Supplementation of postmenopausal women with fish oil does not increase overall oxidation of LDL ex vivo compared to dietary oils rich in oleate and linoleate[*]

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Higdon, J.V., Du, S.-H., Lee, Y.S., Wu, T., and Wander, R.C. 2001. Supplementation of postmenopausal women with fish oil does not increase ex vivo oxidation of LDL comparted to dietary oils rich in oleate and linoleate. J. Lipid Research. 42:407-418.

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Abstract:

Although replacement of dietary saturated fat with monounsaturated and polyunsaturated fatty acids (MUFA and PUFA) has been advocated for the reduction of cardiovascular disease risk, diets high in PUFA could increase low density lipoprotein (LDL) susceptibility to oxidation, potentially contributing to the pathology of atherosclerosis. To investigate this possibility, 15 postmenopausal women in a blinded crossover trial consumed 15 g of sunflower oil (SU) providing 12.3 g/day of oleate, safflower oil (SA) providing 10.5 g/day of linoleate, and fish oil (FO) providing 2.0 g/day of eicosapentaenoate (EPA) and 1.4 g/day of docosahexaenoate (DHA). During CuSO4- mediated oxidation, LDL was depleted of ot-tocopherol more rapidly after FO supplementation than after supplementation with SU ($P = 0.0001$) and SA ($P = 0.05$). In LDL phospholipid and cholesteryl ester fractions, loss of n-3 PUFA was greater and loss of n-6 PUFA less after FO supplementation than after SU and SA supplementation ($P < 0.05$ for all), but loss of total PUFA did not differ. The lag phase for phosphatidylcholine hydroperoxide (PCOOH) formation was shorter after FO supplementation than after supplementation with SU ($P = 0.0001$) and SA ($P = 0.006$), whereas the lag phase for cholesteryl linoleate hydroperoxide (CE18:2OOH) formation was shorter after FO supplementation than after SU ($P = 0.03$) but not SA. In contrast, maximal rates of PCOOH and CE18:2OOH formation were lower after FO supplementation than after SA ($P = 0.02$ and 0.0001, respectively) and maximal concentrations of PCOOH and CE18:2OOH were lower after FO supplementation than after SA ($P = 0.03$ and 0.0006, respectively). Taken together, our results suggest that FO supplementation does not increase the overall oxidation of LDL ex vivo, especially when compared with SA supplementation. Consequently, health benefits related to increased fish consumption may not be offset by increased LDL oxidative susceptibility.—Higdon, J. V., S. H. Du, Y. S. Lee, T. Wu, and R. C. Wander. Supplementation of postmenopausal women with fish oil does not increase overall

[*] Abbreviations: CE, cholesteryl esters; CE18:1n-9, cholesteryl oleate; CE18:2n-6, cholesteryl linoleate; CE20:4n-6, cholesteryl arachidonate; CE20:5n-3, cholesteryl eicosapentaenoic; CE22:6n-3, cholesteryl docosahexaenoate; CEOOH, cholesteryl ester hydroperoxide; CE18:1OOH, cholesteryl oleate hydroperoxide, CE18:2OOH, cholesteryl linoleate hydroperoxide; CE20:4OOH, cholesteryl arachidonate hydroperoxide; CE20:5OOH, cholesteryl eicosapentaenoic hydroperoxide; CE22:6OOH, cholesteryl docosahexaenoate hydroperoxide; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acid; FO, fish oil; GC, gas chromatography; HPLC, high performance liquid chromatography; LDL, low density lipoprotein; LSM, least square mean; MUFA, monounsaturated fatty acid; PC, phosphatidylcholine; PI, peroxidation index; PL, phospholipid; PUFA, polyunsaturated fatty acid; n-3 PUFA, omega-3 polyunsaturated fatty acid; n-6 PUFA, omega-6 polyunsaturated fatty acid; SA, safflower oil; SFA, saturated fatty acid; SU, sunflower oil; 18:1n-9, oleate; 18:2n-6, linoleate; 20:4n-6, arachidonate; 20:5n-3, eicosapentaenoate; 22:6n-3, docosahexaenoate.

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Article:

A large body of research supports the hypothesis that the oxidation of low density lipoproteins (LDL) in vessel walls plays a significant role in the development of atherosclerosis (1, 2). For this reason, factors that influence the oxidative susceptibility of LDL have been the subject of a number of investigations. Despite the favorable effects of diets relatively high in unsaturated fat on lipid profiles (3, 4), concern exists that such diets could increase the oxidative susceptibility of LDL, thereby negating some of their cardioprotective effects.

Generally, the more double bonds present in an unsaturated fatty acid (FA), the more readily it is assumed to oxidize in biological systems. These assumptions are based on the results of investigations of the in vitro oxidation of unsaturated FAs in homogeneous systems (5). However, studies of multiphase systems in vitro (6, 7), which appear to have more similarities to LDL than do homogeneous systems, suggest that oxidative susceptibility may not be directly related to the degree of unsaturation of a FA.

Studies that have examined the effect of consuming specific unsaturated FAs on the oxidative susceptibility of LDL have not resulted in clear support of the notion that the higher the degree of unsaturation of dietary polyunsaturated fatty acid (PUFA), the greater the susceptibility of LDL to oxidation. Although a number of studies have demonstrated that diets high in oleate (18:1n-9) result in LDL that are more resistant to ex vivo oxidation than diets high in linoleate (18:2n-6) (8–10), the effects of increased consumption of the n-3 FAs, eicosapentaenoate (EPA; 20:5n-3) and docosahexaenoate (DHA; 22:6n-3), on LDL oxidative susceptibility have been contradictory. Some studies have suggested that increased dietary intake of EPA and DHA increased LDL susceptibility to ex vivo oxidation (11– 14), while others have found that increasing dietary EPA and DHA intake did not increase the oxidative susceptibility of LDL (15–19). The contradictory nature of these studies may be related to the assays of LDL oxidation as well as the criteria chosen to indicate LDL oxidative susceptibility. For example, shortened lag phase during the conjugated diene assay might be the criterion for increased oxidative susceptibility in one investigation, whereas increased rate or extent of oxidation might be used as the criterion in another investigation. Fish oil (FO) supplementation has been found to shorten lag time as well as, paradoxically, to decrease the rate and extent of oxidation in a number of LDL oxidation studies employing the conjugated diene assay (14, 19, 20).

Because LDL oxidation appears to play an important role in the pathobiology of atherosclerosis, it is important to assess the effect of increasing dietary unsaturated fatty acids on the oxidative susceptibility of LDL. These issues are of particular relevance to postmenopausal women, for whom cardiovascular disease is the major cause of mortality in the United States (21). To evaluate the effects of specific dietary unsaturated FAs on LDL oxidative susceptibility, we isolated LDL from the plasma of postmenopausal women taking daily supplements rich in oleate from sunflower oil (SU), linoleate from safflower oil (SA), and EPA and DHA from FO. We evaluated the oxidative susceptibility of LDL by monitoring the loss of specific FAs, the loss of a-tocopherol, and the formation of phosphatidylcholine hydroperoxides (PCOOH) and cholesteryl ester hydroperoxides (CEOOH) during copper- mediated oxidation.

To measure lipid hydroperoxides, we used a highly sensitive and specific high performance liquid chromatography (HPLC) /chemiluminescence technique that has several advantages over the conjugated diene assay often used to evaluate LDL oxidative susceptibility. Although the conjugated diene assay provides a measure of overall LDL oxidation, it is not specific for lipid classes and it cannot be used to monitor the oxidation of specific fatty acids. HPLC/chemiluminescence methods can be used to evaluate LDL surface and core lipid peroxidation by monitoring the formation of PCOOH and CEOOH, respectively, in addition to allowing for the identification of CEOOH derived from specific FAs (22).

SUBJECTS AND METHODS

Experiment design

To assess the effects of each of three oil supplements on individual subjects, a three-period, three-treatment, blinded crossover trial was used. During each treatment period, subjects consumed 15 g/day of high oleate SU, high linoleate SA, or FO rich in EPA and DHA. Each treatment period lasted 5 weeks and was followed by a 7-week washout interval to minimize any carryover effect from the previous treatment. The initial 16 subjects were randomly assigned to one of six treatment sequences, resulting in at least two individuals taking supplements in each of all possible sequences, each period. The crossover trial was also designed to avoid confounding of period and treatment effects by including each treatment in each period. This design allowed for the statistical assessment of carryover effects, described in the statistical analysis section (23). Total time of participation in the study was 29 weeks.

Blood samples were taken on two separate mornings during the 3 days prior to the start of the treatment and on two separate mornings during the last 3 days of the 5-week treatment period. Compliance was assessed by counting leftover capsules and by evaluating changes in specific FA concentrations in plasma FA profiles.

Subjects

Sixteen postmenopausal women, between 50 and 75 years of age, were recruited from the Oregon State University campus and the surrounding community. The criteria for inclusion in the study and characteristics of the subjects have been discussed previously (24). The study protocol was reviewed and approved by the institutional review board at Oregon State University, and written consent was obtained from each subject prior to beginning the study. Because one subject dropped out after the first period for reasons unrelated to the study, only the data obtained from the other 15 subjects are included in the results.

After the subjects were instructed in the technique of recording all foods and beverages consumed over 3 days (2 weekdays and 1 weekend day), they kept 3-day diet records during each of the three treatment periods. The nutritional content of each subject's diet during each treatment period was analyzed with the computer software, Food Processor Plus (Version 6.0; ESHA, Salem, OR).

Supplements

The FO used in the treatment was obtained from the National Institute of Health's Fish Oil Test Material Program in sealed opaque containers, containing 100 1-g capsules. The SU (generously donated by Humpco, Memphis, TN) and the SA (Arista Industries, Darien, CT) were supplied in

bulk and encapsulated (Professional Compounding Pharmacy, Corvallis, OR) after adjusting their antioxidant content to be equivalent to that of FO. Concentrations of α- and γ-tocopherol in the supplemental oils were measured by normal-phase HPLC, using fluorometric detection (excitation λ 292; emission λ 330) based on a standardized method published by the International Union of Pure and Applied Chemistry (25). After the adjustment, the daily dose of 15 g of each oil supplement supplied approximately 18 mg of RRR-α-tocopherol and 19.5 mg of γ-tocopherol or 20 mg α-tocopherol equivalents (α-TE). Neither the *p*-anisidine value (26) nor the peroxide value (27), measures of lipid peroxidation, increased in any of the supplements by the final period of the study. We have previously shown that storing the capsules under conditions similar to those under in which the subjects stored them did not increase peroxidation (19). FA profiles of the supplemental oils were measured by gas chromatography (GC) (28).

Blood collection

Blood samples were collected from an antecubital vein into tubes containing Na2EDTA (1g/l) after an overnight fast of approximately 12 h. Plasma samples were prepared within 1 hour of blood collection by centrifugation (600 g) for 15 min at 4°C, using a TJ-6 desktop centrifuge (Beckman; Palo Alto, CA). Blood samples were kept in the dark and on ice until centrifugation.

Plasma lipid and lipoprotein profiles

Plasma total cholesterol concentrations were determined enzymatically, using a modification of the method of Allain et al. (29). The cholesterol assay met the National Cholesterol Education Program's performance criteria for accuracy. Plasma triacylglycerol (TG) concentrations were measured using a modification of the method of McGowan et al. (30). High density lipoprotein (HDL) cholesterol concentrations were measured enzymatically after precipitation of LDL and very low density lipoprotein (VLDL) fractions with phosphotungstic acid and MgCl2 (31). LDL cholesterol concentrations were calculated using the formula of Friedewald (32).

LDL isolation and preparation

Immediately after plasma was separated from red cells, LDL was isolated by single spin discontinuous density gradient ultra- centrifugation as described by Chung et al. (33). Briefly, plasma density was adjusted to 1.30 g/ml by adding solid KBr and carefully layered under 0.9% saline in a Quick-seal centrifuge tube (Cat. No. 34413, Beckman). Sealed tubes were centrifuged with a near vertical rotor (NVT 65, Beckman) at 60,000 rpm for 120 min in an L5-75 ultracentrifuge (Beckman) at 7°C. Immediately after isolation, samples for LDL composition were capped with argon and frozen at —70°C, whereas samples for LDL oxidation were dialyzed against phosphate-buffered saline (PBS; 0.15 M NaCl, 0.01 M NaH_2PO_4, pH 7.4) in the dark at 4°C to remove EDTA. The PBS was degassed for 10 min with a water aspirator and purged with nitrogen for 5 min prior to use in order to remove oxygen from the solution. The buffer (LDL/PBS; 1:100 v/v) was changed four times during the 20-h dialysis period.

The protein content of LDL was measured using the method of Lowry et al. (34). After dialysis, each LDL sample was diluted to a concentration of 0.5 mg LDL protein/ml and oxidized using 4.69 μM $CuSO_4$ at 37°C. Aliquots removed just prior to the addition of $CuSO_4$ were designated as samples taken at 0 min of oxidation. Aliquots of LDL were removed at predetermined time points during oxidation for measurement of α-tocopherol, CEOOH, PCOOH, and LDL FA

profiles. Oxidation was terminated by adding EDTA at a concentration of 1.5 mg/ml and placing the samples on ice.

LDL composition by major lipid class

LDL total cholesterol (TC) concentrations were measured enzymatically using a modification of the method of Allain et al. (29), whereas LDL TG concentrations were measured using a modification of the method of McGowan et al. (30) (Sigma; St. Louis, MO). LDL free cholesterol (FC) and LDL phospholipid (PL) concentrations were also measured using enzymatic methods (Wako; Richmond, VA). LDL PLs consist of 62–66% phosphatidyl choline, 24– 28% sphingomyelin, 7–7.4% lysophosphatidylcholine, and 2.3% other (2). The enzymatic assay for PL in LDL measured cholinecontaining PLs, accounting for 98% of the PLs in LDL. LDL cholesteryl ester (CE) concentrations were calculated as (TC – FC) X 1.68 (35). Assays for LDL composition were performed at the end of each of the three treatment periods. The interassay coefficient of variation (CV) for TC, TG, FC, and PL between treatment periods were 3.0%, 5.7%, 9.4%, and 3.6%, respectively.

FA profiles of LDL PLs and CEs

The FA profiles of LDL lipids were analyzed at 0 and 360 min of $CuSO_4$-mediated oxidation. LDL lipids were extracted using a modification of the method of Bligh and Dryer (36). Internal standards of diheptadecanoyl phoshatidylcholine (PC17:0) and cholesterol heptadecanoate (CE17:0) were added to each LDL sample prior to extraction. Lipids were separated using 20 X 20 cm Silica-Gel thin-layer chromatography (TLC) plates (Alltech; Deerfield, IL). Separation of the lipid classes was achieved by elution with diethylether-hexane (15:85, v/v). CE and PL bands were identified by comparison with authentic standards applied to the same TLC plate as the samples (37). Bands corresponding to CE and PL fractions were methylated in the presence of heneicosanoic methyl ester (methyl 21:0) as a standard, and analyzed using GC as described previously (28). Losses of individual LDL FAs were calculated by subtracting the concentration at 360 min of oxidation from the concentration at 0 min. The peroxidation index (PI) is an estimate of the concentration of bisallylic hydrogen atoms present in unsaturated FAs, and may therefore be viewed as an index of their susceptibility to oxidation. The PI was calculated as follows: PI = (YPUFA with 2 double bonds X 1) + (YPUFA with 3 double bonds X 2) + (YPUFA with 4 double bonds X 3) + (YPUFA with 5 double bonds X 4) + (YPUFA with 6 double bonds X 5).

LDL α-tocopherol concentration

LDL α-tocopherol concentrations were measured by reverse- phase HPLC with fluorometric detection (excitation X 292 nm, emission X 330 nm), using external standards of pure α-tocopherol (Sigma) of known concentration (38). Recovery of added α-tocopherol to LDL samples averaged 92% and the interassay CV was 5.6%. The α-tocopherol was extracted from LDL and measured immediately after $CuSO_4$ -mediated oxidation was terminated by the addition of EDTA. LDL α-tocopherol concentrations were compared among the three supplement groups before oxidation and after 10 and 20 min of oxidation. Because the rate of α-tocopherol loss was nearly linear in the majority of cases, the rate of α-tocopherol loss was calculated as the slope of the line of best fit between the three time points. The intercept of the same line with the time axis was used to estimate the time of α-tocopherol depletion of LDL during oxidation. LDL α-to-

copherol concentrations were normalized to LDL protein and to LDL lipid content (the sum of the molar concentrations of TC, TG, and PL in LDL).

PC and CE hydroperoxides in LDL

PCOOH and CEOOH were measured in LDL at 0, 20, 40, 60, 90, 120, 180, 240, 300, and 360 min of CuSO4-mediated oxidation using HPLC with postcolumn chemiluminescence detection, based on the methods of Sattler, Mohr, and Stocker (39). These methods have been demonstrated to be highly sensitive and specific for PCOOH and CEOOH (22).

To remove traces of contaminating metals, Chelex 100 resin (Bio-Rad; Richmond, CA) was added to all aqueous buffers. HPLC solvents were stored in dark bottles at 4°C over a 4 Å molecular sieve (~100 g/l, Aldrich; Milwaukee, WI) to deplete them of hydroperoxides. Hexane was washed to remove trace amounts of hydroperoxides (40).

Lipid hydroperoxides of unoxidized and oxidized LDL were extracted into methanol (PCOOH) and washed hexane (CEOOH). To measure PCOOH, the aqueous methanol extract was filtered and injected onto a NH2 column (LC-NH2, 5µ, 250 X 4.6 mm, Supelco; Bellefonte, PA), using methanol/40 mM NaH2PO4 95:5 (v/v) as a mobile phase, at a flow rate of 1.0 ml/ min. A postcolumn chemiluminescence reagent consisting of 1:1 (v/v) methanol/100 mM sodium borate buffer (pH 10) containing 1 mM isoluminol (6-amino-2,3,-dihydro-1,4-phthalazinedione; Sigma) and 3 mg/l microperoxidase (MP-11; Sigma), at a flow rate of 1.5 ml/min, was added to the eluent. The reaction of hydroperoxides and the postcolumn reagent resulted in the generation of light, which was quantified using a chemiluminescence detector (S-3400, Soma Optics, Japan).

The identification standard for PCOOH was prepared by oxidation of 20 mg of soybean PC (Sigma) in 1 ml of methanol and 2 mM 2,2'-azobis(2-methylpropionitrile) (AIBN; Aldrich) at 37°C for 6 h. Fifteen(S)-hydroperoxyeicosatetraenoic acid [15(S)-HPETE; #44720, Cayman Chemicals, Ann Arbor, MI] was used as an external quantification standard. The lower level of detection of PCOOH was approximately 50-pmol/mg LDL protein, and the interassay CV was 8.3%. PCOOH were extracted from LDL immediately after CuSO4-mediated oxidation was terminated by the addition of EDTA. Extracted samples were capped with argon and immediately frozen at —80° C. They were assayed within 4 weeks of collection.

To measure CEOOH, the hexane extract was evaporated under nitrogen and concentrated in ethanol. Twenty to 100 µl were injected onto a C-18 column (Shim-pack CLS-ODS 5 µ, 250 X 4.6 mm, Shimadzu; Columbia, MD) using a mobile phase of methanol-*tert*-butanol 3:1 (v/v) at a flow rate of 1 ml/min. The postcolumn chemiluminescence reagent was the same as that used in the PCOOH assay at a flow rate of 1.5 ml/min. CEOOH concentrations were quantified using 15(S)-HPETE as an external standard. External standards for the identification of the hydroperoxides of CE18:1n-9 (CE18:1OOH), CE18:2n-6 (CE18:2OOH), CE20:4n-6 (CE20:4OOH), CE20:5n-3 (CE20:5OOH), and CE22:6n-3 (CE22:6OOH) were prepared separately by the oxidation of 20 mg of the pure CE (NuChek Prep, Elysian, MN) in 1 ml of toluene and 2 mM AIBN at 37°C for 6 h. After evaporation of the toluene under nitrogen, the oxidized CE was resuspended in 1 ml of hexane and applied to a hexane-preconditioned Al_2O_3 solid-phase extraction column (Supelclean Alumina N; Supelco). The unoxidized CE was eluted with hexane and the CEOOH was eluted with tert-butylmethyl ether. Purified CEOOH standards were

assayed individually using the HPLC chemiluminescence procedure described in the previous paragraph. A combination of all five CEOOH standards was used to evaluate the separation of different species of CEOOH from one another.

The lower level of detection for CE18:2OOH was approximately 10-pmol/mg LDL protein, and the interassay CV was 4.2%. CEOOH were extracted from LDL immediately after $CuSO_4$ -mediated oxidation was terminated by the addition of EDTA. Extracted samples were capped with argon and immediately frozen at —80°C, and were measured within 6 days of collection.

Hydroperoxide concentrations were plotted over time of CuSO4 -mediated oxidation for CEOOH and PCOOH separately. Maximal rate was determined to be the slope of the line of best fit, using the method of least squares, through the points that defined the steepest slope of the curve. The length of the lag phase was determined to be the time (value for x) at the intersection of the line describing the maximal rate and the line describing the initial rate. The maximal concentration was taken to be the maximal concentration measured during 6 h of oxidation, and the time to one-half of the maximal concentration was determined by drawing a perpendicular line from the oxidation curve at one half the value of the maximal concentration (y axis) to the time (x) axis (41).

Statistical analysis

A 7-week washout period was utilized after each treatment period to decrease the likelihood of carryover effects. Balanced random assignment of at least two subjects to all six possible treatment sequences allowed for the statistical assessment of carryover effects prior to inference regarding direct treatment effects. Carryover effects were estimated and direct treatment effects analyzed, utilizing a combination of between- and within-subjects analysis of variance (ANOVA) procedures as described by Kuehl (23). Briefly, the between-subjects sources of variation consisted of 1) sequence of treatment and 2) subjects nested within sequence; the within-subjects sources of variation consisted of 1) period, 2) treatment (direct), and 3) treatment (carryover). If treatment carryover effects were significant ($P < 0.05$), estimates of differences among treatment means could be adjusted for the carryover effects. If carryover effects were not found to be statistically significant, the ANOVA was performed without the treatment (carryover) effect in the model. No significant treatment carryover effects were found for any of the data presented. Therefore, all least square means (LSM) presented represent direct treatment effects. Because no significant period-treatment interactions were encountered, the period-treatment interaction was not included in the final model. If the ANOVA demonstrated a significant treatment effect, P values for the differences between LSM were adjusted for multiple comparisons using Tukey's studentized range test. Results with P values < 0.05 for a two-sided test were considered statistically significant. Analyses were accomplished using the SAS general linear model (GLM) procedure (Version 6.12; SAS Institute Inc., Cary, NC).

RESULTS

Subject characteristics

All subjects were postmenopausal and taking hormone replacement therapy (HRT). Each subject continued her regimen of HRT, without alteration, for the duration of the study. Although the women's HRT regimens were not identical, they were generally equivalent to 0.625 mg conjugated estrogens daily for hysterectomized women and 0.625 mg conjugated estrogens plus 2.5

mg medroxyprogesterone daily for women who had not had hysterectomies. The subjects did not take any chronic prescription medications other than HRT, nor did they take any nutritional supplements other than calcium or vitamin D for the duration of the study. Compliance with the supplement regimen was estimated to be approximately 95%, based on the return of empty supplement containers and leftover supplement capsules. The results of the plasma FA profiles for each subject during each treatment period were also consistent with a high level of compliance.

Mean age of the subjects (Table 1) was 58 years (range 52–73 years). Although the mean body mass index (BMI) of 25.8 was slightly greater than the desirable upper limit of 24.9, it was well under 30, the level at which morbidity and mortality associated with obesity has been found to increase rapidly (42). All subjects were found to be normolipidemic on the basis of their lipid and lipoprotein profiles (43). The mean weight gain for the participants over the 9-month duration of the study was 0.6 kg.

Dietary content of selected nutrients

Not including the supplements, the subjects consumed an average of 29% of their energy as fat (Table 2), with approximately 12% of that energy as saturated fat, 11% as

TABLE 1. Subject characteristics (N = 15)

	Normal	Mean ± SD
Age (years)	—	57.6 ± 5.8
BMI (kg/m^2)	<25	25.8 ± 3.5
Plasma cholesterol (mmol/l)	<6.20	5.23 ± 0.55
LDL cholesterol (mmol/l)	<4.10	2.98 ± 0.61
HDL cholesterol (mmol/l)	>0.90	1.57 ± 0.39
Plasma triacylglycerol (mmol/l)	<2.30	1.49 ± 0.47

°BMI a25 may indicate obesity (42). Normal ranges for blood lipid concentrations are based on the recommendations of the National Cholesterol Education Program (43).

monounsaturated fat, and 6% as polyunsaturated fat. Of the antioxidant nutrients, mean vitamin E intake was 6 mg α-tocopherol equivalents (α-TE)/day, estimated to be 4.8 mg α-tocopherol daily using the formula: mg α-tocopherol in food = mg α-TE X 0.8 (44). Thus, dietary α-tocopherol intake was considerably below the recently revised recommended dietary allowance of 15 mg/day of α-tocopherol. Mean vitamin C (138 mg/day) and selenium (57 pg/day) intake met recommended levels of 75 mg/day and 55 pg/day, respectively (44).

A FA profile of each of the supplements is presented in Table 3. The daily intakes of seven FAs of interest, from the oil supplements alone and in combination with the subjects' diets, are presented in Table 4. By design, each oil supplement supplied concentrations of specific FAs that were high but attainable through dietary manipulation. Fifteen g/day of SU provided 12.3 g/day of oleate, whereas 15 g of SA provided 10.5 g/day of linoleate. The same quantity of FO provided 2.0 g/day of EPA and 1.4 g/day of DHA.

As a result of the oil supplements, the total dietary intake of specific FAs differed significantly among supplement groups. During the SU supplementation, total 18:1n-9 intake was 11.4 g/day higher than during the SA supplementation ($P < 0.0001$) and 9.7 g/day higher than during the FO supplementation ($P < 0.0001$). During the SA supplementation, 18:2n-6 intake was 9.9 g/day higher than during the SU supplementation ($P < 0.0001$) and

TABLE 2. Average daily intake of selected nutrients from three 3-day diet records° (N = 15)

Nutrient	Recommended	Intake
Energy (kJ)	7,950	7,374 ± 1,916
Protein (g)	50	68 ± 19
Carbohydrate (% of energy)	—	55 ± 9
Total fat (% of energy)	≤30	29 ± 6
Saturated fat (% of energy)	<10	12 ± 3
Monounsaturated fat (% of energy)	—	11 ± 3
Polyunsaturated fat (% of energy)	—	6 ± 2
Cholesterol (mg)	<300	177 ± 90
Vitamin E (mg α-tocopherol)	15[b]	5 ± 2
Vitamin C (mg)	75[b]	138 ± 84
Selenium (μg)	55[b]	57 ± 31

[a] Mean ± SD.

[b] Value represents the recommended dietary allowance (RDA) (44).

TABLE 3.Selected FAs supplied by each oil supplement in mg/g of oil°

Fatty Acid	Sunflower Oil	Safflower Oil	Fish Oil
14:0	NDb	1.3	79.1
16:0	32.7	69.3	144.0
18:0	35.3	24.0	26.3
20:0	3.2	3.3	4.3
Y.SFA[c]	84.0	102.0	267.1
16:1n-7	1.3	122.7	89.4
c-18:1n-9	818.0	697.3	50.6
18:1n-7	ND	10.0	24.4
20:1n-9	2.7	2.0	7.7
Y.MUFA[d]	821.3	136.0	187.0
c,c-18:2n-6	38.7	697.3	14.3
18:3n-3	1.3	1.3	9.7
20:4n-6	ND	ND	7.7
20:5n-3	ND	1.3	131.1
22:5n-3	ND	ND	23.4

22:6n-3	ND	ND	96.1
Y.PUFA[e]	39.3	699.3	392.7

° Values for SU and SA are mean values of samples measured in duplicate. Values for FO were provided by the National Institutes of Health Fish Oil Test Materials Program.

b Not detected.

[c]Sum of the saturated fatty acids = 13:0 + 14:0 + 15:0 + 16:0 + 18:0 + 19:0 + 20:0 + 21:0 + 22:0 + 23:0 + 24:0.

[d] Sum of the MUFAs = 16:1n-7 + t-18:1n-9 + c-18:1n-9 + 18:1n-7 + 20:1n-9 + 24:1.

[e]Sum of the PUFAs = t,t-18:2(n-6) + c,c-18:2(n-6) + 18:3(n-3) + 18:4(n-3) + 20:2(n-6) + 20:3(n-6) + 20:3(n-3) + 20:4(n-6) + 20:5(n-3) + 22:5(n-3) + 22:6(n-3).

9.1 g/day higher than during the FO supplementation ($P < 0.0001$). Because the subjects were asked to exclude fish from their diets throughout the study, the FO supplement greatly increased (approximately 50- to 100-fold) the 20:5n-3 and 22:6n-3 content of the diet when compared with the SU and the SA supplements ($P < 0.0001$ for all four comparisons). During the FO supplementation, 20:5n-3 intake was 1.96 and 1.94 g/day higher than during the SU and SA supplementations, respectively, and 22:n-6 intake was 1.45 and 1.94 g/day higher than during the SU and SA supplementations, respectively. Although total intake of 20:4n-6 was relatively low during all three supplementations, it was significantly higher during the FO supplementation ($P < 0.0001$) owing to the higher 20:4n-6 content of the FO.

LDL composition by lipid class

LDL triacylglycerol content after FO supplementation (0.34 ± 0.03 pmol/mg LDL protein) was 31 % lower than after SU supplementation (0.49 ± 0.05; $P = 0.003$), although it did not differ significantly between the SU and SA (0.43 ± 0.03) and the FO and SA supplementation groups. LDL PL content was 5% higher after SA supplementation (1.01 ± 0.02 pmol/mg LDL protein) than after SU supplementation (0.96 ± 0.02; $P = 0.03$) and 6% higher than after FO supplementation (0.95 ± 0.02; $P = 0.02$), whereas LDL PL content did not differ significantly between the SU and FO supplementation groups. LDL CE and FC content did not differ significantly among the supplementation groups (data not shown).

TABLE 4. Consumption of selected FAs derived from oil supplement and total consumption (oil supplement + dietary content) in g/day during each supplement period° (N = 15)

Fatty Acid	Sunflower Oil		Safflower Oil		Fish Oil	
	Oil	Diet + Oil	Oil	Diet + Oil	Oil	Diet + Oil
16:0	0.49°	7.70 ± 0.57[b]	1.04	8.32 ± 1.25	2.16	9.65 ± 1.03
18:0	0.53	3.99 ± 0.33	0.36	3.67 ± 0.54	0.39	3.69 ± 0.47
c-18:1 n-9	12.27	25.74 ± 1.20[1]	1.84	14.35 ± 2.00[2]	0.76	16.03 ±
c,c-18:2 n-	0.58	6.79 ± 0.90[1]	10.46	16.70 ± 1.06[2]	0.22	7.62 ± 1.371
20:4 n-6	NDc	0.05 ± 0.01[1]	ND	0.07 ± 0.01[1]	0.12	0.17 ± 0.01[2]
20:5 n-3	ND	0.01 ± 0.01[1]	0.02	0.03 ± 0.02[1]	1.97	1.97 ± 0.012
22:6 n-3	ND	0.01 ± 0.01[1]	ND	0.03 ± 0.02[1]	1.44	1.46 ± 0.012

° Values for oil represent measurements made in duplicate; calculations based on 15 g of oil/day.

b Values for diet + oil represent LSM ± SEM. Different superscript numbers represent significant differences in total (diet + oil) fatty acid consumption among oil supplement groups (P < 0.05).

c ND = not detected.

PUFA content of LDL PLs prior to and after 6 h of oxidation

At the end of each supplement period, the FA content of LDL PLs prior to oxidation generally reflected the differences in FA consumption (Table 5). After SU supplementation, LDL PLs contained 37 nmol/mg LDL protein more 18:1n-9 than after SA supplementation (P < 0.0001) and 45 nmol/mg LDL protein more 18:1n-9 than after FO supplementation (P < 0.0001). After FO supplementation, LDL PLs contained 45-nmol/mg LDL protein less 18:2n-6 than after SU supplementation (P = 0.01) and 61 nmol/mg LDL protein less 18:2n-6 than after SA supplementation (P < 0.0001). After FO supplementation, LDL PLs contained approximately 52 nmol/mg LDL protein more 20:5n-3 than after SU and SA supplementation, and approximately 33 nmol/mg LDL protein more 22:6n-3 than after SU and SA supplementation (P < 0.0001 for all four comparisons). After FO supplementation, the PI was 40% higher than after SU or SA supplementation (P < 0.0001 for both comparisons).

Losses of specific FAs from LDL PLs after 6 h of $CuSO_4$- mediated oxidation, determined as the difference between the concentration of a given FA prior to oxidation and the concentration after 6 h of oxidation, are presented in Table 5. The loss of 18:1n-9 and 20:4n-6 from LDL PLs did not differ among supplement groups. The loss of 18:2n-6 from LDL PLs after FO supplementation was 36 nmol/mg LDL protein compared with 57 nmol/ mg LDL protein after SA supplementation (P = 0.05) and 56 nmol/mg LDL protein after SU supplementation (P =

TABLE 5. Specific FA concentrations in LDL PLs and CEs (nmol/mg LDL protein) prior to copper-mediated oxidation, after 6 h of oxidation, and the change in concentration after 6 h of oxidation[c] (N = 15)

	Prior			After 6 h of Oxidation			Change After 6 h of Oxidation								
	Sunflower Oil		Safflower Oil		Fish Oil		Sunflower Oil		Safflower Oil		Fish Oil		Sunflower Oil		Safflower Oil		Fish Oil	
LDL PLs																		
18:1n-9	131	± 51	94	± 62	86	± 52	145	± 71	105	± 72	103	± 62	14	± 4	11	± 6	17	± 6
18:2n-6	222	± 20[1]	238	± 14[1]	177	± 13[2]	166	± 15[1]	182	± 12[1]	141	± 13[2]	-56	± 8	-57	± 8[1]	-36	± 7
20:4n-6	95.5	±	93.4	± 9.0[1]	76.6	± 5.7[2]	37.	± 3.8	38.	± 5.8	34.	± 5.0	-55.0	± 5.2	-55.1	± 8.3	-42.3	± 4.4
20:5n-3	5.4	±	4.0	± 0.8[1]	57.5	± 4.0[2]	0.5	± 0.4[1]	0.9	± 0.8[1]	19.	± 2.6[2]	-4.9	± 0.7[1]	-3.1	± 1.1[1]	-38.1	±
22:6n-3	25.8	±	24.2	± 2.2[1]	58.2	± 3.7[2]	7.6	± 1.1[1]	7.9	± 1.3[1]	18.	± 3.5[2]	-18.2	± 2.0[1]	-16.3	± 2.4[1]	-40.0	±
IPUFA[b]	399	± 29	407	± 24	416	± 26	241	± 20	257	± 20	246	± 25	-159	± 16	-150	± 18	-171	± 15
In-6	354	± 26[1]	367	± 21[1]	275	± 19[2]	227	± 18[1]	243	± 18[1]	194	± 19[2]	-127	± 14[1]	-125	± 16[1]	-82	± 92
In-3	45.3	±	39.3	± 3.4[1]	141	± 10[2]	13.	± 1.9[1]	14.	± 2.1[1]	51.	± 7.4[2]	-31.7	± 2.9[1]	-24.7	± 3.2[1]	-89.4	±
PI[d]	774	± 55[1]	761	± 50[1]	1,06	± 64[2]	382	± 33[1]	400	± 39[1]	491	± 57[2]	-392	± 35[1]	-361	± 43[1]	-574	±
CEs	393	± 21[1]	307	± 11[2]	317	± 15[2]	381	± 28[1]	290	± 11[2]	302	± 15[2]	-12	± 9	-17	± 9	-16	± 8
18:1n-9																		

18:2n-6	964	± 61[1]	1,14	± 44[2]	932	± 46[1]	511	± 46[1]	658	± 68[2]	611	± 51[1,2]	-452	±		-490	± 54[1]	-321	±
20:4n-6	142	± 12	146	± 16	131	± 9	33.	± 5.9	44.	± 9.9	48.	± 4.4	-109	± 6		-101	± 15	-82.9	± 8.1
20:5n-3	10.6	±1.2[1]	8.6	± 1.6[1]	134	± 62	0.6	± 0.7[1]	0.7	± 0.6[1]	34.	± 4.7[2]	-10.0	± 1.2[1]		-8.1	± 1.5[1]	-99.3	±
22:6n-3	10.4	±	11.7	± 1.6[1]	26.5	± 2.5[2]	NDe,1		3.5	± 1.6[2]	4.5	± 1.7[2]	-10.4	± 2.3[1]		-8.2	± 2.2[1]	-21.9	±
IPUFA	1,17	± 71[1]	1,34	± 52[2]	1,25	±	556	± 52[1]	714	± 78[2]	714	± 60[2]	-614	± 47		-632	±61.7	-545	± 45
In-6	1,12	± 67[1]	1,30	± 51[2]	1,07	± 50[1]	549	± 51[1]	706	± 78[2]	665	± 55[2]	-579	± 46	[1]	-602	± 62[1]	-407	±
In-3	42.3	±	38.5	± 4.8[1]	187	± 10[2]	7.5	± 1.7[1]	8.5	± 1.8[1]	49.	± 7.1[2]	-34.9	± 4.9[1]		-30.0	± 5.8[1]	-138	±
PI	1,57	± 99[1]	1,75	± 81[1]	2,07	± 80[2]	635	± 62[1]	828	±	955	± 84[2]	-940	± 65		-925	± 88	-119	± 80
	5		4		4				991,2										

° Values are LSM ± SEM. Different superscript numbers represent significant differences among supplement groups (P < 0.05).
b IPUFA represents the sum of all PUFAs measured in LDL PLs or CEs.
c In-6 PUFA and In-3 PUFA represent the sums of all n-6 and n-3 PUFAs, respectively, measured in LDL PLs or CEs.
[d] PI represents the peroxidation index for LDL PLs or CEs.
e ND = not detected.

0.06). The loss of 20:5n-3 from LDL PLs after FO supplementation was 38 nmol/mg LDL protein compared with 5 nmol/mg LDL protein after SU supplementation and 3 nmol/mg LDL protein after SA supplementation (P < 0.0001 for both comparisons). Loss of 22:6n-3 from LDL PLs after FO supplementation was 40 nmol/mg LDL protein compared with 18 nmol/mg LDL protein after SU supplementation (P = 0.0003) and 16 nmol/mg LDL protein after SA supplementation (P < 0.0001). Loss of 20:5n-3 and 22:6n-3 did not differ between the SU and SA supplement groups.

Although the loss of total PUFA from LDL PLs did not differ significantly among supplement groups, loss of n-6 PUFA from LDL PLs after FO supplementation was 82 nmol/mg LDL protein compared with 127 nmol/mg LDL protein after SU supplementation and 125 nmol/mg LDL protein after SA supplementation (P = 0.02 for both comparisons). Loss of n-3 PUFA from LDL PLs after FO supplementation was 89 nmol/mg LDL protein compared with 32 nmol/mg LDL protein after SU supplementation and 25 nmol/mg LDL after SA supplementation (P < 0.0001 for both comparisons). However, the fraction of n-6 PUFA lost after 6 h of oxidation was about one-third for all three supplement groups, whereas the fraction of n-3 PUFA lost was closer to two-thirds for all three supplement groups. At the end of 6 h of oxidation, the PI for LDL PLs after FO supplementation was 491 compared with 382 after SU supplementation (P = 0.01) and 400 for linoleate-enriched LDL PLs (P = 0.04). The PI for LDL PLs decreased by approximately 50% after 6 h of oxidation for each supplement group, resulting in a significantly greater change after FO supplementation than after SU supplementation (P = 0.01) and SA supplementation (P = 0.003).

PUFA content of LDL CEs prior to and after 6 h of oxidation

FA content of LDL CEs prior to oxidation also reflected the FA consumption during the supplement period (Table 5). The 18:1n-9 content of LDL CEs was 86 nmol/mg LDL protein higher after SU supplementation than after SA supplementation (P = 0.001) and 76 nmol/mg LDL protein higher than after FO supplementation (P = 0.004) . After SA supplementation, the

18:2n-6 content of LDL CEs was 183 nmol/mg LDL protein higher than after SU supplementation ($P = 0.009$) and 215 nmol/mg LDL protein higher than after FO supplementation ($P = 0.002$). The 20:4n-6 content of LDL CEs did not differ significantly among the supplement groups. After FO supplementation, the 20:5n-3 content of LDL CEs was approximately 125 nmol/mg LDL protein higher than after SU and SA supplementation ($P < 0.0001$ for both comparisons), whereas 22:6n-3 content was 16 nmol/mg LDL protein higher after SU supplementation ($P = 0.0002$) and 15 nmol/mg LDL protein higher than after SA supplementation ($P = 0.0004$). The PI for LDL CEs was significantly higher after FO supplementation than after SU supplementation ($P = 0.0007$) and SA supplementation ($P = 0.02$).

Losses of specific FAs from LDL CEs after 6 h of oxidation are also presented in Table 5. The loss of 18:2n-6 from LDL CEs after SA supplementation was 490 nmol/ mg LDL protein compared to only 321 nmol/mg LDL protein after FO supplementation ($P = 0.04$). The loss of 20:5n-3 from LDL CEs after FO supplementation was 99 nmol/mg LDL protein compared with 10 nmol/mg LDL protein after SU supplementation and 8 nmol/mg LDL protein after SA supplementation ($P = 0.0001$ for both comparisons). Loss of 22:6n-3 LDL CEs was 22 nmol/mg LDL protein after FO supplementation compared with 10 nmol/mg LDL protein after SU supplementation ($P = 0.02$) and 8 nmol/mg LDL protein after SA supplementation ($P = 0.004$).

Although the loss of total PUFA from LDL CEs did not differ significantly among supplement groups, loss of n-6 PUFA from LDL CEs after FO supplementation was 407 nmol/mg LDL protein compared with 579 nmol/mg LDL protein after SU supplementation ($P = 0.04$) and 602 nmol/mg LDL protein after SA supplementation ($P = 0.02$). Loss of n-3 PUFA from LDL CEs after FO supplementation was 138 nmol/mg LDL protein compared with 35 nmol/mg LDL protein after SU supplementation and 30 nmol/mg LDL protein after SA supplementation ($P < 0.0001$ for both comparisons). As in LDL PLs, the fraction of n-3 PUFA (69–84%) lost after 6 h of oxidation was greater than that of n-6 PUFA (38–52%). At the end of 6 h of oxidation, the PI for LDL CEs decreased by approximately 55% in each supplement group.

LDL α-tocopherol depletion

When normalized to LDL protein (Fig. 1A), initial LDL α-tocopherol concentrations did not differ among supplement groups. After 10 min of $CuSO_4$-mediated oxidation, FO-supplemented LDL contained less than half of the α-tocopherol found in SU- and SA-supplemented LDL ($P < 0.0001$ for both comparisons). After 20 min of oxidation, the mean α-tocopherol concentration in FO-supplemented LDL was 10 times less than that of SU-supplemented LDL ($P = 0.0003$), wheareas SA-supplemented LDL contained less than half that of SU-supplemented LDL ($P = 0.05$). The rate of loss of α-tocopherol (data not shown) during $CuSO_4$-mediated oxidation from SU-supplemented LDL was 32% slower than from SA-supplemented LDL ($P = 0.04$) and 48% slower than from FO-supplemented LDL ($P = 0.002$). The rate of α-tocopherol loss from SA- and FO-supplemented LDL did not differ significantly. The estimated time of α-tocopherol depletion of LDL during oxidation (data not shown) from FO-supplemented LDL was 16 min compared with 26 min for SU-supplemented LDL ($P < 0.0001$) and 20 min for SA-supplemented LDL ($P = 0.05$). The difference between SU- and SA-supplemented LDL was also statistically significant ($P = 0.002$).

Because LDL lipid content differed between supplement groups, α-tocopherol concentrations were also normalized to LDL lipid content (Fig. 1B). When α-tocopherol concentrations were so normalized, differences among supplement groups prior to oxidation remained nonsignificant. After normalization to LDL lipid content, α-tocopherol

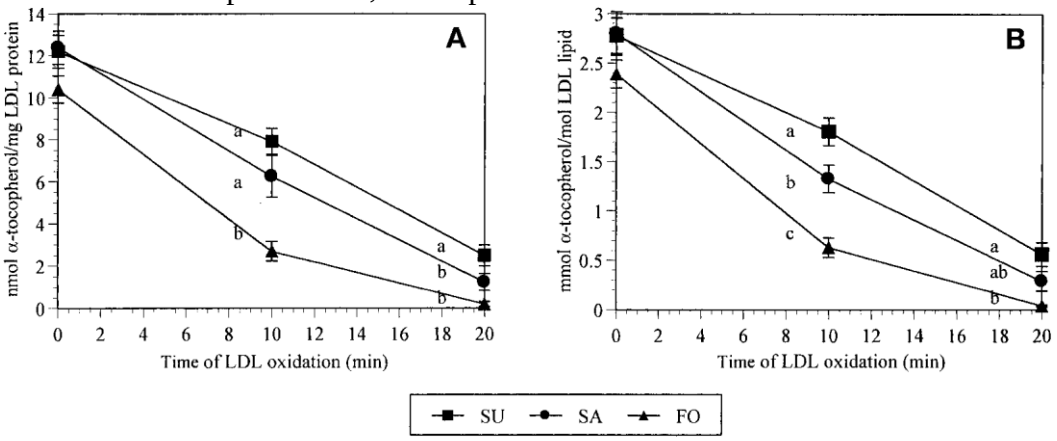

Fig. 1. Loss of a-tocopherol from LDL during copper-mediated oxidation. A: Mean LDL a-tocopherol concentrations at 0, 10, and 20 min of copper-mediated oxidation. Data points represent LDL a-tocopherol concentrations, normalized to LDL protein content, after supplementation of the diets of 15 postmenopausal women with high oleate SU (squares), high linoleate SA (circles), and FO (triangles). Values are LSM ± SEM. Different letters represent significant differences among supplement groups (P < 0.05). B: Mean LDL atocopherol concentrations at 0, 10, and 20 min of copper-mediated oxidation, normalized to LDL total lipid content.

concentrations at 10 min of oxidation were significantly lower in SA-supplemented LDL than SU-supplemented LDL (P = 0.05), and at 20 min of oxidation the difference between SA- and SU-supplemented LDL was no longer statistically significant.

PCOOH formation during LDL oxidation

The formation of lipid hydroperoxides in LDL subjected to CuSO4-mediated oxidation was evaluated using several different measurements: 1) the length of the lag phase, 2) the time to one-half maximal concentration of hydroperoxides, 3) the maximal rate of hydroperoxide formation during the propagation phase, and 4) the maximal concentration of hydroperoxides. The oxidative susceptibility of LDL as determined by measuring the production of PCOOH and CE18:2OOH are presented together in Fig. 2. The duration of the lag phase in the production of PCOOH during CuSO4- mediated oxidation was significantly shorter after FO supplementation than after SU and SA supplementation. Mean lag phase duration after FO supplementation was 27 min less than after SU supplementation (P < 0.0001) and nearly 18 min less than after SA supplementation (P = 0.006). The time required to reach half of the maximal concentration of PCOOH after SU supplementation was 46 min longer than after FO supplementation (P = 0.003). The maximal rate of PCOOH formation during the propagation phase of LDL oxidation was 39% more rapid after SA supplementation than after FO supplementation (P = 0.02). The maximal concentration of PCOOH during $CuSO_4$-mediated LDL oxidation was 28% lower after FO supplementation than after SA supplementation (P = 0.03). Normalization of the maximal

rates and maximal concentrations of PCOOH to LDL PC concentrations did not change the above results.

CE18:2OOH formation during LDL oxidation

Even in LDL enriched with SU or FO, CE18:2n-6 accounted for more than 40% of the CE fraction. During copper-mediated oxidation, CE18:2OOH accounted for more than 80% of the hydroperoxides that could be quantified using the HPLC/chemiluminescence procedure described above. Only CE18:2OOH could be detected in sufficient quantities at all time points to allow for the plotting of kinetic curves. For these reasons, CE18:2OOH concentrations were used to assess the oxidative susceptibility of LDL CEs and are presented in Fig. 2. The duration of the lag phase in the production of CE18:2OOH during CuSO4-mediated oxidation was nearly 18 min longer after SU supplementation than after FO supplementation ($P = 0.03$) but did not differ significantly between SA and FO. The time required to reach half of the maximal concentration of CE18:2OOH was statistically equivalent after all supplements. The maximal rate of CE18:2OOH formation during the propagation phase of LDL oxidation was 31% more rapid after SA supplementation than after SU supplementation ($P = 0.03$) and 76% more rapid than after FO supplementation ($P < 0.0001$). The maximal concentration of CE18:2OOH during CuSO4-mediated LDL oxidation was 30% lower after FO supplementation than after SU supplementation ($P = 0.04$) and 49% lower than after SA supplementation ($P = 0.0006$). Normalization of maximal rates and maximal concentrations of CE18:2OOH to LDL cholesteryl linoleate concentrations eliminated any statistically significant differences among the three supplement groups.

Hydroperoxide formation from CEs of specific FAs

Calculation of the parameters of LDL oxidative susceptibility discussed above using the sum of all CEOOH species measured (CE18:2OOH, CE20:4OOH, CE18:1OOH, and CE22:6OOH) did not result in any significant changes in the results obtained by using only the CE18:2OOH values. The proportions of the maximal concentration of CEOOH, during 6 h of LDL oxidation, contributed by the hydroperoxides of CEs of specific FAs are presented in Table 6. Maximal CE18:1OOH concentrations were 23% higher after SU supplementation than after SA supplementation ($P = 0.03$) and 92% higher than after FO supplementation ($P < 0.0001$), whereas maximal CE18:1OOH concentrations were 56% higher after SA supplementation than after FO supplementation ($P = 0.02$). Maximal CE18:2OOH concentrations are reviewed in the previous section and in Fig. 3B. Maximal CE20:4OOH concentrations were 82% higher after SU supplementation than after FO supplementation ($P = 0.0003$) and 121 % higher after SA supplementation than after FO supplementation ($P < 0.0001$). Maximal CE22:6OOH concentrations were more than 4.5 times higher after FO supplementation than after SU and SA supplementation ($P < 0.0001$ for both comparisons), the results obtained by using only the CE18:2OOH values. The proportions of the maximal concentration of CEOOH, during 6 h of LDL oxidation, contributed by the hydroperoxides of CEs of specific FAs are presented in Table 6. Maximal CE18:1OOH concentrations were 23% higher after SU supplementation than after SA supplementation ($P = 0.03$) and 92% higher than after FO supplementation ($P < 0.0001$), whereas maximal CE18:1OOH concentrations were 56% higher after SA supplementation than after FO supplementation ($P = 0.02$). Maximal CE18:2OOH concentrations are reviewed in the previous section and in Fig. 3B. Maximal CE20:4OOH concentrations were 82% higher after SU supplementation than after FO supplementation ($P = 0.0003$) and 121 % higher after SA sup-

plementation than after FO supplementation ($P < 0.0001$). Maximal CE22:6OOH concentrations were more than 4.5 times higher after FO supplementation than after SU and SA supplementation ($P < 0.0001$ for both comparisons),

Fig. 2. Susceptibility of LDL surface and core lipids to copper-mediated oxidation assessed by the formation of PCOOH and CE18:2OOH. A: The duration of the lag time, B: The time to reach one-half of the maximal concentration of hydro- peroxides, (C) the maximal rate of hydroperoxide formation, and (D) the maximal concentration of hydroperoxides. Bars with different letters over them represent significant differences among groups after 5 weeks of supplementation of the diets of 15 postmenopausal women with 15 g/day of high oleate SU (diagonally striped bars), high linoleate SA (solid bars), and FO (white bars) ($P < 0.05$).

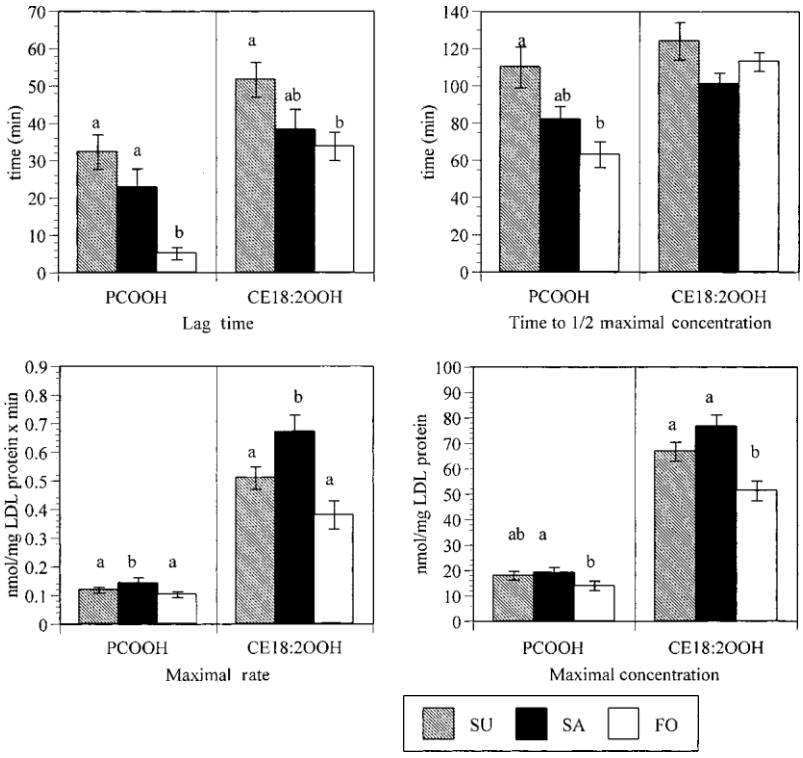

TABLE 6. Maximal concentrations of hydroperoxides of specific CEs (nmol/mg LDL protein) measured during CuSO4-mediated oxidation of LDL after supplementation with oleate-rich SU, linoleate-rich SA, and FO[a] (N = 15)

CEOOH	Sunflower Oil	Safflower Oil	Fish Oil

			nmol/mg LDL protein		
CE18:1OOH	5.8	± 0.3[1]	4.7 ± 0.6[1]	3.0	± 0.6[2]
CE18:2OOH	66.8	± 3.7[1]	76.6 ± 4.6[1]	51.4	± 3.82
CE20:4OOH	4.8	± 0.3[1]	5.8 ± 0.6[1]	2.7	± 0.22
CE22:6OOH	0.7	± 0.2[1]	0.7 ± 0.1[1]	3.8	± 0.32
Sum CEOOH	76.4	± 3.9[1]	86.3 ± 5.4[1]	58.3	± 4.22

a Values are LSM ± SEM. Different superscript numbers represent significant differences among supplement groups (P _- 0.05). though they were still much lower than CE18:2OOH concentrations after all three supplements.

Loss of LDL α-tocopherol during oxidation and the lag phase for hydroperoxide formation
Examination of the relationship between the loss of α-tocopherol and the formation of PCOOH in oxidizing LDL (Fig. 3A) revealed that, on average, significant PCOOH formation occurred before LDL was depleted of α-tocopherol in all three supplement groups. After FO supplementation, LDL was depleted of α-tocopherol more rapidly than after SU supplementation (P = 0.0001) and after SA supplementation (P = 0.05). Unlike SU- and SA-supplemented LDL, the most rapid rate of PCOOH formation in FO-supplemented LDL was observed between 0 and 20 min of oxidation. Once FO-supplemented LDL was depleted of α-tocopherol, the rate of PCOOH formation slowed.

When CE18:2OOH formation was examined with respect to LDL α-tocopherol depletion (Fig. 3B), a distinct lag phase, propagation phase, and plateau could be identified. Unlike PCOOH formation, the propagation phase (maximal rate) for CE18:2OOH formation did not begin until LDL had been depleted ofα-tocopherol in each of the supplement groups. Note that althoughα-tocopherol was lost most rapidly from FO-supplemented LDL, the rate of CE18:2OOH formation in FO-supplemented LDL was slowest.

DISCUSSION
The purpose of the present study was to determine whether increased consumption of FO containing highly

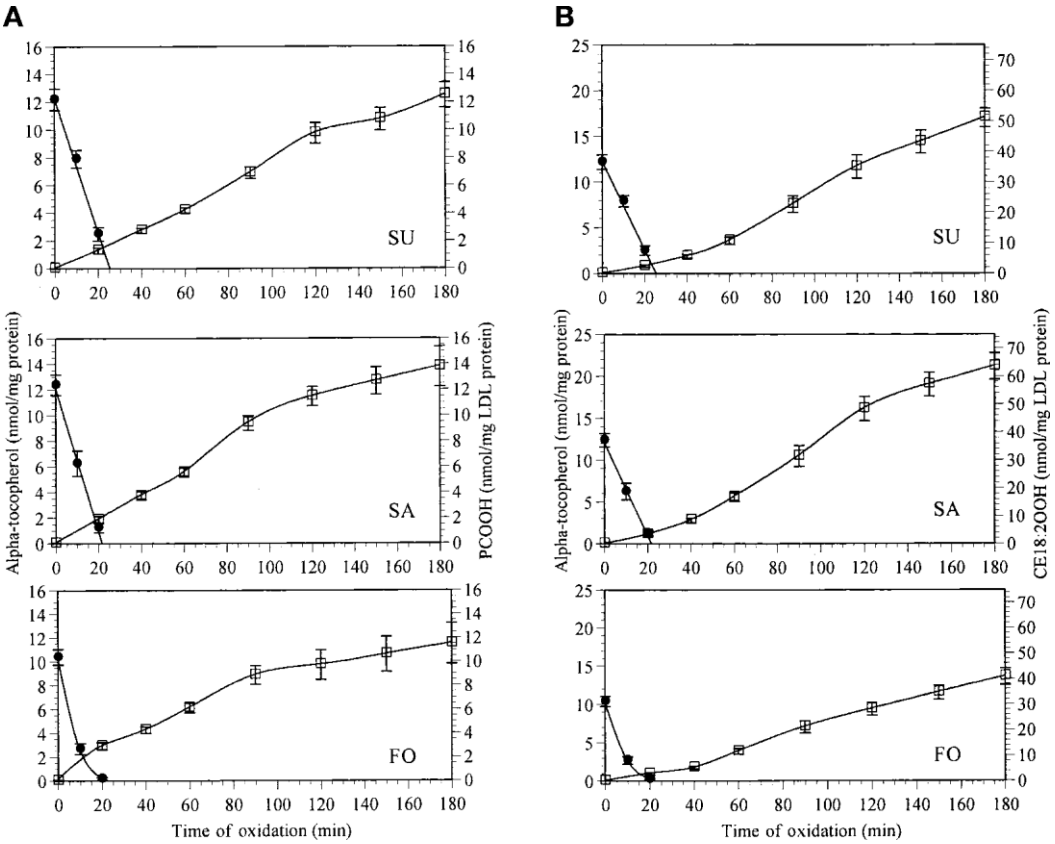

Fig. 3. The relationship between α-tocopherol loss and lipid hydroperoxide formation during copper- mediated LDL oxidation. A: α-Tocopherol concentrations with respect to PCOOH concentrations in oxidizing LDL; values are mean ± SEM. Loss of α-tocopherol is represented by solid circles, and formation of PCOOH is represented by open squares at the end of supplementation of the diets of 15 postmenopausal women with 15 g/day of oil high oleate SU (top graph), high linoleate SA (middle graph), and FO (bottom graph). B: α-Tocopherol concentrations with respect to CE18:2OOH concentrations in oxidizing LDL. Loss of α-tocopherol is represented by solid circles, and formation of CEOOH is represented by open squares at the end of supplementation of the diets of 15 postmenopausal women with 15 g/day of oil high oleate SU (top graph), high linoleate SA (middle graph), and FO (bottom graph).

unsaturated n-3 PUFA increased LDL susceptibility ex vivo oxidation, as compared with the consumption of dietary oils rich in n-6 PUFA (SA) and MUFA (SU) by healthy postmenopausal women. Although the loss of n-6 PUFA from LDL PLs and CEs was lower and the loss of n-3 PUFA greater after FO supplementation than after SU and SA supplementation, the loss of total PUFA did not differ among the three supplement groups. In each supplement group the fraction of n-3 PUFA lost from LDL PLs and CEs was almost twice as high as that of n-6 PUFA, suggesting that n-3 PUFA are more susceptible to oxidation than are n-6 PUFA. However, the extent of oxidation of LDL core and surface lipids, as indicated by the loss of total PUFA from LDL PLs and CEs after 6 h of oxidation, was not significantly greater after FO supplementation.

In our attempt to follow the oxidation of specific CEs in the hydrophobic core of LDL, only CE18:2OOH could be detected in sufficient quantities at all time points to calculate lag time and rate of CEOOH formation. EPA, the n-3 PUFA found in highest concentrations in LDL CE after FO supplementation, could not be accurately measured. As expected, the maximal rate of CE18:2OOH formation was most rapid after SA supplementation, but maximal concentrations of CE18:2OOH were lowest after FO supplementation. The lag phase for the formation of CE18:2OOH was shorter after FO supplementation than after SU supplementation, but did not differ from the lag phase after SA supplementation, suggesting that core lipid oxidation may be initially more rapid in n-3 PUFA-enriched LDL than in MUFA- enriched LDL, but not different than in n-6 PUFA-enriched LDL. Although the assessment of CE18:2OOH formation in the present study did not provide a measure of total lipid peroxidation within the LDL core, the results indicated that FO supplementation did not shorten the lag time or increase the rate of CE18:2OOH formation compared with SA supplementation.

The measurement of total PCOOH formation during LDL oxidation offers a more global assessment of the oxidation of LDL surface lipids than the CEOOH assay did for core lipids. The lag time for PCOOH formation was significantly shorter after FO supplementation than after SU and SA supplementation, suggesting that oxidation of LDL surface lipids was more rapid after FO supplementation than after SU and SA supplementation. However, the maximal rate of PCOOH formation and the maximal concentration of PCOOH were lower after FO supplementation than after SU supplementation. These data could indicate that LDL surface lipids are initially more readily oxidized in LDL supplemented with FO but that the extent of oxidation is not greater in LDL supplemented with FO compared with SA. Although the decreased rate and maximal concentration observed in LDL supplemented with FO could be due to more rapid decomposition of hydroperoxides derived from n-3 PUFA, other mechanisms could also explain these findings.

Studies of aqueous micelles have suggested that the presence of highly unsaturated n-3 PUFA might actually decrease total lipid peroxidation in vitro. Investigations by Yazu et al. (7, 45) demonstrated that the presence of methyl esters of EPA and linoleate in a 1:1 ratio in aqueous micelles reduced the oxidation of total substrate (EPA and linoleate) 5-fold compared with micelles of linoleate alone. Their results indicated that linoleate formed mainly hydroperoxides containing one molecule of O2, whereas EPA formed mainly bicycloendoperoxides containing two molecules of O2. The increased polarity of EPA-derived peroxyl radicals appeared to enhance their diffusion to the micelle surface, increasing the termination reaction rate, decreasing the propagation rate, and ultimately decreasing the rate of total substrate oxidation in the EPA/ linoleate micelles.

The LDL particle consists of an amphipathic surface monolayer and a hydrophobic core, suggesting it may behave more like the biphasic system of the aqueous micelle than like homogeneous solutions of fatty esters. Moreover, a number of FAs with three or more double bonds have been found to form bicyclocendoperoxyl radicals (46). Therefore, in FO-supplemented LDL, the highly unsaturated n-3 FAs might also form more polar radicals, which would be more likely to localize at the surface of LDL particles, resulting in an increased rate of termination and a slower propagation rate.

The loss of LDL α-tocopherol during copper-mediated oxidation was most rapid after FO supplementation, occurring in approximately 16 min rather than 19 and 26 min after SA and SU supplementation, respectively. Examination of the lag time for PCOOH formation and the loss of LDL α-tocopherol during copper-mediated oxidation revealed that the propagation phase of PCOOH formation began prior to the depletion of α-tocopherol from LDL. This phenomenon was most dramatic after FO supplementation, where the rate of PCOOH formation was greatest during the first 20 min of copper-mediated oxidation. With FO supplementation, the rate of PCOOH formation slowed once the LDL particle was depleted of α-tocopherol. These data suggest that increasing the content of highly unsaturated n-3 PUFA in LDL PLs resulted in more rapid consumption of LDL α-tocopherol. The lower rate of PCOOH formation and lower maximal concentration of PCOOH in n-3 PUFA-enriched LDL could be explained by tocopherol-mediated peroxidation (TMP). During TMP, α-tocopherol is the primary lipid peroxidation chain-carrying radical when present in the LDL particle (47). Under relatively mild oxidizing conditions (~5 mol Cu^{2+}/mol LDL in the present study), the rate of lipid hydroperoxide formation is increased when LDL is supplemented with α-tocopherol and decreased when LDL is depleted of α-tocopherol. Thus, using the TMP model, the decreased rate of PCOOH formation and the lower maximal PCOOH concentrations found after FO supplementation could be explained by the relatively rapid depletion of α-tocopherol from LDL enriched with n-3 PUFA.

In the present study, the only parameters suggesting increased oxidative susceptibility in LDL supplemented with FO compared with SA were the shorter lag time in PCOOH formation and the more rapid loss of α-tocopherol. Although these findings likely reflect the increased oxidative susceptibility of n-3 FAs, the finding that hydroperoxide formation and loss of total PUFA were not greater in LDL supplemented with FO compared with SA leaves open the possibility that overall, total oxidation of the LDL particle was not increased by FO supplementation.

A number of studies have shown the consumption of fish to be associated with decreased mortality from cardiovascular disease (48–50). The mechanisms for the observed cardioprotective effects of increased fish consumption are not yet clear. On the basis of the results of this study, the beneficial effects of increased fish consumption do not appear to be significantly offset by an increase in the oxidative susceptibility of LDL. However, these conclusions depend on the assumption that resistance to copper-mediated oxidation ex vivo reflects decreased oxidative susceptibility in vivo. Whether the oxidation of LDL enriched with n-3 PUFA behaves similarly under more physiologically relevant oxidizing conditions remains to be studied.

This work was funded in part by the National Research Institutes Competitive Grants Program/U.S. Department of Agriculture (NRICGP/USDA) #9601081. Tertiary butyl hydroquinone and a- and γ-tocopherol for the oil supplements were generously provided by Eastman Chemical Company (Kingsport, TN). High-oleic acid sunflower oil was generously provided by Humpco (Memphis, TN).

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