Processes Involved in the Site-Specific Effect of Probucol on Atherosclerosis in Apolipoprotein E Gene Knockout Mice

Katherine Choy, Konstanze Beck, Francoise Y. Png, Ben J. Wu, Steven B. Leichtweis, Shane R. Thomas, Jing Y. Hou, Kevin D. Croft, Trevor A. Mori, Roland Stocker

Objective—To elucidate processes by which the antioxidant probucol increases lesion size at the aortic sinus and decreases atherosclerosis at more distal sites in apolipoprotein E–deficient (apoE−/−) mice.

Methods and Results—Male apoE−/− mice were fed high-fat chow with 1% (w/w) probucol or without (controls) for 6 months, before aortic sinus, arch, and descending aorta were analyzed separately for lesion size and composition. Compared with control, probucol significantly increased lesion size by 33% at the sinus, but it inhibited atherosclerosis at the descending aorta by 94%. Sites where atherosclerosis was inhibited contained substantially fewer macrophages, less lipids (cholesterol and cholesteryl esters), and endogenous antioxidant (α-tocopherol), but not oxidized lipids, and the extent to which probucol metabolism occurred was increased. Compared with control, aortic sinus lesions of probucol mice contained a substantially increased content of extracellular matrix, but decreased total cell and macrophage density, comparable levels of lipids and α-tocopherol, and decreased concentrations of oxidized lipids (cholesterol ester hydroperoxides, F2-isoprostanes, and 7-ketocholesterol).

Conclusions—Probucol affects atherosclerosis in apoE−/− mice independent of the accumulation of arterial lipid oxidation products, thereby dissociating the 2 processes. Rather, probucol exerts antiinflammatory activity by decreasing accumulation of macrophages in lesions, and it promotes a more stable lesion composition at the aortic sinus.


Key Words: antioxidants ■ atherosclerosis ■ collagen ■ free radicals ■ inflammation

Atherosclerosis represents a state of heightened oxidative stress characterized by lipid and protein oxidation in the vascular wall.1 The oxidative modification hypothesis of atherosclerosis predicts that low-density lipoprotein (LDL) oxidation is an early event in and that oxidized LDL contributes to atherogenesis.2 Oxidized LDL supports foam cell formation in vitro and other potentially pro-atherogenic activities, the lipid in human lesions is oxidized and contains oxidized LDL, and several different antioxidants inhibit atherosclerosis in animals.3 In addition to LDL oxidation, other relevant oxidative events include the production of reactive oxygen and nitrogen species by vascular cells,3 and oxidative modifications contributing to important clinical manifestations of coronary artery disease such as endothelial dysfunction and plaque disruption.1

However, despite abundant data, fundamental problems remain with implicating oxidative modification as a requisite cause for atherosclerosis.1 These include the poor performance of antioxidants in limiting atherosclerosis or cardiovascular events from it,4 and observations in animals that suggest dissociation between atherosclerosis and lipoprotein lipid oxidation.5–9 To reconcile these discrepancies, the “oxidative response to inflammation” model of atherosclerosis1 considers inflammation as a primary process of atherosclerosis, and oxidative stress as an event secondary to inflammation. However, this model too raises several important questions, including how antioxidants like probucol, that consistently inhibit atherosclerosis and related disorders, actually work.

Probucol is a phenolic antioxidant and rarely used cholesterol-lowering drug that attenuates atherogenesis in animals10 and humans,11 and that protects human coronary arteries from restenosis.12 In animal models of atherosclerosis, probucol alters the cellular composition and proliferation,13,14 and inhibits coronary heart disease and death.15,16 It also prevents intimal thickening after balloon injury independent of cholesterol-lowering and inhibition of lipoprotein lipid oxidation, and instead promotes functional re-endothelialization and inhibits vascular smooth muscle cell proliferation via induction of heme oxygenase-1.17,18

Strikingly, in apolipoprotein E–deficient (apoE−/−) mice, probucol increases lesion size at the aortic sinus19 while at the
same time it strongly inhibits atherosclerosis in the descending aorta.\textsuperscript{6} In the present study, we used this model to elucidate processes by which probucol exerts this site-specific effect.

**Methods**

**ApoE\textsuperscript{−/−} Mice**

Male apoE\textsuperscript{−/−} mice originally purchased from Jackson Laboratories (Bar Harbor, Me) were used at 8 to 10 weeks of age and then fed for 24 weeks ad libitum a high-fat diet containing 21.2 (w/w) fat and 0.15% (w/w) cholesterol (specifications of the Harlan Teklad diet TD88137), without (controls, 103 mice), or with probucol (1% w/w, 87 mice).\textsuperscript{6} The local animal ethics committee approved the study.

**Aortic Sampling for Biochemical Analyses**

Procedures were performed as described.\textsuperscript{5,20} For biochemistry (86 and 70 animals for control and probucol, respectively), hearts and aortas past the femoral bifurcation were excised, cleaned, placed immediately in ice-cold buffer containing protease inhibitors and antibiotics, and stored at \(-80^\circ\text{C}\). Tissues were separated into 2 groups, one for F\textsubscript{2}-isoprostanes and arachidonic acid determination (n=10 for each, control and probucol) and the other for total cholesterol, nonesterified cholesterol (NEC), 7-ketocholesterol (7KC), cholesteryl esters (CE) (defined as the sum of C18:2 plus cholesteryl arachidionate, C20:4), CE-OOH, \(\alpha\)-tocopherol, and probucol and its metabolites (n=76 and 60 for control and probucol, respectively), with analyses as described in detail in supplemental material (please see http://atvb.ahajournals.org).

**Histology**

For intimal lesion assessment, perfusion-fixed hearts and aortas (n=17 for each, controls and probucol) were subjected to blinded morphometry at the sinus, arch, and descending thoracic and abdominal aorta,\textsuperscript{6} as described in detail in supplemental material. For total cell numbers, macrophages, and collagen content, sections from 10 animals each were pooled to obtain 2 pools per treatment per animal (n=76 and 60 for control and probucol) and the other for total cholesterol, NEC and CE, and the antioxidant \(\alpha\)-tocopherol.

**Real-Time Reverse-Transcription Polymerase Chain Reaction**

For reverse-transcription polymerase chain reaction analyses, animals were perfused with cold phosphate-buffered saline and aortas and hearts (n=20 for each control and probucol-treated animals) excised, immediately transferred into RNAlater (Ambion), stored for 24 hours at 4\(^\circ\text{C}\), and then stored at \(-80^\circ\text{C}\). Aortic sinus and thoracic/abdominal aortas were prepared as described and tissues from 10 animals each were pooled to obtain 2 pools per treatment per aortic site. Total RNA was isolated, cDNA prepared, and reverse-transcription polymerase chain reaction performed as described in detail in supplemental material.

**Statistics**

Results are shown as means±SEM. The effect of probucol on lesion size was analyzed by the Mann-Whitney \(U\) test. Biochemical parameters were compared using a 1-way ANOVA or Mann-Whitney \(U\) test. For total cell numbers, macrophages, and collagen content, differences between means were evaluated using the Student \(t\) test. Statistical significance was accepted at \(P<0.05\).

**Results**

**Site-Specific Effect of Probucol on Atherosclerosis**

In apoE\textsuperscript{−/−} mice, probucol affects atherosclerosis nonuniformly.\textsuperscript{6} The results of the present study, using a large number of animals, confirmed this earlier observation (Figure I, available online at http://atvb.ahajournals.org). Probucol significantly increased lesion size by 33% at the sinus (Figure 1; \(P<0.01\)) (Table 1), whereas it visibly inhibited atherosclerosis in other parts of the aorta, including the carotid and femoral arteries (Figure I). Probucol increasingly inhibited disease along the aortic tree, with 36% inhibition at the arch (not significant) and 94% inhibition at the descending aorta (\(P<0.0001\)) (Figure 1 and Table 1).

**Effect of Probucol on Nonoxidized Lipids and Lipid-Soluble Antioxidants**

The ability of probucol to simultaneously promote and inhibit atherosclerosis provides an experimental model to directly relate the extent of lipoprotein lipid oxidation and atherogenesis in different aortic segments of the same animal. To do this, we first determined the concentrations of the nonoxidized lipids, NEC and CE, and the antioxidant \(\alpha\)-tocopherol as measures of lipoprotein lipid accumulation. For control and probucol-treated animals, lesions at the sinus contained more NEC per wet weight (Table I, available online at http://atvb.ahajournals.org) or protein (Table 1) than respective lesions at the arch and thoracic/abdominal aorta. Similarly, in control animals there was more CE per wet weight in the aortic sinus than thoracic/abdominal aorta. This reflects the relatively larger and more mature lesions at proximal than distal sites in apoE\textsuperscript{−/−} mice.\textsuperscript{21} In contrast, the protein-standardized contents of C18:2, C20:4, and \(\alpha\)-tocopherol were not different at the 3 sites in control animals, whereas probucol significantly decreased the tissue content of CE and the vitamin, independent of whether results were expressed per wet weight or protein. Figure 2 is a graphic representation of the protein-standardized results, with data from probucol-
TABLE 1. Aortic Lesion Size and Concentrations of Lipids, Antioxidants, and Oxidized Lipids in ApoE−/− Mice After 24 Weeks of Intervention

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Treatment</th>
<th>Sinus</th>
<th>Arch</th>
<th>Thoracic/Abdominal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lesion area, mm²</td>
<td>Control</td>
<td>0.679±0.035</td>
<td>0.189±0.024*</td>
<td>0.117±0.019*</td>
</tr>
<tr>
<td></td>
<td>Probucol</td>
<td>0.904±0.056</td>
<td>0.121±0.027*</td>
<td>0.007±0.002*</td>
</tr>
<tr>
<td>Lipids, nmol/mgp</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NEC</td>
<td>Control</td>
<td>1033±84</td>
<td>689±54</td>
<td>643±123*</td>
</tr>
<tr>
<td></td>
<td>Probucol</td>
<td>928±72</td>
<td>447±36*</td>
<td>215±27*</td>
</tr>
<tr>
<td>C18:2</td>
<td>Control</td>
<td>57±2</td>
<td>65±3</td>
<td>64±11</td>
</tr>
<tr>
<td></td>
<td>Probucol</td>
<td>61±6</td>
<td>33±4*</td>
<td>10±2†</td>
</tr>
<tr>
<td>C20:4</td>
<td>Control</td>
<td>26±2</td>
<td>27±3</td>
<td>27±3</td>
</tr>
<tr>
<td></td>
<td>Probucol</td>
<td>33±3</td>
<td>17±2*</td>
<td>5±1†</td>
</tr>
<tr>
<td>Antioxidants, nmol/mgp</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-Tocopherol</td>
<td>Control</td>
<td>4.0±0.7</td>
<td>5.6±0.9</td>
<td>6.1±0.5</td>
</tr>
<tr>
<td></td>
<td>Probucol</td>
<td>3.0±0.6</td>
<td>1.6±0.2*</td>
<td>1.2±0.1*</td>
</tr>
<tr>
<td>Probucol metabolites</td>
<td>Probucol</td>
<td>24.4±4.0</td>
<td>7.1±0.8*</td>
<td>4.9±0.5*</td>
</tr>
<tr>
<td>Total Drug</td>
<td>Probucol</td>
<td>3.9±0.6</td>
<td>1.7±0.2*</td>
<td>2.5±0.5</td>
</tr>
<tr>
<td>Oxidized lipids</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CE-OOH, pmol/mgp</td>
<td>Control</td>
<td>115±19</td>
<td>409±174</td>
<td>39±5*</td>
</tr>
<tr>
<td></td>
<td>Probucol</td>
<td>88±13</td>
<td>144±59</td>
<td>20±4</td>
</tr>
<tr>
<td>CE-OOH/CE, mmol/mol</td>
<td>Control</td>
<td>1.38±0.20</td>
<td>4.56±1.92</td>
<td>0.45±0.07†</td>
</tr>
<tr>
<td></td>
<td>Probucol</td>
<td>0.93±0.06</td>
<td>2.70±1.02</td>
<td>1.46±0.36</td>
</tr>
<tr>
<td>7KC, pmol/mgp</td>
<td>Control</td>
<td>0.55±0.28</td>
<td>0.52±0.21</td>
<td>0.40±0.17</td>
</tr>
<tr>
<td></td>
<td>Probucol</td>
<td>0.21±0.11</td>
<td>0.20±0.11</td>
<td>0.10±0.04</td>
</tr>
<tr>
<td>7KC/total cholesterol, mmol/mol</td>
<td>Control</td>
<td>0.30±0.04</td>
<td>0.32±0.14</td>
<td>0.26±0.10</td>
</tr>
<tr>
<td></td>
<td>Probucol</td>
<td>0.16±0.06</td>
<td>0.25±0.20</td>
<td>0.08±0.02</td>
</tr>
<tr>
<td>F2-isoprostanes, pmol/mgp</td>
<td>Control</td>
<td>0.75±0.20</td>
<td>0.42±0.06</td>
<td>0.16±0.02*</td>
</tr>
<tr>
<td></td>
<td>Probucol</td>
<td>0.39±0.06</td>
<td>0.35±0.06</td>
<td>0.14±0.02†</td>
</tr>
<tr>
<td>F2-isoprostanes, μmol/mol arachidonate</td>
<td>Control</td>
<td>14.4±3.3</td>
<td>8.4±2.1*</td>
<td>2.4±0.2†</td>
</tr>
<tr>
<td></td>
<td>Probucol</td>
<td>8.6±2.4</td>
<td>10.4±1.8</td>
<td>3.8±0.7†</td>
</tr>
</tbody>
</table>

Data show mean±SEM. For lesion size, n=17 mice per group. For biochemistry, data from 4 separate pools each containing 19 (control) and 15 (probucol) respective aortic parts are shown, except for F2-isoprostanes that show data for 10 individual sections. CE represents C18:2 plus C20:4.
*†Significantly different from corresponding control (P<0.05).

Figure 2. Site-specific effect of probucol on arterial accumulation of nonoxidized lipids and α-tocopherol. Lesion content of NEC (A), CE (B), and α-tocopherol (C) after 6 months of intervention in probucol-treated mice (black symbols) expressed relative to controls (white symbols). Results show mean±SEM of 4 independent pools containing 19 (control) and 15 (probucol) respective sections. *Significantly different from corresponding control (P<0.05).

Effect of Probucol on Lipid Oxidation in Atherosclerotic Lesions at Different Sites

We used 3 separate measures to assess lipid oxidation, ie, CE-OOH, F2-isoprostanes, and 7KC. CE-OOH represents the extent of lipoprotein lipid oxidation,20 F2-isoprostanes are a general marker of lipid oxidation,22 and 7KC is the most abundant oxysterol in atherosclerotic lesions.23 CE-OOH was the most abundant of these markers of lipid oxidation (Table I and Table 1). In control and probucol-treated mice, 7KC was not different at different sites, irrespective of how data were expressed. In contrast, protein- and parent lipid-standardized concentrations of CE-OOH and F2-isoprostanes were decreased at the thoracic/abdominal site compared with aortic sinus (Table 1). Figure 3 compares the parent lipid-standardized content of oxidized lipids at the 3 sites in control aorta, in parallel with inhibition of disease (Figure 1). However, probucol did not increase the content of NEC (Figure 2A), CE (Figure 2B), and α-tocopherol (Figure 2C) at the sinus.

versus probucol-treated mice. At the sinus, where probucol increased lesion size (Figure 1), the drug decreased the concentrations of CE-OOH (Figure 3A), F2-isoprostanes (Figure 3B), and 7KC (Figure 3C), and this reached statistical significance in the case of F2-isoprostanes and 7KC. In contrast, probucol significantly increased CE-OOH and F2-isoprostanes at the descending aorta where the drug almost completely prevented atherosclerosis. We expressed all 3 parameters of lipid oxidation relative to the respective parent molecule (ie, CE for CE-OOH, arachidonate for F2-isoprostanes, and total cholesterol for 7KC) to distinguish lipid oxidation from lipid load, because the latter was affected significantly by probucol (Figure 2A and 2B). However, even when the lipid oxidation parameters were expressed per wet weight (Table I) or protein (not shown), their concentrations did not reflect the effect of probucol on lesion development.

Tissue Levels of Probucol and Probucol Metabolites

We next assessed whether the site-specific effect on atherosclerosis was related to the drug concentration in the vessel wall. As probucol is metabolized,24 we also determined its known metabolite probucol bisphenol and diphenoquinone. Both probucol and the total amount of the drug were significantly lower in the descending aorta than the sinus (Table I and Table I). This is not surprising, given that probucol is transported in lipoproteins, so that the results reflected the extent of lipoprotein infiltration at these sites. Consistent with this, the amount of probucol was no longer different for the different sites, when the drug concentration was standardized to NEC plus CE (Figure 4A), NEC, or CE (data not shown) rather than protein (Table I). In contrast, the concentration of probucol metabolites varied less at the 3 aortic sites, whether expressed per wet weight (Table I), protein (Table I), or lipid-adjusted (not shown). When expressed relative to parent drug, the metabolites were significantly increased and accounted for nearly one-third of the drug at the descending aorta where atherosclerosis was inhibited compared with the aortic sinus where lesion size was increased (Figure 4B).

Histological Assessment of Aortic Sinus Lesions

Because neither differences in lipid accumulation nor the extent of lipid oxidation explained why lesions at the aortic sinus were larger in probucol-treated than control mice, we next determined the cellular composition at different sites. At the sinus, total cell numbers were similar in control and probucol-treated animals, so that probucol significantly decreased the number of cells per lesion area (Table 2). Similarly, the lesion area covered by macrophages was similar in control and probucol-treated animals, a finding confirmed by the lesion content of mRNA of F4/80 antigen, a specific marker for macrophages.25 As a consequence, probucol significantly decreased the percentage of lesion area covered by macrophages by nearly 50% (Table 2) (Figure II A and II B, available online at http://atvb.ahajournals.org). In contrast, probucol significantly increased the percentage lesion area that stained positive for collagen (Figure IIC to IIF) (66 ± 13% versus 45 ± 10% for probucol-treated versus control mice). At the descending aorta, probucol decreased total cell numbers, macrophages, and mRNA for F4/80 antigen by 90%, a value comparable to the extent of lesion inhibition (Table 2). Despite this, cells per lesion area remained unaltered, because lesions in the descending aorta of apoE−/− mice consisted almost entirely of macrophages (Table 2). Extracellular deposits of collagen were barely detectable, and probucol did not alter its content (Table 2).

Discussion

The present study confirms6 that in apoE−/− mice probucol affects atherosclerosis in a site-specific manner, increasing lesion size at the sinus while at the same time almost completely preventing disease in the descending aorta. Here we used this unique feature as an experimental tool to address how probucol affects atherosclerosis. A novel finding of the present study is that neither the increased lesion size nor the disease-inhibiting effect of probucol can be explained readily by parallel changes in lipid oxidation, as assessed by the accumulation of CE-OOH, 7KC, or F2-isoprostanes at the
different sites. Our results also show that whereas probucol increases the lesion size at the sinus, these lesions are more fibrous and contain, relative to lesion size, fewer inflammatory cells, ie, features that stabilize lesions and therefore can, overall, be seen as protective rather than disease-promoting. Lipoprotein lipid oxidation in the vessel wall is commonly considered a cause of atherosclerosis, although recent observations suggest a dissociation between the 2 processes.5–9 The present study further supports such a dissociation. Most notably, it is the first study to our knowledge in which 3 separate measures of lipid oxidation were applied simultaneously and to both lesion size-enhancing and size-inhibiting conditions. Independent of the marker used, the extent of accumulation of lipid oxidation markers inversely correlated with lesion size, except for 7KC at the thoracic/abdominal aorta (Figure 3). Such inverse relationship is difficult to reconcile with the notion that lipid oxidation causes atherosclerosis, although we assessed lipid oxidation at only one time point during disease development, and as the accumulation of CE-OOH, 7KC, and F2-isoprostanes. However, previous studies in apoE−/− mice20 and humans26 indicate that with increasing disease severity there is net accumulation of aortic oxidized lipids (although oxidized lipids accumulate well after nonoxidized lipids). Thus, even if metabolism of oxidized lipids were to occur, formation of oxidized lipids appears to exceed such metabolism. Metabolism of oxidized lipids is also unlikely to explain our results, because in that case probucol would need to have increased metabolism at the aortic root and decreased it at the descending aorta, for which there is no evidence in the literature. Therefore, the simplest explanation of our data are that probucol’s effects are not explained by changes to aortic lipid oxidation, providing further evidence for dissociation between atherosclerosis and aortic lipid oxidation. Our data are also not immediately consistent with the central element of the oxidative modification theory of atherosclerosis,5 namely that LDL oxidation causes atherosclerosis, because our analyses also included CE-OOH, the single most abundant oxidized lipid in lesion lipoproteins including LDL,20,27 that is also formed in LDL exposed to 2-electron oxidants that favor apolipoprotein B-100 oxidation.28,29 From the inverse relationship between aortic lipid oxidation and lesion size, we do not conclude that lipid oxidation inhibits atherosclerosis. Rather, our contention is that the 2 processes are not causally linked. Previous studies have shown that in apoE−/− mice the aortic content of oxidized lipids, such as F2-isoprostanes30 and CE-OOH,20 increases with increasing lesion size and development. Our results are consistent with this. In control apoE−/− mice, the content of F2-isoprostanes and CE-OOH was higher at the sinus than thoracic/abdominal aorta (Table I and Table 1), and the rate of lesion development along the aortic tree differs, with sinus lesions being larger and more developed than those in the descending aorta.21 However, a direct relationship between lesion size and content of oxidized lipid does not prove a causal relationship between the two parameters. Our studies show that probucol enhanced lesion size at the sinus by means other than increasing lipoprotein lipid accumulation. Similarly, we show that probucol decreased total cell and macrophage numbers per lesion area. By contrast, the drug substantially increased the lesion content of collagen. Given its preponderance, this increase in collagen can explain why probucol, overall, increased lesion size at the sinus. Our findings are consistent with previous reports on compositional changes caused by probucol in coronary arteries of hypercholesterolemic rabbits14,15 and nonhuman primates.13
In these animals, probucol caused more dense and fibrous plaques, with thicker caps and fewer macrophages. Importantly, these changes are associated with a more stable plaque type, less vulnerable to rupture, and in the rabbit have been reported to increase survival. Therefore, the action of probucol at the aortic sinus in apoE−/−, and possibly also LDL receptor−/− mice may be considered beneficial rather than pro-atherogenic.

A striking feature is that in the descending aorta, probucol almost completely prevented atherosclerosis to an extent exceeding that reported for most other interventions. As discussed, this could not be explained by inhibition of lipid or lipoprotein lipid oxidation. Rather, the extent of disease inhibition was reflected in the decreased content of arterial macrophages and nonoxidized lipids. As probucol decreased macrophages (but not lipid) at the sinus and descending aorta, and lesions in the descending aorta are almost entirely composed of macrophages that contain essentially all lipoprotein-derived lipid, we speculate that this antiinflammatory action explains probucol’s antiatherosclerotic activity.

Interestingly in this context, probucol monosuccinate, which prevents restenosis after percutaneous coronary intervention and is being tested as a novel therapeutic agent against atherosclerosis, acts as an antiinflammatory drug by reducing vascular cell adhesion molecule-1 and monocyte chemoattractant protein-1. We are presently investigating whether the apparent antiinflammatory action of probucol is related to its ability to induce heme oxygenase-I.

Unexpectedly, we observed the extent of probucol metabolism to be significantly increased in the aorta compared with the sinus (Figure 4), suggesting that some of the differences in action at the sinus versus descending aorta may be related to probucol metabolism. We do not know at present whether this relates to the previously reported antiinflammatory activity of probucol, ie, inhibition of lipopolysaccharide-induced secretion of interleukin-1 by macrophages. Little is known about probucol metabolism, although it was more extensive in the aorta than liver, as assessed by the respective ratio of bisphenol plus diphenoquinone-to-probucol (not shown), suggesting the involvement of processes other than those mediated by hepatic cytochrome P-450. Nonenzymatic oxidants can convert probucol to its bisphenol and diphenoquinone. We observed recently that during this process a bioactive intermediate is formed that protects vessels against oxidant-induced endothelial dysfunction, suggesting that probucol may act as a pro-drug. Preliminary results suggest that this probucol intermediate has antiinflammatory and antiatherosclerotic activities surpassing those of probucol (BW, PKW, KB, KC, Antony Lau and RS, 2005 unpublished).

Acknowledgments
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References


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Materials and Methods

Materials

Chemicals were obtained from Sigma (St. Louis, MO), except \( \alpha \)-tocopherol was a gift from Henkel Corporation (Sydney, Australia), cholest-5-en-3\( \beta \)-ol-7-one (7-ketocholesterol, 7KC) was from Steraloids Inc. (Wilton, NH), probucol from Medicem (Barcelona, Spain), and 3,3',5,5'-tetra-\textit{ tert}-butyl-4,4'-bisphenol (bisphenol) from Polysciences (Warrington, PA). Hydroperoxide of cholesteryl linoleate (C18:2) was prepared\(^1\) and used as standard for cholesterylester hydroperoxides (CE-OOH). 3,3',5,5'-Tetra-\textit{ tert}-butyl-4,4'-diphenoquinone (diphenoquinone) was prepared from bisphenol.\(^2\) Buffers were prepared from nanopure water, stored over Chelex-100\(^{\circledR}\) (BioRad, Richmond, CA) to remove contaminating transition metals, filtered and argon-flushed immediately prior to use.

Aortic biochemistry

Tissue samples were thawed and each divided into three segments: the sinus (S), arch (A) and thoracic plus abdominal aorta (T+A = remaining aorta). For the sinus material the origin of the aorta was dissected from the surrounding myocardium and used for analysis, whereas the arch was defined as from where the aorta leaves the heart to just distal to the right subclavian artery. For F\(_2\)-isoprostanes and arachidonic acid determination, each aortic segment was analyzed individually. For other biochemical analyses, respective individual aortic segments were pooled (n = 19 and 15 segments per control and probucol pool, respectively) and then analyzed as separate pools (n = 4 for control and probucol). F\(_2\)-isoprostanes were analyzed by GC/MS after solid-phase extraction and HPLC purification. Other analytes were determined by GLC or HPLC as described below.
For F₂-isoprostanes, the thawed aortic segment (≈20 mg wet weight) was blotted dry, weighed and F₂-isoprostanes analyzed by electron capture negative ionization GC/MS after solid-phase extraction and HPLC purification³ using [D₄]-8-iso-prostaglandin F₂α (Cayman Chemical) as internal standard. For arachidonate, phospholipids were separated by thin-layer chromatography, the fatty acid methyl esters then prepared and analyzed by GLC.³,⁴

For other analytes, pooled aortic segments were homogenized and extracted,⁵ and the organic phase analyzed by HPLC with electrochemical (for α-tocopherol), UV (C and CE) and post-column chemiluminescence detection (CE-OOH).⁶,⁷ CE-OOH were measured as a marker of primary lipoprotein lipid peroxidation as they are the primary and major lipid oxidation products formed in lipoproteins from apoE-/⁻ mice undergoing oxidation in the presence of α-tocopherol.⁸ Bisphenol, probucol and diphenoquinone were analyzed by gradient RP-HPLC.¹

For total cholesterol and 7KC, separate 10 μL and 100 μL aliquots, respectively, of the re-dissolved organic extracts were saponified, and subjected to HPLC.⁹,¹⁰ For 7KC, a silica column (0.46 x 15 cm, 120 Å, 5 μm, Supelco) with guard column (3 μm particle size) was eluted with hexane/isopropylalcohol/acetonitrile (94.8:4.6:0.6 v/v/v) at 1.0 mL/min and monitored at 234 nm. For all chromatographic analyses, compounds were quantified by area comparison using authentic standards.

**Histological analyses**

For morphometry, average values of lesion areas were determined for each aortic site for each animal, using the number of sections indicated and taken as follows: 2 cross sections (200 and 350 μm distal from the first appearance of the valves) at the aortic sinus; 9-11 longitudinal sections at the aortic arch, with lesions along the inner curvature of the aorta determined as described by Mach et al.¹¹; 5 to 11 serial cross sections 100 μm apart through the entire lesion
around the third pair of intercostals for the thoracic aorta; and 3 cross sections at the abdominal aorta, the first just distal to the origin of the celiac artery and the others, 100 and 200 µm distal to the first section. The results reported show the mean values of the averages. Mean areas from thoracic and abdominal aortas were similar and therefore were combined.

For total cell numbers, macrophage and collagen content, a single section from 8 animals (total cell numbers) and 6 animals (macrophages and collagen) was analyzed for each experimental group (control and probucol). Sections were taken immediately adjacent to those used for lesion assessment, the results of which are shown in Table 1. For total cell numbers, nuclei were counted in hematoxylin and eosin-stained sections and expressed per lesion area. Macrophages were stained using monoclonal rat-anti-mouse F4/80 antibody and collagen by Sirius red staining as described below.

For macrophages, 4 µm paraffin sections were deparaffinized, rehydrated and endogenous peroxidase quenched with 3% hydrogen peroxide (15 min). Enzymatic antigen retrieval was performed in trypsin (1 mg/mL) solutions pH 7.7 containing 4 mM CaCl₂ and 200 mM Tris for 30 min at 37 °C, followed by a 20 min incubation in 5% normal rabbit blocking serum. Sections were then incubated overnight in a humidified chamber and at 4 °C with monoclonal rat-anti-mouse F4/80 antibody (Caltag Laboratories; dilution 1:20), followed by biotinylated rabbit anti-rat IgG (Vector Laboratories; dilution 1:200, 30 min), Vectorstain Elite ABC reagent (Vectorstain Elite ABC Kit, Vector Laboratories; 30 min), and 3,3’-diaminobenzidine substrate-chromogen (Dako Corporation) with counterstaining using Harris hematoxylin. Images were captured using a Zeiss Axiophot Photomicroscope, and the area staining positive (brown) for F4/80 antigen expressed as a percentage of the total lesion area. Brown staining appeared
intense and distinct and could be distinguished readily from macrophage-free areas without the need for colour threshold.

Collagen was determined as described previously. Briefly, 4 µm sections were deparaffinized, rehydrated and stained with 0.1% Sirius red (Fast red F3B) in saturated aqueous picric acid (pH 2.0) for 1 h at room temperature and then transferred to a solution of 0.01 N HCl for 2 min followed by counterstaining in Harris hematoxylin for 1 min. Total lesion area were measured from bright field images captured with an Olympus BX60 photomicroscope attached with a SPOT digital camera, whereas the birefringent area staining positive for Sirius red was detected using polarization microscopy and expressed as a percentage of the total lesion area at that site.

RT-PCR

Total RNA was isolated using the RNAeasy for fibrous tissues Midi kit (Invitrogen). The tissue was disrupted and homogenized in RLT buffer using a FastPrep machine (BIO 101). Briefly, aortic tissue was cut into small pieces, transferred into Lysin Matrix D centrifugation tubes (Q-Biogene) containing 1 mL RLT buffer, and then disrupted using three 20 sec cycles at speed 6. The homogenate was then transferred into a new centrifugation tube, and proteinase K and column purification steps carried out as indicated by the manufacturer. RNA was eluted in RNase-free H₂O and its concentration and purity determined by measuring the absorption at 260 and 280 nm. RNA preparations were analyzed by denaturing agarose gel electrophoresis and stored at −80°C. On the day of analysis, total aortic RNA (300 ng) was digested with RNase-free DNase (Promega), the DNase then removed by phenol/chloroform extraction, and the RNA precipitated by ethanol and re-suspended in RNase-free H₂O. cDNA was prepared by reverse transcription using the Superscript III first strand synthesis kit (Invitrogen). The provided
random hexamer primers were used to prime RNA and a RNase H step was included to digest the RNA after reverse transcription.

Real time RT-PCR was performed in an ABI 7700 sequence detector using the SYBR Green PCR kit (Applied Biosystems). cDNA corresponding to approximately 10 ng of total RNA was used per 20 µL RT-PCR reaction. The housekeeping gene hydroxymethylbilane synthase (HMBS) was used as internal standard, with the following primer sequences: HMBS forward: 5’ AGATTCTTGATACTGCACTC 3’; HMBS reverse: 5’ TGAAAGACAACAGCATCACA 3’; F4/80 forward: 5’ CTCTGTGGTCCACCTTCAT 3’; F4/80 reverse: 5’ GATGGCCAAGGATCTGAAAA 3’. A minus reverse transcriptase cDNA control was used as negative PCR control. Melting curve analyses were performed to verify the specificity of the amplification. mRNA levels of the candidate genes were determined as the amount of the respective mRNA relative to HMBS mRNA levels using the comparative C_\text{T} method described in the ABI 7700 Sequence Detector User Bulletin 2. Sequence analyses of gel purified PCR products obtained by amplification of two different templates confirmed the specificity of the PCR amplification.

References


Table I. Lipids, antioxidants and oxidized lipids in apoE-/- mice after 24 weeks of intervention

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Treatment</th>
<th>Aortic Site</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Sinus</td>
</tr>
<tr>
<td>Lipids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>Control</td>
<td>55.6 ± 4.0</td>
</tr>
<tr>
<td></td>
<td>Probucol</td>
<td>36.5 ± 3.1</td>
</tr>
<tr>
<td>C18:2</td>
<td>Control</td>
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<tr>
<td></td>
<td>Probucol</td>
<td>2.42 ± 0.3</td>
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<tr>
<td>C20:4</td>
<td>Control</td>
<td>1.38 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>Probucol</td>
<td>1.30 ± 0.2</td>
</tr>
<tr>
<td>Antioxidants</td>
<td></td>
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</tr>
<tr>
<td>α-Tocopherol</td>
<td>Control</td>
<td>0.21 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>Probucol</td>
<td>0.15 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>Probucol</td>
<td>1.3 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Probucol metabolites</td>
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</tr>
<tr>
<td>Oxidized lipids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CE-OOH</td>
<td>Control</td>
<td>6.2 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>Probucol</td>
<td>3.5 ± 0.43</td>
</tr>
<tr>
<td>7KC</td>
<td>Control</td>
<td>0.018 ± 0.005</td>
</tr>
<tr>
<td></td>
<td>Probucol</td>
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</tr>
<tr>
<td>F&lt;sub&gt;2&lt;/sub&gt;-isoprostanes</td>
<td>Control</td>
<td>0.090 ± 0.048</td>
</tr>
<tr>
<td></td>
<td>Probucol</td>
<td>0.039 ± 0.005</td>
</tr>
</tbody>
</table>

Data are given in nmol (lipids and antioxidants) or pmol per mg wet weight (oxidized lipids) and show mean ± SEM from four separate pools each containing 19 (control) and 15 (probucol) respective sections, except for F<sub>2</sub>-isoprostanes that show mean ± SEM of ten individual sections.

CE represents C18:2 plus C20:4. Probucol metabolites refer to probucol bisphenol and its oxidation product probucol diphenoquinone. <sup>a,b</sup>Significantly different from sinus and arch, respectively.
Figure Legends

Figure I. Effect of probucol on atherosclerosis in apoE-/- mice

*In situ* photographs of aortic tree for control (A) and probucol treated (B) male apoE-/- mice fed a high fat diet for six months. For morphometric assessment, sections were taken at the aortic sinus, arch and thoracic and abdominal aorta as described in the Materials and Methods section. The photographs, taken using an Olympus DP10 digital camera and Olympus Flashpath software, are representative of 103 control and 87 probucol-treated mice. Aortic lesions were clearly smaller in probucol-treated versus control mice, except for the aortic sinus, where lesions were smaller in control (C) than probucol-treated mice (D).

Figure II. Macrophage and extra-cellular matrix content in the aortic sinus of atherosclerotic lesions of apoE-/- mice

Aortic sinus lesions of apoE-/- mice fed a high fat diet without (A, C, E) and with 1% (w/w) probucol (B, D, F) were stained for macrophages (A, B) or collagen (C-F) as described in the Methods section. Accumulation of macrophages/macrophage foam cells (brown staining) is evident along the luminal side of the lesions from control and probucol-treated mice. Bright field images (C, D) were used to determine lesion areas, whereas polarization microscopy (E, F) was used to analyze the collagen-containing area that exhibits strong birefringence (red staining). The sections shown are representative of the results seen in six different animals (for control and probucol). Calibration bar represents 2 μm.