Proatherogenic and Antiatherogenic Effects of Probucol and Phytosterols in Apolipoprotein E–Deficient Mice
Possible Mechanisms of Action

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Background—The effects of probucol and a phytosterol mixture (FCP-3PI) on atherosclerotic lesion formation, plasma lipoproteins, hepatic and lipoprotein lipase activities, antioxidant enzyme activities, and plasma fibrinogen were investigated in apolipoprotein E–knockout (apoE-KO) mice.

Methods and Results—Three groups of 8 mice were fed a diet containing 9% (wt/wt) fat (controls) or the foregoing diet supplemented with either 1% (wt/wt) probucol (the probucol group) or 2% (wt/wt) FCP-3PI (the FCP-3PI group) for 20 weeks. Compared with controls, atherosclerotic lesion size was 3 times greater in the probucol group, whereas it was decreased by half in the FCP-3PI group. Probucol treatment resulted in high plasma probucol concentrations, which correlated \( r=0.69 \) with the lesion area. HDL cholesterol was reduced \( (>75\%) \) in the probucol group and slightly increased \( (14\%) \) in the FCP-3PI–treated group. Postheparin lipoprotein lipase (LPL) activity was significantly reduced in both treatment groups, but only FCP-3PI significantly decreased hepatic lipase activity. Plasma fibrinogen was increased 42% by probucol and decreased 19% by FCP-3PI relative to controls. Probucol significantly increased plasma glutathione reductase, glutathione peroxidase, and superoxide dismutase activities \( (P<0.05) \). In contrast to findings in apoE-KO mice, there was no probucol-induced atherosclerosis in their wild-type counterparts fed the same dose for the same period of time.

Conclusions—Antiatherogenic activity of FCP-3PI in apoE-KO mice is associated with an increase in HDL cholesterol concentration along with decreases in hepatic lipase activity and plasma fibrinogen concentrations. Proatherogenic effects of probucol may be related to increased plasma fibrinogen, decreased HDL cholesterol concentrations along with decreased LPL activity, or its direct “toxicity” due to very high plasma concentration. Our studies demonstrate that the antioxidant and cholesterol-lowering properties of probucol do not prevent atherogenesis in this particular animal model.

Key Words: hypercholesterolemia ■ atherosclerosis ■ lipoproteins ■ fibrinogen ■ antioxidants

A polipoprotein E–knockout (apoE-KO) mice have been used extensively to study the relation of hypercholesterolemia and lipoprotein oxidation to atherogenesis.1–7 Phytosterols5,6 and the antioxidant \( N,N' \)-diphenyl-1,4-phenylenediamine7 both reduced the severity of aortic atherosclerosis in apoE-KO mice, with and without reduction in plasma cholesterol concentrations, respectively. In contrast, probucol, an agent with antioxidant and cholesterol-lowering properties, paradoxically promoted atherogenesis in apoE-KO mice8,9 and LDL receptor–deficient mice.10 These findings suggest that factors other than hypercholesterolemia and decreased antioxidant status may be involved in the development of atherosclerotic plaques.

To investigate the mechanisms involved in the pathogenesis of accelerated atherogenesis in this animal model, we compared the effects of probucol with those of a phytosterol mixture, FCP-3PI. Our findings indicate a paradoxical proatherogenic effect of probucol, despite its prominent cholesterol-lowering and antioxidant properties. Increased fibrinogen concentrations, decreased HDL cholesterol concentrations, and diminished lipoprotein lipase (LPL) activity may in part account for the atherogenicity of probucol in the apoE-KO mouse model of atherogenesis.

Methods

Animals and Diets
Twenty-four 4-week-old male C57BL/6J mice homozygous for deletion of the apoE gene (apoE-KO) and 6 wild-type C57BL/6J mice were purchased from the Jackson Laboratory, Bar Harbor, Me. After a 10-day adaptation period, they were divided into 3 groups...
TABLE 1. ApoE-KO Mouse Body Weight at Outset and During Experimental Period

<table>
<thead>
<tr>
<th>Groups</th>
<th>Week 0</th>
<th>Week 6</th>
<th>Week 14</th>
<th>Week 20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>24.9±1.8</td>
<td>31.4±2.0</td>
<td>34.9±3.4</td>
<td>37.4±5.4</td>
</tr>
<tr>
<td>FCP-3PI treated</td>
<td>24.2±1.6</td>
<td>30.6±2.8</td>
<td>35.3±3.9</td>
<td>37.5±4.6</td>
</tr>
<tr>
<td>Probucol treated</td>
<td>23.4±1.5</td>
<td>31.5±1.6</td>
<td>32.9±1.4</td>
<td>32.9±1.3</td>
</tr>
</tbody>
</table>

Values are mean±SD in grams.

(control, probucol treated, and FCP-3PI treated) of 8 (apoE-KO) and 2 (C57BL/6j) mice each, matched for their plasma lipid concentrations and body weight. The mice were fed with Picolab mouse diet 20 (Jamieson’s Pet Food Distributor) (1) without additional supplementation (control), (2) supplemented with 1% (wt/wt) probucol (Hoechst Marion Roussel Inc; probucol treated), or (3) supplemented with 2% (wt/wt) FCP-3PI (phytosterol treated). FCP-3PI (previously named TODPM) extraction and purification and the preparation of supplemented diets have been previously described.5 The phytosterol mixture used contained 69% β-sitosterol, 16% sitostanol, and 15% campesterol. Animals were weighed biweekly, and results are presented in Table 1. Except for the small eruptive skin lesions in the ear area of 1 control and 5 probucol-treated mice, all animals looked healthy and were active during the experimental period. The study was approved by the Animal Care Committee of the University of British Columbia.

Blood Sampling
Plasma and red cells were prepared as previously described5 and used for biochemical analyses.

Histological Examination
Sections from the aortic roots and thoracic aortas were cut and stained with oil red O (ORO), hematoxylin and eosin, and Movat pentachrome as previously described.5

Morphometry
Aortic root sections stained with ORO were used for morphometric measurements as previously described.5 Briefly, using a Quanta BioTech II digitizing system, we determined total lesion area, external circumference of the aortic root, and total lumen area of the aortic root sections. We calculated lesion to lumen ratio by dividing the average lesion area by the average lumen area.

Plasma Probucol Concentrations
The concentration of probucol in the terminal plasma samples was determined by a previously published method.9 The mean of duplicate measurements was calculated for each animal in the probucol-treated group.

Lipoprotein Cholesterol Concentrations
Plasma lipoprotein fractions were separated by a fast protein liquid chromatography system.11 Briefly, aliquots of plasma were injected into the system, and fractions corresponding to VLDL and IDL, LDL, and HDL were collected. Cholesterol was extracted from the pooled fractions of VLDL/IDL, LDL, and HDL and was quantified enzymatically.5

Plasma Lipase Activity
Aliquots of plasma (before and after heparin) were used for measurement of lipase (total, lipoprotein, and hepatic) activity as previously described.12 Briefly, labeled triglyceride ([3H]triolein) was incubated with plasma samples, and liberated fatty acids ([3H]oleate) were quantified by liquid scintillation counting.

Plasma Fibrinogen Analysis
At the final sampling period, heparin (1.5 U/g IP) and pentobarbital (60 mg/kg IP) were administered. Fibrinogen was measured by the established Clauss method.

Red Cell and Plasma Antioxidant Enzyme Analyses
Red blood cells from the terminal blood samples were separated from plasma by centrifugation and washed twice with isotonic saline. Aliquots of plasma samples and red cells were analyzed for the activity of glutathione peroxidase, glutathione reductase, superoxide dismutase, and catalase as previously described.13

Statistical Analyses
Results were analyzed by 1-way ANOVA followed by application of the Tukey test to assess the significance of specific intergroup differences with SPSS software. Data are expressed as mean±SD.

Results
Histological Findings
Sections from aortic roots from all animals showed ORO-positive atherosclerotic lesions by light microscopic examination. However, the extent and severity of the lesions varied markedly among the 3 groups of mice. The probucol-treated animals had the worst lesions with regard to size, severity, and lipid enrichment. Sections from the FCP-3PI–treated animals had lesions with the least volume and apparently less complexity than either the control or the probucol-treated groups. Numerous cholesterol clefts were observed in sections from the probucol-treated and control groups but not in the sections from the FCP-3PI–treated animals. Differences in the extent, severity, and complexity of the lesions from probucol and control groups were more readily appreciated when sections were stained with Movat pentachrome. Figure 1 compares the lesions in representative sections stained with ORO and Movat staining of aortic root sections cut from similar anatomic locations in individual mice from the FCP-3PI–treated (A and B), control (C and D), and probucol-treated (E and F) groups. Figure 1E shows an increased amount of extracellular matrix in the presence of sheaths of apparently proliferative smooth muscle cells, numerous foam cells, and cholesterol crystals indicative of mature and complex lesions in the probucol-treated group. These characteristic features of atherosclerotic lesions are present to a lesser extent in Figure 1C (the control group) and are much less evident in Figure 1A (the FCP-3PI–treated group). Figure 1E also includes evidence of an atherosclerotic aortic aneurysm (arrow). Similarly, as shown in Figure 2, thoracic aortas from the probucol-treated animals showed severe atherosclerotic lesions by various histochemical staining methods (G, H, and I). As evident in a Movat-stained section (Figure 2H, straight arrow) from a representative aorta of the probucol-treated group, all elastic laminae were disrupted, rendering the aorta prone to the development of an atherosclerotic aneurysm and, potentially, to through-and-through rupture of the vessel wall. However, such prominent lesions in thoracic aortas were not observed frequently. In concordance with findings in the aortic roots, the control group also had advanced lesions in their thoracic aortas (Figure 2D, 2E, and 2F). A normal-appearing aorta from an FCP-3PI–treated animal with intact intimal and medial elastic laminae and normal-appearing smooth muscle cells and endothelium is also shown (Figure 2A, 2B, and 2C). In contrast to apoE-KO mice, none of their wild-type counterparts had any evidence of atherosclerotic lesions in their aortic roots.
Aortic Morphometry
Probucol treatment caused a 175% increase (relative to the untreated group) in average lesional area (in square millimeters) of aortic roots, whereas FCP-3PI treatment caused a 50% decrease (relative to the untreated group). These changes paralleled other morphometric measurements such as circumference of the aortic roots (in millimeters) and lesion-to-lumen ratios. Thus, as summarized in Table 2, the greater the lesional area, the greater the circumference and the higher the lesion-to-lumen ratio. As such, both severe intimal disease and remodeling were evident.

Plasma Probucol Concentrations
The mean concentration of probucol in plasma was $404.6 \pm 92.8 \, \mu g/mL \, (n=8)$. Regression analysis showed a strong ($r=0.69, \, n=8$) relationship between the plasma concentrations of probucol and the size of aortic lesions in the probucol-treated mice.

Cholesterol Concentrations of Plasma Lipoprotein Fractions
Both probucol and FCP-3PI treatments significantly reduced plasma VLDL/IDL cholesterol concentrations. However, this reduction was greater in the probucol-treated group (78% versus 37% decrease by probucol and FCP-3PI, respectively, relative to controls). Similarly, the LDL cholesterol-lowering effects of probucol were greater than those of FCP-3PI (50% versus 20%, respectively). On the other hand, HDL cholesterol concentrations were markedly reduced ($>75\%$ decrease) by probucol treatment and slightly increased (by $\approx 14\%$) in the FCP-3PI-treated animals compared with controls. Mean plasma lipoprotein cholesterol concentrations are depicted in Figure 3. In wild-type C57BL/6J animals, plasma total cholesterol concentrations remained unchanged with phytosterol treatment, but they were reduced by $>80\%$ in the probucol-treated animals.

Plasma Lipolytic Activity
Intraperitoneal administration of heparin markedly increased total plasma lipase activity (30 versus 365 mU [the control group], 30 versus 248 mU [FCP-3PI–treated group], and 37 versus 250 mU [probucol-treated group], mean values, $n=8$). Table 3 shows significant reductions in postheparin total, lipoprotein, and hepatic lipase activities in response to both probucol (except hepatic lipase) and FCP-3PI treatments. Probucol and FCP-3PI treatments caused 40% and 31% decreases, respectively, in the average LPL activity compared with the control group ($P<0.05$). Compared with controls, hepatic lipase activity was significantly reduced by 35% in the FCP-3PI–treated group only.

Plasma Fibrinogen Concentrations
Fibrinogen concentrations differed between the 2 treatment groups and as compared with the control group. Probucol treatment caused a significant increase ($42\%$) in fibrinogen concentrations ($2.6 \pm 0.7$ versus $3.7 \pm 0.7 \, g/L; \, n=8; \, P<0.01$), whereas FCP-3PI treatment reduced fibrinogen by 19% ($2.6 \pm 0.7$ versus $2.1 \pm 0.2 \, g/L; \, n=8$) compared with controls.

Antioxidant Enzyme Activities
Table 4 summarizes the effect of each treatment on the activity of antioxidant enzymes in red blood cells and plasma.
Both treatment regimens, particularly probucol, altered the activity of plasma antioxidant enzymes. Among the erythrocyte enzymes examined, glutathione reductase activity increased in both treatment regimens. The extent of the increment in erythrocyte glutathione reductase resulting for probucol was approximately twice that in the FCP-3PI-treated group (24% versus 14%). Similarly, the effect of probucol on increasing plasma glutathione peroxidase and superoxide dismutase activities was 5 times greater than that of FCP-3PI. Probucol and FCP-3PI had opposite effects on the activity of plasma glutathione reductase.

Discussion

Although both probucol and FCP-3PI reduced VLDL and LDL cholesterol, the extent of this effect was more marked with probucol treatment. HDL cholesterol was dichotomously affected by probucol and FCP-3PI. The mechanism of the cholesterol-lowering effect of probucol is poorly understood. Phytosterols, however, are known to inhibit cholesterol absorption.

Probucol is proatherogenic in apoE-KO mice and LDL receptor-deficient mice. One possible explanation is the well-known HDL cholesterol–lowering effect of probucol in various species, including transgenic mice. Because hepatic lipase–deficient mice show increased HDL production, the FCP-3PI treatment–induced slight (14%) increase in HDL cholesterol concentrations is possibly related to the significant decrease in hepatic lipase activity. The observed reductions in both hepatic and LPL activities by FCP-3PI treatment may prevent formation of small atherogenic

TABLE 2. Calculated Morphometry Values for Aortic Roots (Mean±SD per Section)

<table>
<thead>
<tr>
<th>Groups</th>
<th>Lesion Area, mm²</th>
<th>External Circumference, mm</th>
<th>Lesion/Lumen Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=7)</td>
<td>0.4±0.1</td>
<td>5.1±0.4</td>
<td>0.22±0.02</td>
</tr>
<tr>
<td>Probucol (n=8)</td>
<td>1.1±0.6*</td>
<td>6.4±1.0*</td>
<td>0.36±0.08*</td>
</tr>
<tr>
<td>FCP-3PI (n=8)</td>
<td>0.2±0.1</td>
<td>4.3±0.3</td>
<td>0.12±0.04†</td>
</tr>
</tbody>
</table>

Values are mean±SD per section.

*P<0.01 compared with either controls or FCP-3PI group; †P<0.05 compared with controls.
and may also decrease their uptake by the LDL receptor–related protein.\textsuperscript{20}

In addition to decreases in HDL cholesterol concentrations, probucol also alters the size and quality of HDL particles.\textsuperscript{21} Although the mechanisms by which probucol influences the metabolism of HDL particles are not clear, changes in LPL and cholesteryl ester transfer protein (CETP) may play a role. Probucol treatment in humans is associated with an increase in CETP mass, which was correlated with the concomitant decrease in HDL cholesterol concentrations.\textsuperscript{22} Consistent with our findings, probucol caused a significant reduction in postheparin LPL activity and HDL cholesterol concentrations in patients with moderate hypercholesterolemia and in rats.\textsuperscript{23,24} However, the HDL cholesterol–lowering action of probucol in animals (mouse and rat) without CETP\textsuperscript{25,26} suggests that this effect of probucol is independent of CETP activity.

Because probucol treatment was not proatherogenic in wild-type mice (despite its HDL-lowering effects), it seems less likely that it is the major mechanism of its proatherogenic activity in the apoE-KO mouse model.\textsuperscript{9} Moreover, several studies\textsuperscript{27–30} have shown beneficial effects of probucol on atherosclerosis and xanthomatosis in both humans and animals.

![Figure 3. Total cholesterol concentrations (mmol/L, mean±SD) recovered from pooled lipoprotein fractions corresponding to VLDL/IDL, LDL, and HDL after separation by a fast protein liquid chromatography system. n=7, 8, and 6 for untreated controls, FCP-3PI-treated, and probucol-treated groups, respectively. *P<0.05 compared with controls.](image)

**TABLE 3. Plasma Postheparin Lipase Activity**

<table>
<thead>
<tr>
<th>Lipase</th>
<th>Control (n=8)</th>
<th>Probucol (n=8)</th>
<th>FCP-3PI (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total lipase</td>
<td>365.6±49.5</td>
<td>250.6±64.3*</td>
<td>248.4±28.8*</td>
</tr>
<tr>
<td>LPL</td>
<td>244.0±64.4</td>
<td>146.5±68.5*</td>
<td>169.6±23.0*</td>
</tr>
<tr>
<td>Hepatic lipase</td>
<td>121.6±36.0</td>
<td>104.1±27.9</td>
<td>78.8±20.0*</td>
</tr>
</tbody>
</table>

Values are mean±SD in milliunits. *P<0.05 compared with controls.

Although it is not clear whether the increased plasma fibrinogen is a marker of disease or a cause or both, the increased plasma fibrinogen may be considered a possible causal factor for the proatherogenic effect of probucol in apoE-KO mice. Fibrinogen concentrations have been significantly correlated with the likelihood of atherosclerotic cardiac events.\textsuperscript{31,32} In rabbits, probucol showed an antiatherogenic effect in association with a reduction in fibrinogen concentrations (but no effect on cholesterol levels).\textsuperscript{33} In the present study, FCP-3PI–treated animals had lower fibrinogen concentrations and fewer (and less severe) atherosclerotic lesions than either the control or probucol-treated groups. The mechanisms underlying changes in plasma fibrinogen levels caused by probucol and phytosterols remain to be studied.

We have shown herein that treatment with probucol is accompanied by an increase in the activity of several endogenous antioxidant enzymes. However, foam cell formation was increased. This suggests that the antioxidant effects of probucol alone are not sufficient to prevent lesion development.

We have documented that foam cell formation is increased in probucol-treated mice but significantly reduced in FCP-3PI–treated animals. It may be speculated that modification of β-VLDL (a major substrate for foam cell formation) by phytosterols may result in its decreased uptake by macrophages, thereby delaying foam cell formation and plaque development. The opposite may occur in probucol-treated mice. Histological examination revealed many cholesterol clefts in the lesions from the probucol-treated animals; these lesions also contained numerous foam cells and a substantial amount of extracellular matrix, indicating their greater maturity and complexity compared with those from either the control or the FCP-3PI–treated mice. The fact that there is no...
Effects of Probucol and Phytosterols in ApoE-KO Mice

TABLE 4. Antioxidant Enzyme Activities in Red Blood Cells and Plasma

<table>
<thead>
<tr>
<th></th>
<th>Red Blood Cells</th>
<th></th>
<th>Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GPx</td>
<td>GRed</td>
<td>CAT</td>
</tr>
<tr>
<td>Control (n=8)</td>
<td>40.1±10.8</td>
<td>2.1±0.1</td>
<td>5.6±0.7</td>
</tr>
<tr>
<td>Probucol (n=8)</td>
<td>44.8±12.4</td>
<td>2.6±0.2†</td>
<td>5.6±0.7</td>
</tr>
<tr>
<td>FCP-3PI (n=8)</td>
<td>43.3±4.8</td>
<td>2.4±0.2</td>
<td>4.8±0.9</td>
</tr>
</tbody>
</table>

GPx indicates glutathione peroxidase; GRed, glutathione reductase; SOD, superoxide dismutase; and CAT, catalase. GPx and GRed are in nanomoles per minute per milligram of protein/hemoglobin; SOD is in units per milligram of protein/hemoglobin; and CAT is in "k" (reaction rate constant) per milligram of hemoglobin.

Values are mean±SD.

*P<0.05 compared with either controls or FCP-3PI group; †P<0.05 compared with FCP-3PI group; ‡P<0.05 compared with controls.

Evidence of probucol-induced atherosclerotic lesions in wild-type mice\textsuperscript{34,35} suggests specific intracellular effects of probucol\textsuperscript{10} in apoE-KO mice and LDL receptor-deficient mice but not in their wild-type counterparts.

Similar to a recently published study in LDL receptor-deficient mice,\textsuperscript{10} our data indicate a correlation (r=0.69) between the extent of atherosclerotic lesions in the aortic roots and the plasma concentration of probucol. Thus, proatherogenic effects of probucol appear to depend on the dose and duration of the treatment. The average plasma probucol concentration in our study was 405 µg/mL (after 20 weeks of consumption of 1% wt/wt probucol), whereas it was 56 µg/mL in a previous study in which male apoE-KO mice consumed 0.5% (wt/wt) probucol for 3 months.\textsuperscript{9} Similarly, the mean lesion size was greater in the present study than in the aforementioned study.\textsuperscript{9} Probucol concentrations in the present study were also ≈4 times greater than those in Watanabe heritable hyperlipidemic rabbits administered the same dose (1% wt/wt) of probucol for 20 months.\textsuperscript{30} Plasma probucol concentrations observed in the present study were comparable to those reported in Watanabe rabbits treated with 1% probucol for 6 months.\textsuperscript{36} In another study, male LDL receptor-deficient mice also had very high plasma probucol concentrations (497 µmol/L, ≈260 µg/mL) after consumption of 0.5% (wt/wt) probucol.\textsuperscript{10} Given the fact that apoE-KO mice are severely hypercholesterolemic, our goal was to achieve greater lipid lowering through higher plasma probucol concentration than that usually attained in humans. Therefore, on the basis of previous studies,\textsuperscript{5,6,9,17,30,34–41} we estimated that 1% (wt/wt) probucol would result in a plasma concentration twice that seen in humans treated with probucol at a dose of 1 g/d. By the end of the study, plasma probucol concentration was higher than expected. Probucol is a strongly lipophilic agent, and thus it accumulates in lipoprotein particles\textsuperscript{36}; it is also deposited into fat-containing tissues. The half-life of probucol in plasma depends on the rate of clearance of plasma lipoproteins. Mice lacking apoE have a substantial delay in the metabolism of lipoproteins, particularly VLDL. This delay may significantly increase the elimination half-life of probucol. Another factor that could explain the high plasma concentration of probucol concerns the loss of virtually all omental fat in probucol-treated mice, this being the major site of tissue sequestration of probucol. Dietary fat may increase the absorption of probucol; thus, 9% (wt/wt) dietary fat in the present study may also contribute to the high plasma probucol concentration and its proatherogenic effects.

In conclusion, there is no report of a proatherogenic effect of probucol (from 0.2% to 2% wt/wt of diets or up to 800 mg·kg\textsuperscript{-1}·d\textsuperscript{-1} by stomach tube) in other strains of mouse. Additional experiments using treatment with much lower doses of probucol in apoE-KO mice will elucidate whether the proatherogenic effect of the treatment is related to drug toxicity. Our data suggest that increased plasma antioxidant activity alone does not result in decreased foam cell formation, at least in the animal model studied.

Acknowledgments

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