Asymmetric incorporation of dietary n–3 fatty acids into membrane aminophospholipids of human erythrocytes

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Abstract Dietary supplementation with different classes of polyunsaturate fatty acids is known to result in their incorporation into cell membranes, but the effects of this on eicosanoid formation and other cell functions frequently does not correspond to the degree of alteration in total membrane fatty acids. This phenomenon may be related to the compartmentalization of polyunsaturate fatty acids both within the organelles and within membranes. Aminophospholipids are asymmetrically distributed across the membrane bilayers of most human cells. These phospholipids are highly enriched in polyunsaturated fatty acids, and are known to have specific interactions with a number of membrane proteins. To determine whether dietary n–3 fatty acids are preferentially incorporated into membrane lipids in a particular spatial pattern, we have utilized the nonpermeant aminophospholipid probe, trinitrobenzenesulfonic acid, to study the transmembrane molecular species distribution of human erythrocyte ethanolamine phospholipids and phosphatidylserines before and at the end of 4 weeks of dietary supplementation with n–3 fatty acids. Selective incorporation of n–3 fatty acids occurred in the inner membrane leaflet ethanolamine phospholipids, particularly into the alkenyl-acyl species. The n–3 species in phosphatidylserines, particularly 18:0 and 22:6 n–3 (*sn*-1 and *sn*-2, respectively), replaced n–6 and n–9 species. These data may provide a basis for different cell responses to n–3 fatty acid enrichment, and for different degrees of diet-induced alteration in responses involving inner and outer membrane leaflet functions.—**Knapp, H. R., F. Hullin, and N. Salem, Jr.** Asymmetric incorporation of dietary n–3 fatty acids into membrane aminophospholipids of human erythrocytes. *J. Lipid Res.* 1994. **35:** 1283–1291.

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The asymmetric distribution of phospholipids in the bilayer membranes of most mammalian cells is well established (1, 2). Phosphatidylcholine and sphingomyelin are located predominantly in the outer layer, while phosphatidylinositol and the aminophospholipids, phosphatidylethanolamine and phosphatidylserine, are found mainly in the inner layer. The physical properties of the leaflets, including various aspects of molecular motion and packing (3), are determined in part by the cholesterol content and the degree of fatty acid unsaturation. Specific lipid-protein interactions in membranes have also been shown to be influenced by their fatty acid composition, and result in alterations in membrane cation transport systems (4) and hormone receptor activity (5).

Although there have been many reports of dietary influences on erythrocyte membrane phospholipid composition, few have addressed this issue in regard to membrane asymmetry. We have recently found that in contrast to the homogeneous distribution of phosphatidylcholine molecular species across the erythrocyte membrane (6), there are considerable differences in the distribution of ethanolamine phospholipid molecular species in the inner and outer membrane leaflets (7). This suggests that dietary alterations in membrane fatty acids could change the degree of asymmetry, and lead to differential effects on the physical properties and functions of the two leaflets. Dietary enrichment with n–3 fatty acids has been reported to lower blood pressure (8) and suppress cardiac arrhythmias (9), and such effects could be via influences on cell cation transport (10), decreased erythrocyte viscosity (11, 12), and altered signal transduction systems (13, 14). To determine whether altered membrane asymmetry could contribute to these membrane-related effects, the present study examines the incorporation of dietary n–3 fatty acids into aminophospholipids of both the outer and inner membrane leaflets of human erythrocytes.

Abbreviations: BSA, bovine serum albumin; DMA, dimethylacetal; PC, phosphatidylcholine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; PS, phosphatidylserine; PUFA, polyunsaturated fatty acid; RBC, red blood cell; SM, sphingomyelin; TNBS, trinitrobenzenesulfonic acid; TNP, trinitrophenyl-; PRP, platelet-rich plasma; TLC, thin-layer chromatography; HPLC, high performance liquid chromatography; GC, gas chromatography.

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MATERIALS AND METHODS

Red blood cell preparation

Blood was collected by venipuncture from six healthy male volunteers using acid citrate dextrose as anticoagulant (15) both before and after 4 weeks of ingesting menhaden oil (MaxEPA™) providing approximately 9 g eicosapentaenoic acid and 6 g docosahexaenoic acid per day. The blood was kept at 4°C for less than 18 h prior to processing; we have previously shown (7) that this has no effect upon the asymmetric membrane distribution of PE molecular species. After centrifugation at 250 *g* for 20 min at room temperature, the platelet-rich plasma (PRP), the buffy coat, and a small amount of the top layer of RBC were removed. The cells were washed three times in cold isotonic buffer containing 150 mM NaCl, 25 mM glucose, and 20 mM Tris-HCl (pH 7.4) with centrifugation at 1000 *g* for 15 min at 4°C. A small portion of the top of the erythrocyte layer was removed during each cycle in order to avoid leukocyte contamination. These packed RBC were immediately used for TNBS reaction and/or lipid extraction.

Reaction of intact RBC with TNBS and separation of the cell surface trinitrophenyl-PE

The 2,4,6-trinitrobenzenesulfonic acid (TNBS) was purchased from Pierce Chemical Co. (Rockford, IL) and used without further purification. Cell aminophospholipids were labeled with TNBS by a modification of the procedure of Marinetti and Love (16). Unless otherwise stated, 1 ml of packed RBC was reacted with 15 ml of ice-cold freshly prepared TNBS solution (2 mM final concentration) containing 120 mM $NaHCO_3$, 40 mM NaCl, and 4.4 mM glucose, pH 8.3. The pH was adjusted at 0°C. The cells were incubated under nonpenetrating conditions determined as described in detail (17), i.e., for 30 min at 0°C using gentle agitation on an orbital shaker. Light-sheltered tubes were used in order to avoid photodecomposition. Excess reagent was removed by centrifugation at 1000 *g* for 10 min at 4°C and washing the cells twice with ice-cold buffer (pH 7.4). In the first wash, 0.5% fatty acid-free BSA was added to terminate TNBS reaction.

Lipids were extracted twice according to the procedure of Reed et al. (18) after addition of internal standards as previously described (17). The lower phase was concentrated under nitrogen, subjected to TLC, and the lipids were extracted with methanol and subfractionated into molecular species by HPLC as reported (17).

Preparation of the RBC total lipid extracts and determination of plasma membrane aminophospholipids

Washed, packed RBC (1 ml) were initially extracted twice using the solvent ratios described by Reed et al. (18); a mixture of di-14:0-PE and PS was added as internal standards. The pooled chloroform-methanol supernatants were then separated into two phases by addition of water using the solvent ratios described by Bligh and Dyer (19). Aliquots of these chloroform extracts were removed to determine the total phospholipid phosphorus according to a modification of the method of Nelson (20). The remaining samples were then derivatized with TNBS following a modification of the procedure described by Gordesky and Marinetti (21) for TNBS labeling of PE and PS in organic solution as previously described (17). The yellow TNP-PE and TNP-PS derivatives in the chloroform phase were separated by TLC, eluted with methanol, and separated into molecular species by HPLC. These TNP-PE and TNP-PS derivatives corresponded to the total plasma membrane PE and PS composition (7).

Preparation of trinitrophenyl-aminophospholipid standards

Various molecular species of synthetic PE and PS were obtained from Avanti Polar Lipids (Birmingham, AL) and were derivatized with TNBS in organic solution as described above. The yellow TNP-derivatives were further purified by TLC as described above, except that preparative (PLK5) plates were used (Whatman Inc., Clifton, NJ). The molar extinction coefficients of the various molecular species of TNP-PE and TNP-PS at their absorbance maxima of 338 and 342 nm, respectively, in methanol were essentially the same, i.e., 17140 ± 750 (17). These TNP molecular species standards were used to determine the retention behavior on the reversed phase column as well as the quantitative performance of the HPLC method. In addition, di-14:0-PE and PS or the di-14:0-TNP-PE derivatives were used as internal standards in the RBC extraction procedure, as appropriate.

Separation and quantification of molecular species of TNP-PE and TNP-PS by HPLC

Separation of molecular species of TNP-PE and TNP-PS was performed on an HP 1090 liquid chromatograph equipped with an HP 1040 diode array detector and an HP 79995A analytical workstation as previously described (17). TNP-PE and PS were quantified by their absorbance at 338 and 342 nm, respectively. A linear relationship of peak area to molar amount was found for the various TNP molecular species standards.

Fatty acid analysis

The identity of the various molecular species of TNP-PE and TNP-PS from the RBC membrane was previously confirmed by gas chromatographic (GC) analysis of each peak separated after HPLC analysis (7). Fractions collected after HPLC were separated into two phases using the solvent ratios described by Bligh and Dyer (19) and dried under nitrogen. Fatty acids were transmethyl-

ated with boron trifluoride in methanol (14% w/v, Sigma, St. Louis, MO) according to Morrison and Smith (22) and extracted with hexane (from Burdick and Jackson). The combined extracts were evaporated to dryness under nitrogen, redissolved in hexane, and injected into an HP 5880 gas chromatograph equipped with a flame ionization detector. Helium was used as carrier gas with a linear velocity of 35 cm/sec and nitrogen was the make-up gas. A 50 m × 0.25 mm id OV-351 capillary column with a 0.25 μ film-thickness (Analabs, Foxboro, MA) was used with an oven temperature program of 200°–225°C at 1°C/min and held at 225°C thereafter. Fatty acid methyl ester and dimethylacetal (DMA) peaks were identified by their retention times based on a comparison to commercial standard mixtures (Nu-Chek Prep, Elysian, MN). Alkyl species could not be detected using this method.

Statistics

Analysis of variance was performed to compare the various species distributions (Fisher PLSD, Scheffe F-test, Dunnett *t*-test).

RESULTS

Dietary n-3 fatty acids and RBC ethanolamine phospholipids

When the ethanolamine phospholipid fractions derived from the erythrocyte outer membranes and total lipid extracts were examined, the molecular species asymmetry that we previously described (7) was evident both at baseline and after 4 weeks of dietary fish oil supplementation (**Table 1**). Molecular species were assigned previously after fatty acid and fatty aldehyde determination by GC analysis of each HPLC peak, and the identity of fatty aldehyde species was confirmed by their selective acidolysis (17). Molecules containing n-9 unsaturates were located more on the outer surface than were those containing polyunsaturated fatty acids of the n-3 or n-6 classes. The latter groups, particularly the n-6 class, were present in greater proportions in the total lipid extract, and therefore in the inner membrane leaflet. This asymmetry was more marked for some n-3 polyunsaturates than for others; 22:6 n-3-containing species were distributed

TABLE 1. Dietary fish oil effects on selected molecular species of erythrocyte plasma membrane ethanolamine phospholipid

		Baseline		Week 4	
	Species	Outer	Total	Outer	Total
n-3	16:0alk, 22:5	1.68 ± 0.15	2.37 ± 0.13	1.74 ± 0.30	3.04 ± 0.07*
	16:0alk, 22:6	1.64 ± 0.09	1.63 ± 0.10	1.81 ± 0.13	1.74 ± 0.07
	[a]18:0alk, 22:5	1.98 ± 0.16	3.55 ± 0.12	1.93 ± 0.18	4.05 ± 0.08
	[b]18:0alk, 22:6	3.34 ± 0.36	2.77 ± 0.15	2.88 ± 0.26	3.52 ± 0.14*
	18:1alk, 22:6	1.72 ± 0.13	1.25 ± 0.09	1.82 ± 0.09	1.70 ± 0.06*
	[c]16:0, 20:5	0.80 ± 0.09	1.38 ± 0.14	1.61 ± 0.14**	2.00 ± 0.16*
	16:0, 22:6	3.01 ± 0.29	1.63 ± 0.16	3.93 ± 0.37	2.32 ± 0.19
	18:0, 22:6	0.68 ± 0.17	0.98 ± 0.07	0.60 ± 0.11	0.87 ± 0.04
	18:1, 20:5	0.38 ± 0.06	0.40 ± 0.04	0.65 ± 0.09*	1.19 ± 0.07***
	[d]18:2, 20:5	0.26 ± 0.06	0.12 ± 0.01	0.58 ± 0.09**	0.20 ± 0.03
	18:2, 22:5	0.75 ± 0.10	0.62 ± 0.03	0.59 ± 0.09	0.61 ± 0.02
n-6	[e]16:0alk, 20:4	2.80 ± 0.14	5.21 ± 0.16	2.55 ± 0.08	4.67 ± 0.20
	16:0alk, 22:4	5.27 ± 0.37	5.90 ± 0.17	3.93 ± 0.17**	5.04 ± 0.11
	[a]18:0alk, 20:4	5.22 ± 0.32	12.36 ± 0.23	5.19 ± 0.61	11.12 ± 0.35
	[f]16:0, 18:2	5.23 ± 0.33	4.05 ± 0.26	5.09 ± 0.21	4.12 ± 0.19
	16:0, 20:4	2.65 ± 0.19	7.03 ± 0.27	3.16 ± 0.10	6.98 ± 0.28
	[g]16:0, 22:4	0.58 ± 0.02	2.56 ± 0.11	0.56 ± 0.03	2.23 ± 0.06*
	18:0, 20:4	3.37 ± 0.31	6.81 ± 0.26	2.66 ± 0.23	6.21 ± 0.31
	18:1, 20:4	1.85 ± 0.11	4.03 ± 0.17	1.95 ± 0.06	3.59 ± 0.09
	18:2, 18:2	0.78 ± 0.19	0.92 ± 0.13	1.12 ± 0.12	1.14 ± 0.11
n-9	16:0, 18:1	18.80 ± 0.52	10.81 ± 0.33	18.03 ± 0.84	10.59 ± 0.25
	18:0, 18:1	5.10 ± 0.19	2.92 ± 0.17	5.35 ± 0.33	3.13 ± 0.07
	18:1, 18:1	4.90 ± 0.32	3.90 ± 0.14	4.66 ± 0.28	4.68 ± 0.21

Values are mean ± SEM mol% of the RBC aminophospholipid fractions indicated in the outer membrane (Outer) and total lipid extract (Total) obtained from volunteers before or after 4 weeks of fish oil ingestion. Significant differences are indicated at $^{*}P < 0.05$, $^{**}P < 0.02$, and $^{***}P < 0.001$ by ANOVA; alk indicates alkenyl species.

[a]Also contains an unidentified peak.
[b]Also contains 18:0, 22:5 n-3.
[c]Also contains 18:2, 22:6 n-3; 18:2, 20:4 n-6.
[d]Also contains 18:3, 20:4 n-6.
[e]Also contains 16:0, 20:3 n-6.
[f]Also contains 18:1, 22:6 n-3.
[g]Also contains 18:1alk, 22:5 n-3.

TABLE 2. Calculated ethanolamine phospholipid selected molecular species in the inner leaflet of erythrocyte plasma membrane

	Species	Baseline	Week 4	% Change
n-3	16:0alk, 22:5	2.54	3.36	32%↑
	16:0alk, 22:6	1.63	1.72	6%↑
	[a]18:0alk, 22:5	3.94	4.58	16%↑
	[b]18:0alk, 22:6	2.63	3.68	40%↑
	18:1alk, 22:6	1.13	1.67	48%↑
	[c]16:0, 20:5	1.53	2.10	37%↑
	16:0, 22:6	1.29	1.92	49%↑
	18:0, 22:6	1.06	0.94	11%↓
	18:1, 20:5	0.41	1.33	224%↑
	[d]18:2, 20:5	0.09	0.11	22%↑
	18:2, 22:5	0.59	0.62	5%↑
n-6	[e]16:0alk, 20:4	5.81	5.20	10%↓
	16:0alk, 22:4	6.06	5.32	12%↓
	[a]18:0alk, 20:4	14.15	12.60	11%↓
	[f]16:0, 18:2	3.76	3.88	3%↑
	16:0, 20:4	8.13	7.94	2%↓
	[g]16:0, 22:4	3.06	2.65	13%↓
	18:0, 20:4	7.67	7.10	7%↓
	18:1, 20:4	4.58	4.00	13%↓
	18:2, 18:2	0.96	1.15	20%↑
n-9	16:0, 18:1	8.81	8.73	1%↓
	18:0, 18:1	2.38	2.58	8%↑
	18:1, 18:1	3.65	4.69	28%↑

Values are calculated mean mol% based upon PE molecular species distributions in the outer membrane and total lipid extract, assuming 20% of PE are in outer membrane (1); alk indicates alkenyl species.
[a]Also contains an unidentified peak.
[b]Also contains 18:0, 22:5 n-3.
[c]Also contains 18:2, 22:6 n-3; 18:2, 20:4 n-6.
[d]Also contains 18:3, 20:4 n-6.
[e]Also contains 16:0, 20:3 n-6.
[f]Also contains 18:1, 22:6 n-3.
[g]Also contains 18:1alk, 22:5 n-3.

about evenly across the membrane. At baseline, arachidonic acid was present in 15.9% of outer membrane PE molecular species, and calculated to be in 40.3% of inner membrane PE (**Table 2**). For eicosapentaenoic acid, values at baseline were 1.4% of outer and 1.6% of inner membrane PE species. The predominant localization of n-3 fatty acids in the alkenyl-acyl species was again notable.

Diet-induced changes in the molecular species of erythrocyte ethanolamine phospholipids in the outer membrane leaflet and the total lipid extract are also shown in Table 1. That there is a diet-related increase in molecular species asymmetry is evident, and those species containing n-3 polyunsaturates were generally increased at the expense of n-6 and n-9 species. Among the diacyl species, the greatest proportional increases occurred in those containing 20:5 n-3. In contrast, there is a markedly selective enrichment of 22:5 n-3 and 22:6 n-3 in the alkenyl-acyl species, and dietary increases in this group of species take place primarily in the inner membrane. In order to illustrate this, we calculated the mean values for ethanolamine phospholipid species in the inner leaflet fraction from those for the outer leaflet and total lipid extract, assuming that 20% of the ethanolamine phospholipid fraction is in the outer leaflet (1). It can be seen in Table 2 that marked increases in the inner leaflet n-3 alkenyl-acyl species occur during the dietary fish oil period, while nearly all of the species containing n-6 fatty acids are reduced. It was of interest that 18:1, 20:5 n-3 and the alkenyl species of 22:6 n-3 accounted for much of this increase. There were no changes in the total amounts of the alkenyl species after fish oil supplementation (**Table 3**). The diet-induced changes seen in ethanolamine phospholipid molecular species are illustrated in **Fig. 1.**

When the total amount of ethanolamine phospholipid contained in the erythrocyte total lipid extract is known, the actual mass of this class present in the inner, outer, and total membrane fractions can be calculated from our data if one also knows the distribution of total PE between the two leaflets. This distribution has been shown to be 20% and 80% in the outer and inner layers, respectively (1, 2), and the values calculated for the various groups of

TABLE 3. Dietary fish oil and asymmetry of ethanolamine-phospholipid membrane distribution

	Baseline			Four Weeks		
	Outer	Inner	Total	Outer	Inner	Total
Diacyl n-3	11.9	42.4	54.3	15.6 (31%↑)	59.7 (41%↑)	75.3
n-6	50.0	291.5	341.5	47.8 (4%↓)	278.7 (4%↓)	326.4
n-9	60.8	125.4	186.2	59.2 (3%↓)	135.1 (8%↑)	194.3
Total	122.7	459.3	582.0	122.6	473.5	596.1
Alkenyl n-3	24.5	97.7	122.2	24.3 (1%↓)	124.1 (27%↑)	148.4
n-6	33.0	215.8	248.8	31.5 (5%↓)	189.7 (13%↓)	221.2
Total	57.5	313.5	371.0	55.8	313.8	369.6
Mixed n-3/n-6/n-9	17.4	63.4	80.8	17.9	55.6	73.5
Unidentified	13.6	8.9	22.5	15.1	2.0	17.1

Values are expressed as the mean (n = 6) nanomoles of the indicated PE fraction per ml of packed RBC. The inner membrane values were determined by subtraction of outer from total values. Total nanomoles did not differ significantly during the study and the mean value of 1056 nanomoles per ml packed RBC was used for calculations. Percent changes from baseline are given in parentheses.

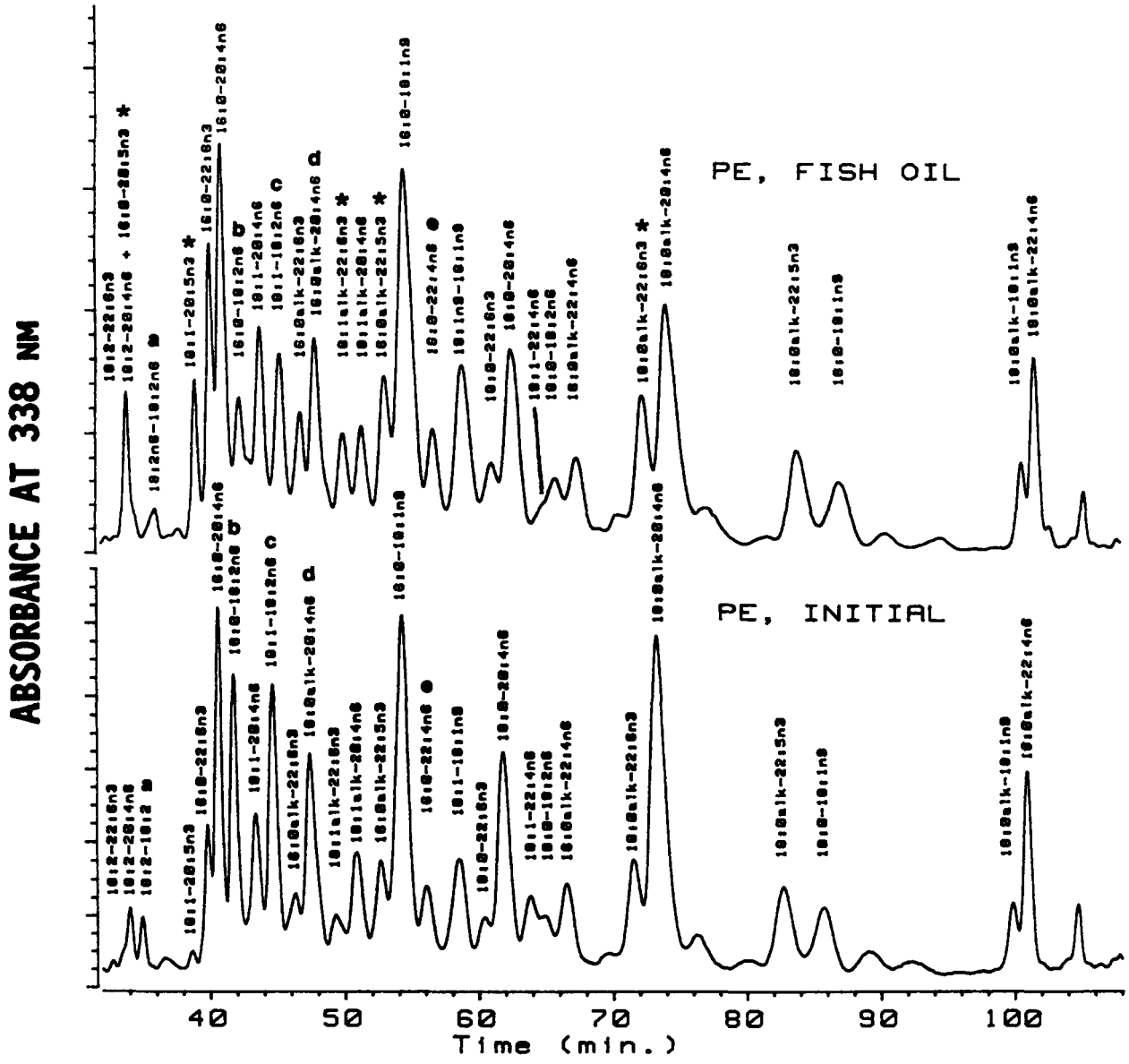

Fig. 1. Shows chromatograms obtained on HPLC of the TNP-PE derivatives from total lipid extracts of human erythrocytes obtained prior to (lower panel, PE, INITIAL) or after 4 weeks of fish oil ingestion (upper panel, PE, FISH OIL). Peaks with an asterisk were those n-3 species with a statistically significant increase during the fish oil period. Peaks with letters also contained a second species: a, 18:2 n-6-20:5 n-3; b, 18:1-22:6 n-3; c, 16:0-22:5n-3; d, 16:0-20:3n-6; e, 18:1alkenyl-22:5n-3.

ethanolamine phospholipid species are presented in Table 3. This analysis assumes that there was no difference in total aminophospholipid between dietary groups; this was confirmed by showing that the amount of TNBS labeling in the intact cells and the total lipid extracts was the same. The total mass of erythrocyte ethanolamine phospholipids did not change during the 4-week fish oil ingestion period, but there was a substantial shift from n-6 and n-9 species to those containing n-3 fatty acids in the inner membrane fraction, while little change occurred in the outer membrane. Similar percentage increases took place in the n-3 content of both alkenyl-acyl and diacyl PE species in the inner leaflet, while only that of the diacyl species was increased in the outer leaflet.

Dietary n-3 fatty acids and RBC phosphatidylserines

No TNP-phosphatidylserines were detected when intact erythrocytes were exposed to the TNBS reagent. This confirmed that the cell membranes were impermeable, as all of this phospholipid class in erythrocytes has been reported to be on the membrane interior (1, 2). There is enrichment in several phosphatidylserine species containing n-3 fatty acids at the expense of those containing n-6 or n-9 unsaturates; the species 18:0, 22:6 n-3 increased

from 6.66 ± 0.24 to 10.53 ± 1.41% ($P < 0.027$) during fish oil ingestion while the fraction containing both 18:0, 20:5 n-3 and 16:0, 22:5 n-3 species increased from 2.54 ± 0.13 to 5.28 ± 1.18% ($P < 0.05$). There were also declines in several n-6 and n-9 molecular species of phosphatidylserines, including the major species, 18:0, 20:4 n-6, but because the decline was distributed among a number of species and there was considerable intersubject variability, these declines did not reach statistical significance. Molecular species were assigned previously after fatty acid determination by GC analysis of the collected peaks (7). In contrast to the ethanolamine phospholipids, the phosphatidylserines were almost entirely in the diacyl form, as we have noted previously (7). As is characteristic for phosphatidylserines (7), the saturated fatty acid (at the *sn*-1 position) was made up almost entirely of stearic (18:0) rather than palmitic (16:0) acid, and no diet-related change in this association was observed (**Table 4**).

Changes in total fatty acid composition of aminophospholipids

In order to compare the degree of n-3 fatty acid incorporation into our erythrocytes with those of previous studies, the fatty acid composition of both the ethanolamine phospholipid and phosphatidylserine fractions from the erythrocyte total lipid extracts are presented in Table 4. The baseline values are similar to those we have published previously for human erythrocytes (7). Because of the small number of subjects, the statistically significant changes are limited to declines in 20:4 n-6, 22:4 n-6, and 18:1 n-9, and increases in the long-chain n-3 fatty acids 20:5 n-3, 22:5 n-3, and 22:6 n-3.

DISCUSSION

The present study demonstrates that dietary n-3 fatty acids are selectively incorporated into plasmalogens and other phospholipid species that are concentrated in the erythrocyte membrane inner leaflet. Our data were obtained using the nonpermeant aminophospholipid reagent TNBS, and earlier work with this probe showed that the aminophospholipids were localized primarily on the membrane inner surface, i.e., 80% of the PE and all of the PS (1, 21). These earlier conclusions were supported by studies using different approaches such as phospholipases (1, 2) or phospholipid exchange proteins (23). Previous studies showed that the membrane asymmetry was dependent upon an ATP-requiring process (24), and our recent demonstration of molecular species asymmetry further supports the idea of a high degree of specificity to the transmembrane distribution of aminophospholipids.

While the asymmetry of phospholipid classes in the membrane can be partly ascribed to charge effects involving the polar headgroups, our finding that aminophospholipid species asymmetry is accentuated by dietary n-3 fatty acids raises the questions of how and why particular fatty acids are compartmentalized in the inner membrane leaflet. We have found (7) that species asymmetry was not maintained in cells stored for >48 h under conditions

TABLE 4. Effect of dietary fish oil on erythrocyte aminophospholipid fatty acids

	Ethanolamine Phospholipids		Serine Phospholipids	
	Baseline	Week 4	Baseline	Week 4
16:0 DMA	3.41 ± 0.41	3.26 ± 0.12		
16:1 DMA	0.41 ± 0.13	0.20 ± 0.02		
16:0	13.05 ± 0.99	12.62 ± 0.31	8.36 ± 1.28	7.73 ± 1.37
18:0 DMA	4.76 ± 1.30	6.57 ± 0.35		
18:1 DMA	1.93 ± 0.54	2.56 ± 0.45		
18:0	7.77 ± 0.63	7.16 ± 0.28	26.20 ± 1.46	29.79 ± 1.77
18:1 n-9/11/12	14.59 ± 0.27	14.45 ± 0.37	9.01 ± 0.42	6.40 ± 0.38**
18:1 n-7	1.57 ± 0.13	1.45 ± 0.02	2.06 ± 0.08	2.09 ± 0.10
18:2 n-6	6.75 ± 0.68	5.63 ± 0.53	6.26 ± 1.08	4.20 ± 0.43
18:3 n-3	0.24 ± 0.03	0.26 ± 0.02		
20:1 n-9/7	0.39 ± 0.02	0.29 ± 0.06	0.18 ± 0.03	0.17 ± 0.02
20:2 n-6	0.36 ± 0.02	0.31 ± 0.01	0.25 ± 0.06	0.20 ± 0.01
20:3 n-6	1.21 ± 0.12	1.01 ± 0.11	2.23 ± 0.24	1.78 ± 0.22
20:4 n-6	22.43 ± 0.33	20.62 ± 0.64	26.73 ± 0.85	21.85 ± 0.65*
20:5 n-3	0.61 ± 0.05	3.10 ± 0.31***	0.51 ± 0.05	4.99 ± 0.91***
22:4 n-6	8.61 ± 0.50	7.67 ± 0.42	6.17 ± 0.52	3.65 ± 0.51**
22:5 n-6	1.04 ± 0.11	0.70 ± 0.11	1.54 ± 0.11	1.11 ± 0.10
22:5 n-3	4.05 ± 0.12	5.30 ± 0.10***	4.05 ± 0.25	5.05 ± 0.31*
22.6 n-3	4.88 ± 0.44	6.85 ± 0.48*	6.04 ± 0.68	10.66 ± 0.66**

Values are mean ± SEM weight % of total RBC fatty acids in the aminophospholipid fractions indicated, obtained from volunteers before or after 4 weeks of fish oil ingestion. Significant differences are indicated at *$P < 0.05$, **$P < 0.02$, and ***$P < 0.001$ by ANOVA; DMA indicates dimethylacetals. Unidentified peaks were included in the calculations but not listed.

leading to lowering of ATP levels. This suggests that an energy-requiring process is continuously reinforcing the asymmetry, and that the molecular species asymmetry is likely to have important consequences for membrane structure and function. In order to study membrane asymmetry with TNBS, it is critical to choose reaction conditions that do not cause membrane leakiness, thereby allowing the probe to react with phospholipids in the membrane inner leaflet. We have recently reported (7) that human erythrocytes stored in buffer or for prolonged periods lose their membrane PE species asymmetry, but that this was preserved when they were kept at 4°C in anticoagulated blood for less than 24 h, as in the present study. The reaction with TNBS must be carried out with 1–5 mM reagent, below 10°C, for less than an hour in order to avoid labeling the phosphatidylserines or inner leaflet ethanolamine phospholipids. Earlier studies that detected only minor differences in the ethanolamine phospholipid fatty acid composition of outer membrane versus total lipid extract in erythrocytes (25) or platelets (26) were performed at room temperature and resulted in a much greater proportion of overall phospholipid labeling than in our study.

There have been a number of reports indicating that dietary enrichment with n-3 fatty acids reduces erythrocyte viscosity (11, 12), and increases erythrocyte flow velocity in human capillaries (27). An improvement in erythrocyte flexibility could contribute by itself to the lowering of blood pressure by n-3 fatty acids (28). Erythrocyte deformation is influenced by interactions between spectrin, calcium, and specific aminophospholipid species (29). Diet-induced changes in erythrocyte membrane fatty acid composition have been shown to influence the activity of several enzymes, including those involved in calcium exchange (30, 31). Also, it was found in earlier studies that calcium pump activity of rat liver membranes could be hormonally manipulated, and the changes corresponded to specific changes in phospholipid fatty acid composition (32, 33). A similar association was reported for developmental changes in calcium pump activity and phospholipid fatty acid composition in chick skeletal muscle (34). Thus, there are prior indications that the diet-induced changes in phospholipid molecular species we have found could be associated with functional effects in erythrocytes and, possibly, in other cell types.

In combination with the general membrane effects of altered fatty acid composition, there have also been observations that alteration of aminophospholipid membrane asymmetry can exert effects on membrane function. Sickled erythrocytes, for instance, have an altered distribution of phosphatidylserines with a considerable exposure of this phospholipid class on the erythrocyte surface, resulting in an increased ability to generate thrombin in the presence of purified coagulation factors (35). Specific preference for interaction of enzymes (36) and cholesterol (37) with particular molecular species of choline phospholipids have been described, and alteration of the membrane distribution of such species could result in functional changes (38). Molecular species asymmetry appears to be a general feature of many cell types, and in the human lymphocyte the degree of asymmetry depends upon the cell cycle (39). Since dietary n-3 fatty acids have clinically apparent effects in some human inflammatory conditions (40) and alter cytokine production (41), it would be of interest to determine whether changes in membrane asymmetry could lead to alterations in signal transduction or specific intracellular messengers to produce these effects. Cell maturation may also be an important factor relating to membrane asymmetry of phospholipid classes or species. In contrast to the asymmetry in the aminophospholipids of the mature human erythrocyte (42), there was no asymmetry in the ethanolamine phospholipid distribution in Friend erythroleukemic cells, and only 80% of the phosphatidylserines were found in the inner leaflet (43). Interestingly, this reduced degree of molecular species asymmetry was suggested to be related to the incomplete skeletal protein network of the Friend cells (43, 44).

The preferential localization of arachidonyl-PE species in the inner cell membrane leaflet is important in many cells, since it has been shown that this phospholipid serves as a precursor pool for the synthesis of eicosanoids (45). Phospholipases specific for hydrolysis of arachidonate from the *sn*-2 position of plasmalogens have recently been reported to be activated during myocardial ischemia (46) and stimulation of pancreatic beta cells (47). Although no cyclooxygenase activity has been detected in erythrocytes, if the same phenomenon occurs to varying degrees in other cell types it could explain how selective replacement of only a portion of the total arachidonic acid by eicosapentaenoic acid in particular phospholipid pools could lead to a marked effect on eicosanoid release in these cells. A similar internal compartmentalization of the arachidonyl species has been observed in platelets (48), and it has been shown that dietary n-3 fatty acids are incorporated preferentially into specific platelet aminophospholipid classes (45). Dietary n-3 fatty acids reduce platelet TxA_2 synthesis from 20:4 n-6 and little TxA_3 is made from 20:5 n-3 in vitro or in vivo (49, 50), while the synthesis of PGI_2 from 20:4 n-6 by the endothelium in vivo is not reduced, and large amounts of PGI_3 can be made from 20:5 n-3 (51, 52). Further work will be needed to determine the role of aminophospholipid membrane asymmetry in these different responses of cell eicosanoid synthesis to dietary n-3 fatty acid supplementation. Finally, our data may provide a biophysical basis for investigating the potential clinical utility of dietary n-3 fatty acids in syndromes where an alteration in erythrocyte membrane properties may be desirable.

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