Effect of Dietary Antioxidant Combinations in Humans

Protection of LDL by Vitamin E but Not by β-Carotene

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Experimental and epidemiological evidence supports the hypothesis that oxidation of low density lipoprotein (LDL) appears to be important in mediating the atherogenicity of LDL. To test this hypothesis in humans, it will be necessary to perform intervention studies in large populations. We performed two studies to assess the effectiveness of supplementation with β-carotene and vitamin E, used alone and in combination with each other, and with vitamin C, to protect LDL from oxidation. In phase 1, after a placebo period, eight subjects were given β-carotene (60 mg/day) for 3 months, then β-carotene plus vitamin E (1,600 mg/day) for another 3 months, and then β-carotene plus vitamin E plus vitamin C (2 g/day) for 3 months. During phase 2, β-carotene and vitamin C were discontinued, and subjects took only vitamin E for 5 months. During each period, LDL samples were isolated, and measurements of susceptibility to oxidation were performed. β-Carotene levels in LDL increased nearly 20-fold, but LDL susceptibility to oxidation did not change. Addition of vitamin E increased LDL vitamin E levels nearly 2.5-fold, and this decreased LDL oxidation 30–40%. During the vitamin C supplementation period, plasma levels of β-carotene and vitamin E rose, but only β-carotene increased in LDL. However, the susceptibility of LDL to oxidation in this period was not decreased further. During phase 2, when subjects took only vitamin E, LDL susceptibility to oxidation was decreased by 50% as measured by thiobarbituric acid-reactive substances, conjugated dienes, and lipid peroxide formation as well as by macrophage degradation. Thus, long-term supplementation with large doses of vitamin E alone, but not β-carotene, conferred increased protection to LDL in in vitro assays of oxidation. These data should be useful in planning therapeutic strategies to test the antioxidant hypothesis in humans. (Arteriosclerosis and Thrombosis 1993;13:590–600)

KEY WORDS • atherosclerosis • antioxidants • vitamin E • β-carotene • vitamin C • LDL • HDL • lipid oxidation • conjugated dienes • macrophages

There is increasing evidence that lipoprotein oxidation plays an important role in the pathogenesis of atherosclerosis. It has been demonstrated that oxidized low density lipoprotein (LDL) has enhanced uptake by macrophages, leading to foam cell formation; is chemotactic for monocytes and inhibits macrophage exodus from the artery; induces endothelial cell damage; and stimulates cytokine and growth factor release from a number of cell types present in the artery wall. These and other properties of oxidized lipoproteins may explain how foam cells of early lesions form as well as how they evolve into complicated plaques (reviewed in References 9 and 10). Atherosclerotic lesions obtained from animal models of atherosclerosis as well as from autopsy and surgical specimens from humans contain oxidized LDL as well as immunoglobulins to oxidized lipoprotein epitopes. In addition, feeding lipophilic antioxidants such as probucol, butylated hydroxytoluene, or N,N'-diphenyl-phenylenediamine to hypercholesterolemic rabbits appears to decrease the development of atherosclerosis. Finally, epidemiological evidence suggests that dietary antioxidants may play a role in preventing coronary artery disease.

A recent National Institutes of Health Consensus Conference examined the body of evidence available and concluded that sufficient evidence was available to encourage clinical trials in humans to test whether antioxidant supplementation would inhibit the development of atherosclerosis. Thus, there is the need to perform studies in humans to determine the optimal modes of antioxidant therapy to use in clinical trials. However, at present there is no clear consensus on the appropriate agent or agents to use in such a trial. Although probucol is an excellent antioxidant, its effects on LDL and high density lipoprotein (HDL) cholesterol levels might complicate interpretation of trials using this agent. Although butylated hydroxytoluene has no known effect on lipoprotein levels, it is not considered safe for human ingestion at high doses. Similarly, N,N'-diphenyl-phenylenediamine cannot be used in humans.
because of toxicity. In contrast, natural antioxidants such as β-carotene, vitamin E (α-tocopherol), and vitamin C can be safely taken at moderately high doses without side effects and with minimal effect on lipoprotein levels.

Preliminary work has suggested that supplementation with vitamin E in humans may decrease the susceptibility of LDL to oxidation ex vivo. Although vitamin C is hydrophilic, in vitro it has demonstrated synergistic activity with vitamin E in protecting LDL from oxidation. Presumably, vitamin C preserves LDL α-tocopherol levels during oxidative stress by converting the α-tocopherol radical back to the reduced state so that it may function again as an antioxidant. Thus, the presence of vitamin C in the plasma (or extracellular fluid) may increase or maintain the content of lipid-soluble antioxidant in LDL when both are given simultaneously. Conflicting reports exist on the effectiveness of β-carotene supplementation in protecting LDL from in vitro oxidation when used alone or in combination with other antioxidants. Although β-carotene is lipid soluble like α-tocopherol and is also carried in lipoproteins, its mechanism of antioxidant activity is believed to be different from that of α-tocopherol. As a singlet oxygen scavenger, it could theoretically provide antioxidant activity to lipoproteins in addition to that provided by free-radical scavengers such as α-tocopherol.

The aims of this study were several. First, to evaluate whether β-carotene supplementation at high doses in humans could decrease susceptibility of LDL to oxidation in vitro. Second, to evaluate whether a combination of β-carotene and vitamin E would provide greater antioxidant protection than either agent used alone. Third, to evaluate whether the addition of a water-soluble antioxidant such as vitamin C would increase concentrations of α-tocopherol or β-carotene in LDL and perhaps further decrease the susceptibility of LDL to oxidation.

**Methods**

**Materials**

Synthetic β-carotene, racemic DL-α-tocopherol (vitamin E), vitamin C, and appropriate placebo capsules were provided by Hoffmann-La Roche Research Institute.

**Patient Population**

Eight healthy nonsmoking mildly hyperlipidemic volunteers (five women and three men), aged 26–65 years, without known medical disease were recruited from the community. All were on average American diets. None were taking medications other than oral contraceptives and occasional aspirin, and all had discontinued use of any vitamin supplementation at least 3 months before beginning the study. The study was approved by the Human Studies Committee of the University of California, San Diego, and was conducted in the outpatient facilities of the University of California, San Diego, General Clinical Research Center.

**Study Design**

The study included two phases: an initial phase in which combinations of antioxidants were evaluated (phase 1) and a second phase consisting of vitamin E alone (phase 2). The study protocol for phase 1 is illustrated in Figure 1. Subjects initially were begun on a 2-month placebo period (period A), during which time patients took placebo capsules for both β-carotene and vitamin E. Plasma and LDL samples obtained during this time were considered baseline samples. During the following 3 months (period B), the patients took β-carotene (30 mg two times per day [b.i.d.]) and vitamin E placebo period (BC + Plac); period C represents the β-carotene (30 mg b.i.d.) and vitamin E placebo period (BC+E); and period D represents the β-carotene (30 mg b.i.d.), vitamin E (800 mg b.i.d.), and vitamin C period (BC+E+C).

**Figure 1.** Line plot showing plasma total (■), low density lipoprotein (●), and high density lipoprotein (□) cholesterol levels during placebo and antioxidant supplementation. Each period (A-D) is represented by one panel, which contains lipid values drawn at two points, 1 month apart, during the indicated period. Each value represents a mean ± SD. Period A represents the placebo period (Plac); period B represents the β-carotene (30 mg two times per day [b.i.d.]) and vitamin E placebo period (BC+Plac); period C represents the β-carotene (30 mg b.i.d.) and vitamin E (800 mg b.i.d.) period (BC+E); and period D represents the β-carotene (30 mg b.i.d.), vitamin E (800 mg b.i.d.), and vitamin C period (BC+E+C).
then was LDL isolated for measurement of its susceptibility to oxidation.

**LDL Isolation and Analysis**

At each visit, blood was collected into tubes containing EDTA (4.0 mmol/L) and cooled immediately on ice, and the plasma fraction was separated. LDL was isolated from each sample by sequential ultracentrifugation and dialyzed extensively against phosphate-buffered saline (PBS) containing 0.27 mmol/L EDTA, as previously described. A final concentration of 0.22 mmol/L gentamycin, 0.15 mmol/L chloramphenicol, 1 μmol/L d-phenylalanyl-l-prolyl-l-arginine chloromethyl ketone, and 2 mmol/L benzamidine was added to plasma and all solutions used subsequently during LDL isolation. Protein was determined by the method of Lowry et al. To adjust for the possibility of interassay variation over the long study period, control LDL samples were also isolated from the same untreated subjects at each time point, and their samples were handled identically to samples from treated subjects. All analyses were completed within 2 weeks of isolation of LDL samples, which were stored at 4°C in the dark. A portion of the LDL protein from each plasma sample was radiiodinated with 125I by the method of Salacinski et al. and the specific activity of each sample was adjusted to approximately 30,000 cpm/μg protein by adding unlabeled LDL from the same sample. For all assays, LDL samples were adjusted to similar protein concentrations with PBS-EDTA. Thus, for each determination of thiobarbituric acid–reactive substances (TBARS) and macrophage degradation assay, each sample had an equal concentration of protein with equal specific activity and equal EDTA content for each experiment, as previously described.

A more extensive evaluation of vitamin E’s effect on LDL oxidation was performed in phase 2, during which time subjects were given only vitamin E for 5 additional months. In addition to TBARS and macrophage degradation experiments performed with 125I-labeled LDL as noted above, conjugated diene and lipid peroxide formation during copper-mediated oxidation of unlabeled EDTA-free LDL was also measured immediately after LDL isolation.

**Vitamin E Content**

Vitamin E was measured by high-performance liquid chromatography according to a modification of methodology described by Kaplan et al. Briefly, vitamin E acetate was prepared in 100% ethanol at 58 μmol/L as an extraction internal standard and for standard curve preparation. Actual concentrations of vitamin E were determined by measuring the absorbance of prepared solutions and calculated concentrations based on known spectral data. Plasma and LDL samples were extracted with petroleum ether and dried with nitrogen. The extract was reconstituted in a mobile phase, which consisted of acetonitrile, chloroform, 2-propanol, and water (78:16:3.5:2.5, vol/vol/vol/vol), and was run at a flow rate of 2 mL/min. The chromatographic analysis was performed on a 5-μm particle C-18 column. Calculations were determined from a standard curve of peak area ratios of sample to internal standard.

**β-Carotene Content**

β-Carotene was measured by a modification of the methodology described by Bieri et al. Briefly, β-carotene standards were made fresh daily, and exact concentrations were verified by absorbance at 450 nm. Echinenone (100 μL), kindly supplied by Dr. P.F. Sonter of Hoffmann-La Roche, Inc., was prepared in ethanol at 0.2 μg/mL and used as the internal standard. Samples and standards were extracted in hexane and dried under nitrogen. The extract was reconstituted in a mobile phase, which consisted of acetonitrile, methylene chloride, and methanol (70:20:10, vol/vol/vol), and was run at a flow rate of 1.7 mL/min on a 5-μm particle C-18 column. β-Carotene calculations were determined from a standard curve of peak area ratios of sample to internal standard.

**Vitamin C Content**

Total vitamin C was measured by the method of Bessey et al. Metaphosphoric acid was added to freshly prepared samples, which were frozen at −70°C, and samples were assayed at the completion of the study.

**Fatty Acid Composition**

LDL lipids were extracted by a modification of the method of Folch et al. The fatty acids were trimethylated and analyzed in a Varian gas chromatograph (model 3700) equipped with a column of 10% Silic 5CP on a Gas Chrom QII, 100/200. For quantitative estimates of fatty acids present in LDL, a C-15 internal standard was added to each sample before extraction. Calculations of fatty acid amounts were determined from a standard curve of peak area ratios of sample to internal standard, as previously described.

**Oxidation of LDL**

The formation of conjugated dienes was measured by incubating 100 μg LDL protein with 5 μmol/L CuSO₄ in 1.0 mL PBS medium. The absorbance at 234 nm was measured continuously in a Uvikon 810 spectrophotometer as previously described. Results are expressed as the absolute increase in absorbance above the initial value, the duration of the lag time before propagation of the oxidation reaction, and the rate of propagation as measured by the slope during the most rapid rise in absorbance at 234 nm. Lipid peroxides generated during LDL oxidation with 5 μmol/L CuSO₄ in PBS for 0, 2, 4, and 6 hours were also measured by the iodometric method of el-Saadani et al.

125I-labeled LDL at a concentration of 100 μg protein/mL was incubated with copper sulfate for 0, 8, and 16 hours or with monolayers of rabbit aortic endothelial cells at 37°C in Ham’s F-10 medium as previously described. Lipid peroxidation as a result of these incubations was assessed by measuring TBARS in the medium. Subsequently, each LDL sample oxidized in this manner was incubated with cultured mouse peritoneal macrophages for 5 hours, and cell-mediated degradation of the LDL was measured by the extent of formation of noniodide trichloroacetic acid–soluble radioactivity.

Thus, for each LDL sample, TBARS and macrophage degradation assays were performed during four different conditions of oxidation: endothelial cell...
oxidation for 8 and 16 hours and copper-mediated oxidation for 8 and 16 hours.

In our experience, there is considerable variability in assays used to measure the susceptibility of LDL to oxidation. Assays that use cultured cells such as endothelial cells to oxidize samples will vary as a result of cell passage, inherent differences in cellular activity, extent of confluence, and other unknown factors. In addition, the macrophage degradation assay is also susceptible to variation because of differences in the activity of different peritoneal macrophage populations. To allow comparison of data from the TBARS and macrophage assays performed at each visit, two steps were taken to adjust for interassay variation: 1) Each macrophage degradation assay was performed under several different conditions of oxidative stress as noted above. At each visit, only those macrophage degradation assays in which the determinations for the control samples were within the range of those present at the baseline evaluation (5–10 μg/5 hr per milligram cell protein) were used. If values for the control LDL samples did not fall into this range, then no data were used for that time point (e.g., copper sulfate oxidation at month 8 and endothelial cell oxidation at month 10). 2) The macrophage degradation data of all treated subjects were expressed as a percentage of control values for that assay. The TBARS assays selected for analysis were those that corresponded to the acceptable macrophage degradation assays, and TBARS data were also expressed as a percentage of control values for that assay.

Statistical Analysis

The BMDP software package was used for all statistical analyses. All paired and unpaired t tests, analyses of variance, and correlations were performed using standard methods. For phase 1, the effects of antioxidants on lipid levels, plasma and LDL antioxidant concentrations, and measures of the susceptibility of LDL to in vitro oxidative modification were examined using a multivariate multiple-regression model. We have previously described a similar model. The current regression analysis estimated fixed effects for each of the three antioxidants examined. The effect of β-carotene was included in the fitting of data for periods B–D, the effect of vitamin E in fitting data for periods C and D, and the effect of vitamin C in fitting period D data only. The regression simultaneously examined all data for all periods. Since the only change in the model between periods B and C is the addition of the vitamin E effect, a test for significance of a vitamin E effect actually compares period C with period B rather than baseline (period A). Similarly, a test for significance of a vitamin C effect compares periods C and D. Because dietary supplements were only added to existing regimens, any effect observed for an added antioxidant will include any interactions with the antioxidants already being taken. For example, the effects of vitamin C (period D) include both independent vitamin C effects and any interaction of vitamin C with β-carotene or vitamin E (period C).

Results

All participants completed the study without significant problems, although all developed persistent hypercarotenodermia after starting β-carotene supplementation. This was so distressing to one subject that her dose was decreased to 30 mg/day at the end of period B. Pill counts of unused capsules indicated that overall compliance was excellent, with less than 5% of the provided capsules returned. Despite the long duration of the study, the body weight of the participants was remarkably steady, with the mean weight for the group ranging from a low of 74.0±12.1 kg to a high of 74.4±11.5 kg. There were no abnormalities in any of the routine chemistry tests, including liver function tests, prothrombin times, or complete blood counts throughout the 14-month study.

Based on the three-day food records, total calories ingested per day for the entire group averaged 1,854±465 kcal/day during the baseline period, with 30% of these calories in the form of fat. Total daily vitamin E and vitamin C contents in the diet were estimated at 18.2±7.9 and 138.1±52.9 mg, respectively. Follow-up food records during the remaining periods of the study showed no marked change in caloric or dietary vitamin intake.

Phase 1

Table 1 contains means and significance testing for all variables obtained during phase 1. Figure 1 shows the plasma lipid and lipoprotein values during periods A–D of phase 1 of the study. There was a very slight, but nevertheless significant, increase in total cholesterol and LDL cholesterol during the phase 1 study. Plasma triglyceride levels varied throughout the study, primarily reflecting the erratic elevations of one subject with moderate hypertriglyceridemia varying from 227 to 815 mg/dL. Increases in total cholesterol corresponded in general with the increases in triglycerides. Comparisons between study periods revealed that HDL cholesterol increased slightly in period C and that total cholesterol and LDL cholesterol levels increased significantly only during period C. The total cholesterol/HDL cholesterol ratio did not change significantly during the study.

Figure 2 shows the mean cholesterol/triglyceride ratio in LDL isolated from subjects during study periods A–D. Although there is large between-subject variation (demonstrated by the large standard deviation), there was no significant change in the LDL cholesterol/triglyceride ratio on any combination of supplements. Similarly, there was no significant change in the fatty acid composition of the LDL isolated from the subjects during periods A–D (Table 2). Similar to our previous data, the majority of the measurable fatty acids in LDL are in the form of 18:1 or 18:2.

Levels of β-carotene and vitamin E in isolated LDL are shown in Figure 3. LDL β-carotene levels rose steadily, reaching a plateau after 3 months of supplementation that was roughly 20-fold higher than baseline values. LDL vitamin E values increased rapidly, essentially doubling within the first 3 months of supplementation (period C). Plasma vitamin C levels were also measured and showed a 71% increase by the end of period D compared with the non–vitamin C–supplemented levels of period C (data not shown). The addition of vitamin C led to a noticeable increase in the β-carotene content of LDL but not in LDL vitamin E levels. However, there was a significant increase in the whole-plasma content of both α-tocopherol and β-carotene during the vitamin C supplementation (Table 1).
Table 1. Mean Values and Significance Tests for All Variables in Phase 1

<table>
<thead>
<tr>
<th>Period</th>
<th>A Mean±SD</th>
<th>B Mean±SD</th>
<th>p (B vs. A)</th>
<th>C Mean±SD</th>
<th>p (C vs. B)</th>
<th>D Mean±SD</th>
<th>p (D vs. C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma chol (mg/dL)</td>
<td>235±29</td>
<td>238±25</td>
<td>0.62</td>
<td>261±42</td>
<td>0.001</td>
<td>253±38</td>
<td>0.18</td>
</tr>
<tr>
<td>HDL chol (mg/dL)</td>
<td>54.3±7.9</td>
<td>53.5±7.4</td>
<td>0.53</td>
<td>56.8±6.1</td>
<td>0.014</td>
<td>53.3±6.9</td>
<td>0.009</td>
</tr>
<tr>
<td>LDL chol (mg/dL)</td>
<td>155±19</td>
<td>152±22</td>
<td>0.96</td>
<td>168±25</td>
<td>0.010</td>
<td>163±28</td>
<td>0.28</td>
</tr>
<tr>
<td>Plasma TG (mg/dL)</td>
<td>133±87</td>
<td>148±90</td>
<td>0.19</td>
<td>180±179</td>
<td>0.39</td>
<td>177±182</td>
<td>0.80</td>
</tr>
<tr>
<td>Plasma chol/HDL chol (mg/mg)</td>
<td>4.4±0.7</td>
<td>4.5±0.6</td>
<td>0.47</td>
<td>4.7±0.8</td>
<td>0.32</td>
<td>4.8±0.8</td>
<td>0.34</td>
</tr>
<tr>
<td>Plasma vitamin E (µg/mL)</td>
<td>0.75±0.58</td>
<td>9.42±5.32</td>
<td>0.001</td>
<td>7.42±3.52</td>
<td>0.31</td>
<td>14.98±7.01</td>
<td>0.001</td>
</tr>
<tr>
<td>Plasma vitamin C (µg/mL)</td>
<td>11.6±2.2</td>
<td>16.1±4.9</td>
<td>0.56</td>
<td>38.9±22.2</td>
<td>0.007</td>
<td>55.6±30.8</td>
<td>0.041</td>
</tr>
<tr>
<td>LDL chol/TG (mg/mg)</td>
<td>9.23±1.5</td>
<td>9.38±3.71</td>
<td>0.81</td>
<td>9.14±3.74</td>
<td>0.61</td>
<td>8.47±3.07</td>
<td>0.17</td>
</tr>
<tr>
<td>LDL β-carotene (µg/mg)</td>
<td>0.26±0.19</td>
<td>5.01±2.00</td>
<td>0.001</td>
<td>6.04±2.60</td>
<td>0.34</td>
<td>9.91±4.47</td>
<td>0.001</td>
</tr>
<tr>
<td>LDL vitamin E (µg/mg)</td>
<td>5.1±1.3</td>
<td>5.0±1.0</td>
<td>0.72</td>
<td>12.2±2.9</td>
<td>0.001</td>
<td>12.9±3.5</td>
<td>0.43</td>
</tr>
<tr>
<td>EC MΦ degradation (% of control)</td>
<td>89±7</td>
<td>133±33</td>
<td>0.001</td>
<td>60±24</td>
<td>0.001</td>
<td>79±17</td>
<td>0.086</td>
</tr>
<tr>
<td>Cu MΦ degradation (% of control)</td>
<td>87±6</td>
<td>140±36</td>
<td>0.001</td>
<td>78±17</td>
<td>0.001</td>
<td>70±24</td>
<td>0.41</td>
</tr>
<tr>
<td>EC TBARS (% of control)</td>
<td>129±15</td>
<td>140±12</td>
<td>0.063</td>
<td>97±18</td>
<td>0.001</td>
<td>105±11</td>
<td>0.20</td>
</tr>
<tr>
<td>Cu TBARS (% of control)</td>
<td>145±19</td>
<td>134±11</td>
<td>0.045</td>
<td>89±12</td>
<td>0.001</td>
<td>94±15</td>
<td>0.33</td>
</tr>
</tbody>
</table>

Throughout phase 1, β-carotene levels in whole plasma were strongly correlated with LDL β-carotene values (r=0.83, p<0.001), as were the correlations of plasma vitamin E levels and LDL vitamin E levels (r=0.75, p<0.001).

Figure 4 and Table 1 show the formation of TBARS during endothelial cell- and copper sulfate-mediated oxidation of LDL isolated from subjects throughout periods A–D. To control for interassay variation, data are presented as the percentage of values measured using control LDL samples that were run simultaneously in each assay, as noted in “Methods.” During period B (β-carotene only), values of TBARS measured after oxidation were, in general, similar to mean values measured during the placebo period. During period C (β-carotene and vitamin E), TBARS generated after endothelial cell- or copper sulfate-mediated oxidation decreased significantly compared with period B values (Table 1). During the period in which subjects consumed 2 g vitamin C (period D) in addition to the other antioxidants, their isolated LDL was not less susceptible to oxidation compared with period C, as measured by TBARS. Compared with values obtained during placebo and active β-carotene supplementation, TBARS generated during both periods C and D were approximately 32% lower.

Macrophage degradation results show a similar pattern (Figure 5 and Table 1). LDL isolated from subjects on β-carotene alone underwent significantly greater macrophage degradation after oxidation than LDL isolated during the placebo phase. In contrast, after the addition of vitamin E to the supplementation regimen, the extent of macrophage degradation was significantly reduced. As with the generation of TBARS, there was little change after vitamin C was added to the regimen of the subjects.
TABLE 2. Low Density Lipoprotein Fatty Acid Content

<table>
<thead>
<tr>
<th>Period</th>
<th>16:0</th>
<th>16:1</th>
<th>18:0</th>
<th>18:1</th>
<th>18:2</th>
<th>20:4</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>20.8±1.5</td>
<td>2.1±0.07</td>
<td>5.9±0.8</td>
<td>18.8±1.9</td>
<td>41.7±3.8</td>
<td>9.1±1.1</td>
</tr>
<tr>
<td>B</td>
<td>20.5±1.9</td>
<td>2.4±0.09</td>
<td>6.6±0.5</td>
<td>17.1±1.8</td>
<td>42.0±3.7</td>
<td>10.8±1.6</td>
</tr>
<tr>
<td>C</td>
<td>21.1±2.4</td>
<td>2.5±1.0</td>
<td>6.8±0.3</td>
<td>19.3±2.3</td>
<td>40.6±4.5</td>
<td>9.0±1.7</td>
</tr>
<tr>
<td>D</td>
<td>22.1±2.6</td>
<td>2.4±0.06</td>
<td>7.1±0.7</td>
<td>18.3±2.0</td>
<td>40.7±4.1</td>
<td>8.6±1.5</td>
</tr>
</tbody>
</table>

LDL, low density lipoprotein; period A, placebo period; period B, β-carotene and vitamin E placebo period; period C, β-carotene and vitamin E period; period D, β-carotene, vitamin E, and vitamin C period. Values are mean±SD.

For each subject during each period, LDL was isolated, and the fatty acid profile was determined as described in “Methods.” No significant change occurred in LDL fatty acid composition during the course of the study.

Phase 2: Vitamin E Alone

Since the antioxidant benefits of vitamin supplementation seemed greatest with the addition of vitamin E, a subsequent detailed evaluation was performed in seven of eight subjects who were able to participate in a second phase of the study (one subject moved from the area after completion of periods A–D). At the completion of period D, the remaining seven subjects continued on 1,600 mg/day vitamin E but discontinued β-carotene and vitamin C. β-Carotene levels were monitored, and only after approximately 5 months did plasma levels of β-carotene return to baseline status.

Plasma lipid levels and LDL antioxidant levels measured on vitamin E alone are shown in Table 3, together with the values obtained at baseline (phase 1, period A) for the same subjects. At the end of this period, LDL cholesterol and HDL cholesterol levels were similar to the placebo period, but total cholesterol levels were slightly higher, presumably because of elevated very low density lipoprotein levels. The total cholesterol/HDL cholesterol ratio was unchanged from baseline. LDL cholesterol/triglyceride ratios were also similar to pretreatment values. LDL β-carotene levels were now markedly reduced from their elevated levels during β-carotene supplementation, although they were still slightly higher than those measured at baseline. LDL vitamin E levels were approximately 2.5-fold higher than during the placebo period.

As a measure of lipid peroxidation, conjugated diene formation during copper sulfate-mediated oxidation

![Figure 3](http://atvb.ahajournals.org/)

**Figure 3.** Line plot showing content of vitamin E and β-carotene in low density lipoprotein (LDL) in periods A–D. Vitamin E (■) and β-carotene (□) levels in LDL were measured at each time point and are shown as the mean±SD. Period A represents the placebo period (Plac); period B represents the β-carotene (30 mg two times per day [b.i.d.]) and vitamin E placebo period (BC+Plac); period C represents the β-carotene (30 mg b.i.d.) and vitamin E (800 mg b.i.d.) period (BC+E); and period D represents the β-carotene (30 mg b.i.d.), vitamin E (800 mg b.i.d.), and vitamin C period (BC+E+C). Vitamin E increased significantly in period C and remained elevated through period D. β-Carotene increased significantly in period B, remained elevated through period C, and increased again in period D (Table 1).

![Figure 4](http://atvb.ahajournals.org/)

**Figure 4.** Line plot comparing the extent of the formation of thiobarbituric acid–reactive substances (TBARS) after endothelial cell– or copper sulfate–mediated oxidation in periods A–D. Period A represents the placebo period (Plac); period B represents the β-carotene (30 mg two times per day [b.i.d.]) and vitamin E placebo period (BC+Plac); period C represents the β-carotene (30 mg b.i.d.) and vitamin E (800 mg b.i.d.) period (BC+E); and period D represents the β-carotene (30 mg b.i.d.), vitamin E (800 mg b.i.d.), and vitamin C period (BC+E+C). Low density lipoprotein (LDL) was isolated from subjects twice during each period and subjected to both endothelial cell (■) and copper sulfate (□) oxidation. Measurements of TBARS were performed and are presented as percentage of control LDL values as described in “Methods.” Each value represents a mean±SD. TBARS decreased significantly only during the addition of vitamin E (periods C and D) (Table 1).
FIGURE 5. Line plot comparing the extent of macrophage degradation after endothelial cell–or copper sulfate–mediated oxidation in periods A–D. Period A represents the placebo period (Plac); period B represents the β-carotene (30 mg two times per day [b.i.d.]) and vitamin E placebo period (BC+Plac); period C represents the β-carotene (30 mg b.i.d.) and vitamin E (800 mg b.i.d.) period (BC+E); and period D represents the β-carotene (30 mg b.i.d.), vitamin E (800 mg b.i.d.), and vitamin C period (BC+E+C). Low density lipoproteins (LDLs) were isolated from subjects twice during each period and subjected to both endothelial cell (m) and copper sulfate (a) oxidation. Measurements of macrophage degradation were performed and are presented as percentage of control LDL values as described in "Methods." Each value represents a mean ± SD. Macrophage degradation decreased significantly only during the addition of vitamin E (periods C and D) (Table 1).

was measured for LDL samples isolated from all seven subjects at the end of phase 2 and compared with LDL isolated from control subjects. For each LDL sample, the delay (lag time) before the propagation phase of the conjugated-diene reaction was determined, as well as the slope of the propagation phase. Lag times were significantly longer in LDL samples isolated from vitamin E–treated subjects compared with control LDL samples (214±33 versus 146±24 minutes, p<0.05). The rate of the propagation phase was also significantly slower in the vitamin E–treated subjects (0.0116±0.0260 versus 0.0175±0.0005 optical density units/min, p<0.01). Similarly, during copper sulfate–mediated oxidation, lipid peroxides were generated significantly more rapidly in control LDL compared with LDL isolated from vitamin E–supplemented subjects (Figure 6).

Figure 7 shows TBARS generated during both endothelial cell– and copper sulfate–mediated oxidation as a percentage of control LDL samples. Although there was considerable individual variation, LDL from the subjects supplemented with vitamin E showed a mean 42% reduction in the amount of TBARS generated during endothelial cell–mediated oxidation and a 68% reduction in TBARS generated during copper sulfate–mediated oxidation compared with control LDL values (p<0.001 and p<0.001, respectively).

The extent of macrophage degradation was also reduced in LDL samples from vitamin E–supplemented subjects compared with samples from control subjects. Again, there was substantial between-subject variation, but overall there was a 45–65% reduction in the extent of macrophage degradation (Figure 8) (p<0.001).

During phase 2 of the study, plasma vitamin E levels were moderately correlated with LDL vitamin E levels (r=0.54, p<0.3). All measures of lipid oxidation were strongly correlated with LDL vitamin E levels: for conjugated-diene lag time, r=0.91 and p<0.005; for conjugated-diene propagation rate, r=−0.76 and p<0.05; for 4-hour lipid peroxide levels, r=−0.91 and p<0.005; and for TBARS, r=−0.93 and p<0.001 (copper sulfate) and r=−0.82 and p<0.05 (endothelial cell).

**Discussion**

The goal of this study was to determine the effectiveness of dietary supplementation with three commonly

<table>
<thead>
<tr>
<th>Period A, placebo period</th>
<th>Vitamin E period</th>
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<tbody>
<tr>
<td>TC (mg/dL)</td>
<td></td>
</tr>
<tr>
<td>234±27</td>
<td>269±48*</td>
</tr>
<tr>
<td>LDL cholesterol (mg/dL)</td>
<td></td>
</tr>
<tr>
<td>156±23</td>
<td>168±38</td>
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<tr>
<td>HDL cholesterol (mg/dL)</td>
<td></td>
</tr>
<tr>
<td>56.2±6.4</td>
<td>56.4±5.7</td>
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<tr>
<td>TG (mg/dL)</td>
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</tr>
<tr>
<td>116±79</td>
<td>193±185</td>
</tr>
<tr>
<td>TC/HDL cholesterol (mg/mg)</td>
<td></td>
</tr>
<tr>
<td>4.2±0.5</td>
<td>4.8±0.8</td>
</tr>
<tr>
<td>LDL TC/TG (mg/mg)</td>
<td></td>
</tr>
<tr>
<td>9.6±3.3</td>
<td>10.6±3.7</td>
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<tr>
<td>LDL β-carotene (μg/mg)</td>
<td></td>
</tr>
<tr>
<td>0.26±0.20</td>
<td>0.37±0.25*</td>
</tr>
<tr>
<td>LDL α-tocopherol (μg/mg)</td>
<td></td>
</tr>
<tr>
<td>5.4±1.4</td>
<td>13.5±3.6f</td>
</tr>
</tbody>
</table>

Period A, placebo period; TC, total cholesterol; LDL, low density lipoprotein; HDL, high density lipoprotein; TG, triglyceride.

*p<0.05 vs. period A.

†p<0.001 vs. period A.
used natural antioxidants to confer antioxidant activity to LDL. At the time this study was initiated, we, as well as other laboratories, had demonstrated that vitamin E was effective in protecting LDL from oxidative stress in vitro, and other reports suggested that dietary supplementation with vitamin E increased LDL vitamin E content and conferred additional protection to the LDL. However, there were no reports on long-term effects of β-carotene nor reports of combined use of these dietary supplements. Because these two antioxidants, as well as vitamin C, have been associated with protection from coronary artery disease in epidemiological studies, we sought to determine their effectiveness, used singly and in combination, on the protection of LDL.

Our first goal was to determine whether dietary supplementation with β-carotene would increase plasma and LDL levels of β-carotene and to determine if this resulted in increased antioxidant activity, as measured by our in vitro assays. Supplementation with 60 mg/day of β-carotene resulted in impressive increases in plasma and LDL of the β-carotene content in all subjects. After 3 months of supplementation, LDL β-carotene levels were increased nearly 20-fold, and despite this, these enriched LDL samples were not more resistant to in vitro oxidation, as measured by generation of TBARS or inhibition of macrophage uptake after being subjected to copper-induced oxidation. In fact, if anything, there were slightly increased rates of macrophage degradation. These results agree with those recently published by Princen et al. In that study, subjects were fed β-carotene (20 mg/day) for 14 weeks, and then LDL was isolated and tested for susceptibility to lipid peroxidation in vitro. β-Carotene content of LDL was increased 17-fold, and despite this, LDL lipids were oxidized nearly as rapidly as LDL isolated from control subjects, as judged by formation of conjugated dienes, both in terms of lag times and rates of propagation. Thus, feeding large doses of β-carotene for 3 months, despite marked enrichment of LDL with β-carotene, did not confer antioxidant protection to the LDL, at least as measured by these assays.

A second goal of these studies was to determine the effect on LDL of combined supplementation with β-carotene and vitamin E. Supplementation with vitamin E (1,600 mg/day) during period C led to a more than doubling of vitamin E content in LDL and substantially increased resistance to oxidation. However, this effect appears to be directly due to the vitamin E alone and not a synergistic effect of the two in combination. During the period of combined supplementation, LDL β-carotene levels did not change when the vitamin E supplementation was initiated. In addition, the fact that significant protection to LDL was seen during phase 2, when β-carotene levels had fallen to baseline values, strongly suggests that it was the α-tocopherol that provided the protection seen during period C. In fact, the effect of vitamin E was even more impressive in phase 2 when the β-carotene was stopped. Additional support for a direct effect of vitamin E is the strong correlation between LDL vitamin E content and measures of LDL oxidation, such as conjugated dienes and TBARS. Importantly, this study demonstrates that the antioxidant activities of vitamin E are persistent, remaining present during 10 months of continuous supplementation. Finally, in a companion article in this issue of Arteriosclerosis and Thrombosis, we demonstrate that supplementation with vitamin E alone, either the racemic mixture used in this study or the naturally occurring vitamin E isomer RRR-α-tocopherol, confers equal degrees of protection, at least when used at 1,600 mg/day. Thus, all of these data strongly support a direct role for vitamin E conferring protection to the LDL.
Vitamin C is a potent water-soluble antioxidant and spares lipophilic antioxidants in LDL in in vitro studies when LDL is exposed to oxidizing conditions.\textsuperscript{32-34} Theoretically, the addition of vitamin C might increase plasma levels of \( \beta \)-carotene and vitamin E as a result of this sparing effect. Indeed, during period D, when vitamin C (2 g/day) was administered, plasma levels of both \( \beta \)-carotene and vitamin E increased significantly (Table 1). \( \beta \)-Carotene content also increased significantly in the LDL, and although vitamin E levels increased slightly as well, this was not statistically significant. Despite this increase in LDL \( \beta \)-carotene levels, however, the LDL isolated from the treated subjects during this time (period D) was not more resistant to oxidation than was LDL isolated during period C (when \( \beta \)-carotene and vitamin E were given alone). It should be appreciated that during the isolation and dialysis of LDL, all the vitamin C present in plasma would be lost, because it is a small hydrophilic molecule. Thus, there would be no vitamin C present in the isolated LDL to provide protection from oxidation as tested in our in vitro assays. By protecting the endogenous content of other lipophilic oxidants in LDL, it was conceivable that the isolated LDL would be better protected. Furthermore, recent data from RetSKy et al\textsuperscript{30} suggest that, at least in vitro, the oxidation product of vitamin C, dehydro-L-ascorbic acid, may actually modify LDL in a way to make it more resistant to oxidative modification. However, our experiments did not demonstrate this.

In these studies, we have used isolated LDL to test the ability of administered antioxidants to protect against oxidation. This strategy makes the assumption that it is the direct protection of LDL that is the mode by which these agents might be conferring the protection against coronary artery disease noted in several epidemiological studies. However, although direct protection to LDL may indeed be one of the mechanisms involved, it by no means excludes effects due to antioxidant activity elsewhere. As recently discussed, the mechanisms by which LDL is oxidized within the artery wall are only poorly understood.\textsuperscript{10} All of the cells in the artery wall, including endothelial cells, smooth muscle cells, and macrophages, have the capacity to initiate oxidation of LDL, and LDL bound to proteoglycans present in the matrix may be subject to copper-mediated oxidation as well.\textsuperscript{31} In the in vivo situation, it is possible that \( \beta \)-carotene, for example, may well have antioxidant properties by altering cell-mediated oxidation of LDL. This may be analogous to the recent demonstration by Parthasarathy\textsuperscript{52} that a water-soluble form of probucol could inhibit the ability of macrophages to initiate oxidation of LDL. Indeed, studies by Navab et al\textsuperscript{53} have recently demonstrated that \( \beta \)-carotene can inhibit the ability of a coculture of smooth muscle cells and endothelial cells to initiate oxidation of LDL and presumably the generation of a minimally modified (oxidized) LDL. Thus, \( \beta \)-carotene and also vitamin E may have the ability to moderate lipid peroxidation within cells in ways that could have profound effects on the ability of cells to initiate oxidation of LDL in vivo. Furthermore, \( \beta \)-carotene is the only naturally occurring antioxidant that has been studied in a double-blind design and shown to have an impact on coronary events. Gaziano et al\textsuperscript{54} have made a preliminary report of 333 physicians who, at the onset of their study, already had evidence of coronary artery disease. They reported that those physicians taking \( \beta \)-carotene (50 mg on alternate days) had approximately 40% fewer coronary events than the group not taking \( \beta \)-carotene. Despite the fact that \( \beta \)-carotene did not confer direct protection to the LDL in our study, it should not be concluded that it has no role as an antioxidant in the prevention of coronary artery disease.

In this study, we administered large doses of these antioxidants for extended periods of time. Although the subjects consumed 60 mg/day \( \beta \)-carotene for 8 months and 1,600 mg/day vitamin E for 10 months, there were no significant abnormalities of any laboratory data nor any clinically adverse effects. All subjects developed some degree of hypercarotenodermia. In only one subject was this a concern, and her dosage was decreased from 60 to 30 mg/day at the end of period B. It is of interest that she had the highest plasma \( \beta \)-carotene levels. Although there are reports that suggest that simultaneous supplementation of \( \beta \)-carotene and vitamin E influences their individual plasma levels,\textsuperscript{55,56} we did not observe this, although our study was not optimally designed to test this effect. The addition of vitamin E did not further increase plasma or LDL \( \beta \)-carotene levels above that reached with \( \beta \)-carotene supplementation alone. Furthermore, LDL levels of vitamin E remained essentially unchanged when both \( \beta \)-carotene and vitamin C supplementation was stopped. In contrast, the additional supplementation with vitamin C (during period D) led to a significant rise in plasma levels of both \( \beta \)-carotene and vitamin E. LDL \( \beta \)-carotene content also rose significantly, and although LDL vitamin E content rose slightly, this was not significant. Although the mechanism by which vitamin C increased levels of \( \beta \)-carotene and vitamin E is unknown, this finding may have important implications for future antioxidant trials and needs further evaluation.

Prior studies that have used vitamin E dosages comparable to those used in the current study have not shown consistent effects on lipoprotein levels.\textsuperscript{27} However, our study found that ingestion of vitamin E was associated with a slight rise in total, LDL, and HDL cholesterol levels. Some or all of this elevation in lipid levels may be related to factors other than vitamin E ingestion. Factors such as relaxed dietary compliance may have played an important role in this long-term study, despite the fact that weight and dietary food records did not document such changes. In our companion article,\textsuperscript{49} in which we used comparable doses of two different preparations of vitamin E, we failed to find any rise in cholesterol levels, although this was a study of shorter duration. Given the current interest in using vitamin E as an antioxidant to prevent atherosclerosis, it will be important to carefully evaluate its effect on lipid levels in a larger population over a range of dosages.

In conclusion, the present study and the companion study\textsuperscript{49} comparing two different forms of vitamin E, clearly document that dietary supplementation with large doses of vitamin E can significantly increase the LDL content of vitamin E and that this, in turn, confers significant protection against oxidative stress. On the other hand, \( \beta \)-carotene supplementation, although significantly increasing LDL content, did not confer such protection. Vitamin C increased plasma levels of both \( \beta \)-carotene and vitamin E and thus, although not pro-
viding enhanced direct protection to LDL, may provide enhanced antioxidant protection through other mechanisms. When taking into account all of the available experimental, epidemiological, and clinical data, we think that it is rational to use a combination of vitamin E, β-carotene, and vitamin C in initial clinical trials to test the antioxidant hypothesis. However, if these agents do confer protection against coronary artery disease, it is likely that they are each working in different ways, and eventually, additional clinical trials with each agent individually may be indicated.

Acknowledgments

We thank Dr. Daniel Steinberg for advice and support and Dr. Lawrence Machlin of Hoffmann-La Roche, Inc., for help and for supplying the vitamins and placebos for this study. We thank Felicidad Almazan, Elizabeth Miller, and Jennifer Patterson for expert technical assistance. Haven Webb provided outstanding help in the conduct of these studies, and Barbara Grasse provided expert dietary counseling.

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Arterioscler Thromb Vasc Biol. 1993;13:590-600
doi: 10.1161/01.ATV.13.4.590
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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