Probuloc Promotes Functional Reendothelialization in Balloon-Injured Rabbit Aortas

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Methods and Results—Aortic balloon-injured New Zealand White rabbits were fed 2% (wt/wt) cholesterol-enriched or normal chow, with 0.75% (wt/wt) probucol (P) or without (controls, C) for 6 weeks. Endothelial denudation of the abdominal aorta was performed at week 3 with a 3F Fogarty embolectomy catheter. The arteries were harvested after week 6 and analyzed for histology, lipids and antioxidants, and endothelial regeneration and function. Probuloc significantly decreased aortic intima-to-media ratio (cholesterol-fed: C, 1.10±0.08 versus P, 0.70±0.10; normal: C, 0.89±0.02 versus P, 0.83±0.05; P<0.05) and the numbers of proliferating intimal smooth muscle cells and lowered serum cholesterol without altering the proportion of aortic lipids that was oxidized. Probuloc promoted endothelial regeneration in the injured aorta in cholesterol-fed rabbits (25% increase in reendothelialization, P<0.05) and in those on normal chow (37% increase, P<0.01). This was associated with both improved endothelial function as assessed by enhanced aortic ring relaxation and cGMP production in response to acetylcholine and decreased intimal thickening.

Conclusions—Probuloc inhibits intimal thickening in balloon-damaged arteries of rabbits by promoting the regeneration of functional endothelium, without affecting the proportion of aortic lipids that was oxidized. This novel in vivo finding helps explain how probucol inhibits restenosis after coronary angioplasty and highlights potential new targets for therapeutic intervention. (Circulation. 2003;107:2031-2036.)

Key Words: angioplasty ■ antioxidants ■ atherosclerosis ■ endothelium ■ restenosis

Angioplasty has revolutionized the treatment of advanced atherosclerosis, although the long-term success of angioplasty remains limited by the occurrence of restenosis. Most conventional drugs have failed to inhibit restenosis, one exception being probucol, a cholesterol-lowering drug with antioxidant properties that consistently protects large1,2 and small3 coronary arteries from renarrowing after balloon angioplasty.

Probuloc affects many vascular processes, although the exact mechanisms underlying its protective activities in vivo remain unclear. Probuloc inhibits atherogenesis in most animal models4 and causes regression of xanthomas in subjects with hypercholesterolemia.5 On the basis of the “oxidation theory,” the oxidation of LDL in the artery wall is an early initiating event and contributes to atherogenesis.6 Probuloc may interfere with this process via its antioxidant activity7 independently of lowering cholesterol. Indeed, probucol inhibits atherosclerosis and copper-induced ex vivo oxidation of LDL in nonhuman primates8 and in Watanabe hyperlipidemic rabbits under cholesterol-clamped conditions.9 Probuloc may also decrease vascular superoxide production, leading to improved endothelial function.10

Probuloc promotes positive adventitial remodeling after balloon angioplasty in humans,11 and it inhibits intimal thickening after balloon injury in swine coronary12 and in rabbit and rat carotid arteries independently of lowering cholesterol.13,14 In addition, probucol inhibits the proliferation of vascular smooth muscle cells (VSMCs) in vitro15 and in vivo16 whereas it promotes the proliferation of endothelial cells in vitro.17 These properties would make probucol an ideal drug for preventing restenosis after angioplasty. Probuloc also possesses anti-inflammatory properties.18 It down-regulates endothelial expression of adhesion molecules19 and decreases tissue macrophages,18,20 secretion of interleukin-1 from macrophages,21 and expression of tumor necrosis factor-α in the vessel wall.22

Given the involvement of endothelial dysfunction and cellular proliferation in atherosclerosis and restenosis,10,23 we...
hypothesized that probucol has broad protective effects on the arterial wall. We provide evidence here that probucol inhibits intimal thickening independently of inhibition of lipoprotein oxidation in the balloon-injured rabbit aorta. We show that probucol promotes the regrowth of functional endothelium associated with an inhibition of intimal proliferation of VSMCs. We propose that the promotion of reendothelialization is a novel protective effect of probucol in vascular proliferative diseases.

Methods

Rabbit Aortic Balloon-Injury Model
Male New Zealand White rabbits (\(\sim 3\) kg; Merunga Farm, Coffs Harbour, Australia) were fed for 6 weeks with chow or without 2\% (wt/wt) cholesterol (USP grade, ICN), with half of each group receiving 0.75\% (wt/wt) probucol (96\% purity). Each rabbit consumed, on average, 150 g chow per day. After 3 weeks, a 3F Fogarty embolectomy catheter (Baxter) was inserted into the right femoral artery, advanced 25 cm proximally, and then withdrawn to the origin with the balloon inflated to 0.2 mL saline, a step repeated twice. At 6 weeks, aorta was harvested. Segments for biochemical analyses were perfused, homogenized, and analyzed by high-performance liquid chromatography; those for histology were pressure-perfused with formalin, stored in 70\% (vol/vol) ethanol, and then embedded in paraffin.

Histology
Cross sections (5 \(\mu\)m) were taken at nonbranched and transverse sections (5 \(\mu\)m) at branch areas and stained with Verhoeff’s hematoxylin, and adjacent sections were immediately stained with HHF-35 and proliferating cell nuclear antigen (Dako), respectively. Intimal foam cells were counted in 3 random high-power fields and expressed as mean number per field. Digital images were obtained, and planimetry (Adobe Photoshop V5.0) was performed with the investigators blinded by tracing the intimal and medial areas and dividing their respective total pixel numbers to determine the intima-to-media ratio. The circumference of the external elastic lamina was traced to assess adventitial remodeling.

Endothelial Regeneration
Evans blue dye (20 mg/kg, Serva) diluted to 5 mL with saline was injected into the carotid artery 30 minutes before euthanasia. Perfused aorta was removed and opened along the midline ventrally. Color photographs of en face aorta were digitalized (Hewlett Packard 5P Scanjet) and combined to reconstruct the whole vessel (Adobe Photoshop). Pixel numbers in blue and nonblue areas were quantified (Video Pro 32 system), and results were expressed as the percentage nonblue of total (blue plus nonblue) area.

Endothelial Function
At 6 weeks, undamaged thoracic (root to 7th intercostal arteries) and abdominal (12th intercostal pair to 5th lumbar arteries) aortas were removed. Cuts were made at the 3rd to 6th intercostal, celiac, superior mesenteric, and 2nd and 3rd lumbar branches. Rings (3 mm) extending distally were used for isometric tension experiments, and those extending proximally were analyzed for nitric oxide synthase (NOS) activity. Briefly, the viability of rings was confirmed by incremental constriction to norepinephrine (2.5 \(\mu\)g load). After preconstriction to 50\% maximal response, rings were exposed to incremental doses of acetylcholine and then sodium nitroprusside. For NOS activity, rings preincubated in isobutylmethylxanthine to inhibit phosphodiesterase were exposed to 1 \(\mu\)mol/L of acetylcholine for 1 minute, snap-frozen, and stored at -80\°C. They were subsequently homogenized and acetylated, and the content of cGMP was determined by enzyme immunoassay (Cayman Chemicals).

Figure 1. Probucol inhibits intimal thickening in balloon-injured aorta. a. Specimens are representative of 20 control (left) and 20 probucol-treated (right) rabbits. b. Intima-to-media ratio in control (open bars) and probucol-treated (solid bars) hypercholesterolemic (2% C, \(n=16\)) and normocholesterolemic (0% C, \(n=24\)) rabbits. *\(P<0.05\) vs control.

\[^{3}H\]Thymidine Incorporation and Cell-Cycle Analysis
Rabbit aortic VSMCs (gift of S. Ylä-Herttuala, University of Kuopio, Finland) (passages 7 to 14) were grown in DMEM containing antibiotics and 10% FBS (Gibco). Primary pig aortic endothelial cells were isolated, maintained in M199 containing 10% FBS, 10 \(\mu\)g heparin/mL, 2 \(mmol/L\) glutamine, and antibiotics, and used before the 4th passage. VSMCs were synchronized in G\(_0\)/G\(_1\) by serum deprivation for 48 hours in the presence of 50 \(\mu\)mol/L BSA and probucol or vehicle (0.1\% ethanol). Cell growth was stimulated with 10% FBS, \[^{3}H\]thymidine (2 \(\mu\)Ci per well, ICN Biomedicals) being added 3 hours before cell harvest. Cells were washed 24 hours after stimulation, an aliquot was used for protein determination, and the remainder was used for radioactivity determination by scintillation counting. Cell cycle distribution was performed by measurement of DNA content with an Epics XL flow cytometer (Beckman Coulter).

Statistical Analysis
Data are expressed as mean±SEM. Unpaired Student’s \(t\) tests were used for between-group comparisons. When appropriate, probability values were adjusted for multiple comparisons by Bonferroni post hoc analysis. Acetylcholine and sodium nitroprusside dose responses were compared by repeated-measures ANOVA.

Results

Intimal Thickening
We conducted identical experiments with and without 2\% (wt/wt) cholesterol supplementation. Probucol decreased serum cholesterol in cholesterol-fed (29.2±10.8 versus 17.2±6.3 mmol/L; \(P<0.05\)) and normal chow–fed rabbits (0.9±0.3 versus 0.5±0.0 mmol/L; \(P<0.05\)). Using an injury score,\(^26\) we found that arterial wall damage was limited to the endothelium and internal elastic lamina (not shown), ie, scores of 0 and 1 that do not affect neointimal thickening.\(^26\) Probucol limited the response to injury (Figure 1a), significantly decreasing the intima-to-media ratio in hypercholesterolemic (1.10±0.08 versus 0.70±0.10, \(P=0.01\)) and normocholesterolemic (0.89±0.02 versus 0.83±0.05, \(P=0.03\)) animals (Figure 1b). Serum cholesterol and intima-to-media ratio correlated only moderately (\(r^2=0.28, n=16\)). Probucol
did not affect adventitial remodeling (11.5±1.7 versus 11.2±1.9 mm, P=NS).

Aortic Lipid
Balloon-injured aorta contained more cholesterol than undamaged aorta, and probucol abolished this difference in cholesterol-fed but not normal chow–fed rabbits (Figure 2a). Comparable results were obtained for cholesterol esters (CE), respectively, in uninjured thoracic (T) and injured abdominal (A) aorta of control (open bars) and probucol-treated (solid bars) rabbits fed 2% cholesterol (n=16) and 0% cholesterol (n=12). mgp indicates mg of protein. *P<0.05 vs control.

Reendothelialization
Regrowth of an intact endothelium is a key repair process in response to arterial injury. We therefore assessed whether probucol promotes endothelial regeneration by administration of Evans blue dye that stains endothelium-denuded areas. Three weeks after injury, nearly half of the previously denuded luminal surface was covered by an intact endothelium (that excluded albumin and therefore appeared gray-white), extending from the arterial side branches and the proximal edge of the injured aorta into the denuded (blue) areas (Figure 3a). A significantly larger proportion of the abdominal aorta was reendothelialized in probucol-treated rabbits (Figure 3a), regardless of whether they received normal or cholesterol chow (Figure 3b). Probucol also promoted the proliferation of endothelial cells in vitro in a time-dependent (not shown) and concentration-dependent manner (Figure 3c).

Vascular Reactivity
We next conducted ex vivo vascular reactivity studies with aortic rings taken from normal thoracic aorta and from damaged abdominal aorta at branch points where endothelial regrowth was maximal. In damaged aorta, probucol significantly improved endothelium-dependent (Figure 4a) but not endothelium-independent relaxation (not shown). Probucol had no significant effect on normal aorta (not shown). In acetylcholine-stimulated abdominal aortic segments immediately adjacent to those analyzed for vascular reactivity, concentrations of cGMP were close to those in nondamaged vessels, indicating that probucol enhanced NOS activity (Figure 4b).

Figure 2. Probucol inhibits lipid accumulation independently of lipid oxidation. a–c, Tissue content of cholesterol, cholesteryl ester hydroxides, and hydroperoxides [CE-O(O)H] standardized for protein (nmol/mgp) and cholesteryl esters (CE), respectively, in uninjured thoracic (T) and injured abdominal (A) aorta of control (open bars) and probucol-treated (solid bars) rabbits fed 2% cholesterol (n=16) and 0% cholesterol (n=12). mgp indicates mg of protein. *P<0.05 vs control.

Figure 3. Probucol promotes reendothelialization in balloon-injured abdominal aorta. a, Descending aorta of control (top) and probucol-treated rabbit (bottom) opened longitudinally along its ventral aspect after in vivo Evans blue staining, showing denuded (blue) areas with areas of reendothelialization (white) around side branches, Celiac (CL), superior mesenteric (SM), and third (L3) and fourth (L4) lumbar arteries are indicated. Specimens are representative of 11 control and 11 probucol-treated animals. b, Proportion of intimal surface (white+blue) of abdominal aorta that is reendothelialized in control (open bars) and probucol-treated (solid bars) hypercholesterolemic (2% C, n=12) and normocholesterolemic (0% C, n=10) rabbits. c, Growth of porcine aortic endothelial cells (EC) in presence of increasing concentrations of probucol. Data are representative of 4 independent experiments, each performed in triplicate. *P<0.05 vs control; **P<0.01 vs control.
Reendothelialization and Intimal Thickening

Previous studies reported that reendothelialized areas in Evans blue–stained aorta consist of a central gray zone surrounded by a white zone that borders with adjacent nonendothelialized (blue) areas (Figure 5a). The endothelium in the central gray zone is oriented to blood flow with little underlying intima, whereas that in the peripheral white zone is irregular, without particular orientation and with thicker intima. We therefore assessed whether probucol also decreased intimal thickening in reendothelialized areas as in denuded areas (Figure 5b). Indeed, probucol significantly decreased maximal intimal thickness in white and gray zones where reendothelialization commences (Figure 5, a and b). Probucol tended to increase medial thickness in these areas, reaching significance in the white zones (Figure 5c).

VSMC Proliferation

The intima and media consisted predominantly of HHF-35–stained VSMCs (not shown), and probucol decreased the numbers of total cells and cells positive for proliferating cell nuclear antigen (Table). Probucol inhibited, in a dose-dependent manner, proliferation of VSMCs in vitro, as assessed by [3H]thymidine incorporation (IC50, 9.2 μmol/L). FACS analysis of propidium iodide–stained cells revealed that probucol caused inhibition of VSMC proliferation by G0+G1 growth arrest (Table). Overall, these findings confirmed that probucol directly limited intimal thickening in injured aortas by inhibiting VSMC proliferation.

Discussion

Here, we show that probucol, a cholesterol-lowering drug with antioxidant properties, inhibits the development of intimal thickening in an animal model of arterial injury. As expected, probucol exerted a lipid-lowering effect in the blood and in the arterial wall. However, our novel finding is that probucol did not decrease the extent to which aortic lipid was oxidized. Instead, probucol promoted the regeneration of a functional endothelium, and this was associated with decreased intimal thickening, demonstrating multiple protective effects on the arterial wall.

Consistent with previous work, probucol lowered serum total and HDL cholesterol, but the correlation between serum cholesterol and intimal thickening was only moderate. This suggests that cholesterol lowering is unlikely to be solely responsible for the protective effects of probucol and that other local arterial wall parameters are probably affected. We found that probucol enhanced reendothelialization to similar

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<tr>
<th>Probucol Inhibits Vascular Smooth Muscle Cell Proliferation</th>
<th>Control</th>
<th>Probucol</th>
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<tr>
<td>Cell count</td>
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<tr>
<td>Total</td>
<td>146±9</td>
<td>72±6*</td>
</tr>
<tr>
<td>PCNA+</td>
<td>45±5</td>
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<td>61±1</td>
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<td>S+G2+M</td>
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PCNA indicates proliferating cell nuclear antigen.

*P<0.01, †P<0.05, control vs probucol.
 extents in normal and cholesterol-fed animals, whereas the hypocholesterolemic effect was much more pronounced in the latter. Indeed, a previous study reported inhibition of intimal thickening in balloon-injured carotid artery by probucol independently of lowering cholesterol. Also, although probucol decreases aortic oxysterols and thiobarbituric acid–reactive substances in balloon-injured rabbits, the precise role of these lipid oxidation products in vascular disease remains unclear. More importantly, we show here that protection by probucol was not associated consistently with a decrease in oxidized lipids, whether expressed per protein or parent lipid. Therefore, inhibition of aortic lipoprotein lipid oxidation does not appear to explain why probucol inhibits intimal proliferation in the animal model used, consistent with the inability of probucol to decrease oxidation-specific epitopes in monkeys and Watanabe heritable hyperlipidemic rabbits at aortic sites at which probucol decreases lesion. However, our results do not rule out the possibility that probucol inhibits oxidative events unrelated to lipoprotein oxidation.

The endothelium plays a central role in regulating processes responsible for intimal thickening, and areas that take longer to reendothelialize after balloon injury have significantly more intimal thickening than areas that are rapidly covered with endothelium. Our observation that probucol promoted reendothelialization and inhibited intimal thickening is consistent with this and with the concept that the regenerated endothelium suppresses proliferation of underlying VSMCs. In addition, probucol moderates the severity of vascular injury by inhibiting VSMC proliferation via G1/S growth arrest, as we have confirmed. A potential mediator in this protective process is carbon monoxide, which can transcriptionally regulate cell-cycle progression genes and activate soluble guanylate cyclase, thereby elevating cGMP levels and leading to VSMC relaxation. Indeed, preliminary studies indicate that probucol increases heme oxygenase activity in balloon-injured rabbit vessels (Y.-M. Deng, B. Wu, and R.S., 2002, unpublished observations). A putative regulation of VSMC proliferation by carbon monoxide may be particularly important when NO production is inhibited or lost because of endothelial denudation.

Endothelial regeneration appears to be dependent on NO, because vascular endothelial growth factor activates NOS and NO promotes endothelial cell migration in vivo. We show here that probucol enhances NO bioactivity in aortic rings, although the precise mechanism responsible for this is unclear. Probucol may upregulate endothelial NOS, like other antioxidants. However, the extent of increased reendothelialization, vascular relaxation, and cGMP production was comparable, suggesting that probucol simply increased the number of functional endothelial cells. Probucol may also attenuate increased vascular production of superoxide associated with balloon injury, and superoxide-derived hydrogen peroxide has been reported to promote VSMCs and to inhibit endothelial cell proliferation.

In our study, probucol did not affect arterial remodeling as seen at 6 months in the Multivitamins and Probucol (MVP) study. Our 3-week model may be too short to observe remodeling that occurs later after balloon injury. Also, we induced injury by withdrawing a compliant balloon inflated at low pressure through a healthy large conduit artery, whereas in human angioplasty, a stationary noncompliant balloon is inflated at high pressure within a diseased medium-size muscular artery. Furthermore, our histological measurement of intimal thickness differs from that of the MVP study, which used intravascular ultrasound to identify neointima and media as one unit, finding no effect by probucol on the combined thickness. These differences make direct comparisons between the studies difficult.

Despite its well-established antirestenotic activity, probucol is rarely used because of its required oral preloading and HDL-lowering effects, although the meaning of the latter is poorly understood. Probucol was voluntarily removed from the US market after the US Food and Drug Administration requested additional clinical trials to clarify its QT-prolongation and potential proarrhythmic effects. Our novel in vivo finding that enhanced regrowth of a functional endothelium contributes to the protection against intimal thickening after arterial injury helps explain the ability of probucol to prevent restenosis after coronary angioplasty and may highlight new mechanistic targets for therapeutic intervention.

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References

9. Carew TE, Schwenke DC, Steinberg D. Antiatherogenic effect of probucol unrelated to its hypocholesterolemic effect: evidence that antioxidant in vivo can selectively inhibit low density lipoprotein degradation in macrophage-rich fatty streaks and slow the progression of atherosclerosis in the Watanabe heritable hyperlipidemic rabbit. Proc Natl Acad Sci U S A 1987;84:7725–7729.
10. Keaney JF Jr, Xu A, Cunningham D, et al. Dietary probucol preserves endothelial function in cholesterol-fed rabbits by limiting vascular oxi-
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