Oxidation of plasma proteins is not increased after supplementation with eicosapentaenoic and docosahexaenoic acids

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ABSTRACT

Background: It is generally thought that as the intake of dietary polyunsaturated fatty acids increases, so should that of α-tocopherol, to protect the polyunsaturated fatty acids from increased in vivo peroxidation. However, there are little quantitative data about the concentration of α-tocopherol that is necessary when eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are consumed.

Objective: The purpose of this study was to measure changes produced in 2 indexes of lipid oxidation after supplementation with EPA and DHA from fish oil and 3 doses of RRR-α-tocopheryl acetate in postmenopausal women.

Design: Daily supplements of fish oil providing 2.5 g EPA and 1.8 g DHA and 0, 100, 200, or 400 mg α-tocopheryl acetate were given to 46 postmenopausal women in a 4-treatment, 4-period crossover design.

Results: The supplements increased plasma concentrations of EPA, DHA, and α-tocopherol. The fish-oil supplement increased the plasma concentration of thiobarbituric acid–reactive substances (TBARS) (P = 0.0001) but not that of oxidatively modified protein, as indicated by the carbonyl content. The α-tocopheryl acetate and fish-oil supplements had no significant effect on plasma concentrations of TBARS or oxidized protein.

Conclusions: Although these data show a small but statistically significant increase in oxidative stress on the basis of plasma TBARS concentrations after the consumption of EPA and DHA, the clinical relevance of this change is questionable. In addition, as supplements of α-tocopheryl acetate were added to the diet, neither the plasma TBARS concentration nor the protein oxidation changed. Consequently, the results of this study indicate that there is no basis for vitamin E supplementation after consumption of EPA and DHA.


KEY WORDS Postmenopausal women, thiobarbituric acid–reactive substances, TBARS, fish oil, lipid oxidation, protein oxidation, vitamin E

INTRODUCTION

Free radicals in biological systems have been implicated in carcinogenesis, inflammation, atherosclerosis, and numerous other diseases and disorders (1). In pure chemical systems, the molecules most susceptible to free radical attack are polyunsaturated fatty acids (PUFAs) and, in general, oxidizability increases as the number of double bonds increases (2–4). In vivo, the same relative oxidative susceptibility is thought to occur. That is, of the various biologically relevant PUFAs, long-chain n–3 fatty acids, such as eicosapentaenoic acid (20:5n–3; EPA) and docosahexaenoic acid (22:6n–3; DHA), oxidize more readily than do less saturated fatty acids, such as linoleic acid (18:2n–6). To prevent in vivo oxidation of PUFAs, when linoleic acid is the primary dietary PUFA, it is recommended that for each gram of PUFA consumed, 0.4 mg RRR-α tocopheryl acetate is needed (5). However, limited information is available to suggest how this quantity should be adjusted if the concentration of the more unsaturated EPA and DHA in the diet is increased. Muggli (6) estimated, using the vitamin E requirements of rats determined by Witting et al (7), that 1.5 and 1.8 mg vitamin E per gram of EPA and DHA, respectively, should be added to the human diet.

Because in vivo oxidation may contribute to the development of atherogenesis, the dietary ratio of vitamin E to PUFA in older

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women is of particular interest. Despite the fact that coronary heart disease (CHD) is a major cause of death in this population (9), little research of CHD risk factors in older women has been conducted (10); most studies have involved middle-aged men. Numerous studies showed that data from men of this age cannot necessarily be extrapolated to women of this age (11–13).

Consequently, the purpose of this study was to measure in vivo oxidation in the plasma of postmenopausal women after the consumption of highly unsaturated fatty acids from fish oil and to determine whether the amount produced would be the consumption of highly unsaturated fatty acids from fish vivo oxidation in the plasma of postmenopausal women after

necessarily be extrapolated to women of this age (11–13).

Experimental design

A double-blind, 4-treatment, 4-period crossover trial was used. Each of the 5-wk treatment periods was followed by a 4-wk washout interval. The total time that a subject was involved in the supplementation phase of the study was 8 mo. During each treatment period, the subjects were given 15 g fish oil and 0, 100, 200, or 400 mg RRR-α-tocopheryl acetate/d. The α-tocopheryl acetate was given in a balanced design that was described previously (14).

Subjects

Forty-eight healthy, nonsmoking, postmenopausal white and Asian women aged 45–75 y participated in the study. Two subjects withdrew because of ill health. Women were considered postmenopausal if they either had not menstruated in the preceding year or had used hormone replacement therapy (HRT) for a comparable length of time. Initial blood chemistry and hematologic profiles were normal and were given in detail previously (14). The only nutritional supplement allowed during the study, other than the study products (fish oil and α-tocopheryl acetate), was calcium. Women who did not consume dairy products were allowed to take a calcium supplement; however, total calcium intake was not to exceed 1200 mg/d. Twenty-four of the women were not receiving HRT. Twenty-two of the women were receiving HRT, primarily 0.625 mg conjugated estrogens and 10 mg medroxyprogesterone daily. Compliance with the study protocol was monitored by counting returned pills, interacting frequently with the subjects, and measuring plasma fatty acid concentrations. The nutritional contents of the subjects’ habitual diets were determined from 3-d diet records by using FOOD PROCESSOR PLUS (version 5.03; ESHA, Salem, OR) and were reported previously (15). The study was approved by the Oregon State University Institutional Review Board and informed consent was obtained from all subjects.

Supplements

The fish oil was obtained from the National Institutes of Health’s Fish Oil Test Material Program and was described previously (15). The fish oil contained no added vitamin E and the effects of HRT and different intakes of α-tocopheryl acetate concurrent with fish oil were evaluated. α-Tocopheryl acetate was given in 4 different sequence patterns (0, 100, 200, and 400 mg; 100, 400, 0, and 200 mg; 200, 0, 400, and 100 mg; and 400, 200, 100, and 0 mg); the numbers of women receiving and not receiving HRT were equally distributed between the 4 sequence-pattern groups. Conounding of treatment and period was avoided by including each treatment in each period. The use of a crossover design prevented between-subject variation in the experimental error because each subject was represented in each treatment. Possible carryover effects between treatments were minimized by the use of a 4-wk washout period. The lack of a carryover effect for α-tocopheryl acetate was indicated by the fact that after the 4-wk washout interval, the plasma concentrations of α-tocopherol at the beginning of the second, third, and fourth periods were equivalent to concentrations measured at the beginning of the first period (baseline), the fatty acid profile of plasma was determined as discussed previously (18) by using heptadecanoic acid as an internal standard. The α- and γ-tocopherol contents of plasma were measured simultaneously by HPLC (Shimadzu, Columbia, MD) with a fluorometric detector (14) and normalized to the lipid content of the plasma. The sum of the concentration of total cholesterol and triacylglycerols was used as an estimate of the total lipid content of plasma (19). Cholesterol was determined enzymatically by using a modification of the method of Allain et al (20). Triacylglycerols were measured by using a modification of the method of McGowan et al (21). Plasma TBARS were measured by using the method of Yagi (22), as reported previously (18). Protein oxidation was determined by measuring the carbonyl groups on the amino acids of plasma proteins (23). With this method, the carbonyl groups are reacted with 2,4-dinitrophenylhydrazine to form 2,4-dinitrophenylhydrazone derivatives, which can be measured spectrophotometrically. The excess reagent is removed by gel filtration with use of HPLC.

Statistical analyses

Data are expressed as least-squares means ± SEMs, unless otherwise indicated. In this 4-treatment, 4-period, double-blind crossover trial, the effect of supplementation with fish oil without additional vitamin E and the effects of HRT and different intakes of α-tocopheryl acetate concurrent with fish oil were evaluated. α-Tocopheryl acetate was given in 4 different sequence patterns (0, 100, 200, and 400 mg; 100, 400, 0, and 200 mg; 200, 0, 400, and 100 mg; and 400, 200, 100, and 0 mg); the numbers of women receiving and not receiving HRT were equally distributed between the 4 sequence-pattern groups. Co-
regardless of the dose during the supplementation interval. A possible carryover effect for fatty acids was evaluated by comparing plasma concentrations at the beginning of the second, third, and fourth periods with baseline values. There was a small carryover effect for EPA, DPA, and DHA. However, this effect was too small to influence the concentrations of these fatty acids after fish-oil supplementation. There was no carryover effect for the dependent variables TBARS and protein oxidation.

Measurements made at the beginning of all 4 treatment periods (before supplementation) in subjects grouped by the dose of α-tocopheryl acetate that they were to receive were compared to determine whether the groups were statistically equivalent initially with respect to the value being assessed. Measurements made at the end of each of the 5-wk supplementation periods, at which time 25% of the subjects had received each of the 4 doses of α-tocopheryl acetate, were used to evaluate the effects of supplementation with α-tocopheryl acetate concurrently with fish oil and HRT. Measurements made when the subjects were not taking supplements of α-tocopheryl acetate were used to determine the effect of fish oil.

Variables were checked to determine whether the assumptions of normal distribution and homogeneity of variances were obeyed (24). For α- and γ-tocopherol concentrations in plasma, where the assumptions were not obeyed, data were log transformed for statistical analysis but are reported as untransformed data.

A Student’s t test was used to determine the effect of fish oil on the values measured when the subjects received no α-tocopheryl acetate supplement (25). Significant effects of HRT and dose of α-tocopheryl acetate were evaluated by using standard analysis for a 4-period, 4-treatment crossover trial (26). The statistical model included dose, HRT status, treatment period, nesting of subjects by HRT status, and the interaction between HRT status and dose. No interactions were observed. Consequently, this interaction term was removed from the statistical model. There was no effect of HRT status on any of the variables, except for small differences in the fatty acid profile, which were reported previously (14). When a dose effect was significant, comparisons between adjacent means were made by using Fisher’s least-significant-difference test as a multiple-comparison procedure with Bonferroni-corrected P values for comparison-wise error rates (24, 25). The effect of age on protein oxidation was evaluated by using analysis of covariance with age as the covariant (24). Statistical analyses were done by using SAS (version 6.12; SAS Institute, Cary, NC).

RESULTS

The characteristics of the subjects at baseline are given in Table 1. Overall, compliance was excellent, as indicated by the almost 30% drop in plasma triacylglycerol concentrations from 1.193 ± 0.077 to 0.842 ± 0.067 mmol/L, the increase in plasma EPA from 0.110 ± 0.001 to 0.734 ± 0.003 mmol/L, and the increase in DHA from 0.283 ± 0.001 to 0.515 ± 0.001 mmol/L (Table 2). The subjects consistently returned their empty supplement containers.

According to the 3-d diet records, the subjects maintained their habitual intake of macronutrients throughout the study (Figure 1). The average intake of macronutrients fell within the recommendations of the Dietary Guidelines for Americans (27). Of the fat present in the diet, 9.2 ± 0.5% was SFAs and 6.5 ± 0.3% was PUFAs. The average dietary intakes of linolenic acid, EPA, and DHA were 0.8 ± 0.06, 0.1 ± 0.01, and 0.1 ± 0.03 g/d, respectively. The fish-oil supplement provided an additional 4.8 g long-chain n-3 PUFAs, 2.46 g EPA, 0.47 g DPA, and 1.80 g DHA daily. The average cholesterol intake was 177 ± 13 mg/d. The diets met or exceeded the recommended dietary allowance (RDA; 5) or DRI (28, 29) for every micronutrient for this age group, except for zinc. The intake of zinc was 9.3 ± 0.5 mg/d and the RDA is 12 mg/d.

As expected, the fish-oil supplement dramatically changed the plasma fatty acid profile (Table 2). There was no effect of α-tocopheryl acetate supplementation on the concentration of plasma fatty acids. Hence, a mean was calculated for each woman by using the values measured at all 4 doses of vitamin E. This value was used to calculate the mean and SE for each fatty acid. The linoleic acid concentration decreased by 19%, whereas those of EPA and DHA increased ≈7-fold and ≈2-fold, respectively.

The concentration of α- and γ-tocopherol in plasma was expressed relative to the total lipid content of plasma. The values before supplementation differed slightly among the women, ranging from 0.283 ± 0.110 to 0.734 ± 0.062 μmol/mmol lipid for α-tocopherol and 0.71 ± 0.04 to 0.84 ± 0.04 μmol/mmol lipid for γ-tocopherol. These differences, however, were not a result of dietary intervention but reflected minor random differences that occurred in the subjects during the course of the study. There was no effect of fish oil on the concentration of α- or γ-tocopherol. The α-tocopherol acetate supplement increased the plasma concentration of α-tocopherol but decreased that of γ-tocopherol.

### Table 1

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
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<tr>
<td>Age (y)</td>
<td>58.4 ± 8.6</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>65.5 ± 8.8</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>24.6 ± 2.7</td>
</tr>
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$^1\overline{x} ± SD.$

### Table 2

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Before supplementation</th>
<th>After supplementation</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0</td>
<td>2.594 ± 0.014</td>
<td>2.316 ± 0.004</td>
</tr>
<tr>
<td>16:1</td>
<td>0.253 ± 0.001</td>
<td>0.207 ± 0.001</td>
</tr>
<tr>
<td>18:0</td>
<td>0.824 ± 0.004</td>
<td>0.796 ± 0.003</td>
</tr>
<tr>
<td>18:1n−9</td>
<td>2.080 ± 0.011</td>
<td>1.493 ± 0.004</td>
</tr>
<tr>
<td>18:1n−7</td>
<td>0.231 ± 0.002</td>
<td>0.193 ± 0.001</td>
</tr>
<tr>
<td>20:2n−6</td>
<td>3.589 ± 0.033</td>
<td>2.911 ± 0.011</td>
</tr>
<tr>
<td>20:3n−3</td>
<td>0.080 ± 0.001</td>
<td>0.066 ± 0.001</td>
</tr>
<tr>
<td>20:3n−6</td>
<td>0.172 ± 0.001</td>
<td>0.089 ± 0.001</td>
</tr>
<tr>
<td>20:4n−6</td>
<td>0.789 ± 0.007</td>
<td>0.636 ± 0.003</td>
</tr>
<tr>
<td>20:5n−3</td>
<td>0.110 ± 0.001</td>
<td>0.734 ± 0.003</td>
</tr>
<tr>
<td>22:5n−3</td>
<td>0.062 ± 0.000</td>
<td>0.115 ± 0.001</td>
</tr>
<tr>
<td>22:6n−3</td>
<td>0.283 ± 0.001</td>
<td>0.515 ± 0.001</td>
</tr>
</tbody>
</table>

$^1\overline{x} ± SE; n = 46$. There was no effect of supplementation with α-tocopheryl acetate on the concentration of plasma fatty acids. Hence, a mean was calculated for each woman by using the values measured at all 4 doses (0, 100, 200, and 400 mg) of α-tocopheryl acetate. This value was used to calculate the mean and SE for each fatty acid.

$^2P < 0.0001$, $^3P < 0.05$. 

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PROTEIN OXIDATION IS NOT INFLUENCED BY EPA OR DHA
(overall P = 0.0001 for both). When no supplement of α-tocopherol acetate was given, the plasma α-tocopherol concentration was 4.15 ± 0.24 μmol/mmol. The plasma α-tocopherol concentration increased to 6.13 ± 0.24 μmol/mmol lipid, an increase of almost 50% (P = 0.0003), after supplementation with 100 mg α-tocopherol acetate. However, the plasma α-tocopherol concentration after supplementation with 200 mg α-tocopherol acetate (6.51 ± 0.24 μmol/mmol lipid) was not significantly different from that after supplementation with 100 mg α-tocopherol acetate. Furthermore, the plasma α-tocopherol concentration after the 400-mg α-tocopherol acetate supplement (6.91 ± 0.24 μmol/mmol lipid) was not significantly different from the concentration measured after the 200-mg supplement.

The consumption of the fish-oil supplement significantly increased lipid peroxidation, as indicated by the plasma concentration of TBARS (P = 0.0001) (Figure 2), but not that of protein oxidation (Figure 3) from the samples obtained when no supplement of α-tocopherol acetate was consumed. The concentration of plasma TBARS went from 1.31 ± 0.03 to 1.52 ± 0.26 nmol/L, an increase of 16%. Protein oxidation was 2.21 ± 0.10 nmol carbonyl/mg plasma protein before supplementation and 2.28 ± 0.08 nmol carbonyl/mg plasma protein after supplementation. There was no significant effect of age on protein oxidation (data not shown) and no significant effect of α-tocopherol acetate supplementation on plasma TBARS or the amount of protein carbonyls (Figures 2 and 3, respectively).

**DISCUSSION**

Current understanding of in vivo oxidation is based on the mechanism proposed by classical auto-oxidation kinetics of purified fatty acids in model systems. In these systems, lipid peroxidation is a free radical process consisting of 3 stages: initiation, propagation, and termination. Initiation occurs by the attack of a species capable of abstracting hydrogen atoms from the fatty acid side chain, producing a carbon-centered radical (L·). This radical then undergoes molecular rearrangement to form a conjugated diene that, in turn, reacts with oxygen to give a conjugated diene peroxy radical (LO2·), which propagates the cycle by abstracting a hydrogen atom from another lipid mole-
cule to make a new carbon-centered radical and LOH. Once initiated, lipid peroxidation accelerates in an autocatalytic manner until radical termination reactions occur. The rate of the propagation reaction is governed by the various carbon-hydrogen bond dissociation energies: 314–335 kJ/(75–80 kcal)/mol for a bisallylic hydrogen, 368 kJ/(88 kcal)/mol for a monoaallylic hydrogen, and 423 kJ/(101 kcal)/mol for an alkyl hydrogen (30).

More than 50 y ago, Holman and Elmer (4) compared relative rates of oxidation of methyl esters of several fatty acids in pure chemical systems by measuring oxygen loss. They found that arachidonate (20:4n−6) was more reactive than was α-linolenate (18:3n−3), which in turn was more reactive than was linoleate. Since then, similar observations have been made using homogeneous solutions of purified methyl esters of fatty acids in chlorobenzene and thermally labile azo compounds to control the rate of initiation (31), a highly specific measure of secondary oxidation products (2), and more physiologically relevant systems, such as immortal cell lines (32–35).

Yazu et al (36), on the other hand, reported that in aqueous, micellar dispersions composed of methyl esters of either EPA or linoleate, the oxidizability of the methyl ester of EPA was lower than that of methyl linoleate. In addition, in a mixed micelle system containing both EPA and linoleate, the oxidizability of linoleate decreased as the amount of EPA in the micelle increased. The EPA micelle had ≥2 molecules of oxygen in the peroxy radical, whereas the linoleate micelle had only 1. Thus, these researchers reasoned that the EPA micelle was more polar. They found, using antioxidants that preferentially locate in different parts of the micelle, that the more polar radical migrated from the lipophilic core of the micelle to the polar surface (37). With this migration, an environment was created that favored the termination and reduced the propagation reactions. Other studies also indicated less oxidation in model systems enriched with EPA and DHA (38–41).

**FIGURE 1.** Least-squares mean (±SEM) intake of macronutrients in postmenopausal women during each of the 4 treatment periods (fish oil and 0, 100, 200, or 400 mg RRR-α-tocopheryl acetate). There were no significant differences between the periods. n = 46.

**FIGURE 2.** Least-squares mean (±SEM) concentrations of thiobarbituric acid–reactive substances (TBARS) in the plasma of postmenopausal women before and after supplementation with fish oil and 0, 100, 200, or 400 mg RRR-α-tocopheryl acetate. The numbers of subjects in each group were 45, 43, 44, and 43 before supplementation and 44, 42, 44, and 40 after supplementation, respectively. Bars with different superscript letters represent values that are significantly different, P ≤ 0.05. The values measured after supplementation with both fish oil and α-tocopherol acetate were compared by using a 4-period, 4-treatment crossover design; there were no significant differences.
Contradictory results about the effect of the consumption of EPA and DHA on oxidation were also obtained in vivo. After rats were fed EPA- and DHA-rich diets, increased concentrations of malondialdehyde (MDA), measured by HPLC as TBARS or more specifically by the adduct that MDA forms with TBA, were found in liver and heart tissue (42–44). Meydani et al (45) found increased plasma TBARS and Piche et al (46) reported increased concentrations of the MDA-TBA adduct in plasma from humans given supplements of fish oil. On the other hand, many studies showed that increased consumption of long-chain n–3 fatty acids did not change the production of lipid peroxides. Red blood cells obtained from rats given 360 mg · kg body wt \(^{-1} \cdot \text{d}^{-1}\) (equivalent to \(\approx 50\) mg in a 60-kg human) of long-chain n–3 fatty acids were not more susceptible to oxidative stress than were those obtained from rats given oleic acid (47). In a previous study (15), we gave postmenopausal women 15-g supplements of EPA and DHA-rich fish oil for 5 wk. Although the concentration of TBARS in the urine was higher after the consumption of the EPA- and DHA-rich fish oil, there was no significant difference in the concentration of the MDA-TBA adduct. In addition, supplements of 100–400 mg \(\alpha\)-tocopheryl acetate/d linearly decreased urinary TBARS concentrations but had no effect on the urinary adduct. Hansen et al (48) fed healthy male volunteers meals that contained 4-g capsules of purified esters of EPA or DHA. Neither changed the plasma concentration of TBARS. There are numerous reasons studies have produced conflicting results about the effect of the consumption of n–3 fatty acids on the in vivo production of lipid peroxides. Paramount among them is the fact that different assays were used to measure the extent of oxidation. Although all of the assays are generally considered to measure lipid peroxides, numerous other products, such as MDA, are often measured and used as a surrogate for lipid peroxides. One of the assays most frequently used to measure lipid peroxidation is the TBARS, or TBA, test. Its popularity stems more from the simplicity of its execution rather than its power for measuring oxidation. The basis for this test is the reaction of MDA, one of several low-molecular-weight end products formed from the decomposition of certain lipid peroxidation products, with TBA. The reaction occurs at a low pH and when there is an elevated temperature, generating an MDA-TBA adduct. There are numerous problems with the assay. MDA is formed in low yields and only certain lipid peroxidation reactions yield it. It reacts with molecules other than TBA and TBA reacts with compounds other than MDA. The test response is influenced profoundly by the reaction conditions. The TBARS test requires corroboration with other indexes of oxidation to be valid and its clinical relevance is questionable.

One assay that might have particular significance in assessing the extent of oxidative damage is the measure of protein oxidation. Damage to proteins may affect the function of such important molecules as receptors and enzymes, as well as contribute to the damage of other biomolecules, so the functional consequences of protein damage are significant. A particularly useful test of oxidative protein damage is the protein carbonyl assay because it evaluates “general” protein damage as opposed to looking at the damage of specific amino acids (49). Limited use has been made of this assay to assess the oxidative damage induced by the consumption of long-chain n–3 fatty acids. Yasuda et al (50) found that, although mice fed DHA-rich fish oil had significantly higher concentrations of TBARS in the liver than did mice fed beef tallow, when fed linoleic acid–rich safflower or \(\alpha\)-linolenic acid–rich perilla oil, the protein carbonyl content in the liver was similar among the 4 dietary groups. This study represented the first use of this assay to measure oxidative stress produced by the consumption of long-chain n–3 fatty acids and vitamin E supplements in humans. Although the plasma concentration of TBARS was modestly increased by the consumption of fish oil, it was not modified by consumption of the vitamin E supplement. As with the study by Yasuda et al (50) in mice, we observed no increase in protein carbonyls after the consumption of fish oil. If fish-oil consumption does not cause an increase in oxidation as measured by protein carbonsyls, then an increased intake of vitamin E is not necessary. Consequently, the results of this study give credence to the argument that EPA- and DHA-rich diets may not lead in vivo to increased oxidation. However, this point must be validated by using diets with different amounts of EPA and DHA and by measuring oxidative stress with several different techniques.

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REFERENCES


