decreased in a dose-dependent manner. Only when the dose was increased to 15 capsules/d was there a reduction in the AA/DCHA ratio in neutrophil lipids. The quantity of AA in neutrophil lipids remained relatively constant at all supplement doses. Taken together, the current study demonstrates the capacity of n-3 fatty acids provided with a Western diet to be rapidly incorporated into neutrophil lipids. However, dietary n-3 fatty acids appear not to significantly reduce arachidonate content within neutrophil phospholipids. Constant arachidonate levels may account for the lack of large reductions in the biosynthesis of lipid mediators by neutrophils after fish-oil supplementation. (*J. Clin. Invest.* 1993. 91:115–122.) Key words: arachidonic acid • eicosapentaenoic acid • docosanexaenoic acid • platelet-activating factor • leukotrienes

Address correspondence to Floyd H. Chilton, Ph.D., Associate Professor, Department of Medicine, Section on Pulmonary and Critical Care Medicine, Bowman Gray School of Medicine, Medical Center Boulevard, Winston-Salem, NC 27157-1054.

Received for publication 5 March 1992 and in revised form 7 July 1992.

J. Clin. Invest.

0021-9738/93/01/115/08 $2.00
Volume 91, January 1993, 115–122

Introduction

In the last 20 years, polyunsaturated fatty acids (n-3) prominent in marine fish oils have been suggested to attenuate the clinical manifestations of certain diseases. Much of the interest in this topic arose from epidemiological studies carried out in the 1970s, which revealed an unusually low incidence of cardiovascular disease in Greenland Eskimos (1–3). This low incidence of cardiovascular disease was attributed to their abundant consumption of fish rich in n-3 fatty acids concomitant with lower ingestion of n-6 fatty acids and saturated fatty acids. Since these initial studies, n-3 fatty acids have been suggested to have beneficial effects in a number of diseases thought to involve chronic inflammation, including arthritis and asthma (4–11).

The mechanism by which these n-3 fatty acids exert their effects in inflammation is not clear at this time. One hypothesis is that n-3 fatty acids abundant in fish oils, eicosapentaenoic acid (EPA)[1] and docosahexaenoic acid (DCHA), reduce the content of arachidonic acid (AA) within inflammatory cells. Studies conducted in humans and in various animal models have provided the basis for this hypothesis (12, 13). It is thought that these n-3 fatty acids compete with AA for enzymes responsible for the esterification of fatty acids into cellular phospholipids (13–20). Once the content of arachidonate within cells is reduced, the cells have a decreased capacity to produce inflammatory mediators, such as eicosanoids and platelet-activating factor (PAF), thereby having a beneficial effect in inflammatory disorders. Alternative hypotheses revolve around the capacity of n-3 fatty acids to participate in enzymatic steps of eicosanoid biosynthesis previously thought to be selective for AA. For example, it has been suggested that n-3 fatty acid–containing phospholipids compete with arachidonate-containing phospholipids for phospholipase A_2 (21, 22). In addition, both EPA and DCHA have been demonstrated to be metabolized by the 5-lipoxygenase pathway in neutrophils to form leukotrienes (LTs) of the B series and their corresponding hydroxyacids (23–27).

In light of an increased understanding of the biochemistry of n-3 fatty acids, many laboratories are supplementing the usual diets of patients suffering from various inflammatory disorders with fish oils and are examining the effects of these dietary supplements on disease activity (7–11). Although some

1. *Abbreviations used in this paper:* AA, arachidonic acid; DCHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; GC/MS, gas chromatography/mass spectrometry; GPC, *sn*-glycero-3-phosphocholine; PAF, platelet-activating factor; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PgB_2, prostaglandin B_2; PI, phosphatidylinositol.

of these studies are yielding promising results regarding the efficacy of fish-oil diets, other studies report that they are without effect. An explanation for this growing controversy may stem from the lack of information in humans on the influence of n-3 fatty acids supplementing usual diets on the in vivo biochemistry of circulating inflammatory cells such as the neutrophil. For example, there are factors such as the kinetics of uptake of dietary n-3 acids, the dose of n-3 acids, the influence of n-3 fatty acids on arachidonate content, and the effect of incorporated n-3 acids on lipid-mediator production in individual blood components that are presently not well understood in the human. The lack of knowledge in these areas often make rational decisions on an appropriate diet impossible. The overall goal of this study was to examine each of these parameters in human neutrophils from volunteers supplementing their diets with various concentrations of n-3 fatty acids. The data presented in this study point out the large capacity of n-3 fatty acids, and EPA in particular, to be rapidly incorporated into neutrophil phospholipids in vivo. However, these data also put into question the capacity of these n-3 fatty acids provided with a usual Western diet to reduce arachidonate content or lipid-mediator production in the human neutrophil.

Methods

Materials. 1-*O*-hexadecyl-2-$^2H_3$-acetyl-*sn*-glycero-3-phosphocholine (GPC), prostaglandin B_2 (PgB_2), AA, EPA, DCHA, and octadeuterioarachidonic acid were obtained from Biomol Research Laboratories, Inc., (Plymouth Meeting, MA). Phospholipid standards used in normal-phase HPLC were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL). LT standards used in reverse-phase HPLC were obtained from Cayman Chemical Co., Inc. (Ann Arbor, MI). Pentafluorobenzyl bromide, diisopropylethylamine, and acetonitrile were from Pierce Chemical Co. (Rockford, IL). Pentafluorobenzoyl chloride, 4-dimethylaminopyridine, and monobasic potassium phosphate (gold label) used to make the buffer for normal-phase HPLC were all obtained from Aldrich Chem. Co. (Milwaukee, WI). Anhydrous methylene chloride was prepared by the passage of methylene chloride (Fisher Scientific, Silver Spring, MD) over anhydrous sodium sulfate (Aldrich Chem. Co.). HBSS (with and without Ca^{++}) was purchased from Biofluids, Inc. (Rockville, MD). Solid phase extraction silica gel columns were purchased from J.T. Baker Chemical Co. (Phillipsburg, NJ). Ionophore A23187 was purchased from Calbiochem-Behring Corp. (San Diego, CA). Ficoll-Paque was purchased from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ). Formic acid, dibasic potassium phosphate used in quantitative phosphorous assay, potassium chloride used in neutrophil isolation, hydrochloric acid, potassium hydroxide, and DMSO were purchased from Fisher Scientific. All solvents were HPLC grade and were purchased from Fisher Scientific.

Protocol I. Short-term supplementation. Four healthy males, 23–33 yr old, gave informed consent to supplement their usual diet with daily consumption of 10 capsules of Promega (Parke-Davis, Morris Plains, NJ) for 12 d. No restrictions were applied to the usual diet regimens of individuals on the diet. Each capsule contains 10 calories, 81% of which are from fat. Total fat is 1 g per capsule. Each capsule also contains 280 mg of EPA, 120 mg of DCHA, and 100 mg of other n-3 fatty acids. In addition, each capsule contains < 1 mg of cholesterol. No significant side effects were reported by any of the subjects during or after the supplementation period. Neutrophils and serum were collected after overnight fasting 1 d before starting supplementation, every 2 d during the supplementation, and at 4 and 8 d after the supplementation was stopped.

Protocol II. Long-term supplementation. Five healthy males, 29–34 yr old, supplemented their usual diet with daily consumption of 10 capsules of Promega for 7 wk. No significant side effects were reported by any of the subjects during or after the supplementation period. Neutrophils were collected before starting and every other week during the supplementation.

Protocol III. Four healthy males, 23–33 yr old, supplemented their usual diet with daily consumption of 10 capsules of Promega for 6 d. Supplementation was then stopped for 10 d; the dosage was then raised to 15 capsules/d for 6 d. Again the supplementation was stopped for 10 d and dosage was then increased to 20 capsules/d for 6 d. No significant side effects were reported by any of the subjects during or after the supplementation periods. Neutrophils were collected before starting the protocol and on the first (before consuming the capsules) and the last day of each supplementation period.

Isolation of neutrophils. Neutrophils were isolated by dextran sedimentation followed by Ficoll-Paque density centrifugation as previously described (28). The purity of neutrophils in this preparation was > 98% and the viability was > 95%. Neutrophils were suspended at a concentration of 5×10^6 cells/ml in HBSS without Ca^{++} and then divided into two 1-ml aliquots. Methanol/chloroform (2:1, vol/vol) was added to each aliquot and the lipids were extracted by the method of Bligh and Dyer (29). One of the aliquots was then split into duplicates containing the extract from 2.5×10^6 cells each. One duplicate was used to determine the mole quantities of AA, EPA, and DCHA in the neutrophil membrane lipids. The other was used to determine the phospholipid content in the neutrophil by a quantitative phosphorous assay (30). The other aliquot was used for separation of phospholipid classes as described below.

Isolation of serum lipids. Serum was collected from 2 ml of whole blood. Lipids were extracted from a 100-μl aliquot of the serum by a modified method of Bligh and Dyer (29), in which a sufficient amount of formic acid was added to bring the pH of the aqueous layer to 3. A small portion (0.6%) of the lipids that were extracted from the serum was used to determine mole quantities of AA, EPA, and DCHA.

Separation of phospholipid classes. Neutrophil phospholipid classes were separated using normal-phase HPLC. Lipid extracts were suspended in 200 μl of a normal-phase injection solvent of hexane/isopropanol/water (4:5.5:0.3, vol/vol). The lipid extract was then loaded onto a silica column (Ultrasphere, 4.6 × 250 mm; Rainin Instrument Co. Inc., Woburn, MA) and eluted with hexane/isopropanol/ethanol/phosphate buffer (pH 7.4)/acetic acid (490:367:100:30:0.6, vol/vol) for 5 min at a flow rate of 1 ml/min (31). The amount of phosphate buffer in the eluting solvent was then increased to 5% over a 10-min period and this solvent composition was maintained until all the major phospholipid classes were eluted from the column. Appropriate peaks were collected on the basis of the elution times of phospholipid standards run in the same system. The isolated phospholipid classes (phosphatidylethanolamine [PE], phosphatidylinositol [PI], phosphatidylserine [PS], and phosphatidylcholine [PC]) were then analyzed for their AA, EPA, and DCHA content as described below.

Analysis of fatty acid content within neutrophil phospholipids. Determination of mole quantities of AA, EPA, and DCHA was accomplished by GC/MS. Octadeuterioarachidonic acid (50 ng) as an internal standard was added to each lipid extract. Fatty acyl chains were then hydrolyzed from glycerolipids with 0.2 N KOH in ethanol/water (75:25, vol/vol) for 30 min at 60°C. After 30 min, an equal amount of water was added and the pH was adjusted to 3 with 6 N HCl. The free fatty acids were extracted from the reaction mixture with hexane/ether (1:1, vol/vol). A fatty acid–enriched fraction was then obtained from the extract by solid-phase extraction using a silica gel column. The silica columns were equilibrated with hexane and then samples were loaded in hexane. The columns were washed with hexane followed by hexane/ether (9:1, vol/vol). The fatty acids were then eluted from the column with hexane/ether (1:1, vol/vol) and converted to pentafluorobenzyl esters, using 20% pentafluorobenzyl bromide and 20% diisopropylethylamine in acetonitrile. The solvents were then removed from the sample with a stream of N_2 and the sample was resuspended in hexane. GC/MS analysis was carried out on a TSQ-700 GC/MS/MS/DS (Finnigan MAT, San Jose, CA) operated as a single-stage quadrupole. The gas chromatography was performed using a 30-m SPB-5

fused silica column (SPB-5; 0.25-mm i.d., 0.25-mm film thickness; Supelco Inc., Bellafonte, PA) threaded within < 1.5 cm of the mass spectrometer ion source. The initial column temperature was 195°C. The column was heated to 235°C at a rate of 40°C/min with subsequent increase in temperature to 267°C at a rate of 3.0°C/min. The column temperature was further increased to 287°C at a rate of 10°C/min. The injector temperature was maintained at 260°C. Each injection was performed in the splitless mode. The splitless mode was maintained for 0.6 min followed by a split of the injector contents at a ratio of 50:1. A volume of 1 μl of 500 μl of recovered material dissolved in hexane was injected. Helium maintained at a pressure field of 70 kPa (10 psi) was used as a carrier gas. The pentafluorobenzyl esters were analyzed using selected ion-recording techniques to monitor for AA (m/z 303), EPA (m/z 301), DCHA (m/z 327), and octadeuterioarachidonate (m/z 311). A standard mixture of the aforementioned fatty acids was injected and analyzed by negative ion chemical ionization-GC/MS before each biological sample to obtain precise retention times.

Cell activation. Neutrophils were suspended at a concentration of 2.0×10^7 cells/ml in HBSS with Ca^{++}. 1-ml aliquots of the cells were challenged with or without A23187 (2 μM) at 37°C for 10 min. The incubations were terminated by lowering the pH of the reaction with 9% formic acid. PgB_2 (250 ng) was added as an internal standard. Cells were immediately removed from supernatant fluids by centrifugation (225 *g*, 8 min, 5°C). The cell supernatant fluids were removed, were acidified with formic acid, and lipids were extracted (2×) with an equal volume of ethyl acetate. The supernatant fluid extract was then analyzed for LTs as described below. The lipids were extracted from the cell pellet by the method of Bligh and Dyer (29) for PAF determination as described below.

1-alkyl-2-acetyl-GPC(PAF) determination. 1-*O*-hexadecyl-2-$^2H_3$-acetyl-GPC (40 ng) was added as an internal standard to the lipid extract of the cell pellet. Solvents were then removed under a stream of N_2 and the lipids resuspended in chloroform. A PAF-enriched fraction was obtained from the extract by solid-phase extraction using a silica gel column. The silica columns were first washed with chloroform. The samples were then loaded onto the columns and the columns were washed successively with chloroform/methanol (3:1, vol/vol) and then with methanol. PAF was then eluted from the column with methanol/water (3:1, vol/vol). Solvents were removed from the sample and the residue was treated with 500 μl of 49% hydrofluoric acid and 1 ml hexane with continuous shaking for 4 h at room temperature (32). This procedure removed the phosphobase moiety of the molecule to form diglycerides. As the diglycerides were formed in the reaction mixtures, they were partitioned into the hexane (upper) phase. The hexane phase containing the diglycerides was then removed and transferred to a clean silanized glass vial. The hexane was removed under a stream of N_2 and pentafluorobenzoyl esters were synthesized by adding 50 μl of pentafluorobenzoyl esterification reagent (2 mg of 4-dimethylaminopyridine and 4 μl of pentafluorobenzoyl chloride in 600 μl of anhydrous methylene chloride) to the residue and heating at 60°C for 5 min. The pentafluorobenzoylglycerides were then analyzed on a TSQ-700 (Finnigan MAT) by selected ion-monitoring technique to record molecular anions at m/z 552, 580, and 578 for 1-alkyl-2-acetyl-GPC containing 16:0 (hexadecyl), 18:0, and 18:1 carbon chain at the sn-1 position, respectively, and m/z 555 for 1-*O*-hexadecyl-2-$^2H_3$-acetyl GPC (33). PAF and its analogs were resolved using a 30-m ASPB-5 fused silica column (0.25 mm i.d., 0.25-μm film thickness) with helium as the carrier gas. The initial column temperature of 205°C was maintained for 1 min and then increased to 245°C at a rate of 40°C/min. The temperature was then increased to 297°C at a rate of 2.0°C/min.

Chromatography and quantitation of LTs in the cell supernatant fluid. LTs were isolated from the ethyl acetate extraction mixture by reverse-phase HPLC. The extract was loaded onto an ODS column (Ultrasphere 2 × 250 mm; Beckman Instruments, Inc., San Ramon, CA) and eluted with methanol/water/phosphoric acid (55:45:0.02, vol/vol), pH 5.7, at 0.25 ml/min. After 5 min, the LTs were eluted from the column by increasing the methanol composition from 55 to 100% over 15 min. Mole quantities of LTB_4, LTB_5, and 20-OH-LTB_4 were determined as a ratio to the internal standard PgB_2 from the absorbance of ultraviolet peaks at 270 nm. Peaks were identified on the basis of the elution times of authentic LT standards run in the same system.

Calculations. AA, EPA, and DCHA content was calculated as a percentage of total fatty acids in the neutrophil phospholipids on the basis of the assumption that there are two fatty acids per molecule of phosphate in each phospholipid.

Results

Influence of short-term supplementation (protocol 1) on neutrophil and serum lipids. Initial studies were designed to examine the kinetics of incorporation of n-3 fatty acids (provided in vivo) into cellular phospholipids of the human neutrophil. In the first set of experiments, healthy males consumed 10 capsules of Promega for 12 d in addition to their regular diets. Neutrophils were collected before starting the supplementation, every 2 d after supplementation, and 4 and 8 d after supplementation was ceased. The data illustrated in Fig. 1 show the ratio of AA to EPA in serum and neutrophil lipids at each collection point. Ingested EPA was rapidly detected in serum lipids; for example, the AA/EPA ratio decreased from 8:1 before the supplementation to ~ 1:1 within 2 d of starting the supplementation. Similarly, the ratio in neutrophil lipids lessened from 49:1 to 8:1 after 6 d and decreased only slightly from

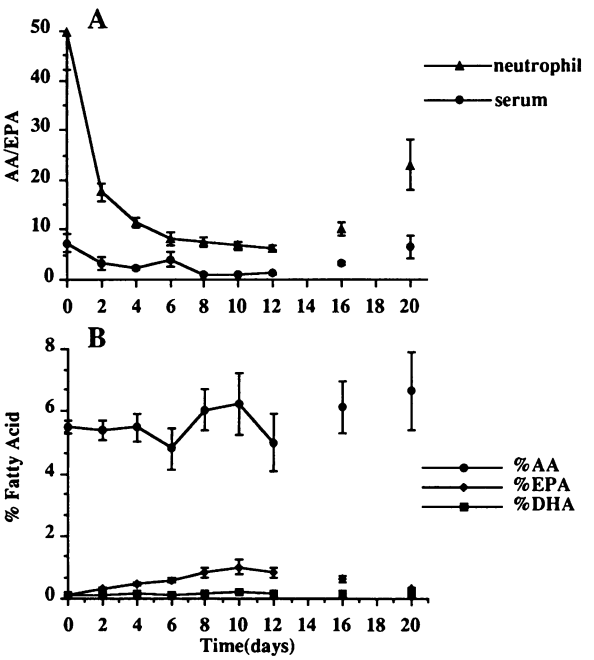

Figure 1. Short-term time course of incorporation of EPA and DCHA into neutrophil and serum lipids. Neutrophils and serum were collected from the same donors before and after Promega supplement (protocol I). Fatty acids linked to complex lipids were hydrolyzed as described. The fatty acid composition of the lipid extracts was determined by GC/MS. (*A*) Ratios of AA to EPA were determined and expressed as the quotient of mole quantity of arachidonate/mole quantity of eicosapentaenoate. (*B*) In addition, the quantities of arachidonate, eicosapentaenoate, or docosahexeanoate were expressed as a percentage of the total fatty acids. The data are presented as mean±SE from four individuals.

that point. Once volunteers had stopped the supplementation, the AA/EPA ratio rapidly increased to > 10:1.

The mole quantities of arachidonate were also measured in neutrophils collected at each day to determine whether the presence of EPA influenced arachidonate levels within cells. Because of the possibility for small inaccuracies in the day-to-day cell counts and the potential for differences in sample-to-sample extraction recoveries, the quantities of arachidonate were standardized to phosphorous content of phospholipids in each sample. Data shown in Fig. 1 *B* demonstrated that increasing the quantity of EPA in neutrophil lipids did not influence arachidonate levels at any time point tested. AA accounted for 5 to 6% of the total fatty acids (assuming two fatty acyl chains per phosphorous molecule) in the cell. The levels of EPA approached 1% of the total fatty acids in the latter days (days 6 to 12) of supplementation. In contrast to EPA, there was little measurable change in the DCHA levels within neutrophil lipids at the doses and time points tested in protocol 1. The changes in the ratio of AA to EPA in the major phospholipid classes were also measured before supplementation, and days 4 and 8 after supplementation. The ratio of AA to EPA in PE and PC decreased from > 10:1 (before supplementation) to ~ 4:1 at 8 d. By contrast, the AA/EPA ratio in PI did not drop below 10:1 at any day tested (data not shown).

Influence of long-term supplementation (protocol 2) on neutrophil lipids. An important difference between the aforementioned experiment and most clinical studies in the literature was the length of time of the supplementation. Therefore, an important question that needed to be addressed was whether a long-term supplementation protocol would influence fatty acid ratios and distribution in a manner that was not observed at 12 d. A 7-wk supplementation study was undertaken to address this question. In this study, males supplemented their usual diet with 10 capsules/d of Promega for 7 wk. Blood was collected from these individuals before supplementation and 1, 3, 5, and 7 wk after supplementation. Fig. 2 shows the ratio of EPA to AA in the major phospholipid classes of the neutrophil. These data confirm that EPA was rapidly incorporated into neutrophil phospholipids of individuals taking the fish-oil concentrate. The ratio of AA/EPA in PE and PC dropped from > 10:1 to < 5:1 after 1 wk and remained at these levels throughout the 7 wk. In agreement with the short-term study (protocol 1), the AA/EPA ratio in neutrophil PI decreased only slightly within the first weeks of fish-oil consumption. Furthermore, this long-term study indicated that the ratios of AA to EPA achieved during the first week were not significantly influenced by prolonging supplementation up to 7 wk.

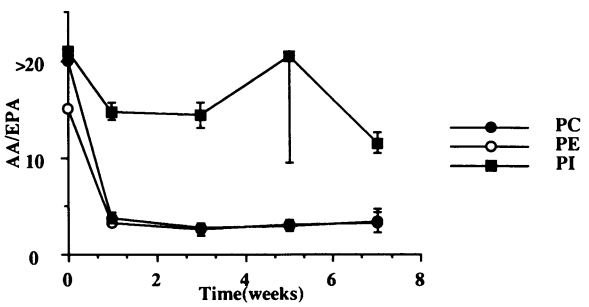

Figure 2. Long-term time course of the incorporation of EPA into neutrophil phospholipids. Neutrophils were collected from the same donors before and after Promega supplementation (protocol 2). The major phospholipid classes were separated by HPLC. Fatty acids linked to complex lipids were hydrolyzed and fatty acid compositions were determined by GC/MS. Fatty acid ratios were determined and expressed as the quotient of mole quantity of arachidonate/mole quantity of eicosapentaenoate. The data are presented as mean±SE from four individuals.

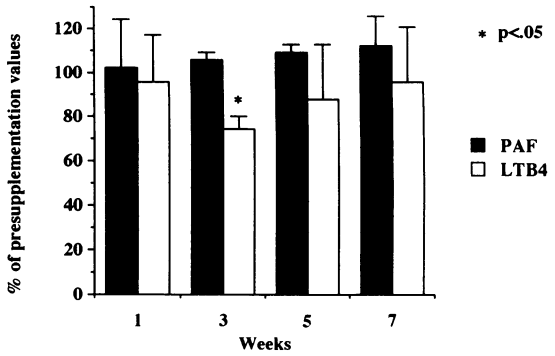

Figure 3. Influence of EPA on PAF and LTB_4 biosynthesis. Neutrophils were collected from the same donors before and after Promega supplementation (protocol 2). Neutrophils were then stimulated and the quantities of PAF and LTB_4 determined as described. The data are expressed as a percentage of the presupplementation values and are the mean±SE from four individuals.

It was clear from the previously described two supplementation studies that n-3 fatty acids provided in vivo have a large capacity to be incorporated into neutrophil phospholipids. The next experiment examined the capacity of the n-3 fatty acids to influence lipid-mediator production by the neutrophil. Neutrophils isolated from volunteers before and at 1, 3, 5, and 7 wk after supplementation were stimulated with ionophore A23187 (2 μM) and lipoxygenase products and PAF were measured as described in Methods. Neutrophils isolated from volunteers produced similar quantities of PAF before and after supplementation (Fig. 3). There was a small (26%) but significant reduction in the quantity of LTB_4 produced by neutrophils at 3 wk after supplementation. In contrast there was a threefold increase in the quantity of LTB_5 produced by neutrophils isolated from volunteers at all time points after the beginning of supplementation. The quantity of LTB_5 increased from 1.3 pmol/10^6 cells before supplementation to 4.5 pmol/10^6 cells after 7 wk of supplementation.

Influence of the dosage of dietary fish oils (protocol 3) on neutrophil lipids. On the basis of information obtained in the previous data, a third study was designed to examine the influence of the dosage of fish-oil concentrates on the levels of n-3 fatty acids and arachidonate in neutrophil lipids. In this protocol, volunteers were directed to consume 10 capsules (2.8 g of EPA, 1.2 g of DCHA, and 1.0 g of other n-3 acids) of Promega/d in addition to their usual diets for 6 d. The volunteers then ceased the supplementation of Promega capsules for 10 d (first wash out); this was then followed by supplementation with 15 capsules (4.2 g of EPA, 1.8 g of DCHA, and 1.5 g of other n-3 fatty acids) of Promega/d for 6 d. Again the volunteer ceased the supplementation with Promega capsules for 10 d (second wash out) followed by consumption of 20 capsules (5.6 g of

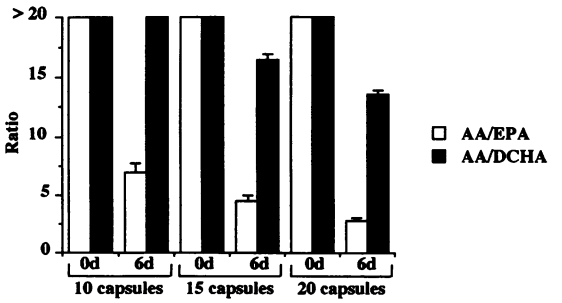

Figure 4. Incorporation of EPA and DCHA into neutrophil lipids as a function of the dose of supplement. Neutrophils were collected from the same donors before and after Promega supplementation (protocol III). Fatty acid ratios were calculated as described in Fig. 1. The data are expressed as mean±SE from four individuals.

EPA, 2.4 g of DCHA, and 2.0 g of other n-3 fatty acids)/d for 6 d. Neutrophils were obtained from each volunteer before starting the study, after each supplementation, and after each wash out period. Fig. 4 shows the AA/EPA and AA/DCHA ratios in neutrophils at each of these points. These data indicated that there was a dose-dependent decrease in the AA/EPA ratio in neutrophils from volunteers on the supplement. At 20 capsules/d, the EPA/AA ratio in neutrophil lipids reached 2.5:1. During each wash out period the AA/EPA ratio returned to > 20:1. In the previously described feeding protocols, the AA/DCHA ratio had not consistently changed during supplementation. However, when the dose of fish oil was increased to 15 capsules/d, the DCHA/AA ratio reached a value of 16:1 and decreased further to 13:1 with the consumption of 20 capsules/d.

It was important to determine at high concentrations of the fish-oil supplement if the decrease in the AA/EPA ratio was due to an increase in the total content of EPA or to a reduction in the quantity of AA relative to EPA. Fig. 5 (*top*) illustrates the percentage of the total fatty acids represented by AA and EPA before and after the 20 capsule/d supplementation. The relative content of AA remained constant between 5 and 6% of the total fatty acids before and after supplementation. In contrast, the levels of EPA increased from 0.2% (before supplementation) to ~ 2.0% (after supplementation) of the total fatty acids in the cell. The increase in EPA resulted in a significant expansion of the total AA + EPA pool within the cell from < 6 to > 7.0%. The production of lipid mediators was also analyzed in neutrophils isolated before and after the highest dosage (20 capsules) of fish oil. It was reasoned that if dietary fish oils have an influence on lipid-mediator production, the highest dosage might be the condition in which we would observe it. Fig. 5 (*middle* and *bottom*) illustrate the quantities of LTB_4, LTB_5, LTB_4 + LTB_5, and PAF synthesized by the neutrophil before supplementation and after 6 d of Promega (20 capsules/d). These data indicated that there was no difference in the quantity of LTB_4 produced by neutrophils from volunteers before supplementation and after 20 capsules of Promega for 6 d. However, the quantity of LTB_5 and LTB_4 + LTB_5 increased significantly after supplementation. PAF was also measured in the same neutrophil preparation used for LT analysis. Neutrophils isolated after supplementation (20 capsules/d) produced slightly higher quantities of PAF compared with cells obtained before supplementation.

Discussion

Substantial evidence has been reported over the last few years that the consumption of n-3 fatty acid may have beneficial effects in a number of inflammatory diseases. Yet little is known about a number of important parameters such as the kinetic and dose dependency of uptake into blood cells of n-3 fatty acids provided in vivo. The present study was designed to examine these parameters in the neutrophil from humans who supplemented their usual diets with n-3 fatty acids. A novel finding of this study was that n-3 fatty acids are rapidly incorporated in the neutrophil phospholipids (within 2 d) and reach maximal levels within 1 wk of dietary supplement. In addition, longer-term supplementation (up to 7 wk) at the same dosage did not alter the quantity of n-3 fatty acids or the distribution of n-3 fatty acids in phospholipid classes. It is not clear at this time whether the neutrophil is unique in its capacity to incorporate n-3 fatty acid provided in vivo into complex lipids in such a

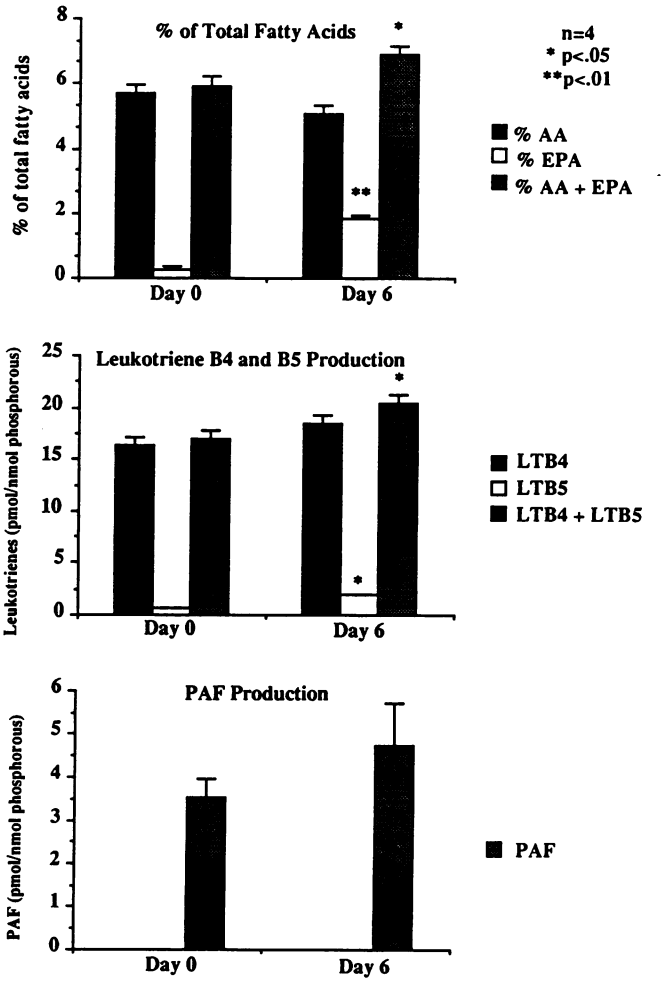

Figure 5. Influence of 20 capsules/d supplementation on fatty acid content and on the synthesis of PAF and LTs. Neutrophils were collected from the same donors before and after Promega supplementation (20 capsules/d). The fatty acid content as well as LT and PAF production were determined as described. The data are expressed as mean±SE from four individuals. In the case of LTB_5, SEs were < 0.05 and therefore were not clearly delineated from the tops of the bars.

rapid manner. For example, von Schacky and Weber (34) have shown that platelets do not incorporate appreciable amounts of EPA until 6 d after consumption. Together, this suggests that it may be possible to differentially incorporate n-3 fatty acid into one cell population such as the neutrophil and not into others.

A second parameter that was examined in this study was the influence of the dose of n-3 fatty acid consumed by the volunteers on the content of n-3 fatty acids which appeared in the neutrophil. Data illustrated in Fig. 4 clearly demonstrate that the amount of EPA and DCHA found in neutrophil phospholipids increased as a function of the dose of n-3 fatty acids provided to the volunteer. The quantity of EPA in neutrophil lipids approached that of AA at doses of EPA of 5.6 g/d (20 capsules/d). The quantity of DCHA in neutrophil lipids did not significantly increase until the dosage of DCHA was increased to 1.8 g/d. These data suggest that EPA is more readily incorporated into neutrophil phospholipids compared with DCHA. Alternatively, the lack of measurable DCHA in membrane phospholipid may indicate that DCHA has been retroconverted to other fatty acids such as EPA (34). Taken together with the kinetic experiments, the data suggest that the neutrophil has the capacity to rapidly incorporate large quantities of n-3 fatty acids provided in vivo into complex lipids. This information may be important in clinical conditions where n-3 fatty acids have been shown to have beneficial effects and it is necessary to rapidly incorporate high levels of n-3 fatty acids into cellular phospholipids. These conditions include coronary revascularization procedures such as thrombolysis and bypass surgery (35, 36). In addition, these data suggest that there may be more flexibility in the feeding strategies (often 4–8 wk) used to study clinical symptoms seen in various chronic inflammatory conditions such as arthritis and asthma. This may especially be the case when the neutrophil is the target cell.

Examination of the EPA/AA ratio in individual phospholipid classes revealed that n-3 fatty acids are preferentially incorporated into PC and PE with little EPA found in PI. Similar findings have been reported in platelets (13, 34). The mechanism for the differential incorporation of n-3 fatty acids into neutrophil phospholipids is not understood at this time. It could involve enzymes responsible for the uptake of fatty acids, such as acyl transferases or enzymes involved in fatty acid remodeling such as the CoA-independent transacylase. The significance of this differential incorporation is not clear at this time. It has been recognized for some time that different phospholipid classes have distinct roles in signal transduction and lipid-mediator production. The data presented here suggest that, if it is possible to regulate lipid-mediator biochemistry with n-3 fatty acids, this may be possible in PC and PE but not PI in peripheral blood neutrophils.

Dietary fish oils have been proposed to alter the inflammatory potential of cells such as the neutrophil by several mechanisms. In some studies it has been suggested that n-3 fatty acids compete with AA for enzymes responsible for the uptake into phospholipids. This competition would lead to a reduction in the quantity of arachidonate found within complex lipids (11, 14). Data from the current study suggest that the quantity of arachidonate remains relatively stable during the time of supplementation at the doses of n-3 fatty acids employed in this study. In addition, it is clear from the present study that the quantity of EPA in the cell does increase and therefore the total AA + EPA pool increases in size. The mechanism that regulates the pool sizes of these fatty acids in cellular lipids is not well understood. However, the expansion of polyunsaturated fatty acid pools within phospholipids appears to be the reason for the lack of reduction of arachidonate observed in the current study. Accordingly, it may be difficult to reduce arachidonate content in cells by merely adding dietary fish oils.

The fact that no reduction in AA content was observed in this study with high doses of Promega fish-oil supplement differs from many studies in the literature with rats on menhaden oil. We found only small differences in the fatty acid composition of Promega and menhaden and therefore do not believe that there is a property unique to Promega that prevents AA depletion. A major difference between rat and human studies revolves around what is consumed by humans or rats in addition to fish oil. In many rat studies, one fat source is substituted for another. Boudreau and colleagues (37) recently demonstrated, in rats on menhaden oil, that the critical factor in reducing AA and eicosanoids is the ratio of n-3 to n-6 fatty acids and not the absolute amounts of n-3 fatty acids consumed. In general, the human volunteers in our studies also consumed diets high in n-6 fatty acids and this may account for the lack of reduction of AA. We are presently carrying out extensive studies in humans on low-fat diets with fish-oil supplement to test whether this will reduce AA levels.

Dietary fish oils have also been suggested to reduce the inflammatory potential of the neutrophil by reducing the quantity of lipid mediators (eicosanoids and PAF) that inflammatory cells produce. However, data from the current study suggest that PAF biosynthesis is not reduced in neutrophils from volunteers supplemented with n-3 fatty acids. These findings are in agreement with those reported by Pickett and colleagues (38) in rat neutrophils. There have been some discrepancies as to whether PAF biosynthesis is reduced during fish-oil supplementation (22, 38, 39). The basis for these discrepancies is unclear at this time. It is possible that the alterations can be attributed to differences in stimulation protocols, cell types, or the enrichment strategies used in these studies. Alternatively, there are differences in the methodologies used to measure PAF in these studies (GC/MS vs. bioassay). The production of LTB_4 was reduced by a small percentage in the current study. The modest inhibition observed in the current study with healthy volunteers is similar to that reported by Strasser and colleagues (25) and less than that seen in other investigations (40). Taken together, our studies suggest that it may be difficult to induce large reductions in PAF or LTB_4 by only increasing EPA levels within cellular phospholipids. This lack of reduction may be explained by biochemical studies that suggest the AA content within precursor phospholipids probably plays a more crucial role in the control of lipid-mediator biosynthesis than an increase in EPA and DCHA content in those phospholipids (22, 41, 42).

Although lipid-mediator production was not attenuated to a large degree in neutrophils from volunteers on dietary fish oils, other mechanisms involving n-3 fatty acids could explain the improvement in clinical symptoms seen in some studies. For example, a number of studies indicate that neutrophils enriched with EPA have a reduced chemotactic and adherence responsiveness as well as a reduced capacity to produce reactive oxygen metabolites (39, 43, 44). The mechanism by which n-3 fatty acids influence these biological effects still has to be elucidated. It may be that metabolites of n-3 fatty acids such as

LTB_5 may play an important role in inhibiting leukocyte function (45). Alternatively, AA and n-3 fatty acids released upon cell activation may themselves be important cellular mediators. AA has recently been proposed to play an important role in signal transduction by regulating cytosolic calcium levels, phagocytosis, and G protein activation (46–48). Additionally, it may be that the synthesis of lipid mediators from other cell types, such as the platelet, may be attenuated in individuals consuming fish-oil–enriched diets. This reduction may eventually be critical to the amelioration of clinical symptoms observed in various studies. Future studies examining these hypotheses will be crucial in evaluating the efficacy of fish-oil diets as a treatment for inflammatory disease.

Acknowledgments

The authors express their gratitude to Dr. David A. Bass for his helpful comments during the project.

This work was supported by National Institutes of Health grants AI-26771 and AI-24985.

References

1. Dyerberg, J., H. O. Bang, E. Stoffersen, S. Moncada, and J. R. Vane. 1978. Eicosapentaenoic acid and prevention of thrombosis and atherosclerosis. *Lancet.* ii:117–119.

2. Dyerberg, J., and H. O. Bang. 1979. Haemostatic function and platelet polyunsaturated fatty acids in eskimos. *Lancet.* ii:443–435.

3. Dyerberg, J., and H. O. Bang. 1982. A hypothesis on the development of acute myocardial infarction in Greenlanders. *Scand. J. Clin. Lab. Invest.* 42:7–12.

4. Leslie, C. A., W. A. Gonnerman, M. D. Ullman, K. C. Hayes, C. Franzblau, and E. S. Cathcart. 1985. Dietary fish oil modulates macrophages fatty acids and decreases arthritis susceptibility in mice. *J. Exp. Med.* 162:1336–1349.

5. Prickett, J. D., D. R. Robinson, and A. D. Steinberg. 1981. Dietary enrichment with the polyunsaturated eicosapentaenoic acid prevents proteinuria and prolongs survival in NZBX $NZWF_1$ mice. *J. Clin. Invest.* 68:556–559.

6. Kelley, V. E., A. Ferretti, S. Izui, and T. B. Strom. 1985. A fish oil diet rich in eicosapentaenoic acid reduces cyclooxygenase metabolites, and suppresses lupus in mRL-1pr mice. *J. Immunol.* 134:1914–1919.

7. Kremer, J. M., A. V. Michalek, L. Lininger, C. Huyen, J. Bigavoette, M. A. Timechalk, R. I. Rynes, J. Zieminski, and L. E. Bartholomew. 1985. Effects of manipulation of dietary fatty acids on clinical manifestations of rheumatoid arthritis. *Lancet.* i:184–187.

8. Sperling, R. I., M. Weinblatt, J.-L. Robin, J. Ravalese, R. L. Hoover, F. House, J. S. Coblyn, P. A. Fraser, B. W. Spur, D. R. Robinston, et al. 1987. Effects of dietary supplementation with marine fish oil on leukocyte lipid mediator generation and function in rheumatoid arthritis. *Arthritis Rheum.* 30:988–997.

9. Arm, J. P., C. E. Horton, B. W. Spur, J.-M. Mencia-Huerta, and T. H. Lee. 1989. The effect of dietary supplementation with fish oil lipids on the airways response to inhaled allergen in bronchial asthma. *Am. Rev. Respir. Dis.* 139:1395–1400.

10. Stenius-Aarniala, B., A. Aro, A. Hakulinen, I. Ahola, E. Seppala, and H. Uapaatalo. 1989. Evening primose oil and fish oil are ineffective as supplementary treatment of bronchial asthma. *Ann. Allergy* 62:534–537.

11. Kremer, J. M., and D. R. Robinson. 1991. Studies of dietary supplementation with omega 3 fatty acids in patients with rheumatoid arthritis. *World Rev. Nutr. Diet.* 66:367–382.

12. Herold, P. M., and J. E. Kinsella. 1986. Fish oil consumption and decreased risk of cardiovascular disease: a comparison of findings from animal and human feeding trials. *Am. J. Clin. Nutr.* 43:566–598.

13. Galloway, J. H., I. J. Cartwright, B. E. Woodcock, M. Greaves, R. G. Russell, and F. E. Preston. 1985. Effects of dietary fish oil supplementation on the fatty acid composition of the human platelet membrane: demonstration of selectivity in the incorporation of eicosapentaenoic acid into membrane phospholipids. *Clin. Sci. (Lond.).* 68:449–454.

14. Mori, T. A., J. P. Codde, R. Vandongen, and L. J. Bellin. 1987. New findings in the fatty acid composition of individual platelet phospholipids in man after dietary fish oil supplementation. *Lipids.* 22:744–750.

15. Ahmed, A. A., and B. J. Holub. 1984. Alteration and recovery of bleeding times, platelet aggregation and fatty acid composition of individual phospholipids in platelets of human subjects receiving a supplement of cod-liver oil. *Lipids.* 19:617–724.

16. von Schacky, C., S. Fisher, and P. C. Weber. 1985. Long-term effects of dietary marine ω-3 fatty acids upon plasma and cellular lipids, platelet function, and eicosanoid formation in humans. *J. Clin. Invest.* 76:1626–1631.

17. Careaga-Houck, M., and H. Sprecher. 1989. The effect of fish oil diet on fatty acid composition of individual phospholipids and eicosanoid production by rat platelets. *Lipids.* 24:477–481.

18. Careaga-Houck, M., and H. Sprecher. 1989. Effect of a fish oil diet on the composition of rat neutrophil lipids and the molecular species of choline and ethanolamine glycerophospholipids. *J. Lipid Res.* 30:77–87.

19. von Schacky, C., W. Siess, S. Fisher, and P. C. Weber. 1985. A comparative study of eicosapentaenoic acid metabolism by human platelets in vivo and in vitro. *J. Lipid Res.* 26:457–462.

20. Payan, D. G., M. Y. S. Wong, T. Chernow-Rogan, F. H. Valone, W. C. Pickett, V. A. Blake, W. M. Gold, and E. J. Goetzl. 1986. Alterations in human leukocyte function induced by ingestion of eicosapentaenoic acid. *J. Clin. Immunol.* 6:402–410.

21. Rosenthal, M. D., and J. R. Hill. 1986. Elongation of arachidonic and eicosapentaenoic acids limits their availability for thrombin-stimulated release from the glycerolipids of vascular endothelial cells. *Biochim. Biophys. Acta.* 875:382–391.

22. Triggiani, M., T. R. Connell, and F. H. Chilton. 1990. Evidence that increasing the cellular content of eicosapentaenoic acid does not reduce the biosynthesis of platelet-activating factor. *J. Immunol.* 145:2241–2248.

23. Prescott, S. M. 1984. The effect of eicosapentaenoic acid on leukotriene B production by human neutrophils. *J. Biol. Chem.* 259:7615–7621.

24. Terano, T., J. A. Salmon, and S. Moncada. 1984. Biosynthesis and biological activity of leukotriene B_5. *Prostaglandins.* 27:217–232.

25. Strasser, T., S. Fisher, and P. C. Weber. 1984. Leukotriene B_5 is formed in human neutrophils after dietary supplementation with eicosapentaenoic acid. *Proc. Natl. Acad. Sci. USA.* 82:1540–1543.

26. Lee, T. H., J.-M. Mencia-Huerta, C. Shih, E. J. Corey, R. A. Lewis, and K. F. Austen. 1984. Characterization and biologic properties of 5,12-dihydroxy derivatives of eicosapentaenoic acid, including leukotriene B_5 and the double lipoxygenase product. *J. Biol. Chem.* 259:2383–2389.

27. Fisher, S., C. V. Schacky, W. Siess, T. Strasser, and P. C. Weber. 1984. Uptake, release and metabolism of docosahexaenoic acid DCHA, C22:6 ω-3 in human platelets and neutrophils. *Biochem. Biophys. Res. Commun.* 120:907–918.

28. Chilton, F. H., J. T. O'Flaherty, J. M. Ellis, C. L. Swendsen, and R. L. Wykle. 1983. Selective acylation of lyso platelet-activating factor by arachidonate in human neutrophils. *J. Biol. Chem.* 258:7268–7271.

29. Bligh, E. J., and W. J. Dyer. 1959. A rapid method of lipid extraction and purification. *Can. J. Biochem. Physiol.* 37:911–917.

30. Martinek, R. G. 1970. Review of methods for determining inorganic phosphorous in biologic fluids. *J. Am. Med. Technol.* 32:337–348.

31. Patton, G. M., J. M. Fasulo, and S. J. Robins. 1982. Separation of phospholipids and individual molecular species of phospholipids by high performance liquid chromatography. *J. Lipid Res.* 23:190–196.

32. Benfenati, E., D. Macconi, M. Noris, G. Icardi, L. Bettazzoli, G. D. Bellis, M. Gavinelli, S. Rotondrol, and G. Remuzzi. 1989. Development of a mass spectrometric method to quantitate platelet activating factor in mouse urine. *J. Lipid Res.* 30:1977–1982.

33. Ramesha, C., and W. C. Pickett. 1986. Measurement of sub-picogram quantities of platelet activating factor by gas chromatography / negative ion chemical ionization mass spectrometry. *Biochem. Mass Spectrom.* 13:107–111.

34. von Schacky, C., and P. C. Weber. 1985. Metabolism and effects on platelet function of the purified eicosapentaenoic and docosahexaenoic acids in humans. *J. Clin. Invest.* 76:2446–2450.

35. DeCaterina, R., D. Giannessi, A. Mazzone, W. Bernini, G. Lazzerini, S. Meffei, M. Cerri, L. Salvatore, and B. Weksler. 1990. Vascular prostacylin is increased in patients ingesting omega-3 polyunsaturated fatty acids before coronary artery bypass graft surgery. *Circulation.* 82:428–438.

36. Braden, G. A., H. R. Knapp, D. J. Fitzgerald, and G. A. FitzGerald. 1990. Dietary fish oil accelerates the response to coronary thrombolysis with tissue-type plasminogen activator: evidence for a modest platelet inhibitory effect in vivo. *Circulation.* 82:178–187.

37. Boudreau, M. D., P. S. Chanmugam, S. B. Hart, S. H. Lee, and D. H. Huang. 1991. Lack of dose response by dietary n-3 fatty acids at a constant ratio of n-3 to n-6 fatty acids in suppressing eicosanoid biosynthesis from arachidonic acid. *Am. J. Clin. Nutr.* 54:111–117.

38. Pickett, W. C., D. Mytko, C. Dondero-Zahn, and C. Ramesha. 1986. The effect of endogenous eicosapentaenoic acid on PMN leukotriene and PAF biosynthesis. *Prostaglandins Leukotrienes Med.* 23:135–140.

39. Sperling, R. I., J.-L. Robin, K. A. Kylander, T.-H. Lee, R. A. Lewis, and K. F. Austen. 1987. The effects on n-3 polyunsaturated fatty acids on the genera-

tion of platelet activating factor-acether by human monocytes. *J. Immunol.* 139:3186–3191.

40. Lee, T. H., R. L. Hoover, J. D. Williams, R. I. Sperling, J. Ravalese, B. W. Spur, D. R. Robinson, E. J. Corey, R. A. Lewis, and K. F. Austen. 1985. Effect of dietary enrichment with eicosapentaenoic and docosahexaenoic acids on in vitro neutrophil and monocyte leukotriene generation and neutrophil function. *N. Engl. J. Med.* 312:1217–1224.

41. Chilton, F. H., J. M. Ellis, S. C. Olson, and R. L. Wykle. 1984. 1-alkyl-2-arachidonoyl-sn-glycero-3-phosphocholine: a common source of platelet activating factor and arachidonate in human polymorphonuclear leukocytes. *J. Biol. Chem.* 259:12014–12019.

42. Ramesha, C. S., and Pickett, W. C. 1986. Platelet activating factor and leukotriene biosynthesis is inhibited in polymorphonuclear leukocytes depleted of arachidonic acid. *J. Biol. Chem.* 261:7592–7595.

43. Parm, J., C. E. Horton, J.-M. Mencia-Huerta, F. House, N. M. Eiser, T. J. H. Clark, B. W. Spur, and T. H. Lee. 1988. Effect of dietary supplementation with fish oils on mild asthma. *Thorax.* 43:84–92.

44. Thompson, P. J., N. L. A. Misso, M. Passarelli, and M. S. Phillips. 1991. The effect of eicosapentaenoic acid consumption on human neutrophil chemiluminescence. *Lipids.* 26:1223–1226.

45. Goldman, D. W., W. C. Pickett, and E. J. Goetzl. 1983. Human neutrophil chemotactic and degranulating activities of leukotriene B_5 derived from eicosapentaenoic acid. *Biochem. Biophys. Res. Commun.* 117:282–286.

46. Randriamampita, C., and A. Trautmann. 1990. Arachidonic acid activates Ca^{2+} extrusion in macrophages. *J. Biol. Chem.* 265:18059–18062.

47. Lennartz, M. R., and E. J. Brown. 1991. Arachidonic acid is essential for IgG Fc receptor-mediated phagocytosis by human monocytes. *J. Immunol.* 147:621–626.

48. Abramson, S. B., J. Leszynska-Piziak, and G. Weissmann. 1991. Arachidonic acid as a second messenger: interactions with a GTP binding protein of human neutrophils. *J. Immunol.* 147:231–236.