Effect of dietary supplementation with alpha-tocopherol on the oxidative modification of low density lipoprotein¹

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Abstract

Much data have accumulated supporting a pro-atherogenic role for oxidized low density lipoprotein (Ox-LDL). Micronutrient antioxidants such as alpha-tocopherol, the principal lipid-soluble antioxidant, assume potential significance because levels can be manipulated by dietary measures without resulting in side effects. Co-incubation of LDL in vitro with alpha-tocopherol inhibits its oxidative modification. Hence the effect of dietary supplementation with alpha-tocopherol on the time course of copper-catalyzed oxidation of LDL was tested in a randomized placebo-controlled single-blind study. Two groups of 12 male subjects were given either placebo or alpha-tocopherol (800 IU/day) for a period of 12 weeks. Alpha-tocopherol therapy did not result in any side effects or exert an adverse effect on the plasma lipid and lipoprotein profile. While the lipid standardized alpha-tocopherol levels were similar at baseline, the supplemented group had 3.3-fold and 4.4-fold higher levels compared to placebo at 6 and 12 weeks, respectively. In the 15 subjects in whom both plasma and LDL alpha-tocopherol levels were quantitated, there was a significant correlation (r = 0.79, P < 0.0001). At baseline there were no significant differences in the time course curves of thiobarbituric acid-reacting substances (TBARS) activity or conjugated diene formation between the alpha-tocopherol and placebo groups. However, at both 6 and 12 weeks the mean levels of TBARS activity and conjugated diene formation were lower in the alpha-tocopherol group; the most significant differences were manifest at the 3-h time point. Also at both 6 and 12 weeks the mean rate of oxidation was lower in the alpha-tocopherol group. There was a significant inverse correlation between both the 3-h time point and the rate of oxidation with plasma lipid standardized alpha-tocopherol levels.

As this study shows that alpha-tocopherol supplementation results in an increase in plasma and LDL alpha-tocopherol levels resulting in a decreased susceptibility of LDL to oxidation, these findings could have major implications in atherosclerosis prevention. — Jialal, I., and S. M. Grundy. Effect of dietary supplementation with alpha-tocopherol on the oxidative modification of low density lipoprotein. J. Lipid Res. 1992. 33: 899-906.

Supplementary key words: LDL • lipid peroxidation • vitamin E • antioxidants • modified lipoprotein

The early lesion of atherosclerosis, the fatty streak, contains predominantly lipid-laden macrophages (1, 2).

Abbreviations: Ox-LDL, oxidized low density lipoprotein; TRARS, thiobarbituric acid-reacting substances; CHD, coronary heart disease.

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The design was that of a placebo-controlled, randomized, Board, University of Texas Southwestern Medical Center. Monitoring for adverse effects. A total ofing criteria to be entered into the study: nonsmoker male, staff conducting the various assays were blinded to the two single-blind study. While both patients and the laboratory were recruited. Participants had to fulfill all of the follow-
creatic, and renal function, no evidence of malabsorption, pan-
faction was studied over an 8-h period. Each time point was arrested by refrigeration and the addition of 200 PM butylated hydroxytoluene.

Two indices of oxidation were used in this study. The lipid peroxide content of oxidized LDL (Ox-LDL) was measured by a modification of the thiobarbituric acid-reacting substances (TBARS) assay of Buege and Aust as described previously (34). TBARS activity was expressed as malondialdehyde (MDA) equivalents using freshly 

PATIENTS AND METHODS

This study was approved by the Institutional Review Board, University of Texas Southwestern Medical Center. The design was that of a placebo-controlled, randomized, single-blind study. While both patients and the laboratory staff conducting the various assays were blinded to the two groups, the principal investigator was not because he was monitoring for adverse effects. A total of 24 volunteers were recruited. Participants had to fulfill all of the following criteria to be entered into the study: nonsmoker male, not on any vitamin supplements for at least 6 months, alcohol intake <1 oz/day, normal plasma glucose hepatic and renal function, no evidence of malabsorption, pancreatic, or biliary disease, and no acute medical condition for at least 3 months. Fasting blood samples were obtained at baseline for assessment of the complete blood count, plasma glucose, plasma proteins, hepatic and renal function, all of which were assayed by standard laboratory techniques. Also, blood was obtained for the lipid and lipoprotein profile, plasma alpha-tocopherol, ascorbate and beta-carotene levels and for LDL isolation. The samples for LDL isolation were collected in tubes containing EDTA (1 mg/ml). All blood samples were collected on ice and the plasma was separated by low-speed centrifugation at 4°C. Thereafter the participants were prescribed either the placebo capsules (soy bean oil) or dl-alpha-tocopherol 800 IU (727.3 mg) a day delivered in soy bean oil capsules for 3 months. The capsules were provided by Hoffman-La Roche Inc. (Nutley, NJ). The two groups were studied in parallel. They were advised to adhere to their usual diet and exercise activity throughout the twelve weeks and to immediately report any side effects. The study subjects returned to the clinic at 6 and 12 weeks. At each visit a clinical examination was performed and blood samples were obtained as described above for the baseline period. Samples for plasma ascorbate were deproteinized with ice-cold 10% metaphosphoric acid and centrifuged; the supernatant was purged with nitrogen, and stored below –20°C in tubes covered with foil. Plasma samples for alpha-tocopherol and beta-carotene were also purged with nitrogen and stored below –20°C in tubes covered with foil.

The lipid and lipoprotein levels were assayed using Lipid Research Clinic methodology except that cholesterol and triglycerides were determined enzymatically (29). The concentrations of alpha-tocopherol and beta-carotene were measured in plasma, after extraction, by reversed phase high performance liquid chromatography (30). The levels of both alpha-tocopherol and beta-carotene were lipid-standardized to total plasma lipids. The sum of cholesterol and triglyceride levels (mg/dl) was taken as an estimate of total lipid (31). Plasma ascorbate levels were determined spectrophotometrically after derivitization with 2, 4-dinitrophenylhydrazine (32). LDL (1.019-1.063 g/ml) was isolated by preparative ultracentrifugation in salt solutions (NaBr, NaCl) containing 1 mg/ml EDTA as previously described (27). The isolated LDL was exhaustively dialyzed against 150 mM NaCl 1 mM EDTA, pH 7.4, filtered, and stored at 4°C under nitrogen. Protein was measured by the method of Lowry et al. (33) using bovine serum albumin as standard. Stock solutions obtained at 6 and 12 weeks were diluted with the saline-EDTA dialysis buffer such that the protein concentration did not vary from the baseline levels by more than 0.5 mg/ml. Oxidation studies were performed within 48 h of LDL isolation. The oxidation of LDL was undertaken after an overnight dialysis against 1 liter of phosphate-buffered saline (PBS). LDL (200 µg/ml protein) was oxidized in a cell-free system using 5 µM Cu²⁺ in PBS at 37°C (27). The time course of oxidation was studied over an 8-h period. Each time point was set up in triplicate. At 0, 1, 3, 5, and 8 h oxidation was arrested by refrigeration and the addition of 200 µM EDTA and 40 µM butylated hydroxytoluene.

Two indices of oxidation were used in this study. The lipid peroxide content of oxidized LDL (Ox-LDL) was measured by a modification of the thiobarbituric acid-reacting substances (TBARS) assay of Buege and Aust as described previously (34). TBARS activity was expressed as malondialdehyde (MDA) equivalents using freshly diluted 1,1,3,3-tetramethoxypropane as the standard. The
amount of conjugated dienes formed was determined by monitoring the absorbance of the oxidized LDL against a PBS blank at a wavelength of 234 nM (25). The data are expressed as the increase in conjugated dienes over baseline (ΔA234). The rate of LDL oxidation was determined from the propagation phase of the time-course curve using a spline function.

All results are expressed as mean ± SEM of multiple determinations unless stated otherwise. A preliminary two-factor repeated-measures analysis of variance was used to assess differences in parameters within groups (baseline, 6 and 12 weeks) and between groups (placebo vs. alpha-tocopherol) Assumptions of homogeneity of variance and normality were tested using Levene's test and the Wilk-Shapiro test, respectively. Comparisons between groups were made using the Mann-Whitney U test. Significance was defined at the 5% level using a two-tail test of significance. Within-group comparisons were made using the Wilcoxon Signed Rank test with the Bonferroni inequality to adjust for multiplicity of testing. Pearson correlations were performed on log-transformed data.

RESULTS

No significant differences were observed between the alpha-tocopherol-supplemented and placebo groups with respect to age, 47.9 ± 5.4 and 47.4 ± 4.5 years (P = 0.95,) and body mass index (27.4 ± 1.2 and 25.3 ± 0.9 kg/m2, P = 0.19). Also there was no significant change in weight in either group throughout the 12-week study period; mean weights for all 24 subjects at baseline, 6, and 12 weeks were 176.6, 175.6, and 175.8 lbs, respectively. None of the participants in either group experienced any side effects as detected by clinical examination and standard laboratory techniques. Furthermore, neither placebo nor alpha-tocopherol supplementation had a significant effect on the plasma lipid and lipoprotein profile (Table 1). Also there were no significant differences between the two groups with respect to plasma lipid and lipoprotein levels.

Plasma ascorbate levels were similar at baseline, 6, and 12 weeks in both groups (Table 2). While the mean levels of lipid-standardized alpha-tocopherol were similar in both groups at baseline, the supplemented group had significantly higher levels at both 6 and 12 weeks. Levels were 3.3-fold higher at 6 weeks and 4.4-fold higher at 12 weeks compared to placebo. Because of insufficient LDL samples after the oxidation studies, only 15 subjects had both plasma and LDL alpha-tocopherol levels assayed at all three time points. In these 15 subjects there was a strong positive correlation between levels of lipid-standardized plasma alpha-tocopherol and LDL alpha-tocopherol (r = 0.79, P < 0.0001).

The levels of plasma beta-carotene were substantially lower than ascorbate or alpha-tocopherol levels in both groups (Table 2). Although beta-carotene levels standardized for plasma lipids were higher in the alpha-tocopherol-supplemented group, throughout the study there was much variability in levels between individuals and within the same individual. Furthermore, no significant increase in plasma beta-carotene levels occurred at 6 and 12 weeks compared to baseline in the alpha-tocopherol-supplemented group (P values 0.09 and 0.43, respectively).

The time-course curves of oxidative modification of LDL at baseline, 6, and 12 weeks in the placebo group are depicted in Fig. 1. No significant differences in the time-course curves of TBARS activity or conjugated diene formation were observed at 6 and 12 weeks compared to baseline. However, at 6 weeks the time-course curves of oxidation of placebo and the alpha-tocopherol-supplemented group were compared at baseline, no significant differences were noted (Fig. 2). The time-course curves of oxidative modification of LDL at baseline, 6, and 12 weeks in the placebo group are depicted in Fig. 1. No significant differences in the time-course curves of TBARS activity or conjugated diene formation were observed at 6 and 12 weeks compared to baseline. However, at 6 weeks the time-course curves of oxidation of placebo and the alpha-tocopherol-supplemented group were compared at baseline, no significant differences were noted (Fig. 2). However, at 6 weeks the time-course curves were definitely shifted to the right in the group supplemented with alpha-tocopherol (Fig. 3). Mean levels for both TBARS activity and conjugated diene formation in the

### Table 1. Effect of placebo and alpha-tocopherol supplementation on the plasma lipid and lipoprotein profile

<table>
<thead>
<tr>
<th>Group</th>
<th>Baseline</th>
<th>6 Weeks</th>
<th>12 Weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/100 ml</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>200.3 ± 11.2</td>
<td>206.0 ± 11.8</td>
<td>201.5 ± 15.2</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>148.1 ± 18.0</td>
<td>145.8 ± 25.5</td>
<td>139.8 ± 18.8</td>
</tr>
<tr>
<td>LDL-cholesterol</td>
<td>130.3 ± 10.1</td>
<td>135.8 ± 10.3</td>
<td>133.9 ± 12.7</td>
</tr>
<tr>
<td>HDL-cholesterol</td>
<td>45.3 ± 2.2</td>
<td>46.0 ± 2.8</td>
<td>44.2 ± 2.5</td>
</tr>
<tr>
<td>Alpha-tocopherol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>199.4 ± 11.1</td>
<td>203.8 ± 9.9</td>
<td>206.8 ± 13.8</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>109.3 ± 13.5</td>
<td>111.7 ± 16.8</td>
<td>110.4 ± 13.8</td>
</tr>
<tr>
<td>LDL-cholesterol</td>
<td>128.1 ± 11.5</td>
<td>134.9 ± 9.6</td>
<td>139.2 ± 13.7</td>
</tr>
<tr>
<td>HDL-cholesterol</td>
<td>49.8 ± 4.0</td>
<td>50.6 ± 3.9</td>
<td>50.5 ± 3.0</td>
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</table>

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alpha-tocopherol group were significantly lower than placebo at both 1 and 3 h. Compared to placebo levels, the percentage decrease in TBARS activity at 1 and 3 h was 56.8% and 64.3%, respectively, and the percentage decrease in conjugated diene formation was 47.8% and 55.2%, respectively.

At 12 weeks the shift in the time-course curves to the right in the alpha-tocopherol group persisted (Fig. 4). In fact, mean levels of TBARS activity were significantly lower than placebo at 1, 3, and 5 h. The mean percentage decreases compared to placebo in TBARS activity at 1, 3, and 5 h were 57.1, 54.6, and 19.9%, respectively. While the shift to the right in the time-course curve of conjugated diene formation persisted at 12 weeks, the mean levels were significantly lower at 3 h; the lower mean levels at 1 h barely escaped significance (P = 0.07).

As is evident in Table 3, while the rate of oxidation was similar in both groups at baseline, the alpha-tocopherol group had significantly lower rates of oxidation at both 6 and 12 weeks.

Throughout the 12-week study period the most significant reduction in TBARS activity and conjugated diene formation occurred at 3 h; hence these values were correlated with the plasma alpha-tocopherol levels for the entire group. There was a significant inverse correlation between plasma alpha-tocopherol and the 3-h TBARS activity as shown in Fig. 5 (r = -0.64, P < 0.0001) and between plasma alpha-tocopherol and the 3-h conjugated dienes (r = -0.59, P < 0.0001). Also, plasma alpha-tocopherol levels correlated with the rate of oxidation (r = -0.47, P < 0.0001).
**Fig. 3.** A comparison of the time-course curves of LDL oxidation at 6 weeks between the placebo and the alpha-tocopherol groups. At 6 weeks, the isolated LDL samples were subjected to copper-catalyzed oxidation as detailed in the Methods section. At the time points shown oxidation was stopped and the samples were assayed for TBARS activity and the formation of conjugated dienes. In the upper panel is shown the TBARS activity and in the lower panel the conjugated dienes. The asterisks denote significantly lower levels in the alpha-tocopherol group: TBARS panel (1 h, $P = 0.031$; 3 h, $P = 0.0004$). Conjugated dienes panel (1 h, $P = 0.0006$; 3 h, $P = 0.0001$).

**DISCUSSION**

Evidence continues to accumulate supporting the hypothesis that the oxidative modification of LDL is a key step in the genesis of the atherosclerotic lesion. Further, several groups have now reported that antioxidants added to in vitro systems can inhibit LDL oxidation. Moreover, dietary supplementation with probucol and butylated hydroxytoluene inhibits lesion formation in rabbits (13–15). However, since probucol has other effects on lipoprotein metabolism such as increasing the activity of cholesteryl ester transfer protein (35), one cannot ascribe its anti-atherogenic effect solely to its antioxidant property. Also, probucol is contraindicated in patients with a prolonged QT interval and syncope of cardiovascular origin (16, 17). This would weigh against it being prescribed as adjunctive therapy in primary prevention. Finally, probucol therapy induces a substantial reduction in HDL-cholesterol levels, the consequences of which are unknown (16, 17). Butylated hydroxytoluene feeding produces severe combined hyperlipidemia in animals (15) and can also be toxic when fed in substantial amounts (18). Therefore, safe and effective antioxidants for human use remain to be developed.

Alpha-tocopherol is the major antioxidant in the LDL particle, and supplementation with doses up to 1000 U/day has not been accompanied by any serious side effects (36). Hence the goal of the present study, to test the effect of alpha-tocopherol supplementation on LDL oxidation, is a logical progression in testing the oxidized LDL hypothesis by extending it to the clinical arena.

Administration of 800 U/day of alpha-tocopherol orally did not produce any detectable side effects over the 3-month period. Furthermore, unlike probucol and

**TABLE 3.** Effect of placebo and alpha-tocopherol supplementation on the rate of LDL oxidation

<table>
<thead>
<tr>
<th>Groups</th>
<th>Placebo</th>
<th>Alpha-Tocopherol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>22.4 ± 1.8</td>
<td>22.0 ± 2.0</td>
</tr>
<tr>
<td>6 Weeks</td>
<td>25.5 ± 2.5</td>
<td>11.3 ± 1.5 *</td>
</tr>
<tr>
<td>12 Weeks</td>
<td>26.2 ± 1.8</td>
<td>15.3 ± 2.4  *</td>
</tr>
</tbody>
</table>

LDL oxidation rate is expressed in nmol MDA/mg protein per h. $^{*}P < 0.001$. $^{*}P < 0.01$. 

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butylated hydroxytoluene, alpha-tocopherol did not deleteriously effect plasma lipid or lipoprotein profiles. This observation is in agreement with published data (36–38).

The finding of at least a 3.3-fold increase in plasma alpha-tocopherol levels with feeding likewise is in accord with the published literature (37, 39). As plasma levels of lipid-standardized alpha-tocopherol and LDL alpha-tocopherol levels were significantly correlated \( r = 0.79 \) in the 15 subjects in whom both levels were assayed, it is reasonable to conclude that the plasma levels of lipid-standardized alpha-tocopherol are a practical surrogate for LDL alpha-tocopherol. Recently, Dieber-Rotheneder et al. (28) obtained a similar correlation \( r = 0.79 \) between plasma total and LDL levels of alpha-tocopherol in a dose response study. Thus data for total plasma levels of alpha-tocopherol obtained in the 28 subjects of this study almost certainly indicate a similar response in LDL levels of this micronutrient.

The present study demonstrated that the oxidation procedure used is highly reproducible for evaluating the susceptibility of LDL to oxidation in clinical studies because the time-course curves in the placebo group at baseline, 6, and 12 weeks were virtually superimposable. At baseline no significant differences were observed between the placebo and alpha-tocopherol groups as to their time-course curves of TBARS activity or conjugated diene formation. However, at both 6 and 12 weeks the alpha-tocopherol group had significantly lower levels of TBARS activity and conjugated dienes. Also the rate of oxidation during the propagation phase was significantly decreased in the group that was supplemented with alpha-tocopherol. The most pronounced lowering was evident at 3 h. Correlations between plasma alpha-tocopherol and both the 3-h time point and rate of LDL oxidation were highly significant. These findings suggest that alpha-tocopherol supplementation significantly decreases the propagation phase of LDL oxidation.

In the only other study that has examined the effect of alpha-tocopherol supplementation on LDL oxidation, Dieber-Rotheneder et al. (28) focused mainly on the oxidative resistance of LDL as measured by the duration of the lag phase of conjugated diene formation over 3 h. Their study showed that supplementation prolonged the lag phase of conjugated diene formation and that LDL alpha-tocopherol levels correlated significantly with the duration of the lag phase. Still, their study was limited by its shorter duration (21 days) and by having only two patients in each supplemented group. Because of this small number of subjects, statistical comparisons were not possible. The present study thus goes considerably further in that it demonstrates that alpha-tocopherol supplementation compared to placebo therapy significantly inhibits the rate of LDL oxidation and that the effect persists for 3 months and does not produce side effects. Since in the present study the major focus was on the time course of oxidation, the lag phase was not studied in detail and hence cannot be commented upon. Although Dieber-Rotheneder et al. (28) focussed only on the lag phase of oxidation whereas the present study included the time course of oxidation, the two studies are consistent in showing that alpha-tocopherol interferes with LDL oxidation.

As both groups of subjects in the current study were age-, sex-, and weight-matched and drawn from the same population pool and had similar lipoprotein, ascorbate, and alpha-tocopherol levels, it would appear that the higher beta-carotene level in the alpha-tocopherol group at baseline is a spurious finding. The higher levels of beta-carotene at 6 and 12 weeks could be attributed to the protective effect of substantially higher alpha-tocopherol levels in the supplemented group. However, the significance of these higher levels of beta-carotene at baseline is unclear; it should be noted that alpha-tocopherol levels were similar between the two groups and the time-course curves of oxidation were not significantly different at baseline. The small increment in beta-carotene levels is unlikely to have exerted an inhibitory effect on LDL oxidation. Furthermore, in accord with the findings of Dieber-Rothender et al. (28), the present study documents much variability in beta-carotene levels between individuals and within the same individual over the 3-month period of study. To obtain a clearer appreciation of the effect of beta-carotene on LDL oxidation, an in vivo study specifically examining the effect of beta-carotene supplementation on LDL oxidation is required.

The inhibitory effect of alpha-tocopherol demonstrated in this study could be more than adequate to protect the LDL in vivo since copper-catalyzed oxidation is a severe oxidative stress and may far exceed that attained in the ar-

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**Fig. 5.** Correlation between plasma lipid-standardized alpha-tocopherol and the 3 h TBARS activity.
terial wall. In fact it has been postulated that in the early phase of oxidation there is formed, in the subendothelial space, minimally modified LDL (MM-LDL) that is mildly oxidized (TBARS activity < 5 nmol MDA) (40, 41). Once formed MM-LDL triggers a sequence of molecular events that result in monocyte binding to endothelium, and its subsequent migration into the subendothelial space where differentiation into tissue macrophages occurs (40, 41). Macrophage products such as reactive oxygen species and aldehydes can then further modify MM-LDL to a more oxidized form which is then recognized by the macrophage scavenger receptor leading to cholesteryl ester accumulation and foam cell formation (42). The level of supplementation in the present study may well prevent the formation of MM-LDL and hence interrupt a key step in atherogenesis.

In conclusion, the present study documents that alpha-tocopherol supplementation in human subjects raises plasma and LDL alpha-tocopherol levels and alpha-tocopherol supplementation in human subjects raises prevention.

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