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 plus vitamin E>oleic acid>linoleic acid plus 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,3-8 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.9,10

In vitro, several cell types have been shown to oxidize LDL.11-14 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

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Methods

Animals and Study Design

Thirty-two homozygous WHHL rabbits were raised by crossbreeding 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 0.005% (wt/wt) vitamin E. Each rabbit was given 100 g 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 μM 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 μM EDTA/L. The oxidation was initiated by the addition of a freshly prepared, 15-μM/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-
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) two-sample 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 triglyceride levels 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 levels both remained stable throughout the study period.

Other Methods

Total cholesterol, free (unesterified) cholesterol, phospholipids, and triglycerides in LDL 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.33 Cholesterol and triglyceride concentrations in plasma were determined with enzymatic methods. Plasma HDL cholesterol was determined by the polyethylene glycol 6000 method.36 The plasma VLDL+IDL fraction (d<1.019 kg/L) was isolated by sequential ultracentrifugation37; LDL was isolated at plasma VLDL+IDL fraction (d<1.019 kg/L) was isolated by sequential ultracentrifugation37; LDL was isolated at

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±2.6‡</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>13.5±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>

WHHL indicates Watanabe heritable hyperlipidemic rabbits. Composition of the diets is as shown in Table 1. Results are expressed in mmol/L. *P<.05, †P<.01, ‡P<.001 vs baseline.

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 (Kon-tro-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 LDL 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.33 Cholesterol and triglyceride concentrations in plasma were determined with enzymatic methods. Plasma HDL cholesterol was determined by the polyethylene glycol 6000 method.36 The plasma VLDL+IDL fraction (d<1.019 kg/L) was isolated by sequential ultracentrifugation37; LDL was isolated at 1.019<d<1.063 kg/L. Fatty acids in LDL were determined by gas chromatography.33 The vitamin E concentrations in LDL and plasma were determined by high-performance liquid chromatography as described previously.36

Table 3. Vitamin E (α-Tocopherol) in Plasma and LDL in Four Groups of WHHL Rabbits on Experimental Diets for 6 Months

<table>
<thead>
<tr>
<th>Diet</th>
<th>Baseline</th>
<th>1 Month</th>
<th>2 Months</th>
<th>4 Months</th>
<th>6 Months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma, mg/L</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oleic</td>
<td>20.2±5.7</td>
<td>25.9±6.5‡</td>
<td>34.8±4.8‡</td>
<td>43.0±8.2†</td>
<td>34.5±5.2‡</td>
</tr>
<tr>
<td>Linoleic</td>
<td>20.2±7.8</td>
<td>14.2±11.5*</td>
<td>13.4±5.5*</td>
<td>17.4±5.8</td>
<td>13.0±2.7*</td>
</tr>
<tr>
<td>Oleic+E</td>
<td>17.8±2.9</td>
<td>66.1±18.7‡</td>
<td>84.6±16.1‡</td>
<td>86.4±13.4‡</td>
<td>51.8±7.8‡</td>
</tr>
<tr>
<td>Linoleic+E</td>
<td>19.0±7.2</td>
<td>48.1±9.0‡</td>
<td>66.3±12.5‡</td>
<td>81.0±9.4‡</td>
<td>62.0±17.1†</td>
</tr>
<tr>
<td>LDL, mg/g LDL protein</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oleic</td>
<td>3.82±0.02</td>
<td>4.58±0.77</td>
<td>5.87±0.85†</td>
<td>6.12±1.51†</td>
<td>6.66±1.13‡</td>
</tr>
<tr>
<td>Linoleic</td>
<td>3.80±0.03</td>
<td>2.24±0.36‡</td>
<td>2.01±0.57‡</td>
<td>2.54±0.54‡</td>
<td>2.11±0.76‡</td>
</tr>
<tr>
<td>Oleic+E</td>
<td>3.94±0.05</td>
<td>12.90±2.58‡</td>
<td>13.74±4.13‡</td>
<td>14.10±4.13‡</td>
<td>10.47±2.52‡</td>
</tr>
<tr>
<td>Linoleic+E</td>
<td>3.81±0.79</td>
<td>9.17±1.42‡</td>
<td>11.20±1.41‡</td>
<td>12.30±1.56‡</td>
<td>10.56±1.80‡</td>
</tr>
</tbody>
</table>

Composition of the diets is as shown in Table 1. n=8 Watanabe heritable hyperlipidemic (WHHL) rabbits for each dietary group. *P<.05, †P<.01, ‡P<.001 vs baseline.
TABLE 4. Fatty Acid Composition of 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></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±3.2</td>
<td>3.5±1.9†</td>
<td>3.2±1.1†</td>
<td>3.8±1.8*</td>
<td>5.1±2.6*</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>18.5±0.7*</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.

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. Inset legend as in Fig 2.

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.

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 oxidizability, the total diene production. At baseline, the mean total diene produc-

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.
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 recently studied another probucol analogue 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 previously 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 increased 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 meet 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 surgery 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 antioxidant hypothesis needs adaptation concerning the quantitative importance of the contributing factors.

Acknowledgments

This work was supported by a grant from the Nijmegen University Research Fund (UOP 1990-4002). We thank An-Si Huijman, Pieternel van Hielst, and Janine Vogelaar for their skillful technical assistance and Jeroen van de Laak for his help with the Kontro-Vidas system. Unilever Research Laboratory, Vlaardingen, the Netherlands, provided the experimental diets.

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