Moderate intake of n−3 fatty acids is associated with stable erythrocyte resistance to oxidative stress in hypertriglyceridemic subjects[1–3]

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ABSTRACT

Background: The important triacylglycerol-lowering capacity of n−3 fatty acids is counterbalanced by their inherent sensitivity to oxidation. Inconsistent results about the latter have been reported in hypertriglyceridemic individuals. After incorporation into cell membranes, n−3 fatty acids may alter membrane-related functions. In view of the distinct composition of hypertriglyceridemic membranes and the prooxidant status in this condition, it can be surmised that cell enrichment with the oxidizable n−3 fatty acids will be associated with an increased hemolytic process.

Objective: We sought to evaluate the effect of fish oil consumption on n−3 fatty acid incorporation into erythrocyte membranes and subsequent ex vivo oxidative-stress-induced hemolysis in normotriglyceridemic and hypertriglyceridemic subjects.

Design: Sixteen normotriglyceridemic and 12 hypertriglyceridemic subjects were given 6 g fish oil/d for 8 wk. Blood samples were collected before and 4 and 8 wk after treatment. Resistance to 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH)–induced hemolysis was assayed in fresh erythrocyte suspensions, and erythrocyte samples were stored at −70°C for later analysis of cholesterol, hemoglobin, fatty acids, vitamin E, and glutathione peroxidase activity.

Results: Fish oil supplementation induced n−3 fatty acid incorporation in normotriglyceridemic and hypertriglyceridemic erythrocyte membranes without decreasing their resistance to AAPH. n−3 Fatty acids significantly protected normotriglyceridemic but not hypertriglyceridemic erythrocytes against hemolysis. In normotriglyceridemic subjects only, the higher resistance to hemolysis correlated with changes in cell vitamin E.

Conclusion: Although they exhibit a high susceptibility to oxidation, n−3 fatty acids may preserve membrane integrity and represent an added benefit in the treatment of hypertriglyceridemic patients. *Am J Clin Nutr* 2001;74:449–56.

KEY WORDS Fish oil, n−3 fatty acids, hemolysis, oxidation, antioxidants, phospholipids, triacylglycerol, hypertriglyceridemia

INTRODUCTION

Research in the past 2 decades has provided considerable evidence that fish or fish oil affects health in relation to either vascular disease or cancer (1–4). In the former, this effect has been attributed to the hypotriglyceridemic (5) and antiaggregatory properties (6) of the long-chain n−3 polyunsaturated fatty acids (PUFAs), ie, eicosapentaenoic acid (EPA; 20:5n−3) and docosahexaenoic acid (DHA; 22:6n−3), which are abundant in fish oil. Nevertheless, potential atherogenic and thrombogenic effects have also been reported (7–10). Furthermore, a recent systematic analysis emphasized the conclusion that fish consumption (40–60 g/d) may be beneficial only in high-risk populations (11). Therefore, the validity of recommendations for dietary supplementation with n−3 PUFAs is not unequivocal and this issue should be investigated further.

Short-term intake of EPA and DHA results in their incorporation into lipoproteins and membrane phospholipids, which can be observed in blood cells such as erythrocytes (12, 13). Prior studies indicated that changes in phospholipid composition or distribution can be associated with functional alterations in membrane erythrocytes and, likely, in other cells (14–17). For instance, membrane lipid peroxidation, which depends on the membrane's fatty acid composition and antioxidant content (mainly vitamin E in cell membranes), together with protein oxidation, leads to the loss of membrane integrity and function, as occurs during hemolysis. Thus, the increased unsaturation index consequent to EPA and DHA incorporation into membranes could alter the red cell response to oxidative threats (18). Similarly, pathologies associated with a prooxidant status, such as hyperlipidemia (19, 20), may significantly affect the behavior of the red cell in response to both oxidative stress and n−3 fatty acid enrichment. Because of the importance of free radical–induced damage in vascular disease and in cancer (21), an assessment of the relation between n−3 PUFAs and oxidative status in humans is warranted. The

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increased n−3 PUFA content of human or rabbit red blood cells that was shown to enhance in vitro lipid peroxidation was also associated with decreased hemolysis (22, 23), suggesting the need to study the entire cell. Although this aspect has been studied in normal erythrocytes, to our knowledge, it has never been studied in cells from hyperlipidemic subjects.

In a previous study of hypertriglyceridemic subjects (24), we showed that the >50% decrease in VLDL triacylglycerol observed in response to 12 g fish oil/d (6 g n−3 fatty acids) was associated with an enhanced LDL susceptibility to in vitro oxidation. However, the question of n−3 fatty acid–induced oxidation remains controversial (25). Thus, the current study was undertaken to assess how a lower dose of fish oil (6 g/d) affects fatty acid incorporation into erythrocyte phospholipids and the cell resistance to free radical–induced hemolysis in normotriglyceridemic and hypertriglyceridemic subjects.

SUBJECTS AND METHODS

Subjects

Sixteen healthy, normolipidemic, volunteer subjects (7 women and 9 men; $\bar{x}$ ± SD age: 34.9 ± 6.8 y) were recruited for the normotriglyceridemic (NTG) group. In a separate study, 12 hypertriglyceridemic patients (all men, 3 with type III dysbetalipoproteinemia and 9 with type IV endogenous hypertriglyceridemia; $\bar{x}$ ± SD age: 47.9 ± 8.5 y) were selected from among the patients seen at our lipid clinic for the hypertriglyceridemic (HTG) group. The study protocol was approved by the Ethics Committee of the Clinical Research Institute of Montreal and informed consent was obtained from each participant.

Normal lipid concentrations were defined as total plasma cholesterol concentrations <5.2 mmol/L and total plasma triacylglycerol concentrations <1.69 mmol/L. Type III dysbetalipoproteinemia was diagnosed according to the following criteria: plasma cholesterol concentrations >5.2 mmol/L, plasma triacylglycerol concentrations >2.3 mmol/L, the presence of β-VLDL on agarose gel electrophoresis, and the apolipoprotein E phenotype E2/2. Type IV hypertriglyceridemic individuals had triacylglycerol concentrations >2.3 mmol/L and LDL-cholesterol concentrations <3.9 mmol/L. All hypertriglyceridemic subjects were free of secondary causes of hypertriglyceridemia, such as diabetes, obesity (>130% of ideal body weight), and excessive alcohol ingestion. None of the subjects had a metabolic disease other than dyslipidemia and none showed signs or symptoms of cardiovascular disease. Selection criteria also included reliability, regular eating habits, normal weight, and nonsmoking status. None of the selected subjects used any vitamins or dietary supplements or any drug known to affect lipid concentrations for ≥8 wk before the start of the study and during the entire experimental period.

A dietary evaluation was conducted by a dietitian at the beginning of the study. Participants in the NTG group were encouraged to maintain a normal, balanced diet; those in the HTG group were encouraged to maintain an appropriate low-saturated-fat, low-cholesterol diet. All participants were encouraged to pursue their normal activities throughout the study period.

Procedure

All subjects received 6 g fish oil/d in the form of 6 capsules of SuperEPA (Bronson Pharmaceuticals, St Louis) over a period of 8 wk. Each 1-g capsule provided 0.671 mg (1 IU) *RRR*-α-tocopherol, 300 mg EPA, and 200 mg DHA. This dose was shown previously to significantly lower triacylglycerol concentrations in normal and hyperlipidemic individuals (26). Drug compliance was verified by capsule count and was confirmed by the phospholipid fatty acid profile. Subjects were weighed at each visit and interviewed by a dietitian to verify dietary compliance. Patients in the HTG group were seen by a physician for follow-up assessment.

Blood sampling

Venous blood samples were drawn immediately before the study and after 2, 4, and 8 wk of fish oil supplementation after the subjects had fasted overnight. Blood was collected in EDTA-containing (1.5 g/L) evacuated tubes and the plasma and blood cells were separated by low speed centrifugation (1180 × *g* for 15 min at 4°C). Washed red blood cells were used immediately for the hemolysis test (described below). Plasma lipid and lipoprotein analyses were performed within 3 d of sampling. Several portions of plasma and washed red blood cells were stored at −70°C for future analyses.

Plasma lipid and lipoprotein determination

Plasma lipoproteins were separated under standard conditions by a combination of ultracentrifugation (320 000 × *g*, 8 h, 4°C) to isolate VLDL at d = 1006 g/L and heparin-manganese precipitation of the apolipoprotein B–containing lipoproteins in the d = 1006 g/L infranatant fluid for measurement of LDL- and HDL-cholesterol concentrations according to the Lipid Research Clinics protocol (27). Plasma and lipoprotein cholesterol and triacylglycerol were measured enzymatically with an automated autoanalyzer (Cobas Mira S; F Hoffmann-La Roche, Ltd, Diagnostica, Basel, Switzerland).

Erythrocyte resistance to hemolysis

The in vitro resistance of intact red blood cells to oxidation was evaluated with 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH; Spiral, Dijon, France). AAPH generates free radicals by spontaneous thermal decomposition (28). The overall antioxidant defense contributes to maintaining cell membrane integrity and function until cell lysis. Isolated blood cells were washed 3 times with saline (0.9% NaCl) and centrifuged for 15 min at 1300 × *g* and 4°C , after which the supernatant fluid and remaining buffy coat were removed. Twelve aliquots of AAPH (final concentration: 40–260 mmol/L) were incubated at 37°C for 150 min with washed erythrocytes adjusted with saline to a hematocrit of 0.15. After centrifugation, the absorbance of the supernatant fluid (index of hemolysis) was measured at 405 nm with a microplate reader (Bio-Tek Instruments, Inc, Burlington, VT). The AAPH concentration corresponding to 50% hemolysis (C_{50}-AAPH in mmol/L) was evaluated with INPLOT software (Prism, GraphPAD Inc, San Diego) and is interpreted as the erythrocyte resistance to free radical attack. The intra- and between-assay CVs were 2.21% (n = 6) and 5.36% (n = 7), respectively.

Fatty acid analyses

Erythrocyte lipids were extracted with isopropanol:chloroform according to Rose and Oklander (29). Phospholipids were separated by thin-layer chromatography (chloroform:methanol:acetic acid:water, 50:30:8:4 by vol) (30). The silica gel corresponding to phosphatidylcholine (PC) and phosphatidylethanolamine (PE) was collected and the phospholipid fatty acids were transmethylated with 14% boron trifluoride in methanol at 100°C for 90 min under

TABLE 1
Effect of supplementation with fish oil (3 g n−3 fatty acids/d) on plasma total and lipoprotein cholesterol and triacylglycerol in normotriglyceridemic (NTG) and hypertriglyceridemic (HTG) subjects[1]

Variable and group	Week 0	Week 4	Week 8
Total cholesterol (mmol/L)			
NTG	4.69 ± 0.17	4.56 ± 0.20	4.67 ± 0.21
HTG	6.76 ± 0.59	5.77 ± 0.47	5.77 ± 0.44
LDL cholesterol (mmol/L)			
NTG	2.80 ± 0.17	2.79 ± 0.19	2.84 ± 0.18
HTG	2.42 ± 0.25	2.75 ± 0.32	3.01 ± 0.30
HDL cholesterol (mmol/L)			
NTG	1.30 ± 0.09	1.28 ± 0.07	1.30 ± 0.08
HTG	0.77 ± 0.06	0.70 ± 0.02	0.73 ± 0.03
Total triacylglycerol (mmol/L)			
NTG	1.05 ± 0.11	0.80 ± 0.07[2]	0.83 ± 0.14[2]
HTG	6.29 ± 1.14	4.58 ± 0.72[3]	3.31 ± 0.36[3]
VLDL triacylglycerol (mmol/L)			
NTG	0.75 ± 0.10	0.48 ± 0.076[2]	0.53 ± 0.11[2]
HTG	5.71 ± 1.12	4.01 ± 0.68[3]	2.77 ± 0.35[3]

[1] $\bar{x}$ ± SEM; n = 16 NTG and 12 HTG subjects.
[2,3] Significantly different from baseline (one-factor ANOVA for repeated measures followed by Bonferroni's procedure for multiple comparisons): [2] $P < 0.05$, [3] $P < 0.01$.

a nitrogen gas atmosphere (31). The fatty acid methyl esters were injected into a gas chromatograph (HP-5880A; Hewlett-Packard, Palo Alto, CA) equipped with a 0.53 mm × 30 m Supelcowax 10 capillary column (Supelco, Mississauga, Canada) and a flame ionization detector, with helium as the carrier gas. Peaks were identified against reference fatty acids (Supelco) and results are expressed as percentages of the sum of all identified peaks.

Erythrocyte antioxidants

The erythrocyte α-tocopherol content was determined by HPLC after lipid extraction of samples that had been kept frozen at −70°C, and results were calculated relative to the cell cholesterol and hemoglobin contents (32, 33). Cholesterol was measured by an enzymatic assay and hemoglobin by colorimetry with a commercial kit (525-A; Sigma Chemical Co, St Louis). Erythrocyte glutathione peroxidase activity was measured with a spectrophotometric assay with use of a commercial kit (Ransel; Randox Laboratories, Mississauga, Canada); results are expressed as units per gram hemoglobin (U/g Hb).

Statistical analyses

Data were analyzed with SAS software (version 6.12; SAS Institute Inc, Cary, NC). For the purpose of this study, data from patients with type III and type IV hypertriglyceridemia were pooled because no specific pattern was found for the 2 types in the response of erythrocytes to fish oil intake. One-factor (time) analysis of variance for repeated measures was performed for the NTG and HTG groups separately, followed by Bonferroni's procedure for multiple comparisons (34). Associations between variables were evaluated by simple linear regression and Pearson's r coefficient was used. In all analyses, $P = 0.05$ was chosen as the threshold of significance.

RESULTS

The fish oil supplement was well tolerated and resulted in no significant changes in body weight (66.9 ± 13.5 and 67.1 ± 13.7 kg in the NTG group and 81.0 ± 8.1 and 81.8 ± 7.8 kg in the HTG group at baseline and after 8 wk, respectively). Subjects generally adhered to their usual eating habits and compliance with the treatment was excellent in both groups. The significant decreases in plasma total and VLDL triacylglycerol noted in both groups after 4 and 8 wk of fish oil treatment are shown in **Table 1**. There were no significant changes in LDL and HDL cholesterol in either group.

Hemolysis

Determination of the resistance of erythrocytes to ex vivo free radical attack showed that the concentration of AAPH needed for 50% hemolysis was significantly higher after 4 and 8 wk of fish oil supplementation than at baseline in the NTG group (**Figure 1**). Although no significant variation in the C_{50} was observed in the HTG group at any time during the study, there was a trend toward an increase over the 8-wk supplementation period. The significance of the changes in the AAPH concentrations required for hemolysis in the NTG subjects after 4 and 8 wk of fish oil supplementation is shown in **Figure 2**; no significant shift in the curves was observed in the HTG group.

Fatty acid composition of erythrocyte membranes

The fatty acid compositions of the 2 main phospholipid species in erythrocytes before and during fish oil supplementation are shown in **Table 2** for the NTG group and in **Table 3** for the HTG group. At baseline, the characteristic dominance of shorter-chain, less unsaturated fatty acids in PC and the high proportion of long-chain PUFAs in PE were found in erythrocyte membranes from both groups (35). Significant incorporation of the n−3 fatty acids EPA and DHA and, less consistently, the intermediate metabolite docosapentaenoic acid (22:5n−3) into erythrocyte phospholipids was observed in both groups. This

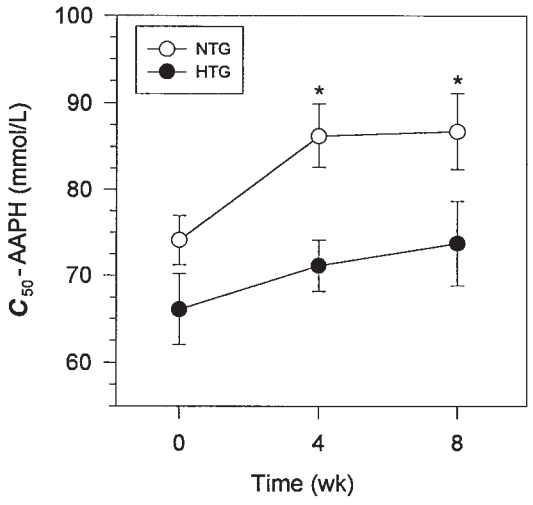

FIGURE 1. The mean (±SEM) 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH) concentration corresponding to 50% hemolysis (C_{50}-AAPH) in normotriglyceridemic (NTG; n = 16) and hypertriglyceridemic (HTG; n = 12) subjects before and after supplementation with fish oil (3 g n−3 fatty acids/d). Erythrocytes were separated from whole blood at each visit, and intact washed cells were submitted to increasing doses of AAPH. *Significantly different from week 0, $P < 0.05$ (one-factor ANOVA for repeated measures followed by Bonferroni's procedure for multiple comparisons).

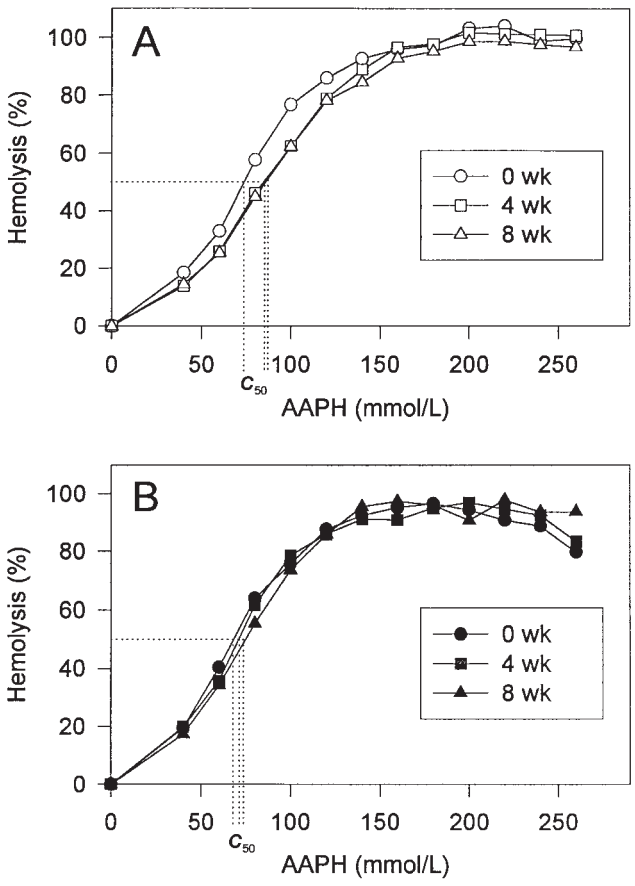

FIGURE 2. Mean hemolysis curves in normotriglyceridemic (A; n = 16) and hypertriglyceridemic (B; n = 12) subjects before and after supplementation with fish oil (3 g n−3 fatty acids/d). Erythrocytes were separated from whole blood at each visit, and intact washed cells were submitted to increasing doses of 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH).

generally occurred at the expense of linoleic (18:2n−6) and arachidonic (20:4n−6) acids. In the NTG subjects, linoleic acid was significantly lower in PC and PE after 4 and 8 wk of fish oil supplementation, whereas arachidonic acid was decreased after 8 wk only. In the HTG subjects, a significant decrease in linoleic acid was observed only in PC after 4 and 8 wk of fish oil supplementation; none of the changes in arachidonic acid in either phospholipid species were significant. As a result of the red cell membrane enrichment in n−3 fatty acids, an increase in the unsaturation index was observed in both groups and in both phospholipid species during the study.

The incorporation of n−3 fatty acids into PC and PE during supplementation is shown in **Figure 3**. In both groups, a rapid rate (initial slope) of EPA and DHA incorporation into PC was observed after 4 wk of fish oil and saturation (plateau) began between weeks 4 and 8 of supplementation. Incorporation of EPA and DHA into PE was progressive and linear, without apparently reaching a plateau, even after 8 wk of supplementation. There was no correlation between the rate of hemolysis and the red blood cell fatty acid composition of NTG or HTG subjects.

Antioxidants

There were no significant changes in erythrocyte α-tocopherol concentrations in either group during this study (**Table 4**). However, α-tocopherol concentrations tended to increase with fish oil intake in the erythrocytes of NTG subjects, the higher mean value being attained after 4 wk. An increase was seen in 12 of 16 participants. Furthermore, changes in erythrocyte α-tocopherol concentrations in the NTG group were strongly and positively correlated with changes in the hemolysis rate after 8 wk of treatment (**Figure 4**). This was observed whether the changes were expressed as absolute or relative values (r = 0.70, P = 0.001 for absolute values as in Figure 4). Such a correlation was not found in the HTG group (r = 0.06, P = 0.85). This suggests that vitamin E was involved in the increased resistance of NTG erythrocytes to hemolysis during fish oil treatment. Even though heterogeneity was observed in the cell vitamin E concentrations of the HTG subjects, the trend for an increase in the α-tocopherol-to-cholesterol ratio in this group suggests that vitamin E may have played a role in preserving the resistance of the red blood cells to hemolysis.

There were no significant changes in erythrocyte glutathione peroxidase activity during the study in either group (Table 4). However, in HTG subjects, the percentage change in glutathione peroxidase activity was positively correlated with the percentage change in hemolysis (r = 0.74, P = 0.01, and r = 0.68, P = 0.05, at 4 and 8 wk, respectively).

TABLE 2
Erythrocyte phosphatidylcholine (PC) and phosphatidylethanolamine (PE) fatty acid composition before and after 4 and 8 wk of supplementation with fish oil (3 g n−3 fatty acids/d) in normotriglyceridemic subjects[1]

	PC			PE		
Fatty acids	Week 0	Week 4	Week 8	Week 0	Week 4	Week 8
	% of total fatty acids			*% of total fatty acids*		
16:0	31.71 ± 0.75	32.47 ± 1.08	32.50 ± 1.11	19.53 ± 1.34	18.88 ± 1.17	18.99 ± 1.00
18:1n−9 + n−7	18.31 ± 0.42	17.55 ± 0.44	18.26 ± 0.37	20.89 ± 0.56	20.91 ± 0.63	20.58 ± 0.48
18:2n−6	17.72 ± 0.55	15.29 ± 0.40[2]	15.22 ± 0.41[2]	6.08 ± 0.33	5.22 ± 0.20[3]	5.08 ± 0.21[2]
20:4n−6	7.52 ± 0.32	7.03 ± 0.21	6.59 ± 0.27[3]	21.63 ± 0.65	20.41 ± 0.63	19.31 ± 0.36[3]
20:5n−3	0.74 ± 0.08	2.61 ± 0.11[2]	2.78 ± 0.20[2]	1.08 ± 0.07	3.18 ± 0.12[2]	4.76 ± 0.18[2]
22:5n−3	0.78 ± 0.05	1.08 ± 0.06[3]	1.17 ± 0.09[2]	3.56 ± 0.27	4.23 ± 0.19	5.04 ± 0.21[2]
22:6n−3	1.62 ± 0.11	2.69 ± 0.15[2]	2.87 ± 0.19[2]	4.41 ± 0.34	5.77 ± 0.35[2]	7.06 ± 0.31[2]
UI	109 ± 2.20	116 ± 2.36[4]	117 ± 3.27[4]	176 ± 5.6	191 ± 5.47[4]	205 ± 4.02[2]

[1] $\bar{x}$ ± SEM; n = 16. UI, unsaturation index = Σ unsaturated fatty acids × number of double bonds.
[2–4] Significantly different from week 0 (one-factor ANOVA for repeated measures followed by Bonferroni's procedure for multiple comparisons): [2] $P < 0.001$, [3] $P < 0.01$, [4] $P < 0.05$.

TABLE 3
Erythrocyte phosphatidylcholine (PC) and phosphatidylethanolamine (PE) fatty acid composition before and after 4 and 8 wk of supplementation with fish oil (3 g n−3 fatty acids/d) in hypertriglyceridemic subjects[1]

	PC			PE		
Fatty acids	Week 0	Week 4	Week 8	Week 0	Week 4	Week 8
	% of total fatty acids			*% of total fatty acids*		
16:0	34.41 ± 0.76	33.24 ± 0.53	34.15 ± 0.87	17.01 ± 0.62	17.85 ± 0.51	16.93 ± 0.49
18:1n−9 + n−7	19.14 ± 0.47	18.91 ± 0.39	19.00 ± 0.34	20.78 ± 1.13	20.21 ± 0.80	20.17 ± 0.56
18:2n−6	17.05 ± 0.43	15.99 ± 0.44[2]	15.62 ± 0.44[2]	6.07 ± 0.23	5.56 ± 0.21	5.60 ± 0.37
20:4n−6	4.88 ± 0.32	4.52 ± 0.27	4.78 ± 0.36	19.51 ± 0.84	18.84 ± 0.44	18.16 ± 0.65
20:5n−3	0.71 ± 0.19	2.55 ± 0.21[3]	2.50 ± 0.15[3]	1.42 ± 0.14	3.05 ± 0.13[3]	4.30 ± 0.26[3]
22:5n−3	0.58 ± 0.09	1.0 ± 0.17	0.90 ± 0.12	4.34 ± 0.26	5.04 ± 0.25[2]	5.90 ± 0.22[4]
22:6n−3	2.17 ± 0.21	3.23 ± 0.26[4]	3.61 ± 0.24[4]	7.57 ± 0.62	8.46 ± 0.60	9.79 ± 0.50[4]
UI	100 ± 2.79	111 ± 2.64[4]	114 ± 4.00[2]	191 ± 4.94	203 ± 5.01	219 ± 5.13[4]

[1] $\bar{x}$ ± SEM, n = 12. UI, unsaturation index = Σ unsaturated fatty acids × number of double bonds.
[2–4] Significantly different from week 0 (one-factor ANOVA for repeated measures followed by Bonferroni's procedure for multiple comparisons): [2] $P < 0.05$, [3] $P < 0.001$, [4] $P < 0.01$.

DISCUSSION

It is widely accepted that, in humans, supplementation with fish oil substantially lowers plasma triacylglycerols and enriches plasma, membranes, and tissues in n−3 fatty acids. The extent to which this occurs depends on the dose and duration of treatment (36, 37). Our finding of a substantial reduction in plasma and VLDL triacylglycerol concentrations after 8 wk of daily supplementation with 6 g fish oil (3 g n−3 fatty acids) is consistent with the results of previous studies in healthy individuals and in hypertriglyceridemic patients (26).

Our study focused on the incorporation of the n−3 fatty acids EPA and DHA into erythrocyte PC and PE, which are the main phospholipids in membranes (35), and on the potential effect of these fatty acids on membrane integrity. The baseline contents of EPA and DHA in red cell membranes in the NTG and HTG groups were consistent with expected values (35). Baseline EPA and DHA contents are likely to reflect circulating plasma lipid concentrations and the metabolic state, which may explain the high DHA concentration observed in the erythrocytes of the HTG subjects, especially in PE (ie, in the inner leaflet), where most n−3 fatty acids are usually incorporated (38). Elevated concentrations of DHA in membranes have also been described in persons with diabetes (39) and in stroke patients (40). In the latter study, the increased proportion of DHA in erythrocytes was associated with a high lipid peroxidation capacity. It is also possible that the baseline EPA and DHA concentrations in PE in the HTG group were the reflection of the patients' diets because these patients are usually encouraged to increase their fish intake. Yet, the high palmitic acid (16:0) concentration observed in their erythrocyte PC does not reflect the low saturated fat component of the usual therapeutic diet.

The daily 6-g dose of fish oil significantly modified membrane erythrocyte fatty acid profiles as expected (12, 41). The significant increase in EPA, DHA, and their intermediate

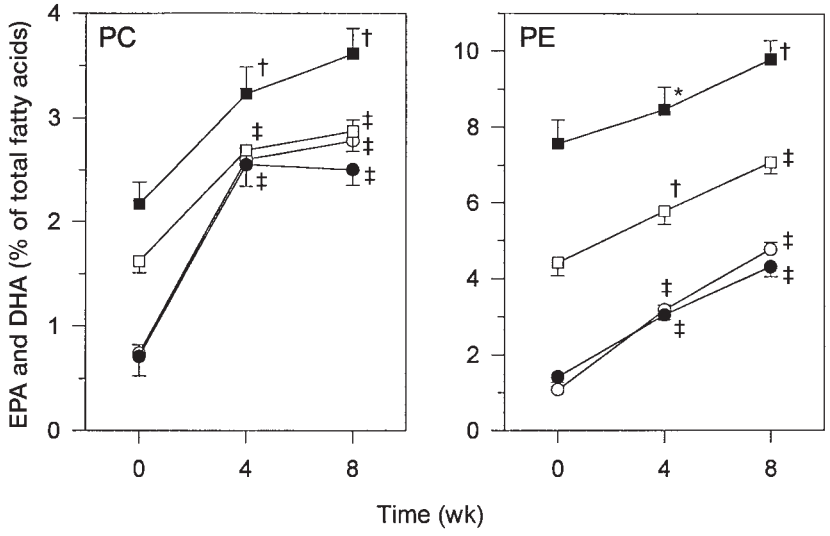

FIGURE 3. Effect of supplementation with fish oil (3 g n−3 fatty acids/d) on mean (±SEM) eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) incorporation into phospholipids of erythrocytes from normotriglyceridemic (NTG) and hypertriglyceridemic (HTG) subjects: ○, EPA-NTG; □, DHA-NTG; ●, EPA-HTG; ■, DHA-HTG. The relative concentrations in EPA and DHA were determined in phosphatidylcholine (PC) and phosphatidylethanolamine (PE) of red blood cells. *,†,‡Significantly different from week 0 (one-factor ANOVA for repeated measures followed by Bonferroni's procedure for multiple comparisons): * $P < 0.05$, † $P < 0.01$, ‡ $P < 0.001$.

TABLE 4
Antioxidant activity in erythrocytes of normotriglyceridemic (NTG) and hypertriglyceridemic (HTG) subjects during supplementation with fish oil (3 g n−3 fatty acids/d)

	α-Tocopherol/cholesterol		GSH-Px	
Week	NTG	HTG	NTG	HTG
	μmol/mmol		*U/g Hb*	
0	0.57 ± 0.06	1.05 ± 0.10	47.38 ± 2.71	44.76 ± 3.61
4	0.68 ± 0.07	1.22 ± 0.16	49.43 ± 2.46	44.72 ± 2.98
8	0.65 ± 0.05	1.18 ± 0.10	47.69 ± 2.90	46.90 ± 3.23

[1] $\bar{x}$ ± SEM, n = 16 NTG and 12 HTG subjects. There were no significant changes over time for either group. GSH-Px, glutathione peroxidase; Hb, hemoglobin.

metabolite docosapentaenoic acid on the one hand and the decrease in linoleic acid on the other hand were observed after 4 and 8 wk in both the NTG and HTG groups. n−3 Fatty acid incorporation into red cell membranes is assumed to come partly from the phospholipid exchange process between plasma and membranes and partly from de novo synthesis as reticulocytes mature into erythrocytes.

The enrichment of the red cell membrane in n−3 fatty acids and the subsequent increase in the total unsaturation index (PC + PE unsaturation index) led to a statistically significant increased resistance of erythrocytes to hemolysis in the NTG group and to a nonsignificant trend in the same direction in the HTG group (Figure 1). The latter group included a smaller number of subjects characterized by a wide range of baseline plasma triacylglycerol concentrations, indicative of various metabolic states possibly accounting for a heterogeneous erythrocyte response to free radical attack. Nevertheless, the absence of significant variation in hemolysis over the supplementation period also suggests that, in this group, the additional n−3 fatty acids did not promote the hemolytic process either. It is likely that the moderate fish oil dose used in this study, which was effective in reducing plasma triacylglycerol concentrations, was low enough to maintain cell integrity. In red cells, the oxidative effect of n−3 fatty acid supplementation has mostly been studied in isolated membranes, leading to conflicting results partly due to the dosages used (18, 36, 42, 43). However, some fish oil studies have provided interesting results on erythrocyte function rather than on membrane structure. In a study conducted in rabbits (23), fish oil prevented hemolysis although it increased lipid peroxidation in the red cell membranes. In the face of this discrepancy, the authors proposed that the more abundant fatty acid substrate in the cell membranes acted as an oxidizable buffer and retarded hemolysis (23). Because the structural integrity of the membrane depends on the biophysical properties of the different phospholipid species, rupturing the membrane would require the alteration of various species. Due to their inherent high oxidizability, n−3 fatty acids may eventually prevent hemolysis by trapping most of the free radicals and reducing the variety of oxidized fatty acids.

We sought to determine whether an altered antioxidant capacity of the cells could explain the hemolysis results in response to fish oil supplementation. We studied vitamin E because it is the main membrane antioxidant and has an early role in blocking the propagation of lipid peroxidation in the bilayer. Baseline vitamin E concentrations in the 2 groups probably reflected the subjects' metabolic states. Vitamin E is transported mainly by VLDL in HTG subjects; thus, the increased number of VLDL particles in this group's plasma results in an increased circulating vitamin E concentration available for incorporation into tissues and cells, including erythrocytes. Fish oil treatment did not trigger any significant changes in the erythrocyte vitamin E content of either group, although there was an apparent (but not significant) mean increase in both groups (Table 4). Yet, in the NTG group, the changes in hemolysis were strongly correlated with the changes in erythrocyte vitamin E concentrations (Figure 4). This suggests that vitamin E contributed to increasing the resistance to hemolysis in the NTG group. In the HTG group, erythrocyte vitamin E concentrations remained high and widely variable during fish oil treatment, even though VLDL, the main carrier of vitamin E in persons with hypertriglyceridemia, was significantly decreased.

The results of studies that used higher and lower doses of n−3 fatty acids than we did are variable (36, 44, 45). Of interest, one study reported that n−3 fatty acids could stimulate vitamin E incorporation into membranes, possibly through the modulation of α-tocopherol binding proteins (45). However, a prooxidant

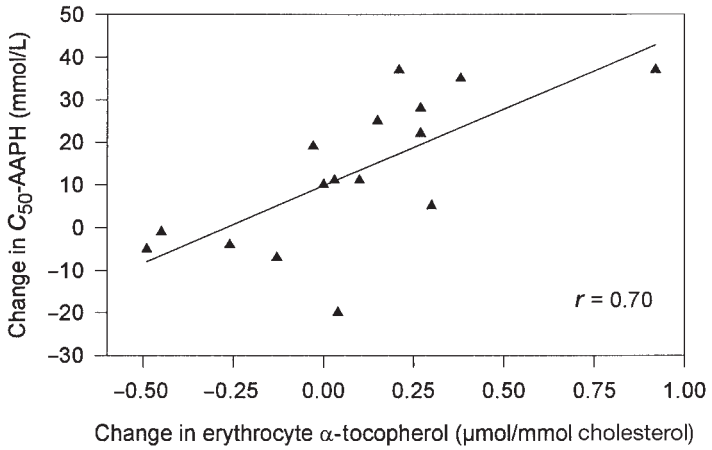

FIGURE 4. Correlation of changes (8 wk − 0 wk) in erythrocyte α-tocopherol (α-tocopherol/cholesterol) with changes in the 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH) concentration corresponding to 50% hemolysis (C_{50}-AAPH) after 8 wk of supplementation with fish oil in normotriglyceridemic subjects.

status has been described in hyperlipidemic subjects, in some cases correlated with plasma triacylglycerol concentrations. For instance, hyperlipidemic subjects were shown to have lower plasma superoxide dismutase activity than control subjects (19) and their mononuclear cells were reported to produce more reactive oxygen species (20). In the HTG group of our study, a prooxidant trend could therefore have counteracted the extent to which n−3 fatty acids stimulated vitamin E incorporation into membranes. This would result in an erythrocyte membrane vitamin E status that was not sufficient to help significantly retard hemolysis, contrary to what occurred in erythrocytes in the NTG group.

Some clinical studies in healthy subjects (46) and in hyperlipidemic patients (47) reported that the incorporation of n−3 fatty acids into erythrocyte membrane phospholipids stimulates glutathione peroxidase activity. We also measured the activity of this enzyme. We found no significant changes in erythrocyte glutathione peroxidase activity, but did observe a trend for an increase in the HTG group (Table 4). In this group, the percentage of change in glutathione peroxidase activity was positively correlated with the percentage of change in hemolysis after both 4 and 8 wk of fish oil treatment. However, the possibility that other antioxidants not measured in the present study were enhanced with fish oil treatment cannot be ruled out. Together with a possible, albeit not generalized, fish oil–induced stimulation of vitamin E incorporation and a trend to higher glutathione peroxidase activity, the n−3 fatty acid enrichment resulted in stable hemolysis rates in the erythrocytes of the HTG group. This confirms that hemolysis is more than a membrane lipid peroxidation process and is likely to depend on the global prooxidant-antioxidant balance of the cell (28).

In summary, a daily 6-g dose of n−3 fatty acids, given for 8 wk, afforded the red blood cells of healthy, normotriglyceridemic subjects some protection against hemolysis. In addition, the triacylglycerol-lowering n−3 fatty acids did not aggravate the hemolytic process in the red cells of subjects with hypertriglyceridemia. We conclude that although n−3 fatty acids are highly susceptible to oxidation, when taken in moderate amounts and incorporated into membranes, these fatty acids do not necessarily impair membrane function. This notion may represent an added benefit in the treatment of patients with hypertriglyceridemia.

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