Low-Dose Eicosapentaenoic or Docosahexaenoic Acid Administration Modifies Fatty Acid Composition and Does Not Affect Susceptibility to Oxidative Stress in Rat Erythrocytes and Tissues

Gabriella Calviello[a], Paola Palozza[a], Piergiorgio Franceschelli[a], and Gianna Maria Bartoli[b,*]

[a]Institute of General Pathology, Catholic University, 00168 Rome, and
[b]Department of Biology, Tor Vergata University, 00133 Rome, Italy

ABSTRACT: In view of the promising future for use of n-3 polyunsaturated fatty acids (PUFA) in the prevention of cancer and cardiovascular diseases, it is necessary to ensure that their consumption does not result in detrimental oxidative effects. The aim of the present work was to test a hypothesis that low doses of eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA) do not induce harmful modifications of oxidative cell metabolism, as modifications of membrane fatty acid composition occur. Wistar rats received by gavage oleic acid, EPA, or DHA (360 mg/kg body weight/day) for a period of 1 or 4 wk. Fatty acid composition and α-tocopherol content were determined for plasma, red blood cell (RBC) membranes, and liver, kidney, lung, and heart microsomal membranes. Susceptibility to oxidative stress induced by *tert*-butylhydroperoxide was measured in RBC. EPA treatment increased EPA and docosapentaenoic acid (DPA) content in plasma and in all the membranes studied. DHA treatment mainly increased DHA content. Both treatments decreased arachidonic acid content and n-6/n-3 PUFA ratio in the membranes, without modifying the Unsaturation Index. No changes in tissue α-tocopherol content and in RBC susceptibility to oxidative stress were induced by either EPA or DHA treatment. The data suggest that EPA and DHA treatments can substantially modify membrane fatty acids, without increasing susceptibility to oxidative stress, when administered at low doses. This opens the possibility for use of low doses of n-3 PUFA for chemoprevention without risk of detrimental secondary effects.
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As a part of a recent study of the biological activity of n-3 polyunsaturated fatty acids (PUFA), we found that dietary supplementation with mixtures of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), in human subjects at high risk for colon cancer, suppressed cytokinetic anomalies in the colonic mucosa (1,2). This indicates that these fatty acids may be protective against cancer and supports our earlier observations and those of others in animal models (3–6). In view of the promising future use for n-3 PUFA in the prevention of cancer and cardiovascular diseases (5,7–9), it is necessary to ensure that their consumption does not result in detrimental side effects. The substitution of n-6 PUFA with potentially unstable n-3 PUFA in the membranes may shift fatty acid composition toward a higher degree of unsaturation and enhance membrane susceptibility to lipid peroxidation (10,11). Moreover, increased uptake of PUFA may affect vitamin E status either by impairing the absorption of the antioxidant (12,13) or by causing increased vitamin E consumption in plasma and tissues owing to enhanced lipid peroxidation (14,15).

We reported earlier that treatment of human subjects with n-3 PUFA at low doses and without significant addition of α-tocopherol did not produce any detrimental effect, whereas high doses resulted in harmful modifications of oxidative metabolism, such as an increase of red blood cell (RBC) susceptibility to lipid peroxidation and changes in endogenous tocopherol content and Mg-K-ATPase activity (16,17).

The aim of the present study was to evaluate in a rat model short- and longer-term effects of low dietary administrations of EPA or DHA without significant supplementation of vitamin E on membrane fatty acid composition of a variety of tissues, on RBC susceptibility to lipid peroxidation, and on tissue α-tocopherol content. n-3 Fatty acids were given separately and at doses comparable to the minimum used in our human studies (1,2,16,17).

MATERIALS AND METHODS

Animals. Thirty male Wistar rats (~250 g) were fed a nonpurified commercial diet (Altromin-Rieper, Rieper Company, Bolzano, Italy). The composition of the diet was: (%, w/w) crude protein, 23; fat, 5.5; fiber, 5; mineral, 8; carbohydrate, 58.5; water, 12. The vitamin mixture added to the diet was 2.5 g/kg (vitamin E, 100 mg/kg), and the mineral mixture was

*To whom correspondence should be addressed at Catholic University, Institute of General Pathology, Lgo F. Vito 1, 00168 Rome, Italy.
E-mail: ibipg@rm.unicatt.it.

Abbreviations: DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; OA, oleic acid; PUFA, polyunsaturated fatty acids; RBC, red blood cell; *t*-BOOH, *tert*-butylhydroperoxide.

0.52 g/kg by weight. The diet provided 7.5 g/kg n-6 PUFA as linoleic acid (18:2n-6) and 1.02 g/kg n-3 PUFA as 18:3, 18:4, 20:5 and 22:6 . The dietary n-3/n-6 PUFA ratio was 0.14 and increased to 1.20 after EPA or DHA administration. Food and water were provided *ad libitum*.

The animals were randomly divided into three groups of 10 animals each. One group received oleic acid ethyl ester (OA, control), a second group received EPA-ethyl ester, and a third DHA-ethyl ester. The oils were given by gastric gavage as a single dose of 360 mg/kg body weight/day. The amount of OA (18:1n-9) in the control group was adjusted to equalize caloric intake among the groups. Preliminary experiments comparing OA-treated animals with untreated animals showed that OA treatment did not modify fatty acid composition and α-tocopherol content in plasma and cell membrane fractions.

The dietary dose (360 mg/kg body weight) was chosen to approximate the daily oral dose (2.5 g) that was found to normalize altered cellular proliferation in the colonic mucosa of patients at high risk for colon cancer (1,2). The same dose was found not to produce any adverse effects (16,17). Body surface area was used as the reference for conversion of the human dosage to the rat (18). An n-3 PUFA dose of 2.5 g/d given to patients weighing about 70 kg corresponded to a daily intake of 300 mg/kg body weight in the rat. In the human study, the n-3 PUFA were taken in three daily subdoses to facilitate absorption from the intestine. We increased the dose to 360 mg/kg body weight/d to compensate for reduced absorption when single doses of n-3 PUFA were used in the rat model.

The α-tocopherol amount (3 mg/g) was added to the oils for prevention of autooxidation. Total intake of α-tocopherol was 1.77 mg/d, (1.5 mg/d from the diet plus 0.27 mg/d from oil supplementation). This approximated the intake in earlier human studies (1,2). The α-tocopherol daily intake during the human studies was the sum of the content in the standard Italian diet (13 mg/d) plus the content in oil capsules (0.9–2.7 mg/d) and was similar to the recommended dietary intake (19,20).

The purity of fatty acids was 92 and 90% for EPA and DHA, respectively. Their α-tocopherol content was verified by high-performance liquid chromatography.

Food consumption and body weight gain for the animals were measured twice a week. Five animals from each group were sacrificed after 1 wk of treatment and the remaining five animals after 4 wk. The animal use protocol was approved by the Ministry of Health, Veterinary Service, Rome, Italy.

Sample collection. The animals were decapitated after ether anesthesia, and the blood was collected in heparinized tubes. Plasma was separated by centrifugation at $1000 \times g$ for 10 min at 4°C. RBC were washed three times in buffered isotonic NaCl solution. RBC membranes were prepared as described by Burton *et al.* (21). Liver, kidneys, lungs, and heart were rapidly excised and immediately frozen in liquid N_2 and stored at −80°C prior to processing. Microsomal membranes were prepared from liver, kidneys, heart, and lungs as described by Bartoli *et al.* (22) and protein concentration was measured by the Bradford assay (23).

Analysis of fatty acids. Lipids were extracted from microsomal membranes by the method of Bligh and Dyer (24), using chloroform/methanol (2:1, vol/vol) containing butylated hydroxytoluene (0.01%), and gravimetrically measured (25). Total phospholipids were separated from neutral lipids by thin-layer chromatography using silica gel 60 plates (Merck, Darmstadt, Germany) and toluene/diethyl ether/ethanol 35:3.5:1 (by vol) as a solvent system. Fatty acids in the phospholipid band were transesterified with the use of methanolic-HCl (3 N) (Supelco, Bellefonte, PA) at 70°C for 2 h. After extraction with *n*-hexane, methyl esters were separated and quantified by means of an HRGC 5300 Megaseries gas–liquid chromatograph (Carlo Erba, Milan, Italy) equipped with a 30 m × 0.25 mm capillary column (Supelco, Omegawax 320) using helium as carrier gas. The oven temperature was programmed at 1°C/min from 180 to 220°C. Retention times were identified with standards (Sigma Chemical Co., St. Louis, MO). Peaks corresponding to 16:0, 18:0, 18:1n-9, 18:2n-6, 18:3n-6, 18:3n-3, 20:3n-6, 20:4n-6, 20:5n-3, 22:4n-6, 22:5n-3, and 22:6n:3 were detected by the system and their areas measured with a Carlo Erba Megaseries integrator. Unsaturation Index was measured as the sum of the percentages of individual fatty acids × number of double bonds.

Oxidative stress induced by tert-*butylhydroperoxide (t-BOOH) in RBC.* A 5% RBC suspension in phosphate-buffered saline (pH 7.4) was incubated with 0.5 mM *t*-BOOH at 37°C under oxygen for 90 min. At each time point, a sample of 1 mL was taken for analysis of both malondialdehyde formation at 533 nm (26) and K^+ efflux. K^+ efflux was determined by flame emission photometry and expressed as percentage of total efflux evoked by lysis of RBC in distilled water (27).

Vitamin E analysis. α-Tocopherol was extracted in hexane from plasma, RBC membranes, and tissue microsomal membranes using, respectively, 25 µL plasma and a membrane equivalent of 7 mg protein. Samples were dissolved in methanol, and 20 µL aliquots were analyzed by high-performance liquid chromatography fluorescence detection on a Perkin-Elmer (Norwalk, CT) 650-LC fluorescence detector at 295 nm excitation, 340 nm emission. α-Tocopherol, as well as the internal standard, tocol, was eluted with 100% methanol on Alltech (Deerfield, IL) C-183-µm column, as indicated in Reference 28.

Statistical analysis. The results are expressed as means ± SE, and significance was assessed by one-way analysis of variance using Minitab software (Minitab Inc., State College, PA). When significant differences were found, *post hoc* comparisons of means were made using Fisher's test. Differences were considered significant at $P < 0.05$.

RESULTS

Food intake and body weight gain were similar in all groups throughout the 4-wk feeding period. Treatments did not modify the content of total lipids in the tissues, as shown by lipid/protein ratios of 0.20 ± 0.01, 1.16 ± 0.11, 0.77 ± 0.04, 0.65 ± 0.05, 1.07 ± 0.12, and 1.39 ± 0.09 in plasma, RBC,

TABLE 1
Fatty Acid Composition (% of total) of Plasma Phospholipids in Rats Treated with Oleic Acid (OA), Eicosapentaenoic Acid (EPA), and Docosahexaenoic Acid (DHA) (for 1 and 4 wk)

Treatment	OA		EPA		DHA	
week	1	4	1	4	1	4
Saturated	32.99 ± 0.41	33.01 ± 1.48	33.24 ± 1.15	34.22 ± 0.95	34.52 ± 1.13	35.00 ± 2.46
Monounsaturated	15.61 ± 1.08	14.05 ± 1.27	17.23 ± 0.67	15.91 ± 1.29	15.85 ± 1.30	15.96 ± 0.78
18:2n-6	26.12 ± 2.01	27.02 ± 2.32	29.28 ± 1.08	30.22 ± 0.81	27.56 ± 1.79	29.80 ± 1.34
18:3n-6	0.27 ± 0.03	0.32 ± 0.02	0.25 ± 0.03	0.29 ± 0.02	0.29 ± 0.06	0.24 ± 0.03
18:3n-3	0.71 ± 0.07	0.67 ± 0.04	1.15 ± 0.09[a]	1.18 ± 0.12[a]	0.87 ± 0.11	1.24 ± 0.06[a]
20:4n-6	14.99 ± 1.66	14.41 ± 1.36	9.60 ± 1.19[a]	11.12 ± 0.90[a]	11.53 ± 0.71[a]	11.09 ± 1.00[a]
20:5n-3	0.91 ± 0.13	0.75 ± 0.04	1.85 ± 0.18[a,b]	1.36 ± 0.13[a,b,c]	1.09 ± 0.06	0.99 ± 0.09[a]
22:4n-6	0.41 ± 0.07	0.54 ± 0.07	0.17 ± 0.10[a]	0.26 ± 0.02[a]	0.18 ± 0.03[a]	0.26 ± 0.05[a]
22:5n-3	0.81 ± 0.05	0.85 ± 0.05	1.22 ± 0.09[a]	1.20 ± 0.15[a,b]	0.53 ± 0.06[a]	0.76 ± 0.13
22:6n-3	3.32 ± 0.15	3.21 ± 0.48	2.57 ± 0.11[a]	2.78 ± 0.38	4.06 ± 0.25[a]	3.70 ±.0.56
Total n-6 PUFA	41.84 ± 1.55	42.58 ± 1.49	39.55 ± 1.84	41.68 ± 0.45	39.32 ± 1.93	41.17 ± 2.02
Total n-3 PUFA	6.30 ± 0.24	6.04 ± 0.52	7.22 ± 0.22[a]	6.33 ± 0.77	6.87 ± 0.46	6.78 ± 0.75
n-6/n-3 PUFA ratio	7.08 ± 0.54	8.23 ± 0.71	6.14 ± 0.25[a]	6.64 ± 0.33[a]	6.51 ± 0.37[a]	7.09 ± 0.82
Unsaturation Index	163.54 ± 7.03	162.48 ± 6.59	151.72 ± 6.05	154.87 ± 4.20	154.74 ± 6.07	156.39 ± 8.78

[a]$P < 0.05$ vs. OA group, same week.
[b]$P < 0.05$ for difference between EPA and DHA group, same week.
[c]$P < 0.05$ vs. first week. Abbreviation: PUFA, polyunsaturated fatty acid. All values are means ± SE of five determinations.

liver, kidney, heart, and lung membranes, respectively. These ratios did not change throughout short (1 wk) or long (4 wk) periods of treatment.

Tables 1–6 show fatty acid composition for total phospholipids extracted from plasma, RBC membranes, liver, kidney, heart, and lung microsomal membranes isolated from rats treated for 1 and 4 wk with OA, EPA, or DHA.

The content of saturated and monounsaturated fatty acids in plasma, RBC, and tissue membranes was unaffected by dietary supplementation with EPA and DHA. On the other hand, the content of total and individual PUFA changed significantly in rats treated with the two n-3 fatty acids as compared to the control group. Alterations in the PUFA pattern became evident within 7 d of treatment. EPA (20:5n-3) was incorporated to different extents in the tissues studied. Its increase in RBC membranes after 1 wk of EPA treatment was the highest (340%), followed by that observed in heart (218%), lung (157%), liver (107%), plasma (103%), and kid-

TABLE 2
Phospholipid Fatty Acid Composition (% of total) of Red Blood Cell Membranes Isolated from Rats Treated with OA, EPA, and DHA (for 1 and 2 wk)

Treatment	OA		EPA		DHA	
week	1	4	1	4	1	4
Saturated	43.12 ± 1.43	45.21 ± 2.27	44.23 ± 1.56	45.20 ± 1.89	42.64 ± 1.32	43.71 ± 1.60
Monounsaturated	11.26 ± 0.35	10.43 ± 0.56	10.40 ± 0.33	10.71 ± 0.46	9.74 ± 0.51	9.76 ± 0.40
18:2n-6	10.33 ± 0.47	10.88 ± 1.03	11.20 ± 0.48	11.25 ± 0.35	10.53 ± 0.45	12.02 ± 0.68
18:3n-6	0.09 ± 0.01	0.12 ± 0.02	0.11 ± 0.01	0.14 ± 0.02	0.08 ± 0.01	0.10 ± 0.02
18:3n-3	0.11 ± 0.01	0.08 ± 0.01	0.10 ± 0.01	0.10 ± 0.01	0.09 ± 0.01	0.09 ± 0.01
20:3n-6	0.51 ± 0.05	0.57 ± 0.08	0.45 ± 0.06	0.55 ± 0.08	0.60 ± 0.07	0.60 ± 0.03
20:4n-6	23.27 ± 0.67	22.88 ± 0.94	21.59 ± 0.63	21.15 ± 0.84	22.18 ± 0.53	21.90 ± 0.25
20:5n-3	0.40 ± 0.08	0.44 ± 0.06	1.76 ± 0.16[a,b]	1.52 ± 0.31[a,b]	0.55 ± 0.05[a]	0.58 ± 0.06[a]
22:4n-6	1.82 ± 0.09	1.55 ± 0.06	1.58 ± 0.09[a]	1.05 ± 0.16[a,c]	1.42 ± 0.07[a]	1.06 ± 0.08[a,c]
22:5n-3	2.22 ± 0.16	1.88 ± 0.14	2.90 ± 0.17[a,b]	3.28 ± 0.37[a,b]	1.83 ± 0.02	1.89 ± 0.18
22:6n-3	3.60 ± 0.22	3.20 ± 0.18	3.24 ± 0.10[b]	3.22 ± 0.45[b]	5.22 ± 0.50[a]	5.17 ± 0.43[a]
Total n-6 PUFA	36.56 ± 0.31	36.03 ± 1.44	35.05 ± 0.88	33.90 ± 0.84	35.84 ± 0.82	35.90 ± 0.56
Total n-3 PUFA	6.65 ± 0.20	5.76 ± 0.36[c]	7.56 ± 0.53[a]	8.15 ± 0.80[a]	8.16 ± 0.47[a]	8.01 ± 0.66[a]
n-6/n-3 PUFA ratio	5.52 ± 0.15	6.35 ± 0.52[c]	4.79 ± 0.45[a]	4.35 ± 0.49[a]	4.46 ± 0.25[a]	4.64 ± 0.46[a]
Unsaturation Index	170.61 ± 2.57	162.90 ± 5.57	168.13 ± 6.48	166.21 ± 4.87	179.86 ± 5.18	173.29 ± 4.50

[a]$P < 0.05$ vs. OA group, same week.
[b]$P < 0.05$ for difference between EPA and DHA group, same week.
[c]$P < 0.05$ vs. first week. See Table 1 for abbreviations. All values are means ± SE of five determinations.

TABLE 3
Phospholipid Fatty Acid Composition (% of total) of Liver Microsomal Membranes Isolated from Rats Treated with OA, EPA, and DHA (for 1 and 4 wk)

Treatment	OA		EPA		DHA	
week	1	4	1	4	1	4
Saturated	41.79 ± 1.17	41.04 ± 1.82	39.82 ± 1.33	39.62 ± 1.64	40.56 ± 2.00	41.20 ± 2.36
Monounsaturated	8.33 ± 0.18	7.62 ± 0.59	8.66 ± 0.49	7.48 ± 0.51	8.03 ± 0.67	7.78 ± 0.35
18:2n-6	15.56 ± 1.82	17.07 ± 0.77	16.14 ± 0.95	19.76 ± 0.90[a,c]	16.63 ± 0.21	19.66 ± 1.33[a,c]
18:3n-6	0.19 ± 0.03	0.19 ± 0.04	0.22 ± 0.04	0.18 ± 0.04	0.27 ± 0.03[a]	0.17 ± 0.04
18:3n-3	0.22 ± 0.05	0.25 ± 0.03	0.21 ± 0.02	0.36 ± 0.08	0.29 ± 0.04	0.24 ± 0.03
20:3n-6	0.75 ± 0.12	0.92 ± 0.03	1.01 ± 0.05[a]	1.07 ± 0.02[a]	1.16 ± 0.08[a]	1.32 ± 0.17[a]
20:4n-6	21.27 ± 0.69	23.39 ± 1.43	20.76 ± 0.88	20.80 ± 0.95[a]	18.52 ± 0.73[a]	19.06 ± 1.36[a]
20:5n-3	0.41 ± 0.11	0.55 ± 0.05	0.85 ± 0.11[a]	0.84 ± 0.11[a]	0.74 ± 0.04[a]	0.73 ± 0.07[a]
22:4n-6	0.38 ± 0.08	0.33 ± 0.03	0.28 ± 0.05	0.26 ± 0.03	0.24 ± 0.02[a]	0.21 ± 0.03[a]
22:5n-3	0.80 ± 0.05	0.81 ± 0.10	2.87 ± 0.39[a,b]	1.66 ± 0.26[a,b,c]	1.12 ± 0.09[a]	1.16 ± 0.10[a]
22:6n-3	5.58 ± 0.65	4.80 ± 0.57	6.29 ± 0.69[b]	5.48 ± 0.53[b]	10.40 ± 1.09[a]	8.90 ± 0.90[a,c]
Total n-6 PUFA	37.97 ± 1.50	41.20 ± 0.62[c]	37.95 ± 1.91	41.46 ± 0.34[c]	36.86 ± 0.891	40.32 ± 0.53[c]
Total n-3 PUFA	8.18 ± 1.53	6.39 ± 0.74	10.13 ± 1.05[b]	8.72 ± 0.92[a]	12.75 ± 0.83[a]	10.44 ± 0.41[a,c]
n-6/n-3 PUFA ratio	5.15 ± 1.63	7.05 ± 0.99	4.21 ± 0.60[a]	5.26 ± 0.76	3.10 ± 0.28[a]	4.39 ± 0.22[a]
Unsaturation Index	179.14 ± 11.18	176.26 ± 7.57	185.66 ± 3.57	181.76 ± 7.65	194.20 ± 4.24	176.79 ± 11.23

[a]$P < 0.05$ vs. OA group, same week.
[b]$P < 0.05$ for difference between EPA and DHA group, same week.
[c]$P < 0.05$ vs. first week. For abbreviations, see Table 1. All values are means ± SE of five determinations.

ney (87%). After 4 wk of EPA treatment, the level of this fatty acid decreased in plasma and heart membranes (Tables 1 and 5). Nevertheless, EPA was still higher than that observed in the control group. In lung (Table 6) it decreased to the control value, whereas in RBC, liver, and kidney membranes it remained relatively unchanged as compared to that observed after 1 wk (Tables 2–4).

EPA treatment did not modify the DHA content in plasma, RBC, and tissue membranes, whereas it significantly increased docosapentaenoic acid (DPA, 22:5n-3), which is a product of EPA elongation. This effect was exaggerated in heart and liver (259 and 127% increase, respectively). DHA did not modify DPA level in any of the tissues except liver, in which a slight (40%) but significant increase was observed (Table 3).

As observed for EPA, but to a lesser extent, increased incorporation of DHA (22:6n-3) occurred in plasma and tissue membranes following dietary supplementation. The increase in DHA was evident after 1 wk and with the exception of

TABLE 4
Phospholipid Fatty Acid Composition (% of total) of Kidney Microsomal Membranes Isolated from Rats Treated with OA, EPA, and DHA (for 1 and 4 wk)

Treatment	OA		EPA		DHA	
week	1	4	1	4	1	4
Saturated	41.90 ± 1.16	41.39 ± 0.10	41.06 ± 0.26	41.61 ± 1.67	42.85 ± 1.46	42.21 ± 1.88
Monounsaturated	10.20 ± 0.45	9.92 ± 0.44	9.69 ± 0.31	10.01 ± 0.72	10.09 ± 0.40	10.62 ± 0.87
18:2n-6	14.06 ± 0.90	14.79 ± 0.98	14.16 ± 0.73[b]	17.06 ± 0.58[a,c]	17.11 ± 0.94[a]	16.79 ± 0.68[a]
18:3n-6	0.18 ± 0.01	0.18 ± 0.01	0.16 ± 0.01	0.16 ± 0.01	0.18 ± 0.10	0.18 ± 0.01
18:3n-3	0.15 ± 0.02	0.21 ± 0.04	0.13 ± 0.06	0.19 ± 0.04	0.19 ± 0.03	0.19 ± 0.03
20:3n-6	1.23 ± 0.03	1.16 ± 0.06	1.05 ± 0.08[a,b]	1.25 ± 0.11	1.35 ± 0.09	1.22 ± 0.02
20:4n-6	26.31 ± 1.08	27.76 ± 0.64	25.12 ± 1.66	23.23 ± 1.30[a]	23.29 ± 1.36[a]	24.12 ± 0.84[a]
20:5n-3	0.52 ± 0.04	0.48 ± 0.08	0.97 ± 0.15[a]	1.24 ± 0.12[a]	1.00 ± 0.21[a]	1.01 ± 0.15[a]
22:4n-6	0.58 ± 0.02	0.52 ± 0.01	0.47 ± 0.17	0.30 ± 0.02[a]	0.37 ± 0.02[a]	0.30 ± 0.04[a]
22:5n-3	0.34 ± 0.03	0.28 ± 0.02	0.58 ± 0.07[a,b]	0.55 ± 0.04[a,b]	0.31 ± 0.02	0.37 ± 0.05
22:6n-3	1.97 ± 0.18	2.12 ± 0.08	2.13 ± 0.13[b]	2.09 ± 0.19[b]	2.50 ± 0.24[a]	2.78 ± 0.37[a]
Total n-6 PUFA	42.28 ± 0.91	44.32 ± 1.32	40.44 ± 1.64	42.31 ± 1.20	42.37 ± 1.66	42.60 ± 1.45
Total n-3 PUFA	2.99 ± 0.06	3.07 ± 0.16	3.43 ± 0.53	4.07 ± 0.22[a]	4.00 ± 0.46[a]	4.36 ± 0.33[a]
n-6/n-3 PUFA ratio	15.91 ± 0.41	16.63 ± 0.38	13.70 ± 1.91	11.79 ± 0.53[a]	12.71 ± 1.54[a]	11.52 ± 0.61[a]
Unsaturation Index	166.36 ± 0.57	173.70 ± 3.40	160.27 ± 4.88	164.63 ± 5.27	165.90 ± 2.66	170.31 ± 3.93

[a]$P < 0.05$ vs. OA group, same week.
[b]$P < 0.05$ for difference between EPA and DHA group, same week.
[c]$P < 0.05$ vs. first week. For abbreviations see Table 1. All values are means ± SE of five determinations.

TABLE 5
Phospholipid Fatty Acid Composition (% of total) of Heart Microsomal Membranes Isolated from Rats Treated with OA, EPA, and DHA (for 1 and 4 wk)

Treatment	OA		EPA		DHA	
week	1	4	1	4	1	4
Saturated	34.96 ± 3.07	34.45 ± 1.10	34.51 ± 1.42	35.49 ± 2.07	35.58 ± 1.91	35.20 ± 1.6
Monounsaturated	8.88 ± 1.89	8.42 ± 0.49	9.76 ± 0.74	8.49 ± 0.61	9.82 ± 0.78	8.16 ± 0.54
18:2n-6	18.86 ± 1.97	20.83 ± 1.94	21.70 ± 1.86	24.64 ± 2.28	20.52 ± 2.13	23.44 ± 2.19
18:3n-6	0.13 ± 0.00	0.14 ± 0.01	0.15 ± 0.01	0.14 ± 0.01	0.15 ± 0.01	0.19 ± 0.03
18:3n-3	0.38 ± 0.09	0.30 ± 0.03	0.35 ± 0.07	0.28 ± 0.03	0.38 ± 0.04	0.29 ± 0.03
20:3n-6	0.52 ± 0.06	0.42 ± 0.04	0.47 ± 0.02	0.39 ± 0.02	0.51 ± 0.03	0.46 ± 0.03
20:4n-6	16.86 ± 0.45	17.57 ± 0.28	15.42 ± 1.00	15.17 ± 0.23[a,b]	14.85 ± 0.44[a]	14.22 ± 0.38[a]
20:5n-3	0.33 ± 0.10	0.23 ± 0.05	1.05 ± 0.09[a,b]	0.55 ± 0.04[a,b,c]	0.33 ± 0.07	0.21 ± 0.08
22:4n-6	1.07 ± 0.15	1.00 ± 0.17	0.91 ± 0.20	0.48 ± 0.02[a,c]	0.92 ± 0.18	0.45 ± 0.06[a,c]
22:5n-3	1.90 ± 0.27	1.73 ± 0.02	4.32 ± 0.09[a,b]	3.85 ± 0.39[a,b]	1.47 ± 0.29	1.40 ± 0.10
22:6n-3	8.13 ± 0.82	9.63 ± 0.71	8.87 ± 0.31[b]	10.15 ± 0.84[b,c]	15.19 ± 1.05[a]	14.56 ± 0.66[a]
Total n-6 PUFA	38.48 ± 2.17	41.45 ± 1.85	38.92 ± 2.13	39.47 ± 2.15	36.60 ± 2.12	38.68 ± 1.80
Total n-3 PUFA	10.85 ± 0.97	12.03 ± 1.17	12.33 ± 1.54[b]	14.39 ± 1.20[b]	17.45 ± 1.02[a]	16.55 ± 0.80[a]
n-6/n-3 PUFA ratio	3.67 ± 0.51	3.30 ± 0.45	3.41 ± 0.66[b]	2.83 ± 0.31	2.12 ± 0.24[a]	2.36 ± 0.20[a]
Unsaturation Index	189.14 ± 5.51	198.94 ± 6.63	190.23 ± 6.77[b]	198.76 ± 5.07	214.44 ± 6.03[a]	211.68 ± 5.96

[a] $P < 0.05$ vs. OA group, same week.
[b] $P < 0.05$ for difference between EPA and DHA group, same week.
[c] $P < 0.05$ vs. first week. For abbreviations, see Table 1. All values are means ± SE of five determinations.

plasma (Table 1), it was also present after 4 wk of treatment. During DHA supplementation the levels of EPA were elevated in plasma, RBC, liver, and kidney but remained unmodified in heart and lung.

The treatments with EPA or DHA were associated with modifications in n-6 PUFA content. In particular, linoleic acid (18:2n-6) was increased in plasma, heart, liver, and kidney (Tables 3 and 4). Nevertheless the change was significant only in liver and kidney. Similarly, a significant reduction in arachidonic acid (20:4n-6) was observed in plasma, liver, kidney, and heart after both EPA and DHA treatments, while no changes were found in RBC and lung.

Fatty acid modification was associated with a significant decrease in the n-6/n-3 PUFA ratio in plasma and in all the tissues examined from both EPA and DHA groups. On the contrary, the Unsaturation Index of plasma, RBC, and tissue fatty acids was unchanged, presumably because the increase of n-3 PUFA was counteracted by a decrease of n-6 PUFA. A slight but signifi-

TABLE 6
Phospholipid Fatty Acid Composition (% of total) of Lung Microsomal Membranes Isolated from Rats Treated with OA, EPA, and DHA (for 1 and 4 wk)

Treatment	OA		EPA		DHA	
week	1	4	1	4	1	4
Saturated	49.98 ± 2.23	47.60 ± 2.34	49.46 ± 1.67	48.82 ± 1.79	48.33 ± 2.30	46.28 ± 1.77
Monounsaturated	17.60 ± 0.93	17.15 ± 0.98	16.95 ± 2.54	18.26 ± 1.56	16.78 ± 1.08	16.10 ± 0.94
18:2n-6	10.34 ± 0.36	10.12 ± 0.31	10.35 ± 0.65	10.47 ± 0.08	11.24 ± 0.34	10.81 ± 0.27
18:3n-6	0.19 ± 0.02	0.23 ± 0.01	0.20 ± 0.05	0.21 ± 0.05	0.18 ± 0.02	0.20 ± 0.04
18:3n-3	0.29 ± 0.03	0.29 ± 0.02	0.26 ± 0.04	0.27 ± 0.01	0.29 ± 0.01	0.27 ± 0.05
20:3n-6	0.89 ± 0.08	0.93 ± 0.01	0.90 ± 0.18	0.98 ± 0.06	0.97 ± 0.06	0.90 ± 0.05
20:4n-6	12.16 ± 0.62	13.18 ± 1.16	11.63 ± 2.26	13.13 ± 0.82	11.04 ± 0.86	12.30 ± 0.87
20:5n-3	0.42 ± 0.09	0.53 ± 0.21	1.08 ± 0.21[a]	0.67 ± 0.13	0.59 ± 0.08	0.60 ± 0.16
22:4n-6	2.77 ± 0.34	2.98 ± 0.36	2.13 ± 0.20[a]	2.60 ± 0.33	2.02 ± 0.27[a]	2.34 ± 0.22
22:5n-3	1.57 ± 0.23	1.08 ± 0.20	3.03 ± 0.22[a,b]	2.49 ± 0.41[a,b]	1.24 ± 0.09	1.48 ± 0.08
22:6n-3	1.77 ± 0.16	1.87 ± 0.01	1.61 ± 0.27[b]	2.12 ± 0.25[b]	4.32 ± 0.80[a]	3.46 ± 0.03[a]
Total n-6 PUFA	26.33 ± 0.69	26.60 ± 2.12	25.69 ± 1.19	27.68 ± 1.96	26.12 ± 0.70	26.73 ± 1.53
Total n-3 PUFA	3.96 ± 0.34	3.58 ± 0.52	5.99 ± 0.50[a]	5.54 ± 0.41[a]	6.46 ± 0.94[a]	5.76 ± 0.19[a]
n-6/n-3 PUFA ratio	6.82 ± 0.52	6.65 ± 1.57	4.90 ± 0.68[a]	5.11 ± 0.63	4.50 ± 0.78a	4.87 ± 0.26
Unsaturation Index	121.99 ± 7.03	127.42 ± 10.75	126.19 ± 3.84	135.83 ± 6.98	132.85 ± 3.75	132.75 ± 3.56

[a] $P < 0.05$ vs. OA group, same week.
[b] $P < 0.05$ for difference between EPA and DHA group, same week.
[c] $P < 0.05$ vs. first week. For abbreviations, see Table 1. All values are means ± SE of five determinations.

TABLE 7
α-Tocopherol Content of Plasma, Red Blood Cell (RBC) Membranes, and Tissue Microsomal Membranes Isolated from Rats Treated with OA, EPA, and DHA (for 1 and 4 wk)

Treatment	OA		EPA		DHA	
week	1	4	1	4	1	4
Plasma (μM)	20.4 ± 2.0	19.3 ± 1.5	20.2 ± 1.3	20.9 ± 1.9	21.7 ± 1.1	22.9 ± 2.6
RBC membranes (ng/mg prot)	574.3 ± 44.7	544.0 ± 37.6	511.2 ± 41.1	443.9 ± 25.5[a]	757.0 ± 75.9[a,b]	461.5 ± 31.7[a,c]
Liver (ng/mg prot)	355.6 ± 31.1	284.0 ± 61.9	311.8 ± 20.7	299.4 ± 23.0	357.1 ± 32.0	287.7 ± 8.1
Kidney (ng/mg prot)	279.9 ± 16.0	282.9 ± 27.2	215.1 ± 15.4	237.2 ± 7.0	270.1 ± 29.3	227.4 ± 38.6
Heart (ng/mg prot)	555.6 ± 37.5	636.5 ± 64.5	509.9 ± 60.7	547.7 ± 79.7	425.1 ± 44.6[a]	632.0 ± 58.9
Lung (ng/mg prot)	1418.7 ± 158.6	1216.1 ± 150.9	1313.0 ± 102.9	1266.2 ± 102.2	1374.4 ± 137.8	1280.1 ± 92.2

[a] $P < 0.05$ vs. OA group, same week.
[b] $P < 0.05$ for difference between EPA and DHA group, same week.
[c] $P < 0.05$ vs. first week, same group. For abbreviations see Table 1. All values are means ± SE of five determinations.

cant increase in Unsaturation Index was observed only in heart membranes after 1 wk of DHA treatment (Table 5).

To evaluate whether the changes in fatty acid composition might account for increased susceptibility to lipid peroxidation, we exposed RBC isolated from animals treated with OA, EPA, and DHA for 4 wk to the prooxidant action of *t*-BOOH (Fig. 1A). RBC were incubated in the presence of 1 mM *t*-BOOH at 37°C for 90 min under oxygen. Lipid peroxidation was measured as malondialdehyde release. Neither EPA nor DHA treatments modified malondialdehyde release in RBC with respect to the control group. Under the same experimental conditions, we compared the time course of *t*-BOOH-induced hemolysis, measured as K^+ leakage, in RBC from OA, EPA, and DHA groups (Fig. 1B). *t*-BOOH-induced hemolysis did not differ in RBC isolated from the three groups.

In view of reports that fatty acid supplementation may influence vitamin E status (29), we analyzed the content of this antioxidant in plasma, RBC, and tissues after dietary treatment with OA, EPA, or DHA for 1 and 4 wk. The results are shown in Table 7. The three groups of animals did not differ in plasma, RBC, and tissue α-tocopherol content prior to dietary treatments. The highest level of α-tocopherol was found in lung, followed by heart, liver, RBC, and kidney. EPA did not induce any modification in α-tocopherol level after 1 wk, and a slight but significant decrease in RBC content was observed after 4 wk. DHA increased α-tocopherol content in RBC membranes after 1 wk and decreased it after 4 wk. Moreover, DHA treatment decreased α-tocopherol content in heart membranes after 1 wk and returned it to control values after 4 wk. This suggests a close relation between α-tocopherol content and Unsaturation Index modifications.

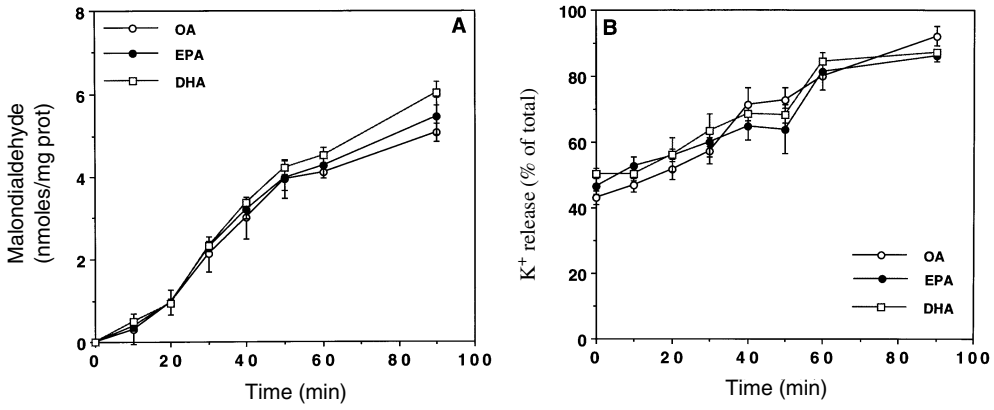

FIG.1. Lipid peroxidation and K^+ release induced by *tert*-butylhydroperoxide (*t*-BOOH) in red blood cells (RBC) isolated from rats treated with oleic acid (OA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA) for 4 wk. RBC were incubated at 37°C for 90 min under oxygen in the presence of 0.5 mM *t*-BOOH. Lipid peroxidation (A) was measured as malondialdehyde formation. Intracellular K^+ release (B) was expressed as percentage of total efflux evoked by lysis of RBC in distilled water. Data are means ± SEM of five experiments.

DISCUSSION

The results show that the individual administration of low doses of EPA or DHA to rats does not change total lipid content but significantly modifies fatty acid composition of membrane phospholipids within 1 wk of treatment. The doses of n-3 PUFA in the present study were 2–10 times lower than those used by other authors (30–33) so as to approximate the dietary dose of the mixture of n-3 PUFA consumed in human studies (1,2,16,17). Beneficial effects of equivalent doses in human subjects at high risk for colon cancer (1,2) were not associated with any side effects (16,17). Moreover, the ratio n-3/n-6 PUFA used in the present study (1,2) was in the range (0.5–2) considered optimal for the expression of n-3 PUFA beneficial effects (34–36).

The changes in phospholipid composition in the rat membranes paralleled those observed in plasma and were directly related to the specific supplementation regimen. EPA was incorporated into the membranes, both directly and indirectly, as 22:5n-3 (DPA), its elongation product. This was reminiscent of the DPA accumulation in various tissues that occurs after fish oil administration (37,38). In agreement with our results, it has been reported that administration of purified EPA led to an increased level of DPA (31,32,39,40). This suggests that DPA represents a temporary storage site for surplus EPA, as postulated by Ackman (41).

Conversely, our data show that EPA supplementation did not lead to DHA (22:6n-3) increases in the tissues. This may be explained by an inhibitory effect of EPA on Δ6-desaturase (31,42) that has been postulated to be involved in the synthesis of 22:6n-3 from 22:5n-3 (38).

DHA treatment induced an increase in EPA (20:5n-3) in plasma, RBC, liver, and kidney. This effect was not observed in heart and lung, where DHA is presumably acylated to phospholipids rather than being retroconverted to 20:5n-3. Our data suggest a lowered capacity for retroconversion from 22:6n-3 to 20:5n-3 in tissues. This is in agreement with Gronn *et al.* (32), who reported a limited ability for retroconversion in heart. We reported earlier that retroconversion from 22:6n-3 to 20:5n-3 occurred in colon mucosa and transplanted hepatocarcinoma from rats (3,4).

Our results show increased levels of 20:5n-3 in plasma and in some membranes (lung, heart, and RBC) during EPA treatment. However, this increase was higher after 1 wk than after 4 wk. On the other hand, the increase of DHA observed in plasma and membranes was similar after 1 or 4 wk. Kinetics of incorporation of EPA and DHA in the various tissues probably reflects differences in metabolism and roles of EPA and DHA in biological membranes of specialized cells. For example it is known that DHA is generally present in mammary tissues at higher levels than EPA (43,44), which is consistent with our results. This observation relates to suggestions that DHA has a role in structural stability, whereas EPA is preferentially utilized for energy purposes, e.g., β-oxidation or eicosanoid synthesis (31,45,46).

Many studies with fish oils or purified EPA or DHA show that, as tissues become enriched in n-3 PUFA, they decrease their content in 20:4n-6 (arachidonic acid) and 18:2n-6 (linoleic acid) (31,38,40,47–49). However, in our experimental conditions, EPA or DHA at low concentrations decreased the content of 20:4n-6 in plasma and different tissues (liver, kidney, and heart) while increasing the level of 18:2n-6 in liver and kidney. This is best explained by the low dose used in our study, because with increasing doses of dietary n-3 PUFA, a large decrease of 18:2n-6 content occurs only with the highest doses (50).

The decrease of 20:4n-6 may be explained either by the known inhibition exerted by n-3 PUFA on the formation of 20:4n-6 from 18:2n-6 at the Δ6-desaturation step (51) or by the competition of n-3 PUFA with 20:4n-6 for acylation on the *sn*-2 position of membrane phospholipids (32). The first mechanism is likely to occur in liver, where Δ6-desaturation is a highly active process (48) and 18:2-n-6 levels are increased (Table 3). On the other hand, in heart, where Δ6-desaturation is limited (51) and 18:2n-6 levels do not change, the second mechanism is more plausible.

Although we found changes in plasma, RBC, and tissue membrane fatty acids as a consequence of EPA or DHA treatment, no significant modifications of Unsaturation Index were observed (Table 5). Only heart membranes showed a slight increase in the Unsaturation Index after 1 wk of treatment with DHA.

Our data show that dietary administration with low doses of n-3 PUFA can induce modifications of composition of phospholipid fatty acids without substantially affecting the degree of membrane unsaturation. This observation is consistent with what we found for α-tocopherol content.

α-Tocopherol content was not changed by EPA or DHA treatments. The only exception was heart membranes, where the treatment with DHA for 1 wk decreased the content of α-tocopherol while increasing the Unsaturation Index, suggesting that these two events are closely related. It is generally reported that dietary administration of n-3 PUFA increases lipid susceptibility for peroxidation, which is observed in membranes as an increase in Unsaturation Index and the consequent decrease of α-tocopherol content (52,53). The lack of change in this relationship found in our study suggests that dietary administration of low doses of n-3 PUFA does not modify susceptibility to oxidative stress.

Treatment with EPA and DHA did not modify the Unsaturation Index of RBC membranes. In contrast, α-tocopherol increased after 1 wk of treatment with DHA and decreased after 4 wk of treatment with both EPA and DHA. This confirms our previous data (16) and that of Berlin *et al.* (54) in human subjects. These studies found that short-term treatment with n-3 PUFA increased RBC membrane content of α-tocopherol and long-term treatment decreased it.

The modification of α-tocopherol in RBC could result from the peculiarity of these cells to freely exchange lipids between plasma and the cell membrane or from differential absorption of α-tocopherol induced by n-3 PUFA treatments.

In the present study, the treatments with EPA and DHA, at

the dose used, did not modify lipid peroxidation and hemolysis susceptibility of RBC exposed to an exogenous source of free radicals. Others reported that both parameters changed owing to oxidative stress induced by supplementation with n-3 PUFA (14,52). Our findings suggest that the overall susceptibility to lipid peroxidation of RBC is not increased and that the dose of n-3 PUFA used is an important variable in the induction of detrimental effects.

We conclude that EPA and DHA, administered at low doses and at an appropriate ratio to n-6 PUFA, modify membrane fatty acids in rat tissues without increasing the susceptibility to oxidative stress. This suggests that n-3 PUFA at low doses are appropriate for use in prevention and/or treatment of cardiovascular diseases and cancer, because detrimental secondary effects on cell membranes are unlikely.

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