Effect of probucol on LDL oxidation and atherosclerosis in LDL receptor-deficient mice

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Abstract Probucol is a powerful inhibitor of atherosclerosis in a number of animal models. However, it is unknown whether this is due to the strong antioxidant protection of low density lipoprotein (LDL), to antioxidant effects in the artery wall, or to cellular effects not shared by other antioxidants. To investigate whether murine models are suitable to study the antiatherogenic mechanisms of probucol, three experiments following different protocols were carried out in 135 male and female LDL receptor-deficient (LDLR−/−) mice. Treatment groups received a high (0.5%) or low (0.025%) dose of probucol, or low-dose probucol plus a high dose (0.1%) of vitamin E for periods ranging from 6 to 26 weeks. In all experiments, probucol strongly protected LDL against ex vivo oxidation (lag times exceeding 1400 min in 0.5% probucol-treated mice). Treatment with 0.5% probucol significantly lowered both HDL-cholesterol and plasma apolipoprotein (apo)A-I concentrations. In all three experiments, treatment with 0.5% probucol consistently increased the size of lesions in the aortic origin, from 1.3-fold (n.s.) to 2.9-fold (P < 0.05) in female mice and from 3.6- to 3.7-fold in males (P < 0.001). Even treatment with 0.025% probucol increased atherosclerosis 1.6-fold in male mice (P < 0.03). Addition of the high dose of vitamin E did not attenuate the pro-atherogenic effect of 0.025% probucol. In conclusion, probucol not only failed to decrease but actively increased atherogenesis in LDLR−/− mice in a dose-dependent manner, even though it provided a very strong antioxidant protection of LDL. This suggests that the reduction of atherosclerosis observed in other animal models is due to intracellular effects of probucol not found in mice, to differences in the metabolism of probucol, and/or to an overriding atherogenic effect of the decrease in HDL in murine models.—Bird, D. A., R. K. Tangirala, J. Fruebis, D. Steinberg, J. L. Witztum, and W. Palinski. Effect of probucol on LDL oxidation and atherosclerosis in LDL receptor-deficient mice. J. Lipid Res. 1998. 39: 1079–1090.

Supplementary key words modified lipoproteins • oxidation • antioxidants • arteriosclerosis • mice

Oxidized lipoproteins may enhance atherosclerosis by a number of mechanisms. These include the recognition of oxidized lipoproteins by macrophage scavenger receptors, their chemotactic and cytotoxic properties, and the modulation of gene expression of vascular cells (reviewed in 1–3). The occurrence of oxidized low density lipoprotein (LDL) within atherosclerotic lesions has been amply demonstrated (4–9), but to date the most convincing evidence for the atherogenicity of LDL oxidation is the fact that powerful antioxidants, such as probucol, butylated hydroxytoluene, and diphenyl-phenylenediamine (DPPD), significantly reduce atherosclerosis in rabbits, hamsters, and primates (10–15). The most potent of these compounds, probucol, strongly protects plasma LDL against ex vivo oxidation. However, the mechanisms by which probucol and some other antioxidants reduce atherogenesis have not been established. Probucol also has a variety of cellular effects which may or may not be due to its antioxidant properties (16–18). For example, probucol down-regulates baseline gene expression of vascular cell adhesion molecule 1 (VCAM-1) and macrophage colony stimulating factor (M-CSF) in rabbit aortas and prevents the up-regulation of VCAM-1 mRNA and protein (18) that occurs during atherogenesis (18–20). Furthermore, it is increasingly recognized that the antiatherogenic effect of antioxidants may not necessarily be due to, or reflected by, the protection of plasma LDL against oxidation. We recently demonstrated in LDL receptor-deficient rabbits that the degree of antioxidant protection of plasma LDL was not a predictor of the anti-atherosclerotic effect of different antioxidants (21). In that study, a combination of antioxidants (vitamin E, a probucol analogue, and a very low dose (0.025%) of probucol) protected plasma LDL to the same extent as 0.091% probucol, yet failed to achieve a significant reduction of atherosclerosis, whereas 0.091% probucol did.

Murine models would be particularly attractive for study.
ing atherogenic mechanisms involving oxidative processes because techniques are readily available to delete or over-express murine genes (22, 23). Two murine strains are available in which extensive atherosclerosis occurs in the entire aortic tree, either spontaneously or induced by high-cholesterol diets: the apoE-deficient (apoE/−/−) mouse (24, 25) and the LDL receptor-deficient (LDLR/−/−) mouse (26). Atherosclerotic lesions in these mice range from fatty streaks to advanced atheromas and show many characteristic features of lesions seen in other animal models and in humans (27, 28). Support for the involvement of lipoprotein oxidation in the atherogenic process in these murine models consists of the immunocytochemical demonstration of “oxidation-specific” epitopes in their lesions and the presence in their plasma of high titers of autoantibodies against epitopes of OxLDL (29–32). However, to date the atherogenicity of lipoprotein oxidation, and conversely, the antiatherogenic effect of antioxidants, have not been conclusively established in mice. An earlier study from our laboratory suggested that an antioxidant, DPPD, can be effective in murine models. ApoE/−/− mice treated with DPPD developed less atherosclerosis (33), but the effect was small and DPPD may have toxic side effects (treated animals gained less weight than controls). In contrast, a recent report by Zhang et al. (34) showed that probucol increased atherogenesis in apo E/−/− mice. However, the mechanisms influencing atherogenesis in this model may be complex, due to the inability of intimal macrophages to secrete apoE and other consequences of apoE deficiency. Furthermore, to study the effects of probucol unrelated to its modulation of lipid metabolism, it would be desirable to match the cholesterol levels in the treatment and control groups. Probucol has a very powerful hypolipidemic effect in mice, which is difficult; if not impossible, to compensate for in apoE/−/− mice, because in control animals extensive hyperlipidemia occurs spontaneously, even on a regular rodent diet. In contrast, extensive hypercholesterolemia and atherosclerosis in LDLR/−/− mice is dependent on administration of a high-fat, high-cholesterol diet (26, 30, 35). This should make it possible to achieve similar plasma cholesterol levels in the treatment and control groups by adjusting the cholesterol content of the respective diets. Therefore, to determine whether murine models are generally suitable to investigate the mechanisms by which probucol and other antioxidants may affect atherogenesis, we carried out a series of intervention studies in LDLR/−/− mice.

METHODS

Experimental protocol

Three consecutive studies (Experiments I–III) were performed using homozygous LDLR/−/− mice with a C57BL/6J × 129Sv background from a breeding colony established from animals originally provided by Jackson Laboratories. The design of these experiments is summarized in Table 1.

In Experiment I, 28 female mice were divided into two equal groups, matched for age (3–4 months), body weight, and plasma cholesterol levels. The first group (high-probucol) was treated with 0.5% (w/w) probucol (a generous gift from the Hoechst Marion Roussel Research Institute, Cincinnati, Ohio). This group was initially fed an atherogenic diet containing 21.2% fat and 1.25% cholesterol (TD96121; Harlan Teklad). The second group (control) was also fed a high-fat diet, but one containing a lower amount of cholesterol. None of the diets contained cholate. Because probucol has a very strong lipid-lowering effect in mice, the cholesterol content of the control diet was adjusted throughout the intervention period to achieve plasma cholesterol levels similar to those in the treatment group. In addition, in order to obtain the same overall cholesterol exposure (area under the curve describing cholesterol levels over time) in both groups, the treatment period of the probucol group had to be extended (142 days vs. 112 days in the control group). One control mouse died during blood sampling, reducing the final number of animals in this group to 13.

Experiment II followed the same experimental approach. Furthermore, two additional groups were included in Experiment II to test the dose dependency of the effect of probucol and to see whether the effect on atherosclerosis correlated with the degree of antioxidant protection of plasma LDL. Sixty-four male LDLR/−/− mice were divided into four groups of 16, matched for age (2–3 months),

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\begin{array}{|c|c|c|c|c|c|}
\hline
\text{Exp} & \text{Group} & \text{Sex} & \text{Probucol} & \text{Cholesterol} & \text{Matched} \\
\hline
\text{I} & \text{Control} & F & \text{none} & 0.08 & \text{Overall Exposure} \\
\text{II} & \text{High probucol} & F & 0.5 & 1.25 & \text{Cholesterol} \\
\text{II} & \text{Control} & M & \text{none} & 0.01 & \text{Exposed Period} \\
\text{II} & \text{Low probucol} & M & 0.025 & 0.83 & \text{All} \\
\text{II} & \text{Vitamin E} & M & 0.025 & 1.12 & \text{By Group} \\
\text{II} & \text{High probucol} & M & 0.5 & 1.78 & \text{F, M} \\
\text{III} & \text{Low cholesterol control} & F, M & \text{none} & 0.01 & \text{Experiment} \\
\text{III} & \text{High probucol} & F, M & 0.5 & 1.25 & \text{F, M} \\
\text{III} & \text{High cholesterol control} & F, M & \text{none} & 1.25 & \text{Experiment} \\
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*This group received 0.1% vitamin E, in addition to probucol. F, female; M, male; Exp, experiment.
body weight, and plasma cholesterol levels (Table 1). The first group was again treated with 0.5% probucol (high probucol). The second group did not receive any antioxidant (control). The third group was treated with a low dose (0.025%) of probucol and a high dose (0.1%) of vitamin E (low probucol/vitamin E). The fourth group received only 0.025% probucol (low probucol). As in Experiment I, the dietary cholesterol content was adjusted periodically throughout the study to compensate for the hypocholesterolemic effect of probucol. More specifically, the cholesterol content of the high probucol group was raised from 1.25% to 2% after 52 days and that of the control group was lowered from 0.02% to 0.01% after 24 days. The dietary cholesterol of the low probucol group was lowered from 1.25% to 1% on day 66, and that of the low probucol/vitamin E group was first reduced to 1% (day 42) and then to 0.5% (day 66) and 0.3% (day 94). In order to achieve the same overall cholesterol exposure in all four groups, treatment of the control and low probucol/vitamin E groups was ended after 119 and 118 days, respectively, whereas treatment of the low probucol group was extended to 139 days and that of the high probucol group to 181 days. During routine blood sampling from the retro-orbital plexus, 1, 1, 2, and 4 mice died from the control, low probucol/vitamin E, low probucol, and high probucol groups, respectively. Also, 2 mice from the low probucol/vitamin E group died of unknown causes.

Experiment III was carried out to compare the effect of probucol in male and female mice and included more than one time point, to test whether the rate of progression of lesion formation was different between sexes. The design of Experiment III was also conceptually different. In Experiments I and II, significantly longer treatment periods of the high probucol groups were necessary to achieve a matched overall cholesterol exposure, because the modulation of the dietary cholesterol by itself had been insufficient to completely compensate for the powerful hypocholesterolemic effect of probucol. In contrast, in Experiment III we assumed that the duration of hypercholesterolemia was more important than an exact match of the cholesterol exposure, and therefore kept the intervention period the same for all groups. In addition, no attempt was made to modulate the cholesterol levels during the intervention phase. The extent of atherosclerosis in mice treated with 0.5% probucol was instead compared to that in two groups of untreated mice fed diets inducing a low or high plasma cholesterol level. In this experiment, 36 female and 24 male mice were divided into three matched groups (age 2–3 months) (Table 1). The first group of 12 females and 8 males (low cholesterol control) was fed a diet containing 21% fat and 0.01% cholesterol. The second group of 12 female and 9 male mice was treated with 0.5% probucol (high probucol) and was fed the high-fat diet containing 1.25% cholesterol. The third group of 12 females and 7 males (high cholesterol control) was fed the high-fat diet with 1.25% cholesterol. After 6 weeks, 6 female mice from each group were killed and analyzed as described below. The remaining mice were treated for 13 weeks. During the intervention phase, 3 mice from the control group and 2 mice from the probucol group died. The size of each group at the end of the study is indicated in Table 2.

Determination of plasma lipids, vitamin E, and probucol levels

Plasma cholesterol and triglyceride levels were determined at 3–4 week intervals, using an automated enzymatic assay (Boehringer Mannheim Diagnostics, Mannheim, Germany) (36). Blood samples for these assays were obtained from the retro-orbital plexus of anesthetized mice and were collected in heparinized tubes. Plasma high density lipoprotein (HDL)-cholesterol levels were determined by precipitating the very low density lipoprotein (VLDL) and LDL with 2 m MgCl2 and 5000 U/ml heparin (37) and then measuring the remaining plasma cholesterol as described above. The plasma concentration of probucol was determined either in terminal plasma samples pooled from 3–4 mice (Experiment I) or in individual samples (Experiments II and III), using an HPLC assay previously described (38). In brief, plasma samples were extracted with methanol–acetone 3:2 after addition of internal standard, 2-pentanone-bis-(3,5-di-t-butyl-4-hydroxyphenyl) mercaptape. HPLC analysis was performed on a C18-reversed phase column. The samples were eluted with acetonitrile–heptane–0.1 M ammonium acetate 92:6:2 (vol/vol/vol), and probucol was measured by the absorbance at 254 nm. The plasma vitamin E levels were determined using a previously described HPLC method with a C18-reversed phase column (38). Briefly, the vitamin E was extracted with heptane and α-tocopherol acetate was added as an internal standard. The mobile phase was acetonitrile–methylene chloride–methanol, 70:20:10 (vol/vol/vol), and detection was by absorbance at 292 nm.

Lipoprotein profiles

Plasma lipoprotein profiles of control and probucol-treated mice were obtained by FPLC using a 50 cm Sepharose 6B column. A plasma aliquot of 100 μl was injected onto the column, and 250 μl fractions were collected and analyzed for cholesterol and triglyceride content, as described above.

Isolation of LDL and HDL

LDL (1.019 < d < 1.063 g/ml) was prepared from terminal plasma samples (pooled from 3–4 mice each) by sequential ultracentrifugation (30,000 rpm, 4°C, 14 h, using a Beckman L7-65 ultracentrifuge with a 50.3 rotor), as previously described (33). HDL (1.21 < d < 1.061 g/ml) was isolated by sequential ultracentrifugation from the plasma of LDLR−/− mice fed a regular rodent diet.

Determination of lag times

The susceptibility of LDL to oxidation was assessed by determining the lag times in the formation of conjugated dienes during copper-mediated oxidation, as previously described (33). LDL was extensively dialyzed against phosphate buffered saline (PBS), adjusted to 100 μg protein/ml with PBS, and CuSO4 was added at a final concentra-

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tion of 5 \mu\text{m}. The formation of conjugated dienes was determined in a UVikon 910 spectrophotometer (Scientific Instruments International, San Diego, CA) at 234 nm. Absorption at the beginning of the reaction was set to zero. Lag times were determined graphically as the time point at which the tangent to the curve during the maximum slope of the propagation phase intercepted the time axis.

**Determination of plasma apoA-I concentrations**

The apoA-I levels in mouse plasma samples of Experiment I were determined by a competitive ELISA using a chemiluminescent detection system. In this assay, HDL isolated from LDLR\(^{-/-}\) mice fed regular mouse chow was plated as antigen by adding 50 \mu\text{l} of a solution of HDL (2 \mu\text{g} protein/\text{ml}) in Tris-buffered saline (TBS) to each well of a 96-well Microfluor "W" microtitration plate (Dynatech Laboratories Inc., Chantilly, VA) and incubated overnight at 4°C. Remaining binding sites on the wells were then blocked by incubating with 3% BSA in TBS for 45 min at room temperature. The antigen-coated wells were then incubated for 1 h at room temperature with 25 \mu\text{l} of a solution of the murine serum (1:500, 1:1,000, and 1:5,000) together with 25 \mu\text{l} of a 1:75,000 dilution of a rabbit anti-mouse apoA-I antibody (Biodesign Inc., Carmel, NY). Plates were washed 4 times with TBS containing 0.27 mm EDTA, 0.02% NaN\(_3\), and 0.001% aprotinin. Each well was then incubated overnight at 4°C. Following washing with TBS, 25 \mu\text{l} of a 30% solution of Lumi-Phos 530 (Lumigen, Southfield, MI) was added to each well. The formation of conjugated dienes was determined in a Uvikon 910 spectrophotometer (Scientific Instruments International, San Diego, CA) at 234 nm.

**Evaluation of atherosclerosis**

The heart and aortic tree were perfusion-fixed with formal–sucrose, dissected, and prepared for morphometry as previously described (39). For each animal, lesion size was measured in 8 sections covering 0.34 mm of the aortic origin, beginning from the appearance of the leaflets of the aortic valve. In addition, the whole aortas including the abdominal and thoracic region were dissected in Experiment II. The aortas were cleaned of adventitia, opened longitudinally, and pinned flat on black wax beds (29). After staining the aortas with Sudan black wax beds (29), the percentage of the aortic surface covered by lesions was determined by computer-assisted image analysis (39).

**Statistical analysis**

All results were analyzed by analysis of variance (ANOVA). Data shown are mean ± SEM.

### TABLE 2. Body weights, plasma lipid and antioxidant concentrations in Experiments I–III

| Exp | Group | Sex | n  | Days | BW  | Overall Cholesterol & TC & TG & Probucol & Vitamin E | Plasma Concentration at the End of the Intervention Period |
|-----|-------|-----|----|------|-----|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|     |       |     |    |      |     | g               | days × mg/dl × 10\(^3\) | mg/dl           | µH              |                 |                 |                 |                 |
| I   | Control | F   | 13 | 112  | 26.3 ± 1.2 | 111.9 ± 5.2 | 903 ± 61 | 86 ± 18 | n.d. | n.d. | 109 ± 109 | 498 ± 88 | n.d. | 117 ± 9 |
|     | High probucol | F   | 14 | 142  | 26.7 ± 1.1 | 103.4 ± 3.7 | 750 ± 76 | 159 ± 24 | 270 ± 11 | 111 ± 7 | 1496 ± 93 | 546 ± 69 | 90 ± 6 | 161 ± 13 |
| II  | Control | M   | 15 | 119  | 39.0 ± 2.3 | 127.1 ± 22.7 | 1386 ± 109 | 498 ± 88 | n.d. | n.d. | 1019 ± 57a | 337 ± 38 | 158 ± 10b | 64 ± 4b |
| Low prob/vit E | M   | 13 | 118  | 43.3 ± 2.0a | 127.2 ± 28.9 | 719 ± 46b | 604 ± 130 | 497 ± 47c | 41 ± 4c |
| Low probucol | M   | 14 | 139  | 42.6 ± 1.6 | 127.4 ± 19.4 | 1019 ± 57a | 337 ± 38 | 158 ± 10b | 64 ± 4b |
| High probucol | M   | 12 | 181  | 41.6 ± 3.1 | 130.9 ± 8.3 | 719 ± 46b | 604 ± 130 | 497 ± 47c | 41 ± 4c |
| III  | Low chol. control | F   | 10 | 91  | 21.2 ± 0.7 | 57.7 ± 1.8 | 589 ± 38 | 171 ± 60 | 0 | 54 ± 14a |
| Low chol. control | F   | 7  | 91  | 33.2 ± 3.8a | 65.9 ± 4.5 | 854 ± 123 | 551 ± 102c | 0 | 83 ± 13b |
| High probucol | F   | 11 | 91  | 24.4 ± 1.3a | 56.3 ± 3.5 | 635 ± 43 | 179 ± 49 | 343 ± 28 | 32 ± 4c |
| High chol. control | F   | 6  | 91  | 35.7 ± 1.5a | 132.3 ± 10.7a | 995 ± 101a | 202 ± 53 | 0 | 59 ± 5a |
|       |       | M   | 6  | 91  | 23.5 ± 1.4 | 92.6 ± 4.7a | 1946 ± 145c | 686 ± 101d | 0 | 116 ± 12c |

In Experiments I and II, n = number of mice at the end of the intervention period. In Experiment III, n = the total number of mice in each group; 6 female mice were killed after 6 weeks, the rest after 13 weeks. Data represent mean ± SEM. F, female; M, male; Exp, experiment; BW, final body weight; TC, total plasma cholesterol; TG, plasma triglyceride; n.d., not determined.

\( aP < 0.05 \) and \( bP < 0.005 \) compared to the control group of the same sex.

\( cP < 0.005 \) compared to the low dose probucol/vitamin E group.

\( dP < 0.05 \) and \( eP < 0.005 \) compared to the female mice of the same group.
RESULTS

Experiment I: Effect of high-dose (0.5%) probucol; equal overall cholesterol exposure.

In order to achieve the same overall cholesterol exposure in the treatment and control groups and in anticipation of a strong hypolipidemic effect of probucol, the probucol group was fed a diet containing 1.25% cholesterol, whereas the control group initially was fed a diet containing only 0.15% cholesterol. In previous studies in LDLR−/− mice, diets containing 1.25% cholesterol had resulted in plasma cholesterol levels of approximately 1100 mg/dl (30, 39). When administered together with 0.5% probucol, in the present study, this diet resulted in plasma cholesterol levels of only 500 mg/dl at day 26, whereas in the control group the levels rose to 750 mg/dl. The cholesterol content of the control diet was therefore reduced to 0.075% after 28 days, and then to zero after 98 days. Although the plasma cholesterol levels in both groups were no longer significantly different during the following 2 months, they remained somewhat lower in the probucol group. For example, at the final time point, plasma cholesterol levels were 903 ± 61 mg/dl in the control group and 750 ± 76 mg/dl in the high probucol group (n.s.) (Table 2). Similar overall cholesterol exposure in both groups (Table 2) was achieved by extending the dietary intervention in the probucol group to 142 days, compared to 112 days in the control group. Plasma triglyceride levels increased in both groups, but were higher in the probucol group at the end of the intervention (Table 2). Probucol treatment had no effect on the body weights and no animal of the treatment group showed signs of adverse drug effects.

The effect of probucol on the extent of atherosclerosis was determined in cross-sections of the aortic origin because the relatively low plasma cholesterol levels and the short duration of the intervention was not expected to, and did not, result in very extensive lesions in the thoracic and abdominal aorta (30, 39). Contrary to expectation, mice treated with high-dose probucol developed more lesions than controls (279.4 ± 28.3 vs. 213.3 ± 34.2 ×10^3 μm^2 per section, respectively) although the difference was not statistically significant (Fig 1A).

Experiment II: Effect of high-dose (0.5%) probucol vs. low-dose (0.025%) probucol with or without vitamin E; equal overall cholesterol exposure

Even though the initial diet fed to the high probucol group in this experiment was the same as in Experiment I and the cholesterol content of the diet fed to the control group was lower than that in Experiment I, the difference in plasma cholesterol levels of these two groups was more pronounced than that in the preceding experiment. Plasma cholesterol levels of the control group were significantly higher than those of the high probucol group after 3 weeks (970 ± 30 vs. 571 ± 31 mg/dl, p < 0.001) and remained that way until the final time point (1386 ± 109 vs. 719 ± 46 mg/dl, P < 0.005), despite the reduction of the dietary cholesterol content of the control diet to 0.01% and the increase of the cholesterol content of the high probucol diet to 2%. To match the overall cholesterol exposure, the treatment period of the high probucol group had to be extended to 181 days (compared to 119 days in the control group). The plasma cholesterol levels of the low probucol/ vitamin E group were similar to those of the control group throughout the study and at the final time point (1496 ± 93 mg/dl). In contrast, the cholesterol levels of the low probucol group (that was initially fed the same diet as the low probucol/ vitamin E group) were significantly lower at several time points and at the end of
the intervention period \((p < 0.05)\). Intervention in this group was therefore extended to 139 days. Thus, an almost identical overall cholesterol exposure was achieved in all four groups (Table 2). As in Experiment I, the plasma triglyceride levels increased over time in all four groups, but did not differ significantly between groups at any given time point. The body weights of the mice in the four groups also increased in parallel with no significant differences except between the final body weight of the control and low probucol/vitamin E groups (Table 2).

Experiment II confirmed the pro-atherogenic effect of probucol observed in Experiment I. In Experiment II, the high probucol group had significantly larger lesions than the control group \((547.9 \pm 47.6 \text{ vs. } 148.8 \pm 14.8 \times 10^3 \mu m^2, P < 0.001)\) (Fig 1B). The pro-atherogenic effect of probucol was dose-dependent. The low probucol/vitamin E and low probucol groups had significantly larger lesions \((254.4 \pm 31.8 \text{ and } 245.2 \pm 24.1 \times 10^3 \mu m^2, \text{ respectively})\) than the control group \((P < 0.01)\), but smaller lesions than the high probucol group.

Quantitation of lesions in the whole aorta (performed only in Experiment II) showed a similar trend, but the differences were much less striking. The percent of aortic surface covered by atherosclerotic lesions was \(8.2 \pm 0.8, 10.6 \pm 1.4, 11.2 \pm 1.0,\) and \(11.6 \pm 1.6\%\) in the control, low probucol/vitamin E, low probucol, and high probucol groups, respectively. Of these, only the high probucol and low probucol groups were significantly different from the control group \((P < 0.05)\).

**Experiment III: Effect of high-dose (0.5%) probucol vs. low-cholesterol and high-cholesterol controls; equal intervention times**

As explained in Methods, Experiment III followed an entirely different protocol (constant intervention period in all groups, as opposed to equal overall cholesterol exposure in Experiments I and II). In both male and female mice, the plasma cholesterol levels of the low cholesterol control and the high probucol groups increased over the first 3 weeks and remained fairly constant thereafter \((589 \pm 38 \text{ and } 635 \pm 43 \text{ mg/dl, respectively, in females, and } 854 \pm 123 \text{ and } 650 \pm 44 \text{ mg/dl, respectively, in males})\). There was no significant difference in plasma cholesterol levels between males and females in these groups. As the treatment period was the same, the overall cholesterol exposure of these two male and two female groups was also similar \((56.3 \text{ to } 65.9 \times 10^3 \text{ days } \times \text{ mg/dl})\) (Table 2). However, the final plasma cholesterol level \((995 \pm 101 \text{ mg/dl})\) and overall cholesterol exposure \((92.6 \pm 4.7 \times 10^3 \text{ days } \times \text{ mg/dl})\) of female mice in the high cholesterol control group were much higher than those in the other two groups of female mice \((P < 0.001)\). The final plasma cholesterol levels \((1946 \pm 145 \text{ mg/dl})\) and overall cholesterol exposure \((1323 \pm 10.7 \times 10^3 \text{ mg/dl})\) of male mice in the high cholesterol control group were even higher and statistically different from those in the male low cholesterol control and high probucol groups, as well as from those in female mice in the high cholesterol control group \((P < 0.001)\). The plasma triglyceride levels of the female mice in all three groups were similar (Table 2). However, triglyceride levels of male mice in the low and high cholesterol control groups were significantly higher \((551 \pm 102 \text{ and } 686 \pm 101 \text{ mg/dl, } P < 0.01, \text{ respectively})\) than those of male mice in the high probucol group \((255 \pm 56 \text{ mg/dl})\) and female mice. There was no difference in body weight among the three female groups throughout the intervention period, except between the low cholesterol control and high probucol groups at the final time point. There was also no difference in body weights among the male groups, although the body weights of males were significantly higher than those of corresponding females (Table 2).

Results of Experiment III confirmed the pro-atherogenic effect of probucol. In female mice, after 6 weeks of treatment, the high probucol group had significantly larger lesions than the low cholesterol control group (in which similar plasma cholesterol levels had been achieved throughout the intervention period) \((65.1 \pm 11.5 \text{ vs. } 30.8 \pm 7.9 \times 10^3 \mu m^2, P < 0.05)\) (Fig 1C). The high cholesterol control group (which had considerably higher cholesterol levels than both of these groups) had more atherosclerosis than the low cholesterol control group, but less than the high probucol group \((49.5 \pm 7.1 \times 10^3 \mu m^2)\), although none of the differences was statistically significant. At 13 weeks, both the high probucol and high cholesterol control groups had significantly larger lesions \((355.6 \pm 80.7 \text{ and } 270.9 \pm 25.2 \times 10^3 \mu m^2, \text{ respectively})\) than the low cholesterol control group \((122.4 \pm 38.6 \times 10^3 \mu m^2, P < 0.05)\) (Fig 1C). Again, the size of atherosclerotic lesions in the high probucol group was also greater than that in the high cholesterol control group, although the difference did not reach statistical significance.

Analysis of male mice at 13 weeks yielded very similar results (see Fig 3C). The high probucol group had significantly more lesion than the low cholesterol control group \((398.0 \pm 37.1 \text{ vs. } 110.3 \pm 13.1 \times 10^3 \mu m^2, \text{ respectively, } P < 0.05)\). Not surprisingly, the high cholesterol control had significantly more lesions \((286.0 \pm 19.1 \times 10^3 \mu m^2, P < 0.001)\) than the low cholesterol control. More importantly, the high cholesterol control group also had significantly smaller lesions than the high probucol group \((P < 0.05)\), even though the control group had been exposed to much higher plasma cholesterol levels.

**Effect of treatment on HDL**

In Experiment I, the terminal plasma samples were used to determine the lipoprotein profiles by FPLC. Representative profiles are shown in Fig. 2. Plasma from control animals that received a small amount of cholesterol (time-averaged: 0.08%) showed patterns similar to those previously reported for LDLR\(^{-/-}\) mice on cholesterol-rich diets \((25)\), i.e., most of the cholesterol was in the LDL peak. Note that under these conditions the HDL-cholesterol peak is much smaller than that seen in LDLR\(^{-/-}\) mice fed regular mouse chow. Profiles of probucol-treated mice resembled those of controls, except for a consistently lower HDL-cholesterol peak. Because the proatherogenic effect of probucol treatment failed to reach statistical signifi-
cance in Experiment I, quantitation of HDL-cholesterol levels was also performed in Experiment III. As shown in Fig. 3, the HDL-cholesterol levels in mice of the high probucol group were significantly lower than those in the low cholesterol control group (that had a similar overall cholesterol exposure) and those of the high cholesterol control group, in both male and female mice treated for 13 weeks ($P < 0.05$ to $P < 0.001$).

ApoA-I concentrations in terminal plasma samples from all animals in Experiment I were determined by competitive ELISA. The plasma apoA-I concentration of control animals was $0.93 \pm 0.27$ mg/ml, compared to $0.51 \pm 0.19$ mg/ml in probucol-treated mice. Although the difference did not reach statistical significance due to a large variance, three separate ELISA determinations of the plasma apoA-I concentrations yielded similar results. In contrast, in Experiment III a significant reduction of the plasma apoA-I concentration was seen in both male and female mice treated for 13 weeks with 0.5% probucol, compared to same sex animals in the low cholesterol control group ($P < 0.05$) (Fig. 4). Together, these data confirmed that treatment with 0.5% probucol leads to a significant reduction of plasma HDL levels in LDLR$^{-/-}$ mice, consistent with observations in other mice (40) and in humans (41–43).

**Plasma concentrations of probucol and vitamin E**

In Experiment I, the probucol concentration in the terminal plasma samples (pooled from 3–4 mice each) from the high probucol group was $270 \pm 11$ nM (Table 2). The average probucol concentration in LDL isolated from the pooled terminal plasma was $45.5$ nmol/mg protein. This was similar to the plasma probucol concentration of female mice receiving 0.5% probucol in Experiment III ($343 \pm 28$ nM). However, male mice receiving the same dose of probucol in Experiments II and III had significantly higher levels of plasma probucol ($497 \pm 47$ and $463 \pm 16$ nM, $P < 0.005$, respectively).
The plasma vitamin E levels in the high probucol groups of Experiments II and III were significantly lower than those of the respective control groups ($P < 0.005$) (Table 2). A decreased plasma concentration of vitamin E was also observed in the low probucol group of Experiment II ($P < 0.005$), even though the plasma concentration of probucol in this group was much lower than in the high probucol group (158 ± 10 vs. 497 ± 47 μm). As expected, there was an increase in plasma vitamin E in the low probucol/vitamin E group compared to the control group (161 ± 13 vs. 117 ± 9 μm), but the increase was relatively mild, considering that the animals were fed a high dose of vitamin E (0.1% of the diet). Remarkably, the plasma level of probucol was also decreased in the low probucol/vitamin E group (90 ± 6 μm, compared to 158 ± 10 μm in the low probucol group that received the same dose of probucol). A similar inverse relationship between the plasma concentrations of vitamin E and probucol has previously been reported (38), and human studies also showed that probucol treatment decreases serum concentrations of other, diet-derived antioxidants (44).

**Effect of treatment on the antioxidant protection of plasma LDL**

The degree of protection of LDL against in vitro oxidation was assessed by determining the lag times in the formation of conjugated dienes, using a standard Cu$^{2+}$ oxidation assay (45). Representative examples from Experiment II are shown in Fig. 5. In all three experiments, the lag times of LDL from the control groups were dramatically lower (range: 95 to 220 min) than those from the high probucol groups (all of which exceeded 1400 min). The degree of antioxidant protection seen in our study was comparable to that conveyed by 1% probucol in WHHL rabbits (38), where probucol achieved a significant reduction of atherosclerosis. Treatment with low-dose probucol yielded a lesser degree of protection. The range of lag times in the low probucol/vitamin E and low probucol groups in Experiment II were 530 to 805 min and 775 to 1180 min, respectively. The finding of longer lag times in mice receiving only the low dose of probucol, compared to mice receiving the same dose of probucol plus a high dose of vitamin E, can be explained by the fact that
DISCUSSION

The present study demonstrated that probucol not only failed to reduce atherogenesis in LDLR−/− mice, but actually enhanced it in a dose dependent manner in both male and female animals. Even the addition of a high dose of vitamin E to a very low dose (0.025%) of probucol did not reverse the atherogenic effect of probucol, possibly because the increase in plasma levels of vitamin E was modest. Our results in LDLR−/− mice are consistent with those obtained in apoE−/− mice by Zhang et al. (34), who found increases in lesion size ranging from 50 to 240% in male and female mice treated with 0.5% probucol for 3 months. In our study, probucol treatment of LDLR−/− mice resulted in a highly significant prolongation of the lag time. The fact that probucol enhanced atherogenesis, even though it strongly increased the resistance of plasma LDL to ex vivo oxidation, provides further evidence that the antiatherogenic effect of antioxidants may not necessarily be reflected by parameters measuring the susceptibility of plasma LDL to oxidation ex vivo (21).

In Experiments I and II, the powerful hypolipidemic effect of probucol could only partially be compensated for by increasing the cholesterol content of the diet of the treatment group and reducing it in the control group. A similar overall exposure to cholesterol in all experimental groups was therefore achieved by extending the treatment period of the probucol groups. This led to a much longer intervention period in some of the probucol-treated groups, in particular the high probucol group of Experiment II. Matching on the basis of the overall cholesterol exposure (i.e., the product of time and plasma cholesterol level) assumes that both the degree of hypercholesterolemia and the length of exposure to hypercholesterolemia have a similar impact on atherogenesis. This assumption may not be true, or at least it may not apply when the differences in these parameters are large. Therefore, it would be tempting to speculate that the apparent increase in atherosclerosis induced by probucol in Experiments I and II may not necessarily reflect the effect of probucol on atherosclerosis, but merely indicate a greater importance of the time factor. In other words, it is conceivable that the much longer exposure to a lower level of plasma cholesterol in the probucol group induced more atherosclerosis than the shorter exposure to a higher cholesterol level in the control group. Experiment III, however, ruled this out and provided persuasive evidence for the atherogenicity of probucol in LDLR−/− mice. In this experiment the intervention period was held constant, and the extent of atherosclerosis in the treatment group was compared to that in two different control groups. One of these, fed a low-cholesterol diet, actually achieved plasma cholesterol levels similar to those in the treatment group throughout the intervention period. The second control group was fed a high-fat, high-cholesterol diet, resulting in much higher plasma cholesterol levels. Nevertheless, the probucol group had consistently more lesions than either of these control groups, both in male and female mice and at two different time points (Fig. 1C).

A potential explanation for the pro-atherogenic effect of probucol in LDLR−/− mice may be its effect on HDL. Our results showed that probucol consistently reduced HDL-cholesterol (Figs. 2 and 3) and plasma apoA-I levels (Fig. 4). This is consistent with the observation of lower HDL levels in some but not all studies on probucol-treated humans (41–43). A significant HDL lowering effect has also been reported in probucol-treated rabbits and primates (15, 38). The fact that probucol was effective in reducing atherosclerosis in these animal models, even though it lowered HDL levels, would seem to speak against this assumption. On the other hand, mice lack cholesterol ester transfer protein (CETP), and the normal lipoprotein profile of LDLR−/− mice on a regular low-cholesterol diet is very different from that of other species and is characterized by a very prominent HDL peak. When LDLR−/− mice are fed an atherogenic diet, HDL loses its function as the main carrier of cholesterol and the HDL peak dwindles in comparison to the VLDL and LDL peaks (26). Furthermore, HDL has an important role in suppressing the oxidation of LDL and in detoxifying proinflammatory oxidized lipids (46). Thus, it is conceivable that because of the dramatic underlying shift caused by the atherogenic diet, any further lowering of HDL by probucol could become a powerful atherogenic factor. However, the protective role of HDL in murine atherosclerosis is far from certain. Zhang et al. (34) reported that mice deficient for both apoE and apoA-I had decreased plasma HDL-cholesterol levels but the same extent of atherosclerosis as apoE−/− mice. On the other hand, overexpression of apoA-I protected apoE−/− mice from atherosclerosis (47, 48).

Another possible explanation for the increased atherosclerosis in the treatment group would be that the effects of probucol at the cellular level are different in mice than in rabbits and primates. It is generally recognized that atherosclerosis may be influenced by many factors expressed by vascular cells, such as adhesion molecules, cytokines, and growth factors (3, 49). Regulation of gene expression of many such factors may involve oxygen radicals and may therefore be affected by a shift in the intracellular redox status, or even by extracellular lipid oxidation. For example, minimally modified LDL (LDL oxidized to an extent that does not lead to recognition by scavenger receptors) induces the expression of chemotactic and colony-stimulating factors in endothelial cells and smooth muscle cells (50–52). Liao and colleagues proposed mechanisms by which lipid peroxidation products, such as those generated during the oxidative modification of LDL, could induce expression of inflammatory mediator genes and activate NF-κB-like transcription factors in vascular cells. It is therefore possible that antioxidants may also directly affect the intracellular redox potential and thus modulate...
cellular processes involved in atherogenesis, in addition to protecting lipoproteins against extracellular oxidation. Indeed, it is increasingly recognized that some antioxidants, including probucol, are capable of modulating the cellular expression of potentially atherogenic factors. In both rabbits and non-human primates, probucol treatment affected the cellular composition of lesions (16, 55) and Northern blot analysis suggested that probucol affects mRNA levels of several growth regulatory molecules (16).

We recently investigated the effects of probucol on the expression of several factors that are likely to influence intimal leukocyte penetration and accumulation, including VCAM-1 and MCP-1, during the early stages of atherogenesis (18). In this study, probucol completely suppressed lesion formation in Watanabe heritable hyperlipidemic (WHHL) rabbits up to 18 weeks of age and prevented the up-regulation of VCAM-1 gene and protein expression that occurs during lesion formation. Probucol also reduced the level of basal VCAM-1 expression found in normal intimal/medial segments of WHHL and NZW rabbits. In contrast, probucol did not affect expression of MCP-1 (18). These results provided the first in vivo evidence for an effect of probucol on VCAM-1, and suggested that an important component of the antiatherogenic effect of probucol in rabbit models may be a down-regulation of VCAM-1. However, it is unknown whether the reduced VCAM-1 expression is due to an inhibition of factors promoting VCAM-1 expression (e.g., products of lipoprotein oxidation) or whether it results from direct antioxidant or other effects of probucol at the intracellular level.

The fact that probucol increased lesion sizes may suggest that the cellular antiatherogenic effects of probucol exerted in rabbits and primates not only are absent in LDLR−/− mice, but that probucol actively enhances atherogenic mechanisms. On the other hand, it cannot be ruled out that differences in the penetration, metabolism, and/or accumulation of probucol in murine vascular cells may have prevented the antiatherogenic effect that probucol exerts in other species, and that the increase in atherosclerosis is a result of HDL lowering rather than a pro-atherogenic cellular effect of probucol.

Neither our results nor those of Zhang et al. (34), however, provide conclusive evidence against the involvement of oxidative processes in atherogenesis, nor do they prove that antioxidants, in general, are ineffective in reducing lesion formation in mice. We have previously shown that treatment of apoE−/− mice with DPPD, a potent antioxidant that does not affect lipid metabolism, decreased atherosclerosis by 36% (33), and a recent preliminary report also suggests that combinations of vitamin E and other antioxidants inhibit lesion formation in LDLR−/− mice (56).

The suitability of murine models to test the antiatherosclerotic efficacy of antioxidants, in general, remains to be established. However, the fact that probucol is antiatherogenic in rabbits and primates but pro-atherogenic in LDLR−/− mice may offer an opportunity to identify the mechanisms by which probucol reduces atherosclerosis in the former models. By comparing the effect of probucol on the expression of specific genes in mice to that in rabbits or primates, one could not only determine the mechanism of probucol, but also obtain valuable information on which genes are important for the progression of lesions. Such genes could then be specifically targeted for intervention.

We thank Florencia Casanada, Richard Elam, Elizabeth Miller, and Jennifer Pattison for excellent technical assistance throughout the study, and Joe Juliano for the determination of the plasma cholesterol and triglyceride levels. These studies were supported by NHLBI grants HL14197 and HL656989 (La Jolla Specialized Center of Research in Molecular Medicine and Atherosclerosis; J. W., D. S., and W. P.).

Manuscript received 4 December 1997 and in revised form 20 January 1998.

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