Atherosclerosis and Lipoproteins

Site-Specific Antiatherogenic Effect of Pro布ucol in Apolipoprotein E–Deficient Mice

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Abstract—The lipid-lowering antioxidant probucol can inhibit atherosclerosis in animals and restenosis in humans. However, probucol has been shown to promote atherosclerosis in the aortic root of apolipoprotein E–deficient (apoE−/−) mice. In the current study, we examined the effects of probucol on both lesion formation at 4 sites along the aorta and lipoprotein oxidation in the plasma and aortas of apoE−/− mice receiving a diet containing 21.2% (wt/wt) fat and 0.15% (wt/wt) cholesterol without or with 1% (wt/wt) probucol. After 6 months, controls had developed lesions at all sites investigated. Lesion development was strongly (P<0.0001) affected by probucol, but this effect was not uniform: lesion size was increased in the aortic root but significantly decreased in the arch, the descending thoracic aorta, and proximal abdominal aorta. Plasma and aortas of probucol-treated mice contained high concentrations of probucol and its metabolites (bisphenol and diphenoxoquinone); increased vitamin C; markedly decreased very low density lipoprotein (but not low density lipoprotein and high density lipoprotein); and decreased cholesterol, cholesteryl esters, triglycerides, vitamin E, and oxidized lipids compared with controls. Interestingly, probucol treatment did not decrease the proportion of aortic lipids that were oxidized. Plasma vitamin C and bisphenol, but not probucol, protected plasma lipids from ex vivo oxidation by peroxy radicals. These results show that as in other species, probucol can inhibit lesion formation in most parts of the aorta of apoE−/− mice. This effect may involve lipid oxidation–independent mechanisms localized within the vessel wall as well as lipid lowering. (Arterioscler Thromb Vasc Biol. 2000;20:e26-e33.)

Key Words: ascorbate ■ atherogenesis ■ lipid peroxidation ■ lipoprotein oxidation ■ probucol

Oxidation of LDL is widely accepted to be critical to the development of atherosclerosis.1 Indeed, oxidized proteins2–4 and lipids5–6 are present in atheroma. For example, in advanced human lesions, ≈5% of cholesteryl linoleate (C18:2), the major form of cholesteryl esters (CEs), are oxidized and evenly distributed as hydroxides and hydroperoxides of CEs (CE-OHs) in the vessel wall increase in parallel with oxides of CEs and triglycerides (LOOHs) and hydroxides of triglycerides among all classes of lipoproteins.7 However, it is unclear to what extent and which oxidized lipids cause atherosclerosis. Lipid hydroperoxides are the primary products formed during the initial stage of lipoprotein oxidation.8,9 They are also the precursors for lipid hydroxides and secondary lipoyxidation products that can contribute to the oxidation of the apolipoprotein B-100 in LDL.10 Antioxidants may attenuate atherosclerosis because they inhibit lipid hydroperoxide formation and hence, lipoprotein oxidation.1

Probucol, a lipid-lowering antioxidant,11 has been extensively studied in experimental intervention studies of atherosclerosis. Its overall effects, however, remain somewhat contentious. Probucol commonly inhibits atherogenesis in hypercholesterolemic rabbits12,13 and nonhuman primates14; inhibits intimal thickening and restenosis after angioplasty in rabbits,15 pigs,16 and humans17; and reverses established plaques in rabbits18 and xanthomas in humans.19 However, in cholesterol-fed rabbits maintained at comparable plasma cholesterol levels, probucol does not attenuate atherosclerosis.20 It is also ineffective in reducing established lesions in mature, LDL receptor–deficient rabbits21 and nonhuman primates.14 Furthermore, probucol promotes atherosclerosis in the aortic origin in LDL receptor–22–24 and apolipoprotein E–deficient (apoE−/−) mice25,26 for reasons presently unknown.

The apoE−/− mouse is a useful tool to assess the role of aortic lipoprotein oxidation in atherosclerosis.27,28 Hydroperoxides of CEs and triglycerides (LOOHs) and hydroxides of CEs (CE-OHs) in the vessel wall increase in parallel with atherosclerosis.28 We therefore examined the effect of probucol on the aortic content of these primary lipoprotein lipid peroxidation products and lesion size at 4 different sites along the aorta.

Methods

Materials

Vitamin E (α-tocopherol, purity 96%) was a gift from Henkel Corp (Sydney, Australia). C18:2 and cholesteryl arachidonate (C20:4), together referred to as CEs, unesterified cholesterol, ascorbate, formalin, EDTA, and BHT were from Sigma Chemical Co, and...
α-tocopherylquinone was from Kodak. Coenzyme Q$_{10}$ (ubiquinone-10) was a gift from Kaneka Corp (Osaka, Japan). Probucol was from Jucker Pharma (Stockholm, Sweden), and 2,2'-azobis(2-amidinopropane)hydrochloride (AAHP) and 3,3',5,5'-tetra-tetra-butylyl-4,4'-bisphenol (bisphenol) were from Polysciences. 3,3',5,5'-Tetra-tetra-butylyl-4,4'-diphenoxyquinone (diphenoxyquinone) was prepared from bisphenol, and α-tocotrienol was purified as described. Authentic hydroperoxides of C18:2, used as a standard for LOOHs and CE-Oh, were prepared as described. Ubiquinol-10 was prepared and used immediately. Dulbecco’s PBS (Sigma) was prepared with nanopure water and stored over Chelex-100 (Bio-Rad) at 4°C for 24 hours to remove contaminating transition metals. Buffers were filtered and argon-flushed.

**Animals and Diet**

Male C57BL/6J mice, homozygous for the disrupted apoE gene (apoE/−/−) and obtained originally from the Jackson Laboratory (Bar Harbor, Me), were bred at The Heart Research Institute (Sydney, Australia). Groups of 36 mice (8 or 9 per cage) were fed standard chow (Laboratory Feed) until the age of 10 weeks. Subsequently, mice were fed ad libitum a high-fat diet containing 22% (wt/wt) fat and cholesterol, respectively, with or without 1% probucol (wt/wt). The high-fat diet (control and probucol-supplemented) was prepared by M.J. Hoxey and Associates, according to the specifications of the Harlan Teklad diet TD88137. Control chow did not contain detectable LOOH (not shown). The local animal ethics committee approved the study.

**Plasma Biochemistry**

Plasma was obtained from control and probucol-treated mice, and aliquots were frozen for subsequent determination of lipids. Separate aliquots were acidified with metaphosphoric acid (5%) to stabilize vitamin C, before freezing and storage at −80°C. The remainder was pooled appropriately and used for ex vivo oxidation initiated by the peroxyl radical generator AAPH and lipoprotein separation by fast protein liquid chromatography with UV$_{260}$ detection, as described previously. Lipid-soluble antioxidants and lipids were quantified by high-performance liquid chromatography (HPLC). For ascorbate, samples were thawed, diluted with Dulbecco’s PBS to adjust the pH to 7.4, and then immediately subjected to HPLC. Plasma triglycerides were determined enzymatically (Boehringer).

**Removal of Aortas**

After being bled, mice were gravity-perfused for 5 minutes with Dulbecco’s PBS containing 20 μmol/L BHT and 1 mmol/L EDTA (buffer A), and their aortas were removed as described. In brief, the heart, ascending aorta, and descending aorta (past the femoral junction) were excised and carefully cleaned. Aortas destined for histology (n = 10 and 9 for control and probucol groups, respectively) were perfusion-fixed with buffer A containing 4% (v/vol) formaldehyde and transferred (with the hearts attached) into formalin. For biochemistry, aortas (n = 22 to 24 for both groups) were not fixed, because adventitious oxidation takes place when standard fixation procedures are used. Once cleaned, the aortas were separated from the heart, taking care to include all aortic material while avoiding heart tissue. To obtain sufficient material for HPLC analysis (ie, 30 to 40 mg wet weight tissue), it was necessary to pool 7 to 8 aortas. Separate pools of aortas (dulbecco’s PBS (Sigma) was prepared immediately frozen in buffer A, and then stored at −80°C until analyses.

**Biochemistry of Aortic Homogenates**

Pooling aortas were snap-frozen in LN$_{2}$, pulverized, resuspended in buffer A, homogenized, and then either treated with metaphosphoric acid (for ascorbate) or extracted; the hexane fraction was then analyzed for lipid-soluble antioxidants, cholesterol, CEs, LOOHs, and CE-Oh by HPLC. LOOH and CE-Oh were measured because they are the primary and major lipid oxidation products formed when lipoproteins from apoE−/− mice undergo oxidation. Our sample work-up procedure recovers 88±4% of a hydroperoxide standard of C18:2 added to mouse aorta and is recovered as LOOH or CE-Oh. To confirm the identity of aortic LOOH, HPLC with postcolumn chemiluminescence detection was used before and after NaBH$_{4}$ treatment of the samples. Bisphenol, probucol, and diphenoxyquinone were analyzed by gradient reverse-phase HPLC with compounds eluting at ≈9, 17, and 27 minutes, respectively. All compounds were quantified by peak area comparison with authentic standards, and protein determined was performed as described.

**Morphometry**

Lesions were assessed in the aortic root, the ascending thoracic aorta, and the proximal abdominal aorta. For 4 sites investigated, cross sections (2 to 3 mm thick) were prepared and stained with Weigert’s hematoxylin–van Gieson’s stain. In the aortic root, 2 sections were cut 200 and 350 mm distal from the point where the aortic valves first appeared, similar to the method used in our previous study. The results are expressed as the mean cross-sectional area of the 2 sections, a measure directly related to the total volume of lesion in the sinuses region (K.P et al, unpublished observations, 1999). There were differences in the rate of lesion development along the aortic tree, with normally more extensive lesions in the aortic arch and the proximal abdominal aorta compared with the descending thoracic aorta. We therefore selected 3 regions for evaluation. In the arch, a major lesion was present at the branching vessels and along the inner curvature of the aorta. Similar to the procedure of Mach et al, we measured only the lesions along the inner curvature because these showed little variation between individuals. Lesion area is reported as the mean of 2 sections, 1 taken just distal to the origin of the brachiocephalic trunk and the other, distal to the origin of the right common carotid artery. In the descending thoracic aorta, lesion development in the nonbranched regions was rare, whereas well-delineated lesions were found around the ostia of the branching intercostal arteries. We therefore estimated the mean cross-sectional area of the lesions around the third pair of intercostals by taking 5 to 11 serial sections 100 mm apart through the entire lesion, estimating the cross-sectional area in each section, and then calculating the mean cross-sectional area of the lesions. In the abdominal aorta, 3 sections were taken, the first just distal to the origin of the celiac artery and the others, 100 and 200 mm distal to the first section, because pilot experiments had indicated that these sites reflected lesions associated with the ostia of the branching celiac artery. However, the location of the branching vessels in this region differed markedly between different mice used in the main study. In addition, plaques associated with branching vessels other than the celiac artery were also found in these sections of most animals, and the lesions had often merged into 1 large(r) plaque. Because the number of branching vessels markedly affects the size of the lesions, such animals were omitted from analysis. When successful, the mean cross-sectional area for the 3 sections was determined, and results are expressed as the mean of the sections. Lesion assessment was carried out in a blinded fashion and with the same equipment as described previously.

**Statistical Analyses**

Data on lesion size are presented as mean±SEM, and effects of drug treatment were analyzed by a 2-way ANOVA (SAS software), with drug treatment and aortic sites used as factors. Because there was a significant interaction term between treatment and site, treatment effect at each site measured was evaluated with Student’s t test. The distribution of lesion size followed a log-normal rather than a normal distribution. A log transformation of the data could not be performed. However, because there were several zero values (in the probucol group), we therefore performed the test after assuming a normal distribution of the values, thereby reducing the sensitivity of the test. Because we obtained only 3 mean values for cross-sectional area from the abdominal aorta in the probucol group, this site was not included in the statistical analyses. Biochemical parameters were compared with the unpaired Student’s t test. Statistical difference was accepted at P<0.05.

**Results**

Representative cross sections from control and probucol-treated groups are shown in Figure I. Consistent with previ-
ous findings, lesions were found at all sites and covered large areas of the vessel, with the exception of the descending thoracic aorta, where lesions were smaller and located around the ostia of the branching intercostal arteries. Although a detailed compositional analysis was not made, the lesion morphology was grossly similar in all regions, with necrotic cores containing cholesterol crystals observed regularly at all sites. Table I summarizes the lesion sizes observed.

ANOVA showed that probucol significantly but site-dependently affected lesion size (Table I), as indicated by a significant interaction term (P=0.001). Direct comparisons showed significantly smaller lesions in the aortic arch and descending thoracic aorta in probucol-treated animals compared with controls. Aortic lesions in probucol-treated mice were clearly smaller in probucol-treated than control mice, except in those taken from the aortic root. Note that in response to the large mass of atherosclerosis in the aortic root in probucol-treated mice, the entire root has grown markedly compared with control (cf a and e). In many mice, probucol almost completely abolished lesion formation in the aorta (eg, g).

Figure I. Cross sections through the aortic root (a and e), aortic arch (b and f), descending thoracic aorta (c and g), and the proximal abdominal aorta (d and h). Sections from controls and probucol-treated animals are shown in a through d and e through h, respectively. Sections were taken in close proximity to branching vessels (see Methods), indicated by arrows. Aortic lesions were clearly smaller in probucol-treated than control mice, except in those taken from the aortic root. Note that in response to the large mass of atherosclerosis in the aortic root in probucol-treated mice, the entire root has grown markedly compared with control (cf a and e). In many mice, probucol almost completely abolished lesion formation in the aorta (eg, g).

Because probucol may inhibit atherosclerosis by inhibiting lipoprotein lipid oxidation, we measured the contents of lipids and antioxidants in the entire aortas of control and probucol-treated mice. As observed previously, feeding apoE/−/− mice a high-fat diet for 24 weeks substantially increased the aortic content of lipoprotein-derived lipids, including C18:2 (the major, readily oxidizable lipid) and α-tocopherol (vitamin E; not shown). Tables II and III show the values expressed per protein for the major lipids and antioxidants obtained after 24 weeks on the high-fat diet. In addition to nonoxidized lipids, aortas also contained LOOH and CE-OH (Table II), despite the presence of substantial amounts of the antioxidant vitamins E and C (Table III). The presence of LOOH was confirmed by HPLC with postchemiluminescence detection, with chemiluminescence-positive signals being eliminated by NaBH₄ treatment of the sample (not shown). Overall, ~1% of the aortic lipid was oxidized (Table II), and aortas also contained ubiquinone-10 and α-tocopherylquinone, the oxidized forms of ubiquinol-10 and α-tocopherol, respectively (Table III). These findings fully agree with a previous report.

Compared with controls, probucol significantly decreased the aortic content of lipids and lipid-soluble antioxidants expressed per protein (Tables II and III). For example, the concentrations of cholesterol, vitamin E, and total coenzyme Q (ubiquinones plus ubiquinols) decreased by 5.6-, 2.9-, and 2.5-fold, respectively. This reduction in aortic lipids is consistent with the lipid-lowering activity of probucol and the

**Table I. Site-Specific Antiatherogenic Effect of Probucol in ApoE−/− Mice**

<table>
<thead>
<tr>
<th></th>
<th>Aortic Root</th>
<th>Arch</th>
<th>Descending Thoracic Aorta</th>
<th>Abdominal Aorta</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>810±20 (10)</td>
<td>319±26 (10)</td>
<td>34±10 (10)</td>
<td>121±31 (9)</td>
</tr>
<tr>
<td>Probucol</td>
<td>1180±260 (9)</td>
<td>140±34 (9)*</td>
<td>5±4 (8)*</td>
<td>12±12 (3)</td>
</tr>
<tr>
<td>Treatment effect</td>
<td>146%</td>
<td>44%</td>
<td>15%</td>
<td>10%</td>
</tr>
</tbody>
</table>

Mice were fed a high-fat diet in the absence (controls) or presence of 1% (wt/wt) probucol for 24 weeks before lesions were assessed at different sites as described in Methods. Results shown represent mean±SEM of lesion cross-sectional areas (μm²×10⁻³) at the indicated sites, with the number of mice evaluated in parentheses. Numbers of cross sections taken at each site are described in Methods. Treatment effect refers to the lesion size in probucol-treated animals expressed as a percentage of the corresponding control value. *P<0.05 vs controls.
Plasma from probucol-treated mice had significantly less lipid (Table II) and vitamin E was decreased by 8% of the drug was converted into bisphenol or diphenoquinone, suggesting that the metabolism of probucol does not take place in the vessel wall. Compared with the aorta, plasma contained only small amounts of CE-OH, and there was no difference between the 2 groups. Furthermore, LOOH and α-tocopherylquinone were absent (Table III), indicating that lipoprotein oxidation in apoE−/− mice occurs within the vessel wall rather than the circulation. Size-exclusion chromatography showed that the majority of the probucol-induced, lipid-lowering action was due to a decrease in VLDL, with LDL and HDL remaining largely unchanged (Figure II). Thus, the content of cholesterol in VLDL from the probucol group was decreased to 25% of the control value, reflecting the situation in plasma (cf Tables 2 and V).

**Plasma Lipids**

 Plasma from probucol-treated mice had significantly less lipid (Table II) and vitamin E was decreased by 13-fold, whereas ascorbate was increased by 1.4-fold and total coenzyme Q remained unchanged (Table III). Similar to the situation in the aorta, probucol was present at an 8% higher concentration than vitamin E, and 8% of the drug was converted into bisphenol or diphenoquinone, suggesting that the metabolism of probucol does not take place in the vessel wall. Compared with the aorta, plasma contained only small amounts of CE-OH, and there was no difference between the 2 groups. Furthermore, LOOH and α-tocopherylquinone were absent (Table III), indicating that lipoprotein oxidation in apoE−/− mice occurs within the vessel wall rather than the circulation. Size-exclusion chromatography showed that the majority of the probucol-induced, lipid-lowering action was due to a decrease in VLDL, with LDL and HDL remaining largely unchanged (Figure II). Thus, the content of cholesterol in VLDL from the probucol group was decreased to 25% of the control value, reflecting the situation in plasma (cf Tables 2 and V).

### Table II. Aortic and Plasma Lipids in ApoE−/− Mice After 24 Weeks of Intervention

<table>
<thead>
<tr>
<th></th>
<th>Aorta, nmol/mg Protein</th>
<th>Plasma, mmol/L</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Probuloc</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>969±295</td>
<td>173±60*</td>
</tr>
<tr>
<td>C20:4</td>
<td>20±5</td>
<td>4.0±2*</td>
</tr>
<tr>
<td>C18:2</td>
<td>64±18</td>
<td>12±6*</td>
</tr>
<tr>
<td>LOOH</td>
<td>0.35±0.16</td>
<td>0.080</td>
</tr>
<tr>
<td>CE-OH</td>
<td>0.69±0.29</td>
<td>0.17±0.08*</td>
</tr>
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LOOH indicates hydroperoxides of cholesteryl esters and triglycerides, none of which were detected (ND) in plasma, with the exception of LOOH determinations in the probucol group, for which the result is the mean of 2 pools of aortas. Aortic parameters are expressed in units of nanomoles per milligram of protein in the homogenate. Plasma data represent mean±SD of 15 animals per group. *P<0.05 vs control.

### Table III. Aortic and Plasma Antioxidants in ApoE−/− Mice After 24 Weeks of Intervention

<table>
<thead>
<tr>
<th></th>
<th>Aorta, pmol/mg Protein</th>
<th>Plasma, μmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Probuloc</td>
</tr>
<tr>
<td>α-Tocopherol</td>
<td>1097±123</td>
<td>380±138*</td>
</tr>
<tr>
<td>α-Tocopherylquinone</td>
<td>183±28</td>
<td>30±2*</td>
</tr>
<tr>
<td>Ubiquinone-9</td>
<td>280±78</td>
<td>114±47*</td>
</tr>
<tr>
<td>Ubiquinol-10</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Ubiquinone-10</td>
<td>64±17</td>
<td>25±10*</td>
</tr>
<tr>
<td>Total coenzyme Q</td>
<td>344±87</td>
<td>139±57*</td>
</tr>
<tr>
<td>Ascorbate</td>
<td>31±18</td>
<td>322±320</td>
</tr>
<tr>
<td>Probucol</td>
<td>...</td>
<td>4127±1977*</td>
</tr>
<tr>
<td>Bisphenol</td>
<td>...</td>
<td>133±56*</td>
</tr>
<tr>
<td>Diphenoquinone</td>
<td>...</td>
<td>315±76*</td>
</tr>
</tbody>
</table>

ApoE−/− mice were fed either a control or a probucol-supplemented diet for 24 weeks before aortas and plasma were analyzed for the parameters indicated, as described in Methods. Results for aortic biochemistry represent mean±SD from 3 independent samples, each sample being a pool of 7 or 8 aortas except for LOOH determinations in the probucol group, for which the result is the mean of 2 pools of aortas. Aortic parameters are expressed in units of picomoles per milligram of protein in the homogenate. Plasma results represent mean±SD of 15 animals per group. α-Tocopherylquinone and ubiquinone-9 were not detected (ND). Total coenzyme Q refers to the sum of ubiquinol-10 plus ubiquinones-9 and -10. *P<0.05 vs control.
HPLC analysis also showed that the lipid-soluble antioxidants and their metabolites were distributed more or less proportionally to the cholesterol content of the lipoproteins (Table V). This scenario could explain why probucol lowered plasma vitamin E.

### Plasma Lipoprotein Oxidizability

Enhanced resistance of plasma lipoproteins to oxidation is often used as a measure of antioxidant efficacy. Therefore, we examined AAPH-induced oxidation of pooled plasma from control and treated animals. Consistent with a previous report, 28 exposure of control plasma to this oxidant resulted in the time-dependent and concomitant consumption of ascorbate (Figure IIIA) and of ubiquinols-9 and -10 (Figure IIIB). Ubiquinols were converted stoichiometrically to the corresponding ubiquinones (data not shown). As expected from the increased starting concentration, the time required for ascorbate depletion was increased somewhat in the probucol group (Figure IIIA), and this divergence was reflected in an increase in the time required for the complete consumption of ubiquinols (Figure IIIB). On depletion of ascorbate and ubiquinols, bisphenol (filled triangles in Figure IIIC) was oxidized to diphenooquinone (not shown). Thereafter, plasma vitamin E (squares in Figure IIIC) decreased, concomitant with the accumulation of CE-O(O)H (Figure IIID), the onset and rate of which were delayed and decreased, respectively, in plasma from probucol-treated mice (Figure IIID). Although probucol significantly lowered plasma \( \alpha \)-tocopherol (Table III), there was no significant difference in the onset of oxidation and the proportion to which the vitamin was consumed in plasma from control and probucol-treated mice (Figure IIIC). Probucol (cross-hatched squares in Figure IIIC) remained unchanged throughout the oxidation period examined. These observations can be explained readily on the basis of the following scenario.

**Figure II.** Plasma lipoprotein profile of apoE \(-/-\) mice fed a high-fat diet without and with 1% probucol for 24 weeks. Plasma was collected from individual mice, pooled, and diluted 1:10 with buffer used for fast protein liquid chromatography, 28 and 300 \( \mu \)L was subjected to size-exclusion chromatography as described in Methods. Chromatograms shown are representative of 2 analyses of independent, pooled plasma samples. Horizontal bars indicate corresponding fractions collected for each lipoprotein pool.

**Figure III.** Probucol decreases ex vivo oxidizability of plasma lipids obtained from apoE \(-/-\) mice fed a high-fat diet. Pooled plasma obtained from control (open symbols) or probucol-treated (closed symbols) mice was exposed to 5 mmol/L AAPH and incubated under air at 37°C. At times indicated, aliquots of the reaction mixture were removed and analyzed for (A) ascorbate (diamonds); (B) ubiquinol-9 plus ubiquinol-10 (inverted triangles); (C) \( \alpha \)-tocopherol (squares), probucol (cross-hatched squares), and bisphenol (filled triangles); and (D) CE-O(O)H (circles) as described in Methods. Initial concentrations of plasma antioxidants are listed in Table III. Data shown are mean \pm SD of a single oxidation experiment performed in triplicate with pooled plasma. Where error bars are not shown, error is smaller than the symbol.
of tocopherol-mediated peroxidation. The data indicate that the increased concentration of ascorbate and the presence of the probucol metabolite bisphenol, rather than probucol itself, afforded an enhanced resistance of plasma lipids to ex vivo oxidation induced by AAPH. Such enhanced resistance of lipids to oxidation was observed despite a substantial decrease in plasma vitamin E for the probucol treatment group, thereby emphasizing the importance of coantioxidants such as vitamin C and bisphenol rather than absolute concentrations of vitamin E as being important in providing effective antioxidant protection to plasma lipoprotein lipids.

**Discussion**

A key and novel finding of the present study is that although probucol enhanced atherosclerosis in the aortic root of apoE<sup>−/−</sup> mice, it increasingly inhibited disease along the aortic arch and descending aorta. Thus, probucol can be antiatherogenic in mice, as it is in other species. A major aim of the present study was to assess and compare the extent of disease and primary lipoprotein lipid (per)oxidation in the vasculature. Given the previous observation that probucol increased lesion size despite decreasing plasma lipid in apoE<sup>−/−</sup> mice, we anticipated to use these animals as a model to study the relationship between antioxidants, lipid peroxidation, and lesion formation in a situation wherein atherogenesis is promoted. Contrary to our expectation, probucol inhibited atherosclerosis overall, and the material used for biochemical analysis in the present study included the entire aorta. Consistent with the histology results, probucol decreased the aortic content of nonoxidized lipids and primary products of lipoprotein oxidation in the form of LOOH and CE-OH. A previous study had shown that probucol decreased the content of oxysterols (that are secondary lipid oxidation products) in rabbit aortas. In contrast, O’Brien et al did not observe a decrease in oxidized LDL in plaques of LDL receptor–deficient rabbits fed probucol. However, in that study, the tissue sections used were fixed, and this process could have caused inadvertent lipid oxidation (see Methods). Together, our results are consistent with, though do not prove, the concept that the absolute amount of oxidized lipids in the vessel may be a trigger for lesion growth.

Importantly however, probucol decreased the amounts of oxidized lipids in the vessel wall by lowering the substrate pool of nonoxidized lipids rather than by acting as an antioxidant (Table IV). This apparent lack of antioxidant protection of polysaturated lipids is consistent with the inability of probucol to inhibit AAPH-induced oxidation of lipoprotein lipids in the presence of vitamin E. Aortic vitamin E was not depleted in control mice, and although substantially decreased in mice treated with probucol, lipid-adjusted concentrations of vitamin E remained high (Tables II and III). This finding, together with the fact that the extent of lipid oxidation measured in the vessel wall was several orders of magnitude higher than that in plasma (Table II), suggests that lipoprotein lipid peroxidation likely proceeded within the aorta and in the presence of α-tocopherol (also see Reference 28). Aortas contained bisphenol (Table III), which inhibits tocopherol-mediated peroxidation and aortic lipoprotein oxidation in apoE<sup>−/−</sup> LDL receptor–deficient mice. However, as judged by their respective plasma levels, the bisphe-

The relative importance of absolute versus proportional extent of lipoprotein lipid oxidation to atherogenesis is not known. If the latter were important, our results would suggest that probucol inhibited disease by means other than inhibition of aortic lipoprotein lipid oxidation. This would represent a second example of dissociation of these 2 processes. We have shown recently that in LDL receptor–deficient rabbits, the accumulation of lipoprotein LOOH and CE-OH is dissociated from atherosclerosis. Future studies will have to address how important aortic lipoprotein lipid oxidation is as a cause of atherosclerosis in apoE<sup>−/−</sup> mice. Interestingly, in humans, probucol prevented restenosis after coronary angioplasty, whereas an antioxidant multivitamin cocktail was without benefit and reversed the effect of probucol. On the basis of this result, the authors argued that the effect of probucol was independent of its antioxidant activity.

The apparent lack of antioxidant protection of aortic lipids by probucol raises the question of how the drug inhibited atherosclerosis so effectively in most parts of the aorta. As in other animals (see, eg, Reference 12), probucol substantially decreased plasma lipids in the apoE<sup>−/−</sup> mice used in the current experiment. This observation represents a confounding factor when considering the relation between lesion formation and lipid (per)oxidation (see above). However, it is not immediately obvious how lipid lowering alone could explain the observed caudal increase in inhibition of atherosclerosis (Table I) or the increase in lesion size in the aortic origin by probucol.

Several explanations have been proposed for the proatherogenic activity of probucol in the aortic root, including the lowering of HDL, probucol toxicity, increased plasma fibrinogen, and decreased plasma lipoprotein lipase activities. However, in the present study, probucol did not lower HDL. Others have suggested that in rabbits, probucol may be antiatherogenic by increasing the activity of CE transfer protein. Although we did not measure this activity, blood parameters alone are not likely to explain the site-specific proatherogenic or antiatherogenic effect of probucol, as all sites are exposed to the same blood.

The site-specific effects of probucol may be explained by local differences in hemodynamic factors, such as shear stress and/or characteristics of the vessel wall, although this hypothesis requires future investigations. Probucol inhibits the secretion of interleukin-1 from macrophages, the expression of vascular cell adhesion molecule-1 and macrophage-colony stimulating factor in the artery wall, and the adhesion of macrophages to the endothelium. Because vascular cell adhesion molecule-1 expression is correlated with and can contribute to the entry of macrophages into the intima, its downregulation by probucol could attenuate atherosclerosis by decreasing the intimal accumulation of macrophages. The expression of adhesion molecules and the distribution of inflammatory cells within the vessel wall are affected by arterial blood flow, so that these local differences in hemodynamic factors could be relevant.
Alternatively, probucol may act only on certain atherogenic processes or on certain stages of atherogenesis, and this may explain the differences observed. For example, there is evidence\cite{12,13,14} that probucol prevents lesion growth but does not cause lesion regression (see, however, Reference 40). Lesion development in apoE−/− mice is observed first in the aortic root and later distally along the aortic tree.\cite{5} Hence, the proatherogenic\cite{22,23,24} and antiatherogenic activity of probucol (this study) at the most proximal and distal sites, respectively, could be explained if lesions existed in the aortic origin but not along the transverse, descending, and abdominal aorta before the intervention. The treatment period of our study was sufficiently long to allow growth of lesions at all sites studied. Lesion growth in the descending thoracic aorta, though later in onset than that in the proximal aorta, is comparatively fast,\cite{28,31} and this difference in growth could explain the more effective antiatherogenic activity of probucol in the dorsal aorta. However, lesions were barely detectable in the aortic root of our apoE−/− mice at 10 weeks of age\cite{25} when the animals started to receive probucol. Therefore, a lack of effect on preexisting lesions is unlikely to explain the proatherogenic effect of probucol at the aortic origin.

Zhang et al\cite{25} were the first to demonstrate a proatherogenic effect of probucol on lesion formation in the aortic root of apoE−/− mice. Those authors also reported that in a subset of 5 probucol-treated female mice, the lesion area in the descending aorta was 4 times larger than that in 4 untreated animals.\cite{25} Moghadasian et al\cite{26} reported that “thoracic aortas from the probucol-treated animals showed severe atherosclerotic lesions,” although those authors did not directly compare the extent of lesion formation in the descending aorta of probucol and control mice. At present, we cannot explain the discrepancy between the proatherogenic effect reported by Zhang et al\cite{25} and the strong antiatherogenic effect observed in the present study for probucol in the descending aorta. A marked difference in the 2 studies is that Zhang et al\cite{25} used normal chow (for 3 months), whereas our animals were fed a high-fat diet (for 6 months). Thus, it is possible that the antiatherosclerotic effect of probucol in apoE−/− mice is related to severe hypercholesterolemia and/or its consequences on the aortic vessel wall.

A limitation of the present study is that the biochemical analyses could not be performed on the aortas used for histology, so that our interpretations rely on comparisons between different animals (albeit treated under identical conditions). Also, the required use of pooled aortas precludes any systematic correlation between lesion size and aortic biochemical parameters. Another limitation is that we did not measure secondary products of lipid peroxidation, such as malondialdehyde-lysine adducts, or those derived from oxidized phospholipids that are present in human and rabbit lesions.\cite{2,4} However, these products derive from primary lipid peroxidation products that we did measure and are formed at substantial quantities only after the depletion of α-tocopherol,\cite{8} whereas aortic vitamin E was clearly not depleted (Table III). Therefore, LOOH fragmentation may not be a major event in lipid protein lipid oxidation in the vessel wall of apoE−/− mice. Nevertheless, it will be important in future studies to test whether probucol affects secondary lipid oxidation and/or protein oxidation and, if so, how this relates to atherogenesis.

The observation that probucol shows site-differential effects on atherosclerosis raises interesting questions about local factors that contribute to the disease. The relevance of the disease-promoting effect of probucol in the aortic root\cite{25,26} is, however, questionable, given that vascular events in humans generally result from lesions in the abdominal aorta and medium-size arteries. Therefore, our finding that probucol inhibits atherosclerosis in the majority of the aorta indicates that this drug can be antiatherogenic in this animal model.

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