Supplementation With Vitamin E but Not β-Carotene In Vivo Protects Low Density Lipoprotein From Lipid Peroxidation In Vitro

Effect of Cigarette Smoking

Hans M.G. Princen, Geert van Poppel, Carla Vogelegang, Rien Buytenhek, and Frans J. Kok

Several lines of evidence suggest that oxidatively modified low density lipoprotein (LDL) is atherogenic and that antioxidants may protect LDL against oxidation. In addition, cigarette smoking is known to induce oxidant stress. We have examined the effect of ingestion of the antioxidants D,L-α-tocopherol (vitamin E) and β-carotene and of smoking on the resistance of LDL against copper-mediated oxidation. Six healthy nonsmoking volunteers ingested 1,000 IU/day D,L-α-tocopherol acetate for 7 days. After vitamin E ingestion concentrations of α-tocopherol in plasma and LDL increased 3.0- and 2.4-fold, respectively. Simultaneously, the oxidation resistance of LDL was elevated significantly (+41%), and the rate of oxidation was decreased significantly (—19%). The increase in α-tocopherol content of LDL and the increase in resistance time were highly correlated (r²=0.89, p=0.014). Eight weeks after termination of the vitamin E intake, α-tocopherol concentrations in plasma and LDL and oxidation resistance of LDL had returned to baseline values. In smokers (n=46), plasma levels of vitamin C (—26%) and concentrations of β-carotene (—44%, —43%) and total carotenoids (—23%, —29%) in plasma and LDL, respectively, were significantly lower compared with nonsmokers (n=23). No differences were found in α-tocopherol content of LDL and the susceptibility of LDL to lipid peroxidation in both groups. Supplementation of a group of smokers in a 14-week randomized, double-blind, placebo-controlled intervention trial with β-carotene resulted in a 16.6- and 5.0-fold increase of LDL β-carotene and total carotenoid content, respectively. Similar increases were found in plasma without changes in other vitamins. Comparison of the changes in the length of the resistance time between the β-carotene group (n=23) and the placebo group (n=23) showed no significant differences. We conclude that ingestion of vitamin E strongly protects LDL against oxidative modification, whereas β-carotene is not effective, and that smoking does not alter the oxidation resistance of LDL.

KEY WORDS • low density lipoprotein oxidation • vitamin E • β-carotene • cigarette smoking

Elevated plasma levels of low density lipoprotein (LDL) are associated with an accelerated development of atherosclerotic lesions, which is one of the main causes of coronary artery disease. Recent experimental evidence strongly suggests that LDL does not accumulate in its native form but that it must be oxidatively modified for its effective uptake by the macrophages that are present in the subendothelial space of the vascular wall. In this so-called scavenger receptor-mediated process, macrophages are transformed into lipid-laden foam cells, thereby giving rise to fatty streaks and plaques. Oxidative modification of LDL has been reported to be effected by cultured endothelial cells, smooth muscle cells, and macrophages themselves. These cells are able to generate reactive free radicals as a first step in the lipid peroxidation of unsaturated fatty acids that are present in LDL. Strong evidence exists that lipid peroxidation of the LDL particle can start only after depletion of all endogenous lipophilic antioxidants, with α-tocopherol acting as the first antioxidant line of defense and β-carotene as the last protective barrier. If so, this would provide an explanation for the observation in epidemiological studies that there is an inverse relation between plasma levels of dietary antioxidants and the risk of cardiovascular disease. Moreover, the LDL oxidation hypothesis may help to explain the well-known association between smoking and cardiovascular disease. Smoking has been recognized as a major risk factor for the development of coronary artery disease, as it accelerates the atherosclerotic process. The underlying mechanisms appear to include changes in vascular tone and cardiodynamics and a decrease in the plasma levels of high density lipoprotein and high density lipoprotein apoproteins, as well as modifications of LDL and other lipoproteins, which make the latter more atherogenic. It has been shown that cigarette smoke contains a large amount of free radicals, with peroxyl radicals being the most abundant.
conceivable that the oxidative challenge during smoking may lead to a (partial) depletion of the antioxidative capacity of LDL in the circulation, rendering it more susceptible to oxidative modification. Indeed, a decrease in the plasma levels of lipophilic β-carotene has been reported in smokers.\(^ {30,31}\)

The cell-mediated oxidative changes can be mimicked by incubation of LDL with ions of transition metals, such as copper or iron.\(^ {6,32}\) It is assumed that the cell-free oxidized LDL resembles biologically modified LDL in many if not all aspects.\(^ {32}\) During oxidation of LDL a complex sequence of reactions takes place. The first stage of these reactions leads to formation of conjugated dienes, lipid hydroperoxides, and aldehydes.\(^ {13}\) The kinetics of the lipid peroxidation in LDL can be measured continuously by monitoring the increase in the absorption at 234 nm, which develops during the conversion of polyunsaturated fatty acids into fatty acid hydroperoxides with conjugated double bonds.\(^ {14}\)

In this study with healthy human volunteers, we have assessed the effects of the ingestion of α-tocopherol on the resistance of LDL isolated from these subjects against in vitro lipid peroxidation. Second, we have compared the susceptibility of LDL from heavy smokers and nonsmokers to in vitro lipid peroxidation. Finally, we used the group of heavy smokers (those who smoked more than 15 cigarettes/day) to evaluate the effect of the ingestion of β-carotene on the resistance of LDL to oxidation. By ingestion the antioxidants are physiologically incorporated into the LDL particle, a method that is preferable to the in vitro addition of these lipophilic compounds to LDL, as has been done by others.\(^ {8,14,33}\)

We found that in vivo supplementation with α-tocopherol but not with β-carotene increased the oxidation resistance of LDL and that smoking did not change the susceptibility of LDL to lipid peroxidation.

Methods

Experimental Protocol: Vitamin E Supplementation

Seven healthy nonsmoking volunteers (male, aged 27–46 years) participated in this study. The subjects were normolipidemic and did not use any medication. For 7 days they ingested a dose of 1,000 IU vitamin E/day in the form of dl-α-tocopherol acetate (1,000 mg; Roche, Mijdrecht, The Netherlands). Vitamin E was taken in capsule form in three equal doses just before breakfast, lunch, and supper. Six participants complied with the study protocol, whereas one person ingested vitamin E during the first 4 days and refrained from taking it afterward. Blood was collected in EDTA-containing Vacutainer tubes (1 mg/ml) in the morning just before, directly after, and 8 weeks after the last dose. The blood was immediately placed on ice and cooled to 4°C, and plasma was prepared, frozen in liquid nitrogen in small portions (leaving as little empty space as possible in the tubes), and stored at −80°C. The procedure was completed within 1 hour.

Experimental Protocol: Nonsmokers, Smokers, and β-Carotene Study

Healthy male employees of the AMEV Insurance Company, the Taxation Office, and the Power Company at Utrecht, The Netherlands, were asked to participate in this study. After completing a questionnaire that documented general anthropometric data, such as age, height, and weight and that assessed smoking habits, medication use, and vitamin intake, 23 nonsmokers and 46 cigarette smokers were selected for this study. Both groups did not use preparations containing retinol, carotenoids, or other dietary antioxidants, and no one reported exposure to chemicals during working or leisure time. Nonsmokers who had never smoked, did not live together with a smoking partner, and were not exposed to smoke during their working time. In the smoking group all participants had smoked at least 15 cigarettes/day for over 2 years. The number of cigarettes (in packs) smoked daily times the number of years of smoking is defined as a pack-year. The smoking volunteers were prestratified according to age and duration and quantity of smoking and were randomly assigned to either β-carotene (20 mg capsules, Roche) or placebo treatment. The participants were instructed to take the capsules daily with the evening meal: 2 capsules/day during the first 2 weeks, followed by 1 capsule/day for the next 12 weeks. All participants completed the trial. Smoking habits and β-carotene intake during the trial were monitored by measurement of the plasma cotinine and β-carotene levels, respectively. Blood was collected into EDTA-containing tubes before and after the 14 weeks of treatment and placed on ice immediately after collection. Plasma was prepared and stored at −80°C. All participants in these studies gave their informed consent.

Preparation and Oxidation of Low Density Lipoprotein

The procedure for preparation and lipid peroxidation of LDL was adapted from the method described by Esterbauer et al\(^ {14}\) with some major modifications. In detail, from each subject 1 ml of frozen EDTA-plasma (1 mg/ml) that had been stored at −80°C was rapidly thawed and used for preparation of LDL by ultracentrifugation (18 hours at 40,000 rpm [285,000g] in a Beckman SW40 rotor in a Beckman L7-55 ultracentrifuge at 4°C) according to Terpstra et al\(^ {34}\) without prestraining with amido black. To protect the LDL against oxidative modification during isolation, 10 μM EDTA was added to each density solution. The entire procedure until the beginning of the oxidation experiment was performed at 4°C. LDL was isolated from the appropriate density fraction (d = 1.019–1.063 g/ml) of the gradient, and a sample was taken for cholesterol and protein determinations, during which time the rest of the fraction was stored under nitrogen in the dark at 4°C. To make use of the protective effect of EDTA against oxidation of LDL\(^ {1,33}\) and to keep the time between isolation and the oxidation experiment short, the LDL was not extensively dialyzed\(^ {34}\) but was used immediately in the oxidation assay after measurement of the protein content. Because dialysis was omitted, we did not add the lipophilic antioxidant butylated hydroxytoluene (BHT) to plasma after it was collected. The LDL-containing fraction was diluted with a solution of the same density (1.18 M NaCl and 10 μM EDTA) to a protein concentration of 0.17 mg/ml, and sodium phosphate (pH 7.4) was added to a final concentration of 10 mM. The assay mixture was brought to 37°C, and oxidation was initiated by the addition of 40 μM CuSO\(_4\). The kinetics of the LDL oxidation was followed by continuously monitoring the change in absorbance at...
234 nm\textsuperscript{14} in a thermostat-controlled (37°C) computerized Kontron-Uvikon 930 spectrophotometer equipped with a 10-position automatic sample changer (Tegimenta AG, Rotkreuz, Switzerland). After setting the initial absorbance to zero, the increase in the 234-nm absorption was recorded every 3 minutes during a 5-hour period. Absorbance curves of LDL preparations obtained from one subject before, immediately after, and 8 weeks after cessation of vitamin E ingestion were determined in parallel. The same was done with LDL preparations obtained before and after \( \beta \)-carotene supplementation and from smokers and nonsmokers. The time profile of the 234-nm absorption shows three distinct phases: 1) a lag phase, during which absorption does not increase or increases only slightly, indicating that the LDL is resistant against oxidation; 2) a propagation phase, during which the absorbance at 234 nm rapidly increases to a maximum value. This period is indicative of the autocatalytic chain reaction of the lipid peroxidation process. After reaching the maximum value, the conjugated dienes slowly decrease by decomposition to form aldehydes [3] the decomposition phase].\textsuperscript{13-15} A tangent is drawn to the steep segment of the curve of the propagation phase and extrapolated to the horizontal (time) axis. The interval between the addition of Cu\textsuperscript{2+} ions and the intersection point on this axis is defined as the lag time and is expressed in minutes. The propagation rate is calculated from the slope of the tangent of the curve during the propagation phase, using a molar extinction coefficient for conjugated dienes of \( \epsilon_{234}=29,500 \text{ M}^{-1} \cdot \text{cm}^{-1}\text{.} \textsuperscript{14} \) and is expressed as nanomoles of dienes formed per minute per milligram of LDL protein.

Each LDL preparation was oxidized in two or three consecutive oxidation runs on the same day. The values shown for lag time and propagation rate are means of the values thus obtained. The intra-assay coefficients of variation for lag time and propagation rate were 2.6\% and 3.1\%, respectively, on oxidation of the same LDL in three consecutive oxidation runs on 1 day.

The interassay coefficients of variation were 4.9\% and 7.4\%, respectively, and were obtained by oxidation of LDL from the same subject that was prepared on different days. In every oxidation run, one reference LDL prepared from a reference plasma stored at \(-80^\circ\text{C}\) was present as a control. Oxidation runs with a deviation higher than 10\% from the mean lag time and propagation rate of former measurements were omitted.

By using this highly standardized method, we found no differences in lag time and propagation rate between LDL prepared from plasma frozen in liquid nitrogen and from freshly collected plasma from the same subject. In addition, no differences in these parameters were found after storage of plasma at \(-80^\circ\text{C}\) for as long as 6 months.

**Analytical Measurements**

Total cholesterol was determined enzymatically by using commercially available reagents (CHOD-PAP, No. 236,691, Boehringer-Mannheim, Mannheim, FRG). The protein content of the LDL preparations was measured according to Lowry et al.\textsuperscript{35} Total \textit{trans}-retinol, \( \alpha \)-tocopherol, \( \beta \)-carotene, and total carotenoids in the plasma and LDL (stored at \(-80^\circ\text{C}\) were assayed by high-performance liquid chromatography with colorimetric detection.\textsuperscript{36} Vitamin C (the sum of L-ascorbic and dehydro-L-ascorbic acids) was assessed in whole blood by high-performance liquid chromatography with fluorimetric detection.\textsuperscript{37} Plasma cotinine levels were determined by gas-liquid chromatography.\textsuperscript{38}

**Statistical Analysis**

Initial baseline values and the changes in these values during the intervention period for the vitamin E and \( \beta \)-carotene supplementation studies were compared by using the Wilcoxon test. Differences between nonsmokers and smokers were tested for significance by the Mann-Whitney two-sample test. To determine the correlations between the increase of \( \alpha \)-tocopherol concentrations in LDL expressed per milligram of protein or per micromole of cholesterol and the increase in lag time, Spearman's rank correlation coefficients (\( r_s \)) were calculated. Values are given as mean±SD. All data analyses were performed using the NCSS software package (version 5.01) developed by Dr. J.L. Hintze, Kaysville, Utah.

**Results**

**Vitamin E Supplementation**

Ingestion of 1,000 IU vitamin E/day for 1 week resulted in a significant increase in the concentration of \( \alpha \)-tocopherol in plasma (3.0-fold) and the \( \alpha \)-tocopherol content in LDL (2.4-fold) without concomitant changes in the concentrations of other antioxidants and plasma cholesterol (Table 1). The rise of the \( \alpha \)-tocopherol content in LDL was also 2.4-fold higher after correction for the cholesterol content of LDL (before intake, 4.2±1.0 and after intake, 10.1±1.7 nmol \( \alpha \)-tocopherol/\( \mu \)mol cholesterol). Only 33.2±8.9\% of the total plasma \( \alpha \)-tocopherol was contained in LDL, and this value was not significantly different after the 7-day supplementation period (30.2±6.7\%). Resistance of LDL against oxidative modification as assessed by determination of the lag time did not show much variation among subjects before vitamin E ingestion (mean, 108±6 minutes; range, 101–114 minutes) (Table 1 and Figure 1). After the intake of vitamin E the lag phase for all subjects was markedly increased (Figure 1), leading to a significant rise in lag time to 152±17 minutes (+41\%). In contrast, the propagation rate decreased significantly (−19\%).

The increase in the \( \alpha \)-tocopherol content of LDL (\( \Delta \alpha \)-tocopherol expressed as nanomoles per milligram of protein) and the increase in lag time were highly correlated in the individual subjects (\( r_s=0.89, p=0.014 \)) (Figure 2). The correlation coefficient was the same after correction for the cholesterol content of the LDL. No significant correlation was found between the decrease in propagation rate and the increase in the LDL \( \alpha \)-tocopherol content in the individual subjects. Eight weeks after termination of the vitamin E intake, the concentration of \( \alpha \)-tocopherol in plasma and LDL, the lag time, and the propagation rate had returned to their initial values before supplementation (Table 1). Again, no changes were found in the other parameters measured, except for plasma all-\textit{trans}-retinol, which was significantly elevated compared with the starting value, possibly because of an increased intake of vitamin A–rich food. When the \( \alpha \)-tocopherol content in LDL from volunteers with baseline levels of
TABLE 1. Effect of Supplementation With Vitamin E In Vivo on Concentrations of Antioxidants in Plasma and on Low Density Lipoprotein, Plasma Cholesterol, and Oxidative Modification of Low Density Lipoprotein

<table>
<thead>
<tr>
<th>Antioxidants in plasma (μM)</th>
<th>Before supplementation</th>
<th>After 7 days' supplementation</th>
<th>8 Weeks after end of supplementation</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Tocopherol</td>
<td>29.5±4.8*</td>
<td>87.7±10.7**†</td>
<td>29.6±6.9†</td>
</tr>
<tr>
<td>(25.1-38.5)</td>
<td>(75.7-99.8)</td>
<td>(23.4-42.3)</td>
<td></td>
</tr>
<tr>
<td>Retinol</td>
<td>1.77±0.18‡</td>
<td>1.81±0.35</td>
<td>2.25±0.50‡</td>
</tr>
<tr>
<td>(1.54-2.03)</td>
<td>(1.34-2.32)</td>
<td>(1.61-3.00)</td>
<td></td>
</tr>
<tr>
<td>β-Carotene</td>
<td>0.44±0.19</td>
<td>0.49±0.25</td>
<td>0.39±0.15</td>
</tr>
<tr>
<td>(0.23-0.79)</td>
<td>(0.10-0.89)</td>
<td>(0.08-0.64)</td>
<td></td>
</tr>
<tr>
<td>Antioxidants in LDL (nmol/mg protein)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Tocopherol</td>
<td>11.7±3.3*</td>
<td>28.0±4.9**†</td>
<td>9.7±1.7†</td>
</tr>
<tr>
<td>(8.2-17.2)</td>
<td>(22.3-35.6)</td>
<td>(6.4-11.6)</td>
<td></td>
</tr>
<tr>
<td>Retinol</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>β-Carotene</td>
<td>0.36±0.22</td>
<td>0.35±0.17</td>
<td>0.31±0.15</td>
</tr>
<tr>
<td>(0.11-0.73)</td>
<td>(0.14-0.64)</td>
<td>(0.11-0.50)</td>
<td></td>
</tr>
<tr>
<td>Plasma cholesterol (mM)</td>
<td>5.14±0.82</td>
<td>5.11±1.01</td>
<td>4.98±0.84</td>
</tr>
<tr>
<td>(3.89-6.18)</td>
<td>(3.55-6.38)</td>
<td>(3.64-6.26)</td>
<td></td>
</tr>
<tr>
<td>Oxidation characteristics</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lag time (min)</td>
<td>108±6*</td>
<td>152±17**†</td>
<td>105±6†</td>
</tr>
<tr>
<td>(101-114)</td>
<td>(135-172)</td>
<td>(98-116)</td>
<td></td>
</tr>
<tr>
<td>Propagation rate (nmol/dienes/min/mg LDL protein)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.5±0.4*</td>
<td>6.1±0.3**†</td>
<td>7.3±0.6†</td>
<td></td>
</tr>
<tr>
<td>(6.8-7.9)</td>
<td>(5.4-6.6)</td>
<td>(6.5-8.1)</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean±SD for six subjects; values for the participant who ingested vitamin E for 4 days only are not included. Range is given in parentheses.

LDL, low density lipoprotein.

*p<0.05; †p<0.05; ‡p<0.05.

α-tocopherol (i.e., before or 8 weeks after the intervention) were compared with the lag time or propagation rate for the same subjects, no correlations could be found. In contrast, both resistance against oxidation ($r_c = 0.88$, $p=0.015$) and maximal oxidation rate of LDL ($r_c = -0.72$, $p=0.039$) were strongly correlated with α-tocopherol levels contained in LDL after 7 days of vitamin E intake.

Effect of Cigarette Smoking

Table 2 summarizes some of the characteristics of the smokers and nonsmokers. Smoking habits are reflected

![Figure 1](http://atvb.ahajournals.org/)

**Figure 1.** Plot showing effect of vitamin E supplementation in vivo on the α-tocopherol content in low density lipoprotein (LDL) and the resistance against oxidation. Lag time and α-tocopherol content (expressed as nanomoles per milligram of LDL protein) were determined as described in "Methods" in LDLs from seven subjects before and after supplementation with 1,000 IU vitamin E/day for 7 days. One participant (○) ingested vitamin E during the first 4 days and refrained from taking it during the following 3 days.

![Figure 2](http://atvb.ahajournals.org/)

**Figure 2.** Scatter plot showing relation between the increase of α-tocopherol content in low density lipoprotein (LDL) and the increase in lag time for individual subjects. Depicted values were obtained by subtraction of the values for α-tocopherol content and lag time of LDL of the seven individual subjects before supplementation from the values after supplementation. The latter values were obtained from Figure 1.
TABLE 2. Characteristics of the Study Groups of Nonsmokers and Smokers

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Nonsmokers (n=23)</th>
<th>Smokers (n=46)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>43.0±10.0 (29.7-61.9)</td>
<td>39.2±9.8 (24.3-61.1)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23.8±2.2 (19.6-29.6)</td>
<td>24.1±2.6 (18.2-30.6)</td>
</tr>
<tr>
<td>Plasma cholesterol (mM)</td>
<td>5.56±1.06 (3.79-7.80)</td>
<td>6.14±1.22 (3.90-8.10)</td>
</tr>
<tr>
<td>Years of smoking</td>
<td>0.7±0.5 (0.1-1.9)</td>
<td>21.2±9.7 (4-44)</td>
</tr>
<tr>
<td>Number of cigarettes/day</td>
<td>0.7±0.5 (0.1-1.9)</td>
<td>20.5±4.9 (15-35)</td>
</tr>
<tr>
<td>Plasma cotinine (ng/ml)</td>
<td>308.9±117.8* (119.0-613.7)</td>
<td></td>
</tr>
</tbody>
</table>

Values are mean±SD, with the range in parentheses. Body mass index (BMI) is body weight in kilograms divided by the square of the height in meters. *p<0.0001 compared with nonsmokers.

by plasma cotinine levels, a metabolite of nicotine. Plasma levels of the antioxidants vitamin C (~26%), β-carotene (~44%), and total carotenoids (~23%) but not of vitamin E were significantly decreased in smokers compared with the nonsmoker group (Table 3). The content of β-carotene (~43%) and total carotenoids (~29%) in the LDL of smokers was also significantly lower than that in nonsmokers. Regardless of these differences, no changes were found in the oxidation resistance and the maximal rate of oxidation of the LDL from smokers compared with nonsmokers (Table 3).

In the smokers' group no significant correlation was found between plasma levels of antioxidants and number of cigarettes per day, years of smoking, and pack-years. A similar observation has been previously reported by others. 30

β-Carotene Supplementation

Table 4 shows that baseline characteristics of both groups before intake of β-carotene or placebo did not differ significantly. In male smokers, supplementation with β-carotene in a 14-week intervention trial resulted in a 16.0- and a 3.4-fold increase in plasma β-carotene and total carotenoids, respectively, without significant changes in other vitamins (Table 4), plasma cholesterol, and cotinine levels (data not shown). The β-carotene and total carotenoid contents of LDL were elevated significantly, to 3.66±2.28 nmol/mg (16.6-fold) and 5.90±2.95 nmol/mg (5.0-fold), respectively, compared with baseline values of 0.22±0.14 nmol/mg for β-carotene and 1.17±0.49 nmol/mg for total carotenoids (n=23). Of the total β-carotene in plasma, about 70-80% is contained in LDL in both conditions. A small and significant prolongation of the lag phase of LDL after β-carotene supplementation was observed. No

Table 3. Effect of Cigarette Smoking on Concentrations of Antioxidants in Plasma and Low Density Lipoprotein and on Oxidative Modification of Low Density Lipoprotein

<table>
<thead>
<tr>
<th>Antioxidants in plasma (μM)</th>
<th>Nonsmokers (n=23)</th>
<th>Smokers (n=46)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin C</td>
<td>55.1±12.3 (38.2-81.6)</td>
<td>40.9±19.3 (8.8-96.1)</td>
<td>0.0012</td>
</tr>
<tr>
<td>α-Tocopherol</td>
<td>28.1±5.4 (19.2-37.8)</td>
<td>31.3±6.6 (19.3-46.0)</td>
<td>NS</td>
</tr>
<tr>
<td>Retinol</td>
<td>2.10±0.35 (1.10-2.96)</td>
<td>2.36±0.66 (1.45-3.33)</td>
<td>NS</td>
</tr>
<tr>
<td>β-Carotene</td>
<td>0.52±0.32 (0.17-1.40)</td>
<td>0.29±0.13 (0.03-0.57)</td>
<td>0.0015</td>
</tr>
<tr>
<td>Total carotenoids</td>
<td>2.22±0.81 (1.13-3.89)</td>
<td>1.70±0.65 (0.57-3.81)</td>
<td>0.0084</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Antioxidants in LDL (nmol/mg protein)</th>
<th>Nonsmokers (n=23)</th>
<th>Smokers (n=46)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Tocopherol</td>
<td>9.7±1.8 (7.1-13.3)</td>
<td>10.7±1.6 (8.4-19.2)</td>
<td>NS</td>
</tr>
<tr>
<td>Retinol</td>
<td>&lt;0.10 (&lt;0.10)</td>
<td>&lt;0.10 (&lt;0.10)</td>
<td>NS</td>
</tr>
<tr>
<td>β-Carotene</td>
<td>0.35±0.16 (0.10-0.65)</td>
<td>0.20±0.12 (0.04-0.48)</td>
<td>0.0108</td>
</tr>
<tr>
<td>Total carotenoids</td>
<td>1.51±0.62 (0.47-2.84)</td>
<td>1.07±0.45 (0.29-2.13)</td>
<td>0.0183</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Oxidation characteristics</th>
<th>Nonsmokers (n=23)</th>
<th>Smokers (n=46)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lag time (min)</td>
<td>106±9 (90-119)</td>
<td>108±13 (71-132)</td>
<td>NS</td>
</tr>
<tr>
<td>Propagation rate (nmol dienes/min/mg LDL protein)</td>
<td>6.4±0.9 (4.9-8.2)</td>
<td>6.4±0.9 (4.9-8.3)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are mean±SD, with the range in parentheses. LDL, low density lipoprotein; NS, not significant.
vitamin E are important in the susceptibility of LDL to oxidative modification. However, after enhancing the antioxidative modification.

LDL from both nonsmokers' and smokers' groups, we found no significant relation between a-tocopherol levels in LDL and its resistance against lipid peroxidation. This may not seem surprising, as a-tocopherol is a chain-breaking antioxidant that protects lipids by reaction with peroxyl radicals without itself reacting in further chain-propagating steps. However, the addition of a-tocopherol to LDL preparations has not been reported to affect the propagation phase of the reaction. The same investigators have shown that lipid peroxidation starts only after LDL is totally depleted of polyunsaturated fatty acids in LDL against lipid peroxidation. In control LDL or LDL prepared from subjects 8 weeks after cessation of vitamin E ingestion and in the LDL from both nonsmokers' and smokers' groups, we found no significant relation between a-tocopherol levels and lag times. A similar finding has been reported by others and suggests that other antioxidants besides vitamin E are important in the susceptibility of LDL to oxidative modification. However, after enhancing the LDL a-tocopherol content to values normally not found among the population, strong and significant correlations were observed between a-tocopherol levels in LDL and its resistance against lipid peroxidation ($r_s=0.89$) and the maximal oxidation rate ($r_s=-0.72$). This observation indicates that at higher, above-normal a-tocopherol concentrations, vitamin E becomes the most important parameter, which determines the oxidation resistance and the oxidation rate of LDL. The duration of the resistance period of LDL was significantly extended by vitamin E supplementation. Using a different system in which macrophage-mediated LDL oxidation was measured, Jessup et al made a similar observation with LDL from only one subject, as did Dieber-Rothneder et al in a recent report. By comparing the differences in vitamin E content and in lag time of LDL in individual subjects before and after supplementation, we found a strong and significant relation ($r_s=0.89$), suggesting that the changes in a-tocopherol levels in LDL are causal for changes in lag time.

We have found, to our knowledge for the first time, that a-tocopherol incorporated in vivo reduces the progression of the lipid peroxidation reaction in LDL. This may not seem surprising, as a-tocopherol is a known chain-breaking antioxidant that protects lipids by scavenging peroxyl radicals without itself reacting in further chain-propagating steps. However, the addition of a-tocopherol to LDL preparations has not been reported to affect the propagation phase of the reaction. The same investigators have shown that lipid peroxidation starts only after LDL is totally depleted of antioxidants, including a-tocopherol. It is difficult to reconcile our finding with the latter observations. It is conceivable that a-tocopherol incorporated in vivo is more homogeneously distributed over the LDL particle than is vitamin E added in vitro, which might be located predominantly at the surface. By supplementation with vitamin E in vivo, the relative amount of a-tocopherol molecules in the interior of the LDL particle may also be increased. When a-tocopherol in the outer layer of LDL is consumed, the autocatalytic chain reaction will begin at the surface of the particle but will be

**TABLE 4. Effect of β-Carotene Supplementation or Placebo on Plasma Concentrations of Antioxidants and Oxidative Modification of Low Density Lipoprotein in Smokers**

<table>
<thead>
<tr>
<th>β-Carotene (n=23)</th>
<th>Placebo (n=23)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Before</em></td>
<td><em>After</em></td>
</tr>
<tr>
<td>Vitamin C</td>
<td>43.4±17.9</td>
</tr>
<tr>
<td>α-Tocopherol</td>
<td>32.1±6.4</td>
</tr>
<tr>
<td>Retinol</td>
<td>2.26±0.54</td>
</tr>
<tr>
<td>β-Carotene</td>
<td>0.31±0.11</td>
</tr>
<tr>
<td>Total carotenoids</td>
<td>1.84±0.72</td>
</tr>
</tbody>
</table>

Values are mean±SD. LDL, low density lipoprotein.

*Baseline characteristics compared with values after a 14-week intervention or placebo.
†β-Carotene group significantly different from placebo group, p<0.0001.
slowed by the α-tocopherol molecules inside the LDL, resulting in a decreased oxidation rate.

β-Carotene is a known antioxidant that may act as an endogenous oxidation resistance factor. Its ability to resist lipid peroxidation at a site is in agreement with the results of others, who, in different assays in which β-carotene was added to LDL in vitro, also found no inhibitory effects. It is possible that the latter experiments were hampered by the low solubility of β-carotene, resulting in less effective incorporation into the LDL particle. By oral administration, as done in our present study, this problem has been overcome and β-carotene is properly incorporated into LDL. Despite a 16.6-fold higher β-carotene content, this LDL showed only a slight tendency to resist lipid peroxidation in vitro. The discrepancy between this observation and the reported capability of scavenging peroxyl radicals by β-carotene might be explained by the fact that these scavenging experiments were performed in a chemical system with high β-carotene concentrations (above 50 μM). It should be noted that the potency of β-carotene as a free-radical scavenger is strongly increased at low oxygen tension.

In our experiments we applied a normal oxygen pressure. For this and other reasons, it is conceivable that β-carotene is physiologically active as an antioxidant either at the low partial pressures of oxygen found in mammalian tissues or by way of its singlet oxygen—quenching potential, thereby regarding the atherosclerotic process. This contention is supported by a study among American physicians with cardiovascular disease who took 50 mg β-carotene or placebo on alternate days. Those who were assigned β-carotene had a 49% reduction in cardiovascular morbidity and mortality. On the other hand, in a recent case–control study no relation was found between the risk of angina pectoris and plasma levels of β-carotene.

Smoking of cigarettes is an established risk factor for heart and vascular diseases, as it accelerates coronary and peripheral atherosclerosis. Although the atherogenic effects of smoking may be multifactorial, there is increasing evidence that oxidative mechanisms play an important role. Cigarette smoke contains a large amount of free radicals, which have been reported to oxidatively modify LDL. Furthermore, plasma levels of the antioxidants vitamin C, β-carotene, and α-tocopherol are lower in smokers than in nonsmokers. In this study we found that the β-carotene and total carotenoid content of LDL was also significantly diminished in smokers. In contrast, no differences were observed in the amount of α-tocopherol contained in LDL and plasma. We have shown that α-tocopherol is a potent resistance factor in LDL, whereas β-carotene and other carotenoids are not, at least against in vitro lipid peroxidation. These findings may explain the absence of differences in oxidation resistance and maximal oxidation rate between the LDL of smokers and nonsmokers. Furthermore, several other mechanisms may be operative in vivo to protect the organism and LDL against the harmful effects of smoking.

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H M Princen, G van Poppel, C Vogezezang, R Buytenhek and F J Kok

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