Pro-Oxidant Effect of Vitamin E in Cigarette Smokers Consuming a High Polyunsaturated Fat Diet

Richard B. Weinberg, Barbara S. VanderWerken, Rachel A. Anderson, Jane E. Stegner, Michael J. Thomas

Abstract—Dietary polyunsaturated fats and vitamin E are associated with reduced risk for atherosclerosis, but in smokers, they could promote lipid oxidation. Therefore, we examined the effects of a high polyunsaturated fat diet and vitamin E supplementation on measures of lipid oxidation in cigarette smokers. Ten subjects who smoked >1 pack of cigarettes per day were sequentially fed the following: a baseline diet in which the major fat source was olive oil, a diet in which the major fat source was high-linoleic safflower oil, and finally, the safflower oil diet plus 800 IU vitamin E per day. LDL oxidation lag time and rate and plasma total F2-isoprostanes and prostaglandin F2α (PGF2α) were determined after 3 weeks on each diet. The safflower oil diet increased total F2-isoprostanes from 53.0±7.2 to 116.2±11.2 nmol/L and PGF2α from 3.5±0.2 to 5.5±0.5 nmol/L, without changing LDL oxidation parameters. Addition of vitamin E prolonged mean LDL oxidation lag time but, paradoxically, further increased F2-isoprostanes to 188.2±10.9 nmol/L and PGF2α to 7.8±0.4 nmol/L. These data suggest that vitamin E may function as a pro-oxidant in cigarette smokers consuming a high polyunsaturated fat diet. (Arterioscler Thromb Vasc Biol. 2001;21:1029-1033.)

Key Words: LDL ■ lipid oxidation ■ antioxidants ■ α-tocopherol ■ F2-isoprostanes

Atherosclerotic cardiovascular disease (ASCVD) is the leading cause of morbidity and mortality in the developed world. Peroxidation of plasma LDLs by oxygen-derived free radicals has been ascribed a central role in the etiology of ASCVD.1 The corollary to this theory is that agents that can protect LDL from peroxidation should decrease the risk of developing ASCVD, attenuate its progression, or even reverse established disease.2 Indeed, epidemiological studies have suggested that antioxidants, such as α-tocopherol, confer dose-dependent protection against ASCVD,3–5 yet prospective intervention trials6–8 have failed to observe a protective effect.

Tobacco use is one of the most potent risk factors for ASCVD. The high concentration of free radicals in tobacco smoke9 and the fact that the impact of smoking on the incidence ASCVD correlates with plasma LDL levels10,11 are consistent with the concept that LDL peroxidation by cigarette smoke promotes atherogenesis.12 Indeed, measures of lipid oxidation, such as thiobarbituric acid–reactive substances (TBARS) in plasma,13 LDL,14–16 and red blood cells17; breath alkane excretion18–20; and F2-isoprostanes in urine21 and plasma,22 are increased in cigarette smokers. Conversely, plasma levels of antioxidant vitamins in smokers, which could protect LDL against the damaging effects of free radicals, are depressed.23

Cigarette smokers thus constitute a high-risk population in which intervention could reduce the risk of ASCVD. Diets high in monounsaturated fatty acid (MUFA) or polyunsaturated fatty acid (PUFA) effectively lower plasma LDL,24 but high PUFA diets constitute a theoretical risk, because PUFAs are excellent substrates for lipid peroxidation.25 Indeed, studies in cigarette smokers15 and in nonsmokers26,27 have found that PUFAs increase LDL oxidation rate and also breath pentane excretion.28

The deleterious impact of dietary PUFA on lipid oxidation, in theory, could be mitigated by concomitant supplementation with antioxidant vitamins. However, although antioxidant vitamins are generally considered to be nontoxic,29 vitamin E is a redox reagent that can function as a pro-oxidant under certain conditions.30–32 This has led some to voice concerns that antioxidant vitamins could function as pro-oxidants in vivo.33,34 The fact that prospective studies have failed to show a protective effect of vitamin E against ASCVD3,8,35 and have even suggested that it increases the risk of cardiovascular events in smokers6,35,36 lends credence to these concerns and warrants an examination of the interaction of vitamin E and diet in cigarette smokers.

To this end, we have investigated the effects of a high PUFA diet and vitamin E supplementation on oxidation stress in cigarette smokers by using 2 informative measures: the kinetics of in vitro copper-catalyzed LDL peroxidation, the most frequently used method to assess the effects of diet and antioxidants on lipid oxidation,37–42 and the plasma concentration of F2-isoprostanes, which are biologically active
prostanoids formed by free radial–initiated rearrangement of arachidonic acid, and which may be a much better indicator of global in vivo oxygen-derived free radical stress.

Methods

Subjects

The present study was approved by the Institutional Review Board of the Wake Forest University School of Medicine. All subjects signed a statement of informed consent. Ten subjects (7 female, 3 male) who smoked >20 cigarettes per day and used no medications except oral contraceptives were recruited. The subjects’ mean age (±SE) was 37.4 ± 1.1 years; mean weight was 69.8 ± 3.5 kg. Prestudy plasma lipids were as follows: total cholesterol 4.94 ± 0.31 mmol/L, LDL 2.84 ± 0.41 mmol/L, and HDL 0.96 ± 0.08 mmol/L (mean ± SE).

Study Design

The present study was conducted in the General Clinical Research Center of the Wake Forest University School of Medicine. During the study, the subjects ate only food prepared by the metabolic kitchen and used no ad libitum vitamin supplements. Daily energy requirements were estimated by the Harris Benedict equation. Subjects were weighed daily, and the total caloric content of each diet was adjusted, if necessary, to maintain weight within ±1 kg. All diets provided the following: 45% of total calories as carbohydrate, 20% as protein, and 35% as fat; 300 mg cholesterol per day; and no alcohol. The majority of fat energy at each meal was provided by a single food item made with vegetable oils that contained no added vitamin E (Hain). Diets were modified in 3 sequential phases; each phase was 3 weeks long. In phase 1, the major fat source was olive oil. In this diet, 7.5% of the total calories came from saturated fat; 20%, from MUFA; and 7.5%, from PUFA. In phase 2, the major fat source was high linoleic safflower oil. In this diet 7.5% of the total calories came from saturated fat; 7.5%, from MUFA; and 20%, from PUFA. Phase 3 continued the phase 2 diet with the addition of a 400 IU dl-α-tocopheryl acetate soft-gel (Eckerd Drug Co) at lunch and dinner.

Measurement of Plasma Lipids, Lipoproteins, Cotinine, and F2-Isoprostanes

Venous blood was collected in sodium EDTA Vacutainers after an overnight fast, kept on ice, and centrifuged at 4°C. Plasma for lipid analysis was stored at 4°C under argon and assayed within 24 hours for total, LDL, and HDL cholesterol by a Technicon RA-1000 analyzer. Plasma for isoprostane measurements was stored at −70°C under argon and assayed within 1 week of collection. Plasma total F2-isoprostanes and prostaglandin F3α (PGF3α) were quantified as pentafluorobenzyltrimethylsilyl ether derivatives by gas chromatography–mass spectrometry, and d3-PGF3α (Cayman Chemical Co) was used as an internal standard. Plasma cotinine was measured by radioimmunoassay.

Preparative Isolation of Plasma LDL

Fasting venous blood was collected into 3 mmol/L sodium EDTA, pH 7.4, 1.5 mmol/L sodium azide, 0.175 mmol/L gentamicin SO4, and 0.25 mmol/L chloramphenicol. Plasma was made to 1 mmol/L with benzamidine and phenylmethylsulfonyl fluoride. LDL was isolated by ultracentrifugation at 4°C at a density range of 1.006 to 1.063 g/mL, stored at 4°C in the dark under nitrogen, and studied within 48 hours. Protein concentration was determined by using bicinchoninic acid with BSA used as a standard.

Measurement of LDL Fatty Acid and α-Tocopherol Concentration

The fatty acid composition of LDL was determined by gas chromatography with pentadecanoic acid used as an internal standard. The α-tocopherol concentration in plasma and LDL was measured by high-pressure liquid chromatography with tocol used as an internal standard.

<table>
<thead>
<tr>
<th>Table 1. Plasma Cotinine, Lipids, and Vitamin E</th>
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<tbody>
<tr>
<td>MUFA</td>
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<tr>
<td>Cotinine, μmol/L</td>
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<tr>
<td>Total cholesterol, mmol/L</td>
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<td>LDL cholesterol, mmol/L</td>
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<tr>
<td>HDL cholesterol, mmol/L</td>
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<td>Vitamin E, μmol/L</td>
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Values are mean ± SE. Values in each row with different superscripts are significantly different as indicated.

Measurement of LDL Oxidation Kinetics

LDL oxidation was measured at the end of each phase by fluorescence spectroscopy. Immediately before each assay, LDL was dialyzed against PBS, pH 7.4, at 4°C and diluted to 0.2 g/L. LDL was incubated at 37°C with 200 μmol/L CuSO4, and the fluorescence intensity at 435 nm was monitored with excitation at 370 nm. Oxidation lag time was determined at the intercept of the linear segments of the initial lag phase and the propagation phase; oxidation rate was calculated from the slope of the propagation curve. Coefficients of variation were as follows: for lag time, intra-assay 3.5%, interassay 4.4%; for oxidation rate, intra-assay 7.5%, interassay 7.4%.

Statistical Analysis

The significance of differences in plasma lipoprotein cholesterol and total vitamin E, LDL fatty acid and vitamin E composition, LDL oxidation parameters, and plasma F2-isoprostanes across the 3 diet phases was determined by 1-way repeated-measures ANOVA with Tukey post hoc testing. The significance of relationships among parameters was determined by linear correlation analysis.

Results

So that the effect of PUFA and vitamin E on oxidation parameters could be referenced to a standard diet, the subjects were first placed on a baseline MUFA diet for 3 weeks, because MUFA are oxidized less readily than are PUFAs. The subjects were next fed a high PUFA diet for 3 weeks and then, finally, a high PUFA diet supplemented with 800 IU vitamin E for 3 weeks. Plasma cotinine levels did not change across the 3 phases of the study (Table 1), indicating that there was no change in cigarette consumption. After 3 weeks on the PUFA diet, mean plasma total, LDL, and HDL cholesterol decreased (Table 1), consistent with the well-known effects of PUFA on plasma lipoproteins. There was no change in mean plasma (Table 1) or LDL (Table 2) vitamin E concentration. The mole fraction of linoleic acid in LDL increased with a reciprocal decrease in oleic acid; the mole fraction of arachidonic acid, 43 and which may be a much better indicator of global in vivo oxygen-derived free radical stress.

<table>
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<tr>
<th>Table 2. LDL Arachidonate, Vitamin E, and Oxidation Kinetics</th>
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<tr>
<td>MUFA</td>
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<tr>
<td>Vitamin E, mol/mol</td>
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<tr>
<td>Arachidonate, mol/mol</td>
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<tr>
<td>Lag time, min</td>
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<tr>
<td>Oxidation rate, U/min/1000</td>
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</tbody>
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Values are mean ± SE. Values in each row with different superscripts are significantly different as indicated.
Nonetheless, mean plasma total $F_2$-isoprostane and PGF$_{2\alpha}$ concentration increased by 58% (Table 3), suggesting that the increase in dietary PUFA had promoted in vivo free radical oxidation reactions.

Addition of 800 IU vitamin E per day to the PUFA diet resulted in no change in plasma lipoprotein levels, in keeping with previous observations, or in LDL fatty acid composition. However, mean plasma vitamin E concentration and mean LDL molar vitamin E content increased significantly (Tables 1 and 2). Mean LDL oxidation lag time increased, reflecting the increase in LDL antioxidant content, but there was no change in the mean LDL oxidation rate. Nonetheless, mean plasma total $F_2$-isoprostane and PGF$_{2\alpha}$ levels increased further by 62% and 47%, respectively, suggesting that vitamin E had a pro-oxidant effect in the setting of a high PUFA intake.

PGF$_{2\alpha}$ may be formed either enzymically or by free radical oxidation of arachidonic acid. In each diet phase, there was a tight linear relationship between plasma concentrations of total $F_2$-isoprostanes and PGF$_{2\alpha}$ (Figure 2), suggesting that PGF$_{2\alpha}$ was derived from lipid oxidation. A plot of plasma $F_2$-isoprostane levels, normalized by the mole fraction of LDL arachidonate, versus total plasma vitamin E concentration gave a linear correlation, with $r=0.84$ and $P<0.001$ (Figure 3), further suggesting that isoprostane synthesis was related to vitamin E availability.

**Discussion**

It is well established that vitamin E can function as a pro-oxidant in vitro under certain specific conditions (particularly, a constant low-level flux of initiator free radicals and the absence of co-antioxidants, such as vitamin C) that can regenerate $\alpha$-tocopherol from tocopheryl radicals. These conditions may exist in cigarette smokers. The finding that vitamin E increased total plasma $F_2$ isoprostanes and PGF$_{2\alpha}$ in smokers consuming a high PUFA diet suggests that in this setting, vitamin E can function as a pro-oxidant in vivo. Moreover, the correlation between plasma total $F_2$ isoprostanes and vitamin E concentration suggests that the rise in plasma isoprostane levels may have been a global tissue response to increased vitamin E availability.

Mean plasma $F_2$-isoprostane concentration nearly doubled on the PUFA diet, despite the fact that (as reflected by LDL fatty acid composition) there was no change in dietary arachidonate intake. This suggests that dietary arachidonate did not serve as a substrate for increased isoprostane production. Rather, the high PUFA diet, by increasing the intake of oxidizable linoleate, may have increased the flux of free radicals available to initiate isoprostane synthesis from arachidonate already present in peripheral tissues. Alternatively, incorporation of dietary linoleate into cell membranes may have created a more fluid lipid microenvironment that promoted faster diffusion of free radicals. In either case, with the addition of vitamin E to the PUFA diet, the increased tissue availability of vitamin E, functioning in a pro-oxidant mode, may have further enhanced the synthesis of $F_2$-isoprostanes.

Although some studies in smokers have found that vitamin E decreases breath pentane excretion and plasma or red blood cell TBARS, other studies have noted no effect of vitamin E, or antioxidant cocktails containing vitamin E, on breath alkane excretion, plasma TBARS, LDL oxidation rate, or urinary $8$-oxo-PGF$_{2\alpha}$ excretion, plasma oxysterols, or plasma $F_2$-isoprostane concentration. The disparities among these studies and our present observations may reflect a sensitivity of oxidation status in smokers to factors such as the intensity of smoking, the source and dose of vitamin E, the plasma concentration of co-antioxidants such as ascorbate, and dietary fatty acid intake. In this regard,
it is important to note that no other study has used an experimental diet with a PUFA content as high as we provided in the present study.

In vitro determination of LDL oxidation kinetics has been widely used as a measure of lipoprotein oxidation susceptibility and of the in vivo efficacy of antioxidants. However, given the presence of potent aqueous phase antioxidants such as urate and ascorbate, it seems unlikely that LDL oxidation could occur in the plasma or interstitial compartments. Moreover, the radical flux rate in vivo is likely orders of magnitude smaller than that generated by chemical catalysis in vitro. Furthermore, in vitro copper-catalyzed oxidation is highly sensitive to the copper/LDL ratio, so that very different oxidation kinetics can be obtained depending on the conditions selected. Therefore, our observation that vitamin E raised plasma F$_2$-isoprostane levels despite prolonging LDL lag time suggests that ex vivo measurement of LDL oxidation may not provide a physiologically relevant measure of global oxidation status.

Whereas some studies have noted that PUFAs alter LDL oxidation kinetics, we, like others, noted no effect of increasing dietary PUFA on LDL oxidation rate. Similarly, although studies in nonsmokers have noted a salutary effect of vitamin E on LDL kinetic parameters, we noted no effect of vitamin E on the mean LDL oxidation rate. Again, these disparities may be a function of the specific conditions of each study. Indeed, because copper-catalyzed oxidation is sensitive to the number of copper-binding sites on the LDL surface and LDL phospholipid fatty acid composition, the large interindividual variability in these factors will tend to obscure all but the largest differences in oxidation kinetics among different studies.

There are several important caveats to the present study. First, we did not evaluate the effect of vitamin E on lipid oxidation on the MUFA diet, so we cannot determine whether its pro-oxidant effect in smokers was a unique consequence of its coadministration with a high-PUFA diet. However, because there appears to be no synergistic antioxidant effect of vitamin E supplementation and a high MUFA intake, we believe that vitamin E would be less likely to function as a pro-oxidant in this setting.

Second, we did not conduct parallel studies in nonsmokers, so we cannot conclude that vitamin E acts as a pro-oxidant exclusively in smokers consuming a high PUFA diet. Finally, the copper/LDL molar ratio we used in our in vitro oxidation assay (500:1) is higher than that used in other studies, and as noted above, oxidation kinetics are highly sensitive to the copper/LDL ratio.

In summary, our data suggest that supplementing cigarette smokers with vitamin E, in the setting of a high PUFA diet, promotes in vivo lipid oxidation, as evidenced by increased plasma F$_2$-isoprostane levels. Even though it is unlikely that many smokers would consume a diet containing the high content of PUFA that we used in the present study, because F$_2$-isoprostanes are vasoactive and increase platelet activation, our findings suggest caution in the use of high-dose vitamin E supplementation as a therapeutic means to reduce the risk of ASCVD in cigarette smokers.

Acknowledgments

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