Effect of n-3 Fatty Acid Supplementation on Lipid Peroxidation and Protein Aggregation in Rat Erythrocyte Membranes

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ABSTRACT: Human erythrocytes in the circulation undergo dynamic oxidative damage involving membrane lipid peroxidation and protein aggregation during aging. The present study was undertaken to determine the effect of n-3 fatty acid supplementation on lipid peroxidation and protein aggregation in the circulation and also the *in vitro* susceptibility of rat erythrocyte membranes to oxidative damage. Wistar male rats were fed a diet containing n-6 fatty acid-rich safflower oil or n-3 fatty acid-rich fish oil with an equal amount of vitamin E for 6 wk. n-3 Fatty acid content in erythrocyte membranes of rats fed fish oil was significantly higher than that of rats fed safflower oil. The degree of membrane lipid peroxidation and protein aggregation of rats fed fish oil was not significantly higher than that of rats fed safflower oil when the amounts of phospholipid hydroperoxides, thiobarbituric acid-reactive substances, and detergent-insoluble protein aggregates were measured. When isolated erythrocytes were oxidized under aerobic conditions in the presence of Fe(III), the degree of membrane lipid peroxidation of erythrocytes from rats fed fish oil was increased to a greater extent than that of rats fed safflower oil, whereas the degree of membrane protein aggregation of both groups was increased in a similar extent. Hence, n-3 fatty acid supplementation did not affect lipid peroxidation and protein aggregation in membranes of circulating rat erythrocytes, and the supplementation increased the susceptibility of isolated erythrocytes to lipid peroxidation, but not to protein aggregation, under the aerobic conditions. If a sufficient amount of vitamin E is supplied, n-3 fatty acid supplementation may give no undesirable oxidative effects on rat erythrocytes in the circulation.
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Fish oils which contain high amounts of the n-3 fatty acids, eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3), have been suggested to decrease the risk of developing cardiovascular diseases and cancer (1). Epidemiological studies have shown an apparent beneficial effect of fish intake in reducing mortality from heart disease (2). On supplementation of n-3 fatty acids, arachidonic acid (20:4n-6) in phospholipids of cell membranes in various tissues of animals is replaced by these n-3 fatty acids (3,4).

Fish oils containing highly unsaturated n-3 fatty acids are readily susceptible to autoxidation under aerobic conditions (5). Glavind *et al*. (6) have reported that lipid peroxides appear in the plaque of human atheroma and that the degree of atheroma correlates with the extent of lipid peroxidation of the plaque. Since those initial observations, lipid peroxidation products have been considered to be related to various disorders in humans including atherosclerosis, diabetes, burn injury, and retinopathy (7). In relation to possible undesirable effects of fish oil intake, several studies have been done. Studies on human urine (8,9) and plasma (10–14) have shown that levels of lipid peroxidation products were slightly increased in persons eating a diet with high fish oil content. Studies on experimental animals have shown a decrease in tissue vitamin E content (15–17) and an increase in *in vitro* susceptibility to lipid peroxidation of tissues on n-3 fatty acid supplementation (15–22).

Our previous studies have shown that human erythrocytes in the circulation undergo dynamic oxidative damages including membrane lipid peroxidation (23) and protein aggregation (24) during aging. Membrane protein aggregation leads to aggregation of cell surface carbohydrate chains of band 3 glycoprotein resulting in recognition by anti-band 3 autoantibody (25). This process may be related to clearance of oxidatively damaged senescent erythrocytes by macrophages (26). It is important to elucidate whether the increased unsaturation of fatty acids in erythrocyte membranes makes them more susceptible to lipid peroxidation and protein aggregation in the circulation, and whether lipid peroxidation precedes induction of protein aggregation. The present study was undertaken to determine the effect of n-3 fatty acid supplementation on lipid peroxidation and protein aggregation of rat erythrocyte membranes in the circulation, and to clarify whether protein aggregation results from lipid peroxidation.

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Abbreviations: BHT, butylated hydroxytoluene; $C_{12}E_8$, octaethylene glycol *n*-dodecyl monoether; DPBS, Dulbecco's phosphate-buffered saline; DPBS(–), Ca^{2+}- and Mg^{2+}-free DPBS; HPLC, high-performance liquid chromatography; PCOOH, phosphatidylcholine hydroperoxide; PEOOH, phosphatidylethanolamine hydroperoxide; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; TBA, thiobarbituric acid; TBARS, TBA-reactive substance.

It was found that n-3 fatty acid supplementation did not affect lipid peroxidation and protein aggregation of rat erythrocyte membranes in the circulation. The supplementation increased the susceptibility of isolated erythrocytes to lipid peroxidation but produced little change in the susceptibility to protein aggregation under aerobic conditions.

MATERIALS AND METHODS

Materials. ADP was obtained from Oriental Yeast Company (Tokyo, Japan). Phosphatidylcholine (egg yolk) and phosphatidylethanolamine (egg yolk) were obtained from Nippon Fats and Oils Liposome Company (Tokyo, Japan). Phosphatidylcholine hydroperoxide (PCOOH) and phosphatidylethanolamine hydroperoxide (PEOOH) were prepared just before use according to the method previously described (27). Peroxide values of PCOOH and PEOOH were estimated to be 1380–1680 and 770–840 neq/mg, respectively. Cytochrome c and luminol were purchased from Sigma Chemical Company (St. Louis, MO) and Wako Pure Chemical Industries (Osaka, Japan), respectively. Thiobarbituric acid (TBA) and Silica gel 60 for thin-layer chromatography were obtained from Merck (Darmstadt, Germany). Phenylmethylsulfonyl fluoride and diisopropyl fluorophosphate were obtained from Sigma. Octaethylene glycol *n*-dodecyl monoether ($C_{12}E_8$) was from Nikko Chemical Company (Tokyo, Japan).

Animals and diets. Sixteen 4-wk-old Wistar male rats weighing 50–70 g were supplied by Japan Laboratory Animals Inc. (Tokyo, Japan). Four animals were housed together in a stainless steel cage in a room of controlled temperature at 23 ± 1°C, humidity at 55 ± 5%, and lighting of 12 h dark–light cycle. The animals were allowed free access to food. The animals were fed a normal solid diet, Clea rodent diet CE-2 containing 4.5% soybean oil and 7.0 mg% vitamin E (Clea Japan Corp., Tokyo, Japan) during 1 wk for acclimatization. Rats were divided into two groups of eight animals each, and each group was fed a diet with safflower oil or with fish oil for 6 wk. Weights of rats after feeding were 350–450 g. A powdered diet with AIN-76 composition, in which corn oil was replaced by cornstarch, and whose vitamin E content was 4.56 mg% (Oriental Yeast Company), was stored at 4°C before use. Safflower oil and fish oil with the fatty acid composition shown in Table 1 were stored at –20°C before use. Each oil contained 32.5 mg% vitamin E. Peroxide and acid values of safflower oil were 1.1 neq/mg and 0.2, respectively, and those of fish oil were 1.2 neq/mg and 0.2, respectively. Diets for feeding were freshly prepared every day by uniformly mixing 427.5 g of the powdered AIN-76 diet, 22.5 g of each oil (5% by weight), and 62.5 mL of deionized water. The overall content of vitamin E in both diets was equal at 5.93 mg% dried solid.

Erythrocyte preparation. At the end of the feeding period, eight rats of each diet group (six for lipid and protein aggregate analysis and two for vitamin E analysis) were anesthetized with an air/diethyl ether mixture, and blood (10–12 mL) was collected over citrate-phosphate-dextrose by cardiac puncture. Blood was immediately centrifuged at 320 × *g* and 4°C for 10 min to remove plasma and buffy coats. Erythrocytes were washed four times by centrifugation at 650 × *g* and 4°C for 5 min with Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline (DPBS) to make a cell pellet.

In vitro *oxidation of isolated rat erythrocytes*. Oxidation of erythrocytes was carried out according to the method previously described (28). Five milliliters of an erythrocyte suspension in DPBS (40% hematocrit) was mixed with an equal volume of a solution of an iron catalyst ADP/Fe(III) (a mixture of 3.4 mM ADP and 0.2 mM $FeCl_3$ in isotonic saline) and incubated under aerobic conditions at 37°C for 90 min. The cells were pelleted by centrifugation, washed twice with DPBS(–) and twice with isotonic saline to make a cell pellet.

Determination of hemoglobin. To 10 μL of the erythrocyte pellet was added 3.0 mL of water, and the lysate was centrifuged at 9,600 × *g* for 20 min. Absorbance of the supernatant at 523 nm was measured, and the hemoglobin concentration was calculated using a molecular extinction coefficient of 7,880 and a molecular weight of 64,000 (28).

Determination of vitamin E. Vitamin E contents of erythrocytes from two rats of each diet group were determined by high-performance liquid chromatography (HPLC) according to the method previously described (29). The amount of vitamin E in the sample was estimated by comparing the peak area with those of the calibration curve of the standard *dl*-α-tocopherol. Vitamin E content in erythrocytes was expressed per g hemoglobin.

Fatty acid composition of phospholipids of erythrocyte membranes. Erythrocytes were lysed and extracted with chloroform/methanol (2:1, vol/vol). The phospholipid fraction on silica gel thin-layer chromatography was methylated with 3% hydrochloric acid/methanol and analyzed by gas chromatography using an Hitachi G-5000 gas chromatograph (Tokyo, Japan) equipped with a fused-silica capillary column DB-225 (0.25 mm i.d. × 30 m) (J&W Scientific Company, Inc., Folsom, CA) and a flame-ionization detector with helium as a carrier gas.

Lipid peroxidation products in erythrocyte membranes. To 1.0 mL of the erythrocyte pellet, 1.0 mL of water was added and the mixture was left for 15 min. To the lysate, 11 mL of 2-propanol was added and the mixture was shaken occasionally for 1 h, after which 7 mL of chloroform was added to the mixture and shaken occasionally for another hour. The organic layer (18 mL) was collected by centrifugation at 1,500 × *g* for 15 min, filtered through a glass wool column, and 17 mL was isolated (30). The layer was divided into two 8-mL fractions. Both fractions were evaporated at below 30°C to dryness for determination of PEOOH, PCOOH, and TBA-reactive substances (TBARS). The amounts of lipid peroxidation products were expressed per g hemoglobin.

PEOOH and PCOOH. Contents of PCOOH and PEOOH in the lipid fraction of erythrocyte membranes were determined by a HPLC-chemiluminescence method (27,31). The lipid fraction was dissolved in 5.0 mL of chloroform/

methanol (2:1, vol/vol) and washed twice with 1 mL of 0.05 M KCl to obtain 3.5 mL of the organic layer. A 3.0-mL portion of the organic layer was evaporated to dryness at below 30°C and redissolved in 50 μL of chloroform/methanol (1:9, vol/vol). A 40-μL portion of this extract was analyzed. The amounts (neq) of PCOOH and PEOOH were determined by comparing their peak areas with a calibration curve of the peak areas of the standard solutions of PCOOH and PEOOH (0–100 neq).

TBARS. TBARS in the lipid fraction of erythrocyte membranes were determined according to the method previously described with added butylated hydroxytoluene (BHT) and in the absence and presence of EDTA (23,32). The lipid fraction of membranes was suspended in 0.9 mL of water for analysis.

Protein aggregates in erythrocyte membranes. $C_{12}E_8$-insoluble protein aggregates in erythrocyte membranes were isolated according to the method previously described (24,33). The amounts of protein in erythrocyte membranes and isolated aggregates were determined by the Lowry method (34). SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of SDS-solubilized aggregates was done by the method of Laemmli (35) using 10% polyacrylamide gel under reducing conditions. The gel was stained by Coomassie brilliant blue R-250.

Statistical analysis. Data were analyzed by the Student's *t*-test.

TABLE 1
Fatty Acid Composition of Safflower Oil and Fish Oil[a]

Fatty acid	Safflower oil	Fish oil
14:0		5.8
16:0	6.9	16.4
16:1n-7	0.1	5.9
17:0		1.2
18:0	2.4	3.9
18:1n-9	12.3	15.8
18:1n-7		3.4
18:2n-6	78.0	1.3
18:3n-3	0.3	1.4
20:1n-9		1.2
18:4n-3		3.1
20:5n-3		9.6
22:1n-9		2.7
22:5n-3		2.2
22:6n-3		10.2
Unknown		15.9
Total saturated fatty acid	9.3	27.3
Total MUFA	12.4	29.0
Total n-6 PUFA	78.0	1.3
Total n-3 PUFA	0.3	26.5
Total n-6 + n-3 PUFA	78.3	27.8
UI	168	168
PI	78	114

[a]Expressed as percentages (w/w) of total fatty acids present. Abbreviations: UI, unsaturation index (sum of percentages of individual fatty acids × number of double bonds); PI, peroxidizability index (sum of percentages of individual fatty acids × number of active methylenes); MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

RESULTS

Two groups of Wistar male rats were fed a diet with 5% safflower oil or 5% fish oil for 6 wk. Fatty acid compositions of safflower and fish oils are shown in Table 1. As expected, safflower oil was rich in n-6 fatty acids (n-6 total: 78.0%, and n-3 total: 0.3%) and fish oil was rich in n-3 fatty acids (n-6 total: 1.3%, and n-3 total, 26.5%). While the unsaturation index (the sum of the percentages of individual fatty acids × number of double bonds) of the fish oil was equal to that of the safflower oil, the peroxidizability index, (the sum of the percentages of individual fatty acids × number of active methylenes) of the fish oil was higher than that of safflower oil, indicating that fish oil may be more readily peroxidized. Both diets contained an equal amount (5.93 mg% dried diet) of vitamin E; the safflower oil diet had 1.5 mg vitamin E/g polyunsaturated fatty acids and the fish oil diet had 4.3 mg vitamin E/g polyunsaturated fatty acids. Erythrocytes were isolated from each of six rats of both rat groups. Fatty acid compositions of phospholipids of erythrocyte membranes of both rat groups were determined (Table 2). It was found that phospholipids of erythrocytes of rats fed safflower oil contained higher amounts (39.0%) of n-6 fatty acids than those of rats fed fish oil (13.0%), and those of rats fed fish oil contained higher amounts (22.3%) of n-3 fatty acids than those of rats fed safflower oil (1.9%). Both the unsaturation index and the peroxidizability index of the erythrocyte membrane phospholipids from rats fed a diet containing fish oil were higher than those from rats fed a diet containing safflower oil. Vitamin E content of erythrocytes was estimated to be 12% lower in two rats fed fish oil (10.3 and 10.5 μg/g hemoglobin) than in two rats fed safflower oil (11.6 and 12.0 μg/g hemoglobin).

Contents of phospholipid hydroperoxides, PCOOH and PEOOH, in erythrocyte membranes of both rat groups were determined by an HPLC-chemiluminescence method (27,31). Representative HPLC chromatograms are shown in Figure 1A. When the mean content of these phospholipid hydroperoxides of six rats of both the groups were compared (Fig. 2A), no significant differences between two groups were observed. Concentrations of PCOOH and PEOOH of both rat groups were lower than 0.7 nmol/g hemoglobin. The TBARS in erythrocyte membranes of both rat groups were estimated. TBA assay of Ohkawa's procedure at pH 3.5 was performed in the presence of BHT in order to prevent lipid peroxidation while heating under the acidic assay conditions (23,32). It has been claimed that the TBA assay in the presence of EDTA reflects exclusively malonaldehyde derivatives that liberate malonaldehyde under the assay conditions, and that the assay in the absence of EDTA reflects not only malonaldehyde derivatives but also the derivatives of alka-2,4-dienals and alk-2-enals (32,36). Representative absorption spectra of the TBA assay mixtures of erythrocyte membranes shown in Figure 3A indicate that absorption spectra from both rat groups were similar to that of standard TBA red pigment without disturbing colored pigments. When the mean values of contents of TBARS of six rats of two rat groups were compared (Fig.

TABLE 2
Fatty Acid Composition of Phospholipids of Erythrocyte Membrane of Rats Fed a Diet Containing Safflower Oil or Fish Oil[a]

Fatty acid	Safflower oil[a] Mean[b] ± SD	Fish oil[a] Mean[b] ± SD	P^c
14:0	0.2 ± 0.1	0.3 ± 0.2	0.263
15:0	0.3 ± 0.2	0.3 ± 0.0	0.758
16:0	26.2 ± 1.3	30.2 ± 1.3	0.000
16:1n-7	0.5 ± 0.1	0.9 ± 0.6	0.167
17:0	0.3 ± 0.0	0.6 ± 0.0	0.000
17:1 n-7	0.1 ± 0.0	0.2 ± 0.0	0.000
18:0	17.7 ± 1.2	13.4 ± 0.3	0.000
18:1n-9	4.8 ± 0.2	7.7 ± 0.6	0.000
18:1n-7	3.8 ± 0.2	4.2 ± 0.1	0.002
18:2n-6	7.4 ± 0.4	1.6 ± 0.1	0.000
18:3n-6	0.1 ± 0.0	0.1 ± 0.0	0.570
20:0	0.2 ± 0.0	0.3 ± 0.1	0.279
20:1n-12	0.0 ± 0.0	0.1 ± 0.0	0.000
20:1n-9	0.1 ± 0.0	0.3 ± 0.0	0.000
20:2n-6	0.3 ± 0.1	0.1 ± 0.0	0.011
20:3n-6	0.5 ± 0.1	0.3 ± 0.2	0.186
20:4n-6	28.2 ± 0.6	10.7 ± 0.5	0.000
20:5n-3	0.0 ± 0.0	11.4 ± 0.8	0.000
22:0	1.0 ± 1.2	0.8 ± 1.6	0.529
22:1n-9	0.4 ± 0.6	0.3 ± 0.3	0.584
22:2n-6	0.1 ± 0.1	0.1 ± 0.1	0.089
22:4n-6	2.6 ± 0.2	0.1 ± 0.1	0.000
23:0	0.0 ± 0.0	0.1 ± 0.1	0.046
22:5n-3	0.4 ± 0.1	3.8 ± 0.3	0.000
22:6n-3	1.4 ± 0.1	7.1 ± 0.4	0.000
24:0	1.9 ± 0.2	2.4 ± 0.4	0.015
24:1n-9	1.5 ± 0.2	2.6 ± 0.3	0.000
Total saturated fatty acid	47.8	48.4	
Total MUFA	11.2	16.3	
Total n-6 PUFA	39.0 ± 0.8	13.0 ± 0.6	0.000
Total n-3 PUFA	1.9 ± 0.1	22.3 ± 1.3	0.000
Total n-6 + n-3 PUFA	40.8	35.3	
UI	162	183	
PI	110	131	

[a]Expressed as percentages (w/w) of total fatty acids present.
[b]Values are means for six rats.
[c]Significance of difference by Student's *t*-test between data for safflower oil- and fish oil-fed rats. For abbreviations see Table 1.

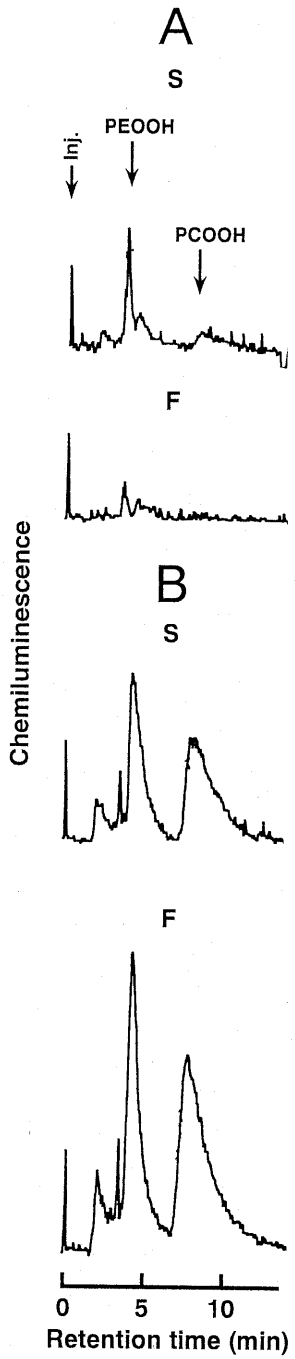

FIG. 1. Representative chromatograms of high-performance liquid chromatography-chemiluminescence assay of phosphatidylcholine hydroperoxide (PCOOH) and phosphatidylethanolamine hydroperoxide (PEOOH) in rat erythrocyte membranes. (A) Membranes of erythrocytes of a rat fed a diet containing safflower oil (S) or fish oil (F); (B) membranes of erythrocytes of a rat fed a diet containing S or F which were oxidized *in vitro* with ADP/Fe(III) at 37°C for 90 min.

4A), no significant differences between the groups were observed. Contents of the substances in the presence of EDTA of both rat groups were estimated to be about 2 nmol/g hemoglobin, and those of the substances in the absence of EDTA of both rat groups were estimated to be much higher at 35 nmol/g hemoglobin. Hence, erythrocyte membranes of rats fed fish oil did not show enhanced lipid peroxidation in the circulation as compared to rats fed safflower oil.

Membrane protein aggregates insoluble in detergent $C_{12}E_8$, whose presence indicates oxidative damage to erythrocyte membranes (24,33), were obtained from erythrocytes of both rat groups. The aggregates were solubilized with SDS, and their protein contents were measured (Fig. 5A). Contents of the aggregates of erythrocytes from rats fed fish oil were similar to those from rats fed safflower oil. SDS-PAGE of the aggregates from both rat groups revealed a similar pattern (Fig. 6A). Erythrocyte membranes of rats fed fish oil did not exhibit increased oxidative protein aggregation in the circulation when compared to the membranes of rats fed safflower oil.

Erythrocytes from the two rat groups were oxidized *in vitro* at 37°C for 90 min in the presence of ADP/Fe(III) (28) under aerobic conditions. Figure 1B shows representative HPLC chromatograms of PCOOH and PEOOH, and Fig-

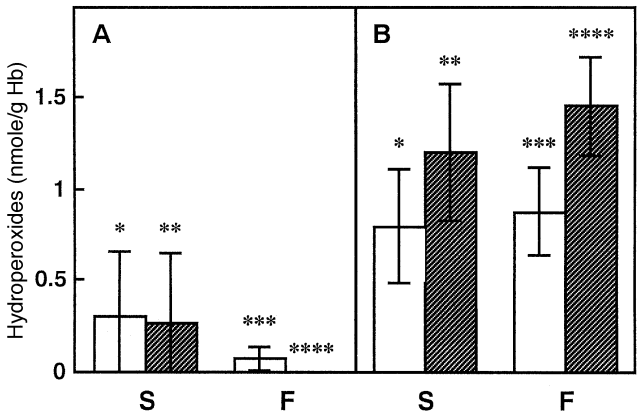

FIG. 2. Contents of PCOOH (striped bars) and PEOOH (open bars) in rat erythrocyte membranes. (A) Membranes of erythrocytes of rats fed a diet containing S or F. (B) Membranes of erythrocytes of rats fed a diet containing S or F which were oxidized *in vitro* with ADP/Fe(III) at 37°C for 90 min. Mean values ± SD for six rats are given. $*P < 0.01$; $**P < 0.02$; $***P < 0.001$; $****P < 0.001$. Hb, hemoglobin; for other abbreviations see Figure 1.

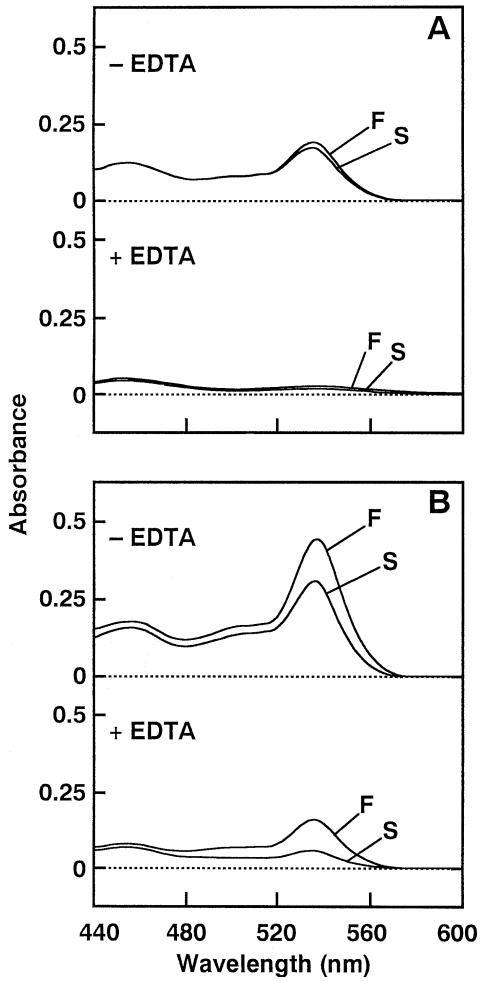

FIG. 3. Representative absorption spectra of thiobarbituric acid (TBA) reaction mixtures with and without EDTA of rat erythrocyte membranes. (A) Membranes of erythrocytes of a rat fed a diet containing S or F; (B) membranes of erythrocytes of a rat fed a diet containing S or F which was oxidized *in vitro* with ADP/Fe(III) at 37°C for 90 min. For abbreviations see Figure 1.

ure 2B shows the concentrations of PCOOH and PEOOH from six rats of both rat groups. The hydroperoxide levels of both rat groups were extensively increased by the *in-vitro* aerobic oxidation, and the degree of the increase of the group fed fish oil was higher than that fed safflower oil. Figure 3B shows representative absorption spectra of the TBA reaction mixtures, and Figure 4B shows the mean levels of TBARS of six rats of both groups. The levels of TBARS in the presence and absence of EDTA of both groups were increased by oxidation, and the levels of the rat group fed fish oil were increased more. The TBARS of the rat group fed fish oil were increased over that of the safflower-fed group twofold in the assay without EDTA and fivefold in the assay with EDTA. The results indicate that fish oil supplementation caused enhanced *in vitro* susceptibility to lipid peroxidation of erythrocyte membranes. $C_{12}E_8$-insoluble membrane protein aggregates were obtained from erythrocytes of both rat groups oxidized *in vitro*. The amount of aggregates from both rat groups was increased by the oxidation, and the increased levels of the rat group fed fish oil was similar to those of the rat group fed safflower oil (Fig. 5B). SDS-PAGE of the aggregates from both rat groups revealed a similar pattern (Fig. 6B). Erythrocyte membrane proteins of both rat groups were similarly aggregated by the *in vitro* oxidation, and the degree of aggregation did not correspond to the degree of lipid peroxidation of membrane.

DISCUSSION

Wistar rats were fed a diet containing safflower oil or fish oil with an equal amount of vitamin E, which was carefully prepared to prevent lipid peroxidation before feeding. n-3 Fatty acid content of isolated erythrocyte membranes of rats fed fish oil was significantly higher than that of rats fed safflower

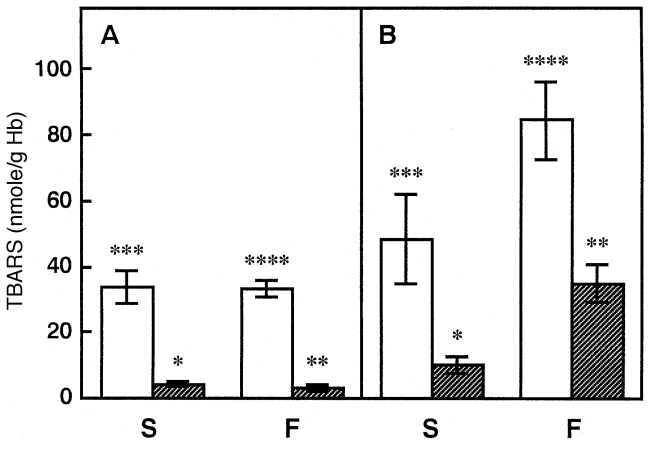

FIG. 4. Contents of TBA-reactive substances (TBARS) in the TBA assay with (striped bars) and without EDTA (open bars) of rat erythrocyte membranes. (A) Membranes of erythrocytes of a rat fed a diet containing S or F; (B) membranes of erythrocytes of a rat fed a diet containing S or F which was oxidized *in vitro* with ADP/Fe(III) at 37°C for 90 min. Mean values ± SD for six rats are given. $*P < 0.001$; $**P < 0.001$; $***P < 0.02$; $****P < 0.001$. For abbreviations see Figures 1 and 3.

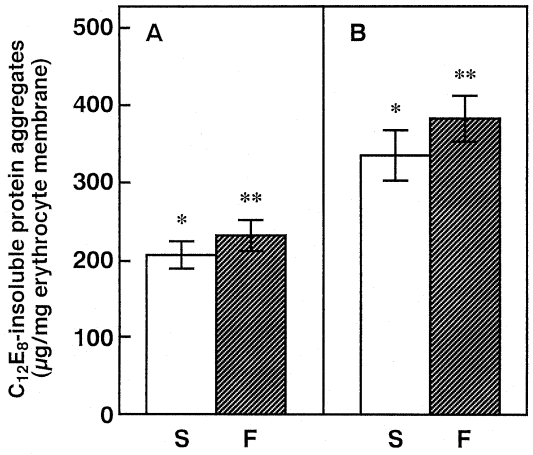

FIG. 5. Amount of detergent-insoluble protein aggregates in rat erythrocyte membranes. (A) Membranes of erythrocytes of rats fed a diet containing S or F; (B) membranes of erythrocytes of rats fed a diet containing S or F which were oxidized *in vitro* with ADP/Fe(III) at 37°C for 90 min. Mean values ± SD for six rats are given. $*P < 0.001$; $**P < 0.001$. $C_{12}E_8$, octaethyleneglycol *n*-dodecylmonoether; for other abbreviations see Figure 1.

oil. When lipid peroxidation products, phospholipid hydroperoxides, and TBARS of isolated erythrocyte membranes were determined, the degree of membrane lipid peroxidation of isolated erythrocytes with high n-3 fatty acid content was similar to that of erythrocytes with low n-3 fatty acid content. In the studies on TBARS, great attention was paid to prevent lipid peroxidation under the aerobic conditions of the assay. The assay was always conducted in the presence of antioxidant BHT (32,36), and a modified Ohkawa's method at pH 3.5 with and without EDTA (23,32) was employed. In this study, it is conceivable that water-soluble TBARS in membranes may be released into plasma while in the circulation or into the medium during the isolation of membranes, and such losses could not be determined. However, it seems unlikely that a large amount of water-soluble TBARS was released from membranes because a substantial amount of TBARS in the erythrocyte membranes oxidized *in vitro* stayed in the membranes (Figs. 3B and 4B). Vitamin E in membranes of erythrocytes with high n-3 fatty acid content was slightly decreased when compared with that of erythrocytes with low n-3 fatty acid content, indicating that vitamin E was consumed to prevent lipid peroxidation of n-3 fatty acids during the circulation, as has been suggested in earlier studies (15–22). It was found in this study that the amount of vitamin E supplemented was high enough to prevent lipid peroxidation of erythrocyte membranes in the circulation. The degree of membrane protein aggregation, as assessed by detergent $C_{12}E_8$-insoluble aggregates (24,33) and SDS-PAGE of the aggregates, was similar between erythrocytes with high and low n-3 fatty acid contents. Hence, fish oil supplementation that caused high n-3 fatty acid content in erythrocyte membranes did not cause increased lipid peroxidation and protein aggregation while in the circulation. The results indicate that n-3 fatty acid supplementation did not increase the oxidative damage of circulating rat erythrocyte membranes, which may be reasonably explained by the fact that the oxygen pressure in the rat vasculature is lower than atmospheric. A sufficient supply of vitamin E prevents oxidative damage to erythrocytes with high n-3 fatty acid content in the circulation.

The degree of membrane lipid peroxidation of erythrocytes with high n-3 fatty acid content was increased under aerobic conditions with Fe(III) ion to a higher extent than that of erythrocytes with low n-3 fatty acid content, indicating that the n-3 fatty acid supplementation increased the susceptibility of erythrocyte membranes to lipid peroxidation under aer-

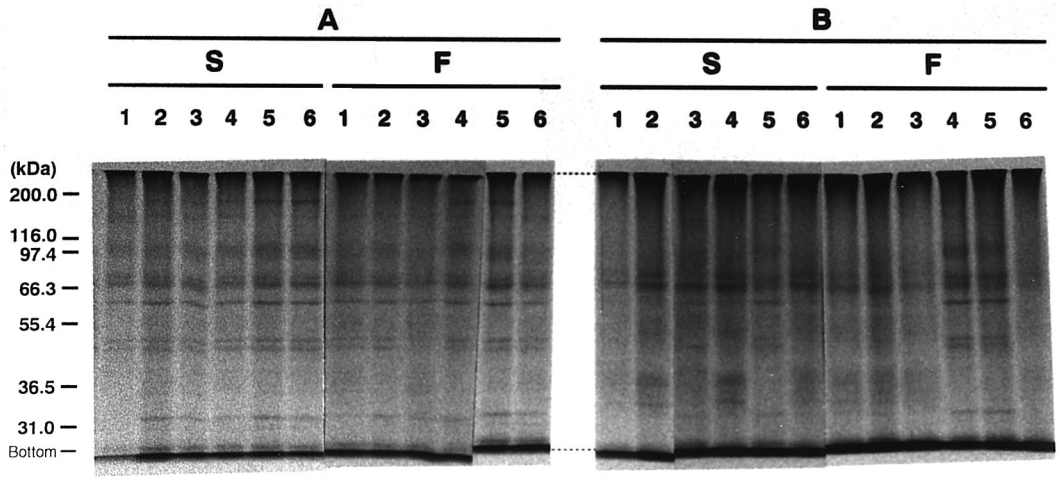

FIG. 6. SDS-polyacrylamide gel electrophoresis of detergent-insoluble protein aggregates in rat erythrocyte membrane. (A) Membranes of erythrocytes of rats fed a diet containing S or F; (B) membranes of erythrocytes of rats fed a diet containing S or F which were oxidized *in vitro* with ADP/Fe(III) at 37°C for 90 min. All the aggregates of six rats of each group were electrophoresed. Bands were visualized by staining with Coomassie brilliant blue R-250. Protein molecular-weight-marker (Iwaki Glass Corporation, Chiba, Japan) was used for indication of the protein positions of molecular weight. For abbreviations see Figure 1.

obic conditions. The amount of vitamin E present in membranes under these conditions may be too small to prevent oxidation of membranes with high n-3 fatty acid content. The increased susceptibility to lipid peroxidation, which was tested under the artificial aerobic conditions, does not reflect the circulation conditions of the erythrocytes *in vivo.* The degree of membrane protein aggregation of erythrocytes with high vs. low n-3 fatty acid content was increased to a similar extent by the *in vitro* oxidation, indicating that oxidative membrane protein aggregation did not correspond to lipid peroxidation, and that lipid peroxidation was not a causative factor for membrane protein aggregation.

Several studies suggesting undesirable effect of dietary n-3 fatty acid intake for humans have appeared. Measurement of human urinary and plasma levels of lipid peroxidation products in relation to increased n-3 fatty acid intake has been carried out. Daily urinary TBARS levels in humans fed a diet containing n-3 fatty acids for 40–50 d are slightly increased (8,9), but the same authors suggested the increase was caused by the lipid peroxidation products formed in food before its consumption (8). The present authors have shown in previous studies that human urinary TBARS levels of each subject eating regular Japanese food exhibited a 1.5–3-fold variation daily and 2–3-fold variation within a day (37), suggesting that the urinary TBARS level is not a good index for lipid peroxidation studies of the whole human body. Several reports show that high intakes of n-3 fatty acids led to increased human plasma TBARS and lipid hydroperoxide levels (10–14). However, in most of these studies (10,11,13) the TBA assay was carried out without addition of BHT, and TBARS could be produced during the aerobic assay procedures. Furthermore, TBARS in serum or plasma are usually very low and could not yet be identified (38). Hackett *et al*. (39) strongly argued that the TBA asssay of plasma is not reliable. By contrast, Kinsella (40) has suggested that intake of n-3 fatty acid preparations which contain vitamin E should not exert any deleterious effects *via* peroxidation when adequate levels of vitamin E are consumed (40).

Studies on experimental animals supplemented with n-3 fatty acids have shown a decrease in vitamin E content and an increased susceptibility to *in-vitro* lipid peroxidation of tissues (15–22). Supplementation of herring oil to rats increased TBARS levels in liver microsomes and susceptibility to *in vitro* lipid peroxidation (19). Supplementation of menhaden oil to rats increased TBARS levels in heart and liver and their susceptibility to *in vitro* lipid peroxidation (20). In most of these previous studies (19–21), the TBA assay was carried out without addition of any antioxidants, and there is a possibility that artifactual lipid peroxidation took place during the assay under aerobic conditions.

In conclusion, n-3 fatty acid supplementation did not affect lipid peroxidation and protein aggregation in rat erythrocyte membrane and may not enhance dynamic oxidative damages of the cells. It is likely that if a sufficient amount of vitamin E is supplied, n-3 fatty acid supplementation does not bring about undesirable effects in relation to lipid peroxidation.

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