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Vitamin E and Fatty Acid Intervention Does Not Attenuate the Progression of Atherosclerosis in Watanabe Heritable Hyperlipidemic Rabbits


Abstract We investigated the effect of different interventions on aortic atherosclerosis in Watanabe rabbits. Four groups of rabbits were fed either an oleic acid–enriched diet (80% of total fat intake) with or without vitamin E supplementation (250 IU/kg) or a diet enriched in linoleic acid with or without vitamin E supplementation for 6 months. At the start of the study, plasma cholesterol concentration was 21.4 ± 3.6 mmol/L (n = 32). The diets did not influence the mean plasma lipids and lipoprotein concentrations except for HDL cholesterol, which was increased more on the oleic acid–enriched diets than on the linoleic acid–enriched diets. Vitamin E levels in plasma and LDL were increased on the oleic acid diet and reduced on the linoleic acid diet. On the latter diet, supplementation of vitamin E was quantitatively less effective in raising plasma or LDL vitamin E levels. The susceptibility of LDL to oxidation was determined in vitro. Both oleic acid–enriched diets increased the lag time by 140% from baseline. The linoleic acid diet supplemented with vitamin E increased lag time by 59%. Linoleic acid alone, however, decreased the lag time by 30%. Similar but inverse effects were seen on LDL oxidation rate. Thus, intervention protected LDL to oxidation in the following order: oleic acid > vitamin E > oleic acid > vitamin E > linoleic acid. Despite the differences in LDL oxidizability induced by the four experimental diets, assessment of aortic atherosclerosis at the end of the 6-month dietary study period revealed no differences among the four study groups. These results suggest that a decrease in the oxidative susceptibility in vitro alone is not sufficient to attenuate atherogenesis when cholesterol levels remain markedly elevated.

Key Words • LDL oxidation • unsaturated fatty acids • antioxidants • atherosclerosis • WHHL rabbits

Oxidation of LDL is believed to be an important step in the process of atherogenesis.1,2 Several lines of evidence support this concept, but the strongest evidence comes from studies in which treatment with the synthetic antioxidant probucol slowed the progression of atherosclerotic lesion formation in Watanabe heritable hyperlipidemic (WHHL) rabbits, an animal model for homozygous familial hypercholesterolemia.3-8

In vitro, several cell types have been shown to oxidize LDL.9-14 The cell-mediated oxidation can be mimicked in a cell-free system with copper as a prooxidant.15 LDL oxidation can be monitored continuously by measuring diene production as a measure of lipid peroxidation. This method allows a sensitive assessment of various indexes that together describe the oxidizability of LDL. Typically, oxidation of LDL can be divided into three consecutive phases: (1) a lag phase during which there is almost no diene production, (2) a propagation phase characterized by a rapid increase in diene production, and (3) a decomposition phase during which diene production reaches its maximum and then the dienes start to decompose. Copper-induced in vitro oxidation of LDL has been used widely to evaluate the effectiveness of antioxidant therapy and dietary intervention on LDL oxidizability. It has been shown that in vitro and in vivo supplementation of LDL with vitamin E, the major lipophilic antioxidant, increases its resistance against oxidation.16-18 This suggests that the oxidation resistance of LDL is determined primarily by its vitamin E (antioxidant) content; in other words, the lag phase should be predictable from the vitamin E (antioxidant) status of a particular LDL sample. However, in our assay with highly reproducible oxidizability, we found no such correlation.15 This indicates that other factors contribute to the oxidizability of LDL.

Traditionally, dietary advice has included a reduction in total fat and replacement of saturated fat by unsaturated fat.19 Diets in which saturated fatty acids are substituted for polyunsaturated fatty acids lower plasma cholesterol levels.20-22 A recent meta-analysis of 27 trials clearly showed that substitution of saturated fat for unsaturated fat created a more favorable lipoprotein risk profile for coronary heart disease.23 Diets rich in monounsaturated fatty acids have also been shown to lower plasma LDL cholesterol levels.20,22-26 Comparatively little is known about the effect of dietary enrichment with unsaturated fatty acids on atherosclerosis. A recent study in African green monkeys showed that dietary polyunsaturated fatty acids reduced atherosclerosis development compared with saturated fat.27 Similar effects in African green monkeys have been observed for dietary fish oil and monounsaturated fat.28,29

One of the first steps in the oxidative modification of LDL is the peroxidation of polyunsaturated fatty acids.16 Theoretically, LDL rich in polyunsaturated fatty acids...
TABLE 1. Fatty Acid Composition and Vitamin E Content of Experimental Diets

<table>
<thead>
<tr>
<th>Component</th>
<th>Oleic</th>
<th>Linoleic</th>
<th>Oleic+E</th>
<th>Linoleic+E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fatty acid</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Palmitic acid (16:0)</td>
<td>6.7</td>
<td>7.0</td>
<td>6.7</td>
<td>7.0</td>
</tr>
<tr>
<td>Stearic acid (18:0)</td>
<td>4.3</td>
<td>2.6</td>
<td>4.3</td>
<td>2.6</td>
</tr>
<tr>
<td>Oleic acid (18:1)</td>
<td>74.6</td>
<td>13.1</td>
<td>74.6</td>
<td>13.1</td>
</tr>
<tr>
<td>Linoleic acid (18:2)</td>
<td>12.8</td>
<td>76.0</td>
<td>12.6</td>
<td>76.0</td>
</tr>
<tr>
<td>Linolenic acid (18:3)</td>
<td>0.1</td>
<td>0.3</td>
<td>0.1</td>
<td>0.3</td>
</tr>
<tr>
<td>Vitamin E, mg/kg</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>d-α-Tocopherol</td>
<td>50.9</td>
<td>46.5</td>
<td>50.9</td>
<td>48.5</td>
</tr>
<tr>
<td>DL-α-Tocopherol acetate</td>
<td></td>
<td></td>
<td>250</td>
<td>250</td>
</tr>
</tbody>
</table>

Four diets were studied: an oleic acid–enriched diet (Oleic), a linoleic acid–enriched diet (Linoleic), and two similar diets, each supplemented with 250 mg vitamin E per kg (Oleic+E and Linoleic+E, respectively). Vitamin E in the diets is natural d-α-tocopherol (1 mg=1.49 IU vitamin E), and 20% oleic acid (18:1). The standard chow contained 0.007%' (wt/wt) fat. The diets were prepared fresh each month and stored at —40°C until use. The content of thiobarbituric reactive substances (TBARS), mainly malondialdehyde (MDA), in plasma was measured by recording the fluorescence spectrum of the thiobarbituric acid-

should be more easily oxidized and therefore may be more atherogenic. Plasma LDL of rabbits and humans fed a diet rich in oleic acids is more resistant to oxidation compared with linoleic acid.25,30,31 A recent dietary study by Bonanome et al32 showed that the in vitro peroxidation rate of plasma LDL was inversely correlated with the ratio of oleic acid to linoleic acid in the LDL particle. We recently found that the peroxidation rate of plasma LDL also is determined in the basal state by this ratio.33 Moreover, the resistance of LDL against oxidation (lag time) and the extent of oxidation (total diene production) were shown to be highly correlated positively and negatively, respectively, with the ratio of oleic acid to linoleic acid.33 If LDL oxidation proves to be an important step in atherogenesis, then diets rich in monounsaturated fatty acids supplemented with lipophilic antioxidants would be the strategy of choice in providing sufficient protection against oxidative modification. In the present study, we evaluated the effect of such a combination on the susceptibility of LDL to oxidation and the development of aortic atherosclerosis in the WHHL rabbit.

Methods

Animals and Study Design

Thirty-two homozygous WHHL rabbits were raised by crossing and backcrossing with New Zealand White (NZW) rabbits. The animals were housed individually in wire-bottom stainless-steel cages under controlled light. Until the start of the study, the rabbits were fed a standard rabbit chow (LK04 diet, Hope Farms) containing 3% (wt/wt) fat, with 40% linoleic acid (18:2) and 20% oleic acid (18:1). The standard chow contained 0.007%' (wt/wt) DL-α-tocopherol acetate (1 mg=1 IU). The rabbits were divided into four groups of eight animals with matched ages, body weights, and serum cholesterol and serum triglyceride concentrations. Their mean age at the start of the study was 5.0±1.7 months (range, 3.5 to 7 months). The animals were housed individually in wire-bottom stainless-steel cages under controlled light. Until the start of the study, the rabbits were fed a standard rabbit chow (LK04 diet, Hope Farms) containing 3% (wt/wt) fat, with 40% linoleic acid (18:2) and 20% oleic acid (18:1). The standard chow contained 0.007%' (wt/wt) DL-α-tocopherol acetate (1 mg=1 IU vitamin E). The groups were fed semisynthetic diets containing 10% fat. The oils were prepared fresh each month and stored at —40°C until use. The diet components were calcium caseinate (186 g/kg), mineral mixture (39.5 g/kg), fat mixture (100 g/kg), starch (453.5 g/kg), fiber (166 g/kg), grass meal (50 g/kg), and vitamin mixture (5 g/kg). The diets contained 30% of calories as fat, 50% as carbohydrates, and 20% as protein. The diets differed only in their fatty acid composition and vitamin E content (Table 1). Two groups received a linoleate (safflower oil)-enriched diet containing approximately 75% linoleic acid; the two other groups received an oleate (Trisun 80)-enriched diet that contained approximately 75% oleic acid. One linoleate-enriched diet and one oleate-enriched diet were supplemented with extra vitamin E (DL-α-tocopherol acetate); the final vitamin E concentration in the supplemented diets was 250 mg/kg (0.025%, wt/wt). The nonsupplemented diets contained 0.005% (wt/wt) vitamin E. Each rabbit was given 100 g of their respective diets daily for 6 months. Water was provided ad libitum. Dietary consumption and weight gain did not differ among the animals of the four groups during the study period.

Blood Sampling

Fasting blood samples were obtained from the marginal ear vein at the start of the study; after 1, 2, and 4 months; and at the end of the study period (6 months). Blood samples were collected into tubes containing 0.1 mL 15% (wt/vol) EDTA (Merck). Plasma was stored at —80°C until assayed. Before the samples were frozen, saccharose (6 mg/mL) was added to the plasma to stabilize the lipoproteins.

LDL Isolation and Oxidation

LDLs were isolated by a short-run ultracentrifugation method followed by in vitro copper-induced LDL oxidation. In brief, after isolation the LDL was dialyzed for 24 hours in the dark at 4°C against phosphate-buffered saline (pH 7.4) containing 10 μmol/L EDTA. The buffer was made oxygen-free by vacuum degassing followed by purging with nitrogen. The LDL-containing sample was filtered through a 0.45-μm filter and diluted with dialysis buffer to a final concentration of 0.05 mg LDL protein per milliliter and 1 μmol EDTA/L. The oxidation was initiated by the addition of a freshly prepared, 15-μmol/L CuCl2 solution. The kinetics of the oxidation of LDL was determined by monitoring the change of the 234-nm diene absorption on a UV spectrophotometer (Lambda 5, Perkin-Elmer) equipped with a six-position automatic sample changer at 30°C. The change of absorbance at 234 nm versus time was divided into three consecutive phases, i.e., lag, propagation, and decomposition phases. Lag time, maximal rate of oxidation, and total amount of conjugated dienes formed per 1 mg LDL protein were calculated as described. Addition of 3 or 10 μg vitamin E dissolved in 10 μL isopropanol per milliliter of LDL did not quench the absorbance (at 234 nm) of control LDL.

In Vivo Lipid Peroxidation

The content of thiobarbituric reactive substances (TBARS), mainly malondialdehyde (MDA), in plasma was measured by recording the fluorescence spectrum of the thiobarbituric acid-
Table 2. Plasma Lipids and Lipoproteins at Baseline and After 6 Months in Four Groups of Eight WHHL Rabbits on Experimental Diets

<table>
<thead>
<tr>
<th>Study Diet</th>
<th>Baseline</th>
<th>Oleic</th>
<th>Linoleic</th>
<th>Oleic+E</th>
<th>Linoleic+E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol</td>
<td>21.4±3.6</td>
<td>23.7±4.0*</td>
<td>21.9±3.8</td>
<td>25.4±4.6</td>
<td>24.2±4.5</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>2.72±1.51</td>
<td>1.50±0.57*</td>
<td>1.36±0.37*</td>
<td>1.40±0.40*</td>
<td>2.03±1.04</td>
</tr>
<tr>
<td>VLDL+IDL cholesterol</td>
<td>6.4±3.4</td>
<td>11.6±2.8‡</td>
<td>9.4±3.2</td>
<td>12.4±4.4†</td>
<td>10.6±3.5*</td>
</tr>
<tr>
<td>LDL cholesterol</td>
<td>14.8±3.5</td>
<td>11.8±2.4±</td>
<td>12.3±2.8</td>
<td>12.7±2.7†</td>
<td>13.3±3.3</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>0.20±0.05</td>
<td>0.36±0.17*</td>
<td>0.25±0.10*</td>
<td>0.37±0.09‡</td>
<td>0.25±0.08</td>
</tr>
</tbody>
</table>

*P<.05, †P<.01, ‡P<.001 vs baseline.

MDA complex between 500 and 600 nm on a Shimadzu RFP-5000 recording spectrophotometer (Shimadzu), with a constant interval of 14 nm maintained between excitation and emission wavelengths. The fluorescence intensity was measured at 533 nm after subtraction of the baseline value (owing to the Rayleigh diffusion). This synchronous fluorescence method was found to be free from interfering compounds.

Extent of Aortic Atherosclerosis

At the end of the study period, the rabbits were anesthetized intramuscularly with 0.1 mL/kg Hypnorm (Janssen Pharmaceutica). The entire aorta from the aortic valve to the iliac bifurcation was removed, cleaned of excess adventitial tissue, and rinsed with saline. The aortas were opened by a longitudinal incision over the total length and fixed between two glass plates for at least 24 hours in 10% buffered formaldehyde solution. After fixation, the aortas were stained with Sudan IV (Sigma Chemical Co) to identify lipid-containing atherosclerotic plaques. The area covered with atherosclerotic lesions was quantified with a computer-assisted planimetry system (Kontron-Vidas system) coupled to a video camera (Sony). The extent of atherosclerotic lesions was quantified for the total aorta and for the three segments divided by the first intercostal artery and the celiac artery: the aortic arch, the descending thoracic aorta, and the abdominal aorta.

Other Methods

Total cholesterol, free (unesterified) cholesterol, phospholipids, and triglycerides in plasma and tissue samples were determined by commercially available enzymatic methods (numbers 237574, 310328, and 691844, Boehringer-Mannheim, and number 6639, Miles Laboratories, respectively). LDL protein was determined by the method of Lowry et al.

Cholesterol and triglyceride concentrations in plasma were determined with enzymatic methods. Plasma HDL cholesterol was determined by the method of Lowry et al. The plasma VLDL+IDL fraction (d<1.019 kg/L) was isolated by sequential ultracentrifugation; LDL was isolated at 1.019<d<1.063 kg/L. Fatty acids in LDL were determined by gas chromatography. The vitamin E concentrations in LDL and plasma were determined by high-performance liquid chromatography as described previously.

Statistical Analysis

Results are expressed as mean±SD. Unless otherwise indicated, differences among group means were analyzed by one-way ANOVA. Differences among group means that developed during the 6-month study period were analyzed by multivariate analysis of variance. For comparisons among groups, Student's (unpaired) t test was used. For paired observations, data were analyzed by Student's t test. A two-tailed probability value of less than .05 was considered significant. Statistical analyses were performed with procedures available in the spss/pc+ statistical package (SPSS Inc.).

Results

Plasma Lipids and Lipoproteins

Table 2 shows the effects of the four diets on plasma lipid and lipoprotein levels. Total plasma cholesterol concentration in the rabbits was strongly elevated throughout the study; the basal concentration was 21.4±3.6 mmol/L. Except for the rabbits on the oleic acid–enriched diet (Oleic), total cholesterol concentrations were not changed after the 6-month study period. In contrast, plasma triglycerides declined in almost all dietary groups. Also, LDL cholesterol tended to be lower during the study period; in the rabbits on the oleic acid–enriched diet (Oleic), this decrease was significant. VLDL+IDL cholesterol and HDL cholesterol concentrations increased most markedly on the oleic acid–enriched diets.

Only the effects of the four diets on plasma HDL cholesterol were significantly different; ie, the oleic acid–enriched diets resulted in higher HDL cholesterol concentrations than did the linoleic acid–enriched diets (Oleic...
Vitamin E in Plasma and LDL

Table 3 summarizes plasma and LDL vitamin E levels. The Oleic diet resulted in a significant increase in both plasma and LDL vitamin E levels. After 4 months, plasma vitamin E reached a peak at 43.0±8.2 mg/L, an increase of 113% from baseline levels. Vitamin E in LDL was maximal at 6 months with 6.66±1.13 mg/g LDL protein, an increase of 70% from baseline. In contrast, the Linoleic diet resulted in a decrease of plasma vitamin E and the vitamin E content of LDL. After 6 months on this diet, plasma vitamin E levels had decreased 36% to 13.0±2.7 mg/L. In the same period, LDL vitamin E content was reduced 46% to 2.11±0.76 mg/g LDL protein. The effects of the Oleic diet on plasma vitamin E and on vitamin E in LDL were significantly different from those of the Linoleic group (both P<.001). Both vitamin E-supplemented diets resulted in comparable increases of vitamin E concentrations in plasma and LDL.

Fatty Acid Content and Chemical Composition of LDL

Table 4 shows the fatty acid composition of plasma LDL. No difference in fatty acid composition of LDL was found between the Oleic group and the Oleic+E group; neither was a difference observed between the Linoleic group and the Linoleic+E group. The oleic acid and linoleic acid content of LDL in the Oleic groups differed significantly from those of the Linoleic group. Oleic acid was approximately 45% of total fatty acids in LDL from rabbits on the Oleic diet; in rabbits on the Linoleic diets, this was <20%. The linoleic acid content in the rabbits fed Oleic diets was approximately 25%; in rabbits fed the Linoleic diets, the linoleic acid content was approximately 50%.

Besides the changes in oleic acid and linoleic acid content, all diet groups showed a decrease in palmitic acid (16:0) and linoleic acid (18:3) compared with baseline. Except for the group on the Linoleic diet, arachidonic acid (20:4) content of LDL was also reduced. The groups on the Linoleic diets showed an additional decrease of the stearic acid (18:0) content of their LDL.

The change from normal rabbit chow to synthetic diets resulted in a general alteration in the chemical composition of LDL (Table 5); this change was basically the same for all diet groups. Both free cholesterol and cholesterol ester content of LDL increased on the synthetic diets; in contrast, the relative content of triglycerides decreased. Protein content was unchanged in all groups, slightly decreased in the group on the Oleic diet, and slightly increased in the group on the Linoleic+E diet.

LDL Oxidation

Fig 1 shows typical examples of LDL oxidation curves. This figure clearly shows the effect of a monounsaturated fatty acid–enriched diet and a polyunsaturated fatty acid diet on the oxidation profile of LDL. Fig 2 shows the effects of the four different diets on lag time. At baseline, the mean lag time was 105.0±14.9 minutes (n=32). After only 1 month on the synthetic diets, the differential effects were apparent. LDL of the rabbits that received the Linoleic diet appeared significantly less resistant against oxidation; in general, lag time was reduced approximately 50% compared with baseline. The other three diet groups showed an increase in lag time; for the Linoleic+E group, the increase was 59% after 6 months. Although the Oleic+E diet was more

Table 4. Fatty Acid Composition of Plasma LDL at Baseline and After 6 Months in Four Groups of WHHL Rabbits Fed an Experimental Diet

<table>
<thead>
<tr>
<th>Fatty Acid</th>
<th>Baseline (n=32)</th>
<th>Oleic</th>
<th>Linoleic</th>
<th>Oleic+E</th>
<th>Linoleic+E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmitic acid (16:0)</td>
<td>22.0±1.8</td>
<td>17.7±1.1†</td>
<td>18.9±1.4†</td>
<td>18.4±0.8‡</td>
<td>18.1±1.1‡</td>
</tr>
<tr>
<td>Stearic acid (18:0)</td>
<td>8.7±0.5</td>
<td>8.0±0.7*</td>
<td>8.5±0.5</td>
<td>8.0±0.3*</td>
<td>8.6±0.6</td>
</tr>
<tr>
<td>Oleic acid (18:1)</td>
<td>22.8±2.3</td>
<td>46.7±2.1†</td>
<td>17.8±1.8*</td>
<td>43.7±2.0‡</td>
<td>16.7±0.7‡</td>
</tr>
<tr>
<td>Linoleic acid (18:2)</td>
<td>38.7±2.2</td>
<td>23.6±1.9*</td>
<td>49.4±2.9†</td>
<td>25.4±1.5‡</td>
<td>52.4±1.5‡</td>
</tr>
<tr>
<td>Linolenic acid (18:3)</td>
<td>3.0±0.5</td>
<td>0.7±0.2‡</td>
<td>1.0±0.2*</td>
<td>1.1±0.3‡</td>
<td>0.7±0.3‡</td>
</tr>
<tr>
<td>Arachidonic acid (20:4)</td>
<td>4.8±1.0</td>
<td>3.4±0.7†</td>
<td>4.5±0.8</td>
<td>3.4±1.1†</td>
<td>3.6±1.1†</td>
</tr>
</tbody>
</table>

 Experimental diets are as shown in Table 1. n=8 Watanabe heritable hyperlipidemic (WHHL) rabbits for each group. Results are expressed as weight percentage of total fatty acids. *P<.05, †P<.01, ‡P<.001 vs baseline.

Table 5. Chemical Composition of LDL at Baseline and After 6 Months in Four Groups of WHHL Rabbits Fed Different Experimental Diets

<table>
<thead>
<tr>
<th>Experimental Diet</th>
<th>Baseline (n=32)</th>
<th>Oleic</th>
<th>Linoleic</th>
<th>Oleic+E</th>
<th>Linoleic+E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free cholesterol</td>
<td>9.1±0.5</td>
<td>10.3±0.7*</td>
<td>9.7±0.3‡</td>
<td>10.0±0.5†</td>
<td>9.8±0.7†</td>
</tr>
<tr>
<td>Cholesterol esters</td>
<td>42.1±4.3</td>
<td>46.9±2.3*</td>
<td>46.8±1.8*</td>
<td>46.8±2.6*</td>
<td>44.6±3.4</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>7.5±3.9</td>
<td>3.5±1.9†</td>
<td>3.2±1.1†</td>
<td>3.8±1.8*</td>
<td>5.1±2.8*</td>
</tr>
<tr>
<td>Phospholipids</td>
<td>18.6±0.9</td>
<td>18.4±0.6</td>
<td>18.0±0.5</td>
<td>18.3±7.8</td>
<td>17.4±0.7*</td>
</tr>
<tr>
<td>Protein</td>
<td>22.7±1.5</td>
<td>20.9±1.4*</td>
<td>22.3±1.1</td>
<td>21.2±1.2</td>
<td>23.7±1.4*</td>
</tr>
</tbody>
</table>

 Experimental diets are as shown in Table 1. n=8 Watanabe heritable hyperlipidemic (WHHL) rabbits for each group. Results are expressed as weight percentage of total LDL. *P<.05, †P<.01, ‡P<.001 vs baseline.
Fig 1. Graph shows typical examples of LDL oxidation curves as determined by continuous measurement of the diene absorbance during copper-induced in vitro oxidation. LDL was isolated from Watanabe heritable hyperlipidemic rabbits at the beginning of the study (•), after 1 month on a linoleic acid-enriched diet (○), and after 1 month on an oleic acid-enriched diet (∇). Lag times of the curves are indicated. Effects of the dietary interventions on oxidation rate (slope of the curve) and on the extent of the oxidation (maximal diene absorbance) are clearly shown.

effective than the Oleic diet in the first 4 months of the study period (lag time of LDL at 4 months on the Oleic+E diet was 316.3±73.7 minutes), both diets resulted in a similar 140% increase in lag time after 6 months. All differences in lag time between the diet groups were significant (P < .01) except for the effect of Oleic versus Oleic+E on lag time after 6 months.

Similar but inverse effects were seen on maximal rate of diene production (Fig 3). At baseline, the mean oxidation rate was 11.2±1.8 nmol·min⁻¹·mg⁻¹ LDL protein (n=32). The Linoleic diet increased the rate maximally by 46% (after 4 months). In contrast, the Linoleic+E, Oleic, and Oleic+E diets resulted in a decrease in the oxidation rate of 10%, 55%, and 68%, respectively.

Fig 4 shows the effect of the synthetic diets on the third parameter of LDL oxidizablity, the total diene production. At baseline, the mean total diene production was 590.8±52.7 nmol/mg LDL protein (n=32). The Oleic diet resulted in a 40% decrease in diene production; the Linoleic diet gave a 10% to 20% increase in diene production. In both cases, vitamin E supplementation had no additional effect on total diene production.

Lipid Peroxidation

As an indication of in vivo lipid peroxidation, TBARS were determined in plasma at baseline and after 6 months on the experimental diets. The mean initial plasma TBARS concentration was 0.73±0.14 nmol/mL. At the end of the study period, TBARS in the Oleic group were significantly (P=.004) reduced to 0.56±0.10 nmol/mL compared with baseline. TBARS in the Linoleic, Oleic+E, and Linoleic+E diet groups were 0.86±0.13, 0.66±0.13, and 0.62±0.06 nmol/mL, respectively. ANOVA revealed that the differences among the groups after 6 months were significant (P<.001). Statistical testing indicated that this difference was caused by the TBARS increase in the Linoleic group.

Fig 2. Bar graph shows the effect of antioxidative intervention on the lag time of LDL oxidation. Four groups (n=8) of Watanabe heritable hyperlipidemic rabbits were fed a diet enriched in oleic acid, linoleic acid, oleic acid plus vitamin E, or linoleic acid plus vitamin E. At the indicated time points, LDL was isolated and the susceptibility to copper-induced in vitro oxidation was determined.

Fig 3. Bar graph shows the effect of antioxidative intervention on maximal oxidation rate of LDL. Four groups (n=8) of Watanabe heritable hyperlipidemic rabbits were fed a diet enriched in oleic acid, linoleic acid, oleic acid plus vitamin E, or linoleic acid plus vitamin E. At the indicated time points, LDL was isolated and the susceptibility to copper-induced in vitro oxidation was determined. Inset legend as in Fig 2.

Fig 4. Bar graph shows the effect of antioxidative intervention on maximal diene production during LDL oxidation. Four groups (n=8) of Watanabe heritable hyperlipidemic rabbits were fed a diet enriched in oleic acid, linoleic acid, oleic acid plus vitamin E, or linoleic acid plus vitamin E. At the indicated time points, LDL was isolated and the susceptibility to copper-induced in vitro oxidation was determined. Inset legend as in Fig 2.
Aortic Atherosclerosis

At the end of the study period, all rabbits were killed, their aortas were removed, and the extent of aortic atherosclerosis was determined. No significant differences were observed in the extent of atherosclerotic lesion formation among the four groups (Table 6). This accounts for the total aorta and the three defined subregions of the aorta.

Discussion

Strongest evidence for the oxidation hypothesis of atherosclerosis came from the probucol studies in WHHL rabbits. Feeding 1% (wt/wt) probucol (0.4 g/kg body weight) to WHHL rabbits for 6 months retarded the progression of atherosclerosis when started at the age of 2 months; this was independent of the cholesterol-lowering effect of probucol. This antiatherogenic effect of 1% probucol feeding was also demonstrated in cholesterol-fed rabbits and monkeys. In one study in cholesterol-fed rabbits, a protective effect of probucol could not be demonstrated. Mao et al reported the effects of a probucol analogue without a cholesterol-lowering effect in attenuating atherosclerosis in WHHL rabbits. In accordance with the present study, antioxidant therapy reduced the susceptibility of LDL to oxidation but failed to affect atherosclerosis development significantly. Fruebis et al recently studied another probucol analogue with an antioxidant potency that in vitro was equal to that of probucol. The bioavailability of the probucol analogue was much greater than that of probucol. When this analogue was given at 0.05% (wt/wt) to WHHL rabbits, similar plasma levels were achieved as with 1% (wt/wt) probucol. Despite these similar plasma levels, lesion development was not affected by treatment with the analogue, whereas probucol slowed lesion progression by almost 50%. These studies with probucol analogues strongly suggest that the antiatherogenic effect of probucol is probably less related to its antioxidant properties than was initially believed and that other biological effects contribute to its antiatherogenic potential.

The present study differs in several aspects from earlier reports that showed an antiatherogenic effect of antioxidant therapy. In most animal experiments, the antioxidant dosages applied, usually in the range of 0.5% to 1.0% (wt/wt), are unrealistic for evaluation in humans. It is obvious that the efficacy of lower doses should preferably be evaluated in combination with other measures, such as an optimal composition of the fatty acids in the diet. Therefore, we evaluated in the present study in WHHL rabbits the efficacy of 0.025% (wt/wt) of vitamin E in the food, which corresponds to a daily dose of 10 mg/kg body weight. The rationale for choosing vitamin E was that in WHHL rabbits, this natural product in a dose of 0.025% was at least as effective as probucol in decreasing the susceptibility of LDL to oxidation in vitro. As an additional preventive measure, we evaluated the exchange of linoleic acid with oleic acid. For maximal response and discriminating power, we enhanced the amount of fat in the diet from 3% to 10%. On this diet, total plasma cholesterol did not show a systematic difference compared with the basal diet, whereas plasma triglycerides decreased approximately 40%. This decrease probably induced a shift of the apolipoprotein B-100-containing lipoproteins toward a lower density range accompanied by increased concentrations in the VLDL+IDL fraction and a decrease in LDL cholesterol (Table 2). In the present study, we confirmed the HDL cholesterol sparing effect of oleic acid versus linoleic acid as observed previously in humans.

It appeared that three of the four diets—the linoleic acid–enriched diet with additional vitamin E and the oleic acid–enriched diets with and without additional vitamin E—resulted in an increased resistance to in vitro oxidation; the linoleic acid–enriched diet without vitamin E supplementation showed a decreased resistance against oxidation. The improved resistance of LDL against oxidation was clear not only from an increased lag time but also from a decreased maximal oxidation rate. These are both known to be beneficially influenced by an increased vitamin E content and an increased ratio of oleic acid to linoleic acid content in LDL. Reduced oxidizability is achieved not only by delaying the onset of oxidation but also by reducing the amount of available substrate for oxidation. The latter is reflected in the decreased amounts of dienes produced in the LDL of the oleic acid–supplemented animals. However, despite the fourfold difference in lag time and oxidation rate and the twofold difference in total diene production, we failed to observe any difference in the aortic lesion surface after 6 months of intervention with these diets. We found similar results recently when we compared antioxidant treatment in three groups of WHHL rabbits. Two groups were treated with low doses of either vitamin E or probucol; the third group acted as control. Despite significant reductions in the susceptibility of LDL to oxidation, antioxidant treatment did not influence atherosclerosis development compared with the control group. From these results, it is clear that many more factors determine the outcome of atherosclerosis intervention studies by antioxidants, especially

### Table 6. Percentage of Aortic Surface Covered With Atherosclerotic Lesions in Four Groups of WHHL Rabbits Fed Different Experimental Diets for 6 Months

<table>
<thead>
<tr>
<th>Experimental Diet</th>
<th>Oleic</th>
<th>Linoleic</th>
<th>Oleic+E</th>
<th>Linoleic+E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aortic arch</td>
<td>91±3</td>
<td>88±3</td>
<td>90±3</td>
<td>89±7</td>
</tr>
<tr>
<td>Thoracic aorta</td>
<td>77±10</td>
<td>67±25</td>
<td>65±20</td>
<td>62±32</td>
</tr>
<tr>
<td>Abdominal aorta</td>
<td>22±14</td>
<td>25±11</td>
<td>24±17</td>
<td>22±16</td>
</tr>
<tr>
<td>Total aorta</td>
<td>65±9</td>
<td>59±18</td>
<td>59±14</td>
<td>56±23</td>
</tr>
</tbody>
</table>

Experimental diets are as shown in Table 1, n=8 Watanabe heritable hyperlipidemic (WHHL) rabbits (age 5.0±1.7 months) for each group. Lesions were evaluated after Sudan IV staining of the aorta.
when physiological dosages are administered. One very important factor is the age at which the prevention is started. For a realistic evaluation, we deliberately initiated intervention in WHHL rabbits of approximately 5 months of age. Another important factor is the magnitude of the plasma cholesterol concentration in the WHHL rabbits; the concentrations in our rabbits were almost twofold higher than reported in the other two studies. At these concentrations, plasma TBARS were elevated compared with the levels in healthy human volunteers and NZW rabbits (P.N.M.D., unpublished observations), indicating significantly increased lipid-peroxidized lipoproteins in the circulation of WHHL rabbits, in agreement with previous results. At these high serum cholesterol concentrations, a successful intervention, especially when applied in adult rabbits at a physiological antioxidant concentration, probably needs a duration of more than 6 months, if not several years. It could also be that atherosclerosis is attenuated only when antioxidants are given in relatively high amounts and that in the present study, the degree of antioxidative protection was insufficient to influence atherogenesis. Fruebis et al suggested that there is a threshold level of protection that would require the minimum necessary to exert an antiatherogenic effect. In this context, they mentioned a lag time of more than 400 minutes. Interestingly, such an increase was not achieved in our study. Of course, additional research is needed to test this hypothesis. Given the positive results concerning retardation of atherosclerosis regression in WHHL rabbits after partial ileal bypass or after treatment with 3-hydroxy-3-methylglutaryl coenzyme A inhibitors, it is obvious that normalization of plasma lipids, especially of cholesterol, remains the first choice in a program preventing atherosclerosis. This is especially true because polyunsaturated fatty acids, despite their increased oxidizability in vitro and in vivo, are effective in preventing atherosclerosis and coronary heart disease. From this study, it is obvious that the antioxidation hypothesis needs adaptation concerning the quantitative importance of the contributing factors.

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