Comparative Study on the Effect of Low-Dose Vitamin E and Probucol on the Susceptibility of LDL to Oxidation and the Progression of Atherosclerosis in Watanabe Heritable Hyperlipidemic Rabbits

Henne A. Kleinveld, Pierre N.M. Demacker, Anton F.H. Stalenhoef

Abstract The diet of Watanabe heritable hyperlipidemic (WHHL) rabbits was supplemented with a low dose (0.025% [wt/wt]) of the antioxidant vitamin E or probucol. The effect of 6 months of treatment on the susceptibility of low-density lipoproteins (LDLs) to oxidative modification and on established atherosclerotic lesions was studied. Vitamin E administration had no effect on plasma lipid levels; probucol supplementation decreased plasma total cholesterol. Vitamin E levels in plasma and LDL increased threefold in the course of treatment with this antioxidant. Six months of treatment with vitamin E and probucol increased the lag time of conjugated diene formation of LDL subjected to in vitro oxidation by 54% (P<.001) and 51% (P=.019), respectively. In this LDL-oxidizability assay, only vitamin E reduced the maximal rate of conjugated diene production (~24%, P<.001). Neither vitamin E treatment nor probucol therapy reduced the amount of thiobarbituric acid-reactive substances in plasma. Vitamin E treatment reduced the specific LDL apolipoprotein B-100 fluorescence (~10%, P=.035) compared with controls. Probucol was without effect on this index of in vivo LDL oxidation. At the end of the 6-month study, the mean±SD percentage area of aorta covered with plaques was 58.7±10.1% in control animals, 62.7±12.0% in the probucol-treated animals, and 48.9±13.8% in the animals treated with vitamin E; these differences were not significant. This study demonstrates that at this low dosage, vitamin E is a more effective antioxidant than probucol. (Arterioscler Thromb. 1994;14:1386-1391.)

Key Words • atherosclerosis • vitamin E • probucol • LDL • WHHL rabbits
extent of aortic atherosclerotic lesion formation in the rabbits was investigated.

### Methods

#### Animals and Study Design

Twenty homozygous WHHL rabbits (age, 5 to 7 months) were divided into three groups with matched body weights and serum cholesterol and serum triglyceride concentrations. The control group was given a standard rabbit diet (LK04 diet containing 0.008% wt/wt DL-α-tocopherol acetate; Hope Farms). The vitamin E group received a standard diet supplemented with 0.025% wt/wt DL-α-tocopherol acetate (1 IU/mg). The probucol group received a standard diet supplemented with 0.023% wt/wt probucol. Each rabbit was given 100 g of its respective diet daily for 6 months. Dietary consumption and weight gain did not differ among the three groups of animals during the study period. Three rabbits (two in the vitamin E group and one control) died in month 4 of the study period. All deaths were caused by a Pasteurella multocida infection of the lungs and the upper respiratory tract, as shown by autopsy.

#### 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 of 15% wt/vol EDTA (Merck). Plasma was stored at −80°C until assayed. Before the plasma was frozen, saccharose (6 mg/mL) was added to stabilize the lipoproteins.

#### LDL Isolation and Oxidation

LDL was 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 (Merck). Plasma was stored at −80°C until assayed. 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 per liter. The oxidation was initiated by 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, ie, a lag phase, a propagation phase, and a decomposition phase. Lag time, maximal rate of oxidation, and total amount of conjugated dienes formed per milligram of LDL protein were calculated as described.

#### In Vivo Lipid Peroxidation

The content of thiobarbituric acid–reactive substances (TBARS), mainly malondialdehyde, in plasma was measured by recording the fluorescence spectrum of the thiobarbituric acid–malondialdehyde complex between 500 and 600 nm on a Shimadzu RFF-5000 recording spectrofluorometer (Shimadzu Corp), keeping a constant interval of 14 nm between excitation and emission wavelengths. The fluorescence intensity was measured at 553 nm after subtraction of the baseline value (because of Rayleigh diffusion). This synchronous fluorescence method was found to be free of interfering compounds. The specific apolipoprotein B-100 fluorescence of isolated LDL was determined according to Esterbauer et al. The relative 430-nm fluorescence intensity at an excitation of 360 nm was measured. Addition of 3 or 10 μg of either vitamin E or probucol, dissolved in 10 μL isopropanol per milliliter LDL, did not quench the absorbance (at 234 nm) or fluorescence (excitation, 360 nm; emission, 430 nm) of control LDL.

#### Extent of Aortic Atherosclerosis

At the end of the study period, the rabbits were anesthetized with a 10 mL/kg IM mixture of fentanyl citrate and fluonisone (Hypnorp, Janssen Pharmaceutica). Immediately thereafter, their aortas were removed from the arch to the iliac bifurcation. The aortas were cleaned of excess adventitial tissue and rinsed with saline. They were opened by a longitudinal incision over the total length and fixed for at least 24 hours in 10% buffered formaldehyde. After fixation, the aortas were stained with Sudan IV (Sigma Chemical Co) to identify lipid-containing atherosclerotic plaques. The area covered by atherosclerotic lesions was quantified with a computer-assisted planimetry system (Kontro-Vidas system) coupled to a video camera (Sony). Repeated measurements of the area covered by atherosclerotic plaques, performed in a blinded fashion by a skilled technician, resulted in an intra-assay coefficient of variation for this system of <5%. The extent of atherosclerotic lesions was quantified for the total aorta as well as for three subregions: the ascending aortic arch, the descending thoracic aorta, and the abdominal aorta. Analyses of the extent of atherosclerosis were all done by one skilled technician in a blinded fashion.

#### Other Methods

Total cholesterol, free (unesterified) cholesterol, phospholipids, and triglycerides in LDL samples were determined by commercially available enzymatic methods (Boehringer-Mannheim, No. 237574, 310328, and 691444, and Miles Laboratories, No. 6639, respectively). LDL protein was determined by the method of Lowry et al. Cholesterol and triglyceride concentrations in plasma were determined by enzymatic methods. 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. Probucol concentrations in LDL and plasma were determined by the method of Mao et al.

#### Statistics

Results are expressed as the mean±SD. Differences in the extent of aortic atherosclerosis between treatment groups and aortic regions were evaluated by analysis of variance using the SPSS/PC+ statistical package. Statistical evaluation of paired data was performed by Student’s t-test. For unpaired data, Student’s two-sample t-test was used. A two-tailed value of P<.05 was considered significant.

### Results

#### Serum Lipids and Lipoproteins

Supplementing the diet of WHHL rabbits with 0.025% wt/wt probucol resulted in a significant decrease of the plasma total cholesterol concentration (Table 1). This significant hypocholesterolemic response to probucol was evident throughout the 6-month treatment period. The plasma triglyceride concentration in probucol-treated rabbits remained stable. Control WHHL rabbits and WHHL rabbits that received extra vitamin E in their diet showed no significant changes in their plasma cholesterol and triglyceride concentrations throughout the 6-month study period.

#### Antioxidant Concentration in Plasma and LDL

The plasma vitamin E levels in control WHHL rabbits and in the probucol-supplemented WHHL rabbits at the start of the study were 17.0±4.1 and 15.1±1.7 mg/mL, respectively. The baseline vitamin E content of
LDL was 3.24±0.51 and 3.28±0.40 mg/g LDL protein for control and probucol-treated rabbits, respectively. These remained stable throughout the study. The vitamin E concentrations in plasma and in LDL of WHHL rabbits fed a diet supplemented with 0.025% wt/wt vitamin E were approximately threefold higher than in control rabbits. In general, this increase in vitamin E concentration was already attained after 1 month. In 6 months, the plasma levels increased significantly (P<.05), from 18.2±5.0 to 46.7±11.4 mg/mL, and the LDL content had increased from 3.95±0.85 to 9.67±2.34 mg/g LDL protein (P<.05). In the probucol-treated rabbits, the mean plasma concentration of this synthetic antioxidant after 6 months was 289±67 μmol/L. In the same period, the probucol content of LDL amounted to 57±22 nmol/mg LDL protein.

**Composition of LDL**

Both chemical composition and fatty acid content of LDL were determined at the various time points during the study period. Typically, LDL from control WHHL rabbits (n=20) contained 8.1±0.6% free cholesterol, 40.0±4.1% cholesterol esters, 10.7±3.4% triglycerides, 17.1±1.0% phospholipids, and 24.1±3.2% protein. The fatty acid composition of control LDL was palmitic acid 23.5±1.3%, stearic acid 9.4±0.9%, oleic acid 19.7±3.2%, linoleic acid 38.3±2.4%, linolenic acid 3.3±0.4%, and arachidonic acid 5.8±1.1%. Treatment with either vitamin E or probucol during 6 months changed neither the chemical composition nor the fatty acid composition of LDL significantly.

**LDL Oxidizability**

The oxidizability of LDL was determined in vitro by continuously measuring the conjugated-diene production induced by incubation with copper. Supplementing the rabbit diet with the antioxidants vitamin E and probucol resulted in an =50% increase in lag time after 1 month of treatment (Fig 1). Prolonged treatment did not lead to a further significant increase. After 6 months of treatment with vitamin E, the lag time had increased from 116.4±13.3 to 180.0±24.6 minutes. Six months of treatment with probucol led to an increase of the lag time from 112.7±18.5 to 171.3±13.6 minutes. Remarkably, only vitamin E supplementation led to a significant decrease in the maximal rate of diene production (Fig 2). Again, this was already apparent after 1 month of treatment and did not decrease further after prolonged treatment. The total amount of dienes formed during in vitro LDL oxidation did not change as a consequence of treatment with either antioxidant (Table 2). LDL oxidizability of control WHHL rabbits did not alter significantly during the 6-month study period.

**In Vivo Lipid Peroxidation**

The concentration of TBARS in plasma was determined as an indication of lipid peroxidation in vivo (Table 3). Both in the vitamin E–treated group and in the probucol-treated group, plasma TBARS were similar to the controls.

The 430-nm fluorescence of LDL when excited at 360 nm was measured as an indication of in vivo LDL oxidation (Table 3), and more specifically, of the oxidative modification of apolipoprotein B-100. The fluorescence of LDL from vitamin E–fed rabbits was significantly lower than that of the rabbits fed the control diet or the probucol-supplemented diet. Six months of supplementation with vitamin E resulted in a 10% reduction of specific LDL fluorescence compared with control values (P=.035). The LDL fluorescence of the probucol-fed group was not different from the fluorescence of the control group.

**Aortic Atherosclerosis**

At the end of the 6-month study period, the rabbits were killed and their aortas removed to quantify the extent of atherosclerotic lesion formation. The aortas of the control rabbits had an average surface of 2059.5±131.4 mm², of which 58.7±10.1% was covered with atherosclerotic lesions. In general, the aortic arch was the subregion most heavily covered with atherosclerotic lesions; toward the thoracic aorta, the relative area covered with lesions decreased. Typically, the abdominal aorta was least heavily covered with plaques (Table 3).

![Graph showing lag time of conjugated-diene formation in low-density lipoprotein (LDL) subjected to copper-induced in vitro oxidation as described in "Methods." LDL was isolated from control Watanabe heritable hyperlipidemic (WHHL) rabbits, vitamin E–fed WHHL rabbits, and probucol–fed WHHL rabbits at the indicated time points. Rabbits were fed 10 mg antioxidant per day per kilogram body weight. *P<.01 for treatment group vs control group (Student's two-sample t test).](image-url)
Discussion

Ever since it was shown that probucol, through its antioxidant activity, could attenuate the atherosclerotic process in WHHL rabbits, much interest has been focused on the possible role of antioxidants in preventing the oxidative modification of LDL and retarding the atherosclerotic process. Clearly, there is a need to evaluate the effectiveness of several antioxidants that could eventually be used clinically in humans, the most promising ones in this respect being the naturally occurring vitamin E and the synthetic antioxidant probucol.

In studies showing that probucol attenuates atherosclerotic lesion development in WHHL rabbits, large doses of the antioxidant usually were used. Typically, the diet was supplemented with 1% wt/wt probucol. A recent study by Williams et al. who showed that vitamin E attenuates early lesion development in WHHL rabbits, also used a relatively large dose of the antioxidant: 0.5% wt/wt supplemented in the diet. In general, rabbits consume approximately 100 g food a day; in the above-mentioned studies, this meant a daily antioxidant intake of ~200 to 400 mg/kg body wt. If similar doses were applied in humans, this would amount to 14 to 28 g of antioxidants a day for a person of normal weight (70 kg). Clearly, such high doses are impractical and undesirable because of possible side effects.

In the present study, we compared the effectiveness of a small dose of the natural antioxidant vitamin E with a similar dose of the synthetic antioxidant probucol in WHHL rabbits. Rabbits were fed a diet supplemented with 0.025% wt/wt of either vitamin E or probucol, corresponding to a daily intake of 10 mg/kg body wt. This vitamin E treatment increased both plasma and LDL vitamin E levels threefold. In the study by Williams et al., plasma vitamin E levels rose fourfold despite a 20-fold higher vitamin E dose. This suggests a certain saturation level above which an increase in vitamin E dose does not result in an increase in plasma concentration. Probably the optimal dietary dose of vitamin E is near the 0.025% wt/wt, as was used in the present study. The probucol levels in plasma and LDL obtained in the present study are similar to those reported by Mao et al. for WHHL rabbits fed 1% probucol. Probably a similar saturation mechanism is at work for probucol as for vitamin E. This is supported by a report from Dachet et al. showing that the levels of probucol found in plasma and LDL of patients with familial hypercholesterolemia who received probucol (1000 mg/d) for 6 months were similar to those found in the present study. Both low-dose vitamin E and low-dose probucol treatment increased the lag time of the copper-induced in vitro oxidation of LDL. Remarkably, only vitamin E significantly reduced the maximal oxidation rate of LDL. This suggests a difference in antioxidative capacity between these two antioxidants. This difference is also reflected in the lower apoprotein B-100 fluorescence of LDL observed in the vitamin E-treated group compared with the probucol-treated group. The latter suggests a reduced in vivo oxidation and again points to a greater (in vivo) antioxidative capacity of vitamin E compared with probucol. The higher relative in vivo antioxidant action of vitamin E could be due to an increased uptake and incorporation of vitamin E into the LDL particle compared with probucol. However, the significant reducing effect of vitamin E on the in vitro oxidation rate of LDL makes it more likely that the difference in antioxidant capacity between the two antioxidants is caused by a higher recycling efficiency from its radical form (the chromanoxyl radical) by vitamin E. Therefore, vitamin E seems more effective in protecting LDL than probucol.

Table 2. Susceptibility to In Vitro Oxidation of Low-Density Lipoproteins From WHHL Rabbits Fed a Diet Supplemented With 0.025% Wt/Wt Vitamin E or Producol for 6 Months

<table>
<thead>
<tr>
<th>Comp</th>
<th>Control (n=6)</th>
<th>Vitamin E (n=7)</th>
<th>Probucol (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lag time, min</td>
<td>108.2±14.3</td>
<td>121.4±20.5</td>
<td>116.4±13.3</td>
</tr>
<tr>
<td>Rate, nmol dienes·min⁻¹·mg LDL protein⁻¹</td>
<td>11.2±1.2</td>
<td>9.9±1.2</td>
<td>10.2±1.2</td>
</tr>
<tr>
<td>Diene production, nmol/mg LDL protein</td>
<td>647.8±42.5</td>
<td>617.1±36.7</td>
<td>614.8±41.6</td>
</tr>
</tbody>
</table>

WHHL indicates Watanabe heritable hyperlipidemic. Values are mean±SD.

*P<.05 vs baseline (Student’s t test).
†P<.05 vs control (Student’s two-sample t test).
‡P<.05 vs probucol (Student’s two-sample t test).

Fig 2. Graph showing maximal rate of conjugated-diene production in low-density lipoprotein (LDL) subjected to copper-induced in vitro oxidation as described in “Methods.” LDL was isolated from control Watanabe heritable hyperlipidemic (WHHL) rabbits (○), vitamin E–fed WHHL rabbits (△), and probucol–fed WHHL rabbits (●) at the indicated time points. Rabbits were fed 10 mg antioxidant per day per kilogram body weight. *P<.05 for vitamin E group vs control and probucol groups (Student’s two-sample t test).
when given in equimolar concentrations. It is remarkable that in the few studies in which the antioxidant capacities of vitamin E and probucol were compared, probucol usually appeared more effective, although all these studies compared the antioxidants after in vitro addition to the respective test systems. The present study points to a certain discrepancy between the in vitro and in vivo antioxidant capacities of vitamin E and probucol.

Probucol is suggested to exert its antiatherogenic effect not only through its antioxidant action but also via several other mechanisms. Foremost among these additional effects of probucol is its cholesterol-lowering action, an effect also observed in the present study. Other actions of probucol related to its antiatherogenic effect include inhibition of interleukin-1 secretion and stimulation of reverse cholesterol transport. Despite these diverse actions described for probucol, we did not observe an antiatherogenic effect in the present study. It is thus suggested that in the dose used, probucol is unable to attenuate the progression of atherosclerotic lesion formation in the WHHL rabbit despite a significant hypcholesterolemic and antioxidant effect. The absence of a probucol-induced antiatherogenic effect in the present study might be due to the relative old age (5 to 7 months) of the rabbits used. The studies by Carew et al and Kita et al used rabbits approximately 2 months old, at which age no significant atherosclerotic lesions have yet developed. In a recent study, regression of established atherosclerotic plaques had been achieved by supplementing the diet of 8-month-old WHHL rabbits with 1% wt/wt probucol for a period of 6 months. Taken together, the present results and those obtained by others show that the antiatherogenic potential of probucol depends not only on the age of the rabbits used but also on the dose at which the antioxidant is supplemented, although a lack of bioavailability could not completely explain the absence of an antiatherogenic effect of probucol in the present study. Furthermore, we found indications that probucol and vitamin E differ in their antiatherogenic potential. Low-dose vitamin E treatment of relatively old WHHL rabbits resulted in a tendency to reduce aortic atherosclerotic lesion development, especially in the descending aorta (although not significantly, possibly because of the limited number of rabbits studied and the large variation in the extent of aortic atherosclerosis within the group). A power determination using a value of \( P = .05 \) for the chance of a type I and type II error, aimed at detecting a 10% reduction in atherosclerosis, revealed that to observe a significant effect of vitamin E on atherosclerosis development, the number of rabbits should be expanded to at least 30. Other studies in which rabbits were fed an atherogenic diet supplemented with vitamin E gave conflicting results in this respect; some reports showed an inhibitory effect on lesion formation, but others found an aggravating effect. The study by Williams et al concluded that the claimed antiatherogenic effect of vitamin E was attributable not only to its antioxidant action but also to its hypcholesterolemic effect. The latter effect of vitamin E could not be confirmed in the present study, possibly because of the lower dose used. Taken together, low-dose vitamin E treatment resulted in a strong decrease in susceptibility of LDL to oxidative modification and a trend toward reduced aortic lesion development, despite the absence of a hypcholesterolemic effect.

In conclusion, we have demonstrated stronger antioxidant properties of vitamin E than probucol in WHHL rabbits. Furthermore, in relatively old WHHL rabbits, vitamin E treatment resulted in a marked tendency toward a reduction of aortic atherosclerotic lesion development without evident cholesterol lowering. In contrast, low-dose probucol lowered plasma cholesterol without an apparent effect on atherosclerosis development. Recently, it was shown that low-dose probucol therapy in humans provided marked antioxidant activity, but it also retained substantial HDL cholesterol-lowering effects. Therefore, in agreement with the latter study and extending on our own observations, clinical trials to test the antioxidant hypothesis in humans should be designed with a safe and strong antioxidant that does not possess cholesterol-lowering activity. In this respect, vitamin E seems to be the first choice.

Acknowledgments

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References

1. Carew TE, Schwenke DC, Steinberg D. An antiatherogenic effect of probucol unrelated to its hypcholesterolemic effect: evidence that antioxidants in vivo can selectively inhibit low density lipoprotein degradation in macrophage-rich fatty streaks slowing down

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**Table 3. Parameters Indicating In Vivo LDL Oxidation in WHHL Rabbits**

<table>
<thead>
<tr>
<th></th>
<th>Control (n=6)</th>
<th>Vitamin E (n=7)</th>
<th>Probucol (n=4)</th>
</tr>
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<tbody>
<tr>
<td>TBARS, nmol/mL</td>
<td>0.60±0.09</td>
<td>0.66±0.14</td>
<td>0.57±0.11</td>
</tr>
<tr>
<td>Fluorescence, %</td>
<td>64±5.2</td>
<td>58.1±6.9*</td>
<td>68.0±11.3</td>
</tr>
</tbody>
</table>

WHHL indicates Watanabe heritable hyperlipidemic.

**Table 4. Percentage of Aortic Surface Covered With Atherosclerotic Lesions in WHHL Rabbits Fed a Diet Supplemented With 0.025% Wt/Wt Vitamin E or Probucol for 6 Months**

<table>
<thead>
<tr>
<th></th>
<th>Control (n=6)</th>
<th>Vitamin E (n=7)</th>
<th>Probucol (n=4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aortic arch</td>
<td>93.5±2.1</td>
<td>92.6±2.8</td>
<td>95.9±1.5</td>
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<tr>
<td>Thoracic aorta</td>
<td>65.1±18.0</td>
<td>50.7±18.9</td>
<td>66.9±15.5</td>
</tr>
<tr>
<td>Abdominal aorta</td>
<td>23.3±10.2</td>
<td>19.1±17.7</td>
<td>30.3±17.2</td>
</tr>
<tr>
<td>Total aorta</td>
<td>58.7±10.1</td>
<td>48.9±13.8</td>
<td>62.7±12.0</td>
</tr>
</tbody>
</table>

WHHL indicates Watanabe heritable hyperlipidemic.

*Lesions were evaluated after Sudan IV staining of the aorta.*
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