Effect of Probucol Dosage on Plasma Lipid and Lipoprotein Levels and on Protection of Low Density Lipoprotein Against In Vitro Oxidation in Humans

Peter D. Reaven, Sampath Parthasarathy, William F. Beltz, and Joseph L. Witztum

To determine whether probucol's ability to confer antioxidant protection to low density lipoprotein (LDL) could be dissociated from its ability to lower high density lipoprotein (HDL) cholesterol, 17 hypercholesterolemic patients were treated with either a standard dose, 1 g/day (4 tablets), or a low dose, 250 mg/day (1 tablet), of probucol for a 6-month period. Effects of therapy on lipoprotein levels and on susceptibility of LDL to in vitro oxidation were measured at frequent intervals. Probucol levels in plasma LDL rose less rapidly in the 1-tablet group but were nearly 50% of levels in the 4-tablet group after 6 months. HDL cholesterol and apolipoprotein A-I decreased 17.6% and 27.9%, respectively, in the 1-tablet group compared with 28.0% and 38.3%, respectively, in the 4-tablet group (p=0.07 and p=0.06). In the 4-tablet group, LDL was protected from copper and endothelial cell–mediated oxidation after 2 months of therapy. In the 1-tablet group, equal degrees of protection occurred, but only after 6 months of therapy. In the whole study group, the decrease in LDL susceptibility to copper or endothelial cell–mediated oxidative modification was correlated with the content of probucol in LDL (r=0.73, r=0.65, p<0.005). Additionally, the decrease in HDL cholesterol level was correlated with the increase in protection to LDL from oxidative modification (r=0.67 for copper, r=0.58 for endothelial cells, p<0.05 for both) and also with the content of probucol in LDL (r=0.6, p=0.01). In conclusion, a low dose of probucol provides impressive protection to LDL against in vitro oxidation and modification, with less marked HDL cholesterol lowering. However, these effects are not completely dissociated from each other, and in fact, the extent of HDL cholesterol lowering was correlated with the LDL probucol content and the resulting antioxidant protection.

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KEY WORDS • macrophage degradation • antioxidants • lipid peroxidation • high density lipoprotein cholesterol

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here are several lines of evidence supporting the hypothesis that oxidatively modified low density lipoprotein (LDL) is important in atherosclerosis, and this has recently been reviewed by Steinberg et al. This hypothesis has led to great interest in the development and evaluation of antioxidants as potential antiatherosclerotic agents. One such antioxidant, probucol, is very lipophilic, has a relatively long half-life, and is carried in blood, primarily in LDL. This combination of properties makes probucol an excellent antioxidant for LDL. In support of this, probucol administration to hypercholesterolemic rabbits and humans has been demonstrated to decrease the susceptibility of their LDL to in vitro oxidative modification.

Probucol has also been shown to inhibit atherosclerosis in rabbits, independent of its cholesterol-lowering effects. Additionally, probucol has been reported to induce regression of tendon xanthomas in homozygous familial hypercholesterolemia patients. Thus, from the point of view of its antioxidant properties, probucol would be an excellent agent to use in a clinical trial designed to test the hypothesis that antioxidant therapy will inhibit atherogenesis. However, probucol also lowers plasma levels of LDL and high density lipoprotein (HDL) cholesterol, both effects that would complicate the interpretation of the results of a clinical trial. Lowering of LDL levels, if sufficient, could conceivably explain any antiatherosclerotic effects of probucol, independent of its antioxidant activity. However, probucol's lowering of LDL cholesterol is modest, and its ability to further lower LDL levels when used in combination with other lipid-lowering agents is minimal. Thus, it is conceivable that a trial could be designed to control for probucol's LDL-lowering effect. In contrast, when used at its standard dose of 1 g per day, probucol consistently lowers HDL cholesterol by 20–30%. If in this clinical situation HDL cholesterol lowering promotes atherosclerosis, probucol's antioxidant effects could be diminished or even negated.
Because probucol is so lipophilic and distributes throughout the body's lipid stores, we reasoned that prolonged administration of even low doses might be sufficient to yield effective antioxidant protection to LDL but might not alter lipoprotein levels. Improved understanding of the relations between probucol dose, its antioxidant effect, and its HDL and LDL cholesterol-lowering effects would be important for the design of future clinical trials with this promising antioxidant. The current study compares the effect of low- versus standard-dose probucol on plasma lipids and LDL oxidation and modification in humans.

Methods

Study Design

Seventeen patients (10 female, seven male) who were followed in the University of California San Diego (UCSD) Research Lipid Clinic for therapy of hyperlipidemia were recruited for this study, which was approved by the UCSD Human Subjects Review Board. Four patients who were concurrently taking other lipid-lowering medications (lovastatin plus colestipol HCl) continued with these medications and were assigned to the 250 mg/day (1 tablet) probucol group. Of the remaining 13 patients, seven were randomly assigned to the 1-tablet-probucol group and six to the 500 mg twice a day (4 tablets) group. After a 6-week baseline assessment period, therapy was started at the assigned dose and maintained for 6 months. At the end of 6 months, all patients on 250 mg/day probucol had their dosage increased to 500 mg/day for an additional 3 months. All subjects were healthy at the time of recruitment, were on American Heart Association stage 1 or 2 diets, and were instructed to maintain these diets through the study. At several points during the study, subjects completed 3-day food records, which were reviewed by a dietician to help maintain adherence to the dietary regimen. Pill counts were performed monthly to assess compliance. No participants were taking vitamin supplements when recruited, and all were instructed to avoid supplemental vitamins. Subjects were enrolled in the study in two groups 1 month apart. Subjects on 1 or 4 tablets per day were evenly distributed in each group. For each group, blood was drawn at two separate baseline visits and approximately every month during the treatment phase of the study. Fasting blood from all subjects in a given group was drawn on the same day, and LDL samples were isolated from all subjects simultaneously. At each blood draw for LDL isolation, blood was also drawn from a healthy untreated control subject and was treated identically to samples from the study subjects. The same control subject was used throughout the study. This control LDL therefore allowed adjustment for the interassay variation that occurred over the long study period. At both baseline visits and at 2, 4, 6, and 9 months into the study, assays of antioxidant protection of LDL isolated from participants were performed as described below.

Lipoprotein Quantification and Isolation

After an overnight fast, plasma was collected into tubes containing EDTA (1 mg/ml) from each patient and an untreated control. Plasma was separated within several hours and used within 3 days for lipid and lipoprotein quantification, using standardized enzymatic techniques as previously described.15 The laboratory is under the continuous standardization program of the Centers for Disease Control, Atlanta, Ga. Apolipoproteins (apos) A-I and B were measured by Dr. Richard Smith (Johnson & Johnson Biotechnology, La Jolla, Calif.) using radioimmunoassay techniques as previously described.16,17 LDL was isolated from each individual by stepwise ultracentrifugation.2,18 The final LDL "wash spin" was performed at a density of 1.085 g/ml to allow concentration of the LDL in the range 8–15 mg/ml. LDL iodination was performed using [125I]NaI as described previously18 so that all samples had approximately 200 cpm/ng protein. All lipoprotein samples were dialyzed against phosphate-buffered saline containing 0.01% EDTA and were used within 48 hours after removal from dialysis. LDL protein was determined as described by Lowry et al.19 Each subject's 125I-LDL was diluted with his/her unlabeled LDL and dialysis buffer to a concentration of 6.7 mg/ml and approximately 60 cpm/ng protein before assay. Therefore, all LDL samples used for cell culture studies were of equal protein concentration and specific activity and contained similar concentrations of EDTA.

Probucol Measurements

Probucol levels in plasma LDL were determined by high-performance liquid chromatography (HPLC) after extraction with methanol/acetone by a method supplied by Merrell Dow Research Institute, Cincinnati, Ohio. Isolated LDL samples were extracted into methanol/acetone (3:2, vol/vol) with 2-pentanone-bis(3,5-di-4-buty-4-hydroxyphenyl)mercaptole as an internal standard, partitioned into heptane, and analyzed by HPLC on a C18 reversed-phase column eluted with acetonitrile/heptane/0.1 M ammonium acetate (92:6:2, vol/vol/vol). Probucol samples assayed in our laboratory yielded levels that were, on average, 14% lower than levels measured on identical samples by Merrell Dow Research Institute. Reported values were not adjusted for this variation.

Cells

Rabbit aortic endothelial cells (ECs) were maintained in culture as previously described.18 Cells were used at confluence and were washed three times in F-10 medium before addition of LDL. Mouse resident peritoneal macrophages were prepared,18 and 1.4×10^6 cells were placed into each well of 24-well dishes in Dulbecco's modified Eagle's medium (DMEM) containing 50 μg/ml gentamicin and 2.5 μg/ml fungizone. After overnight incubation, nonadherent cells were removed and degradation assays were performed in DMEM.

Tests of Low Density Lipoprotein Oxidation and Biologic Modification

Thiobarbituric acid–reactive substances (TBARS) were measured as an index of lipid peroxidation. In preliminary studies, we demonstrated that under the conditions used for these experiments, incubation of LDL with ECs for 16 hours produced oxidative modification nearly equivalent to that produced by incubation of LDL with 5 μM copper sulfate (Cu) for 8 hours. Therefore, each 125I-LDL sample (100 μg/ml) was incubated in F-10 with Cu for 8 hours or with ECs for 16
TABLE 1. Effect of Probucol on Plasma Lipids and Lipoproteins

<table>
<thead>
<tr>
<th>Lipid/lipoprotein</th>
<th>1 Tablet/day (n=11)</th>
<th>4 Tablets/day (n=6)</th>
<th>p value (column 3 vs. column 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline (1)</td>
<td>Final (2)</td>
<td>Mean change, percent (3)</td>
</tr>
<tr>
<td>Total chol</td>
<td>275±48</td>
<td>255±44</td>
<td>~6.8†</td>
</tr>
<tr>
<td>LDL chol</td>
<td>192±42</td>
<td>187±41</td>
<td>~3.0</td>
</tr>
<tr>
<td>HDL chol</td>
<td>53±13</td>
<td>42±7</td>
<td>~17.6†</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>148±125</td>
<td>129±96</td>
<td>~7.7</td>
</tr>
<tr>
<td>Apo A-1</td>
<td>147±32</td>
<td>106±23</td>
<td>~27.9†</td>
</tr>
<tr>
<td>Apo B</td>
<td>88±20</td>
<td>103±17</td>
<td>19.5†</td>
</tr>
</tbody>
</table>

Each value represents mean±SD (milligrams per deciliter) of values obtained at two monthly visits before initiation of drug therapy (baseline) and two values measured at the fifth and sixth months of therapy (final).

Chol, cholesterol; apo, apolipoprotein.

*Significant at p<0.05, final vs. baseline.

†Significant at p<0.01, final vs. baseline.
incubation with ECs for 16 hours or Cu for 8 hours. As a measure of lipid peroxidation, we measured the formation of TBARS before and after the incubation of LDL with ECs or Cu. The zero-time TBARS showed no difference between groups at any time point during the study (data not shown). The extent of TBARS generated after EC or Cu oxidation was much less in the LDL isolated from patients after 2 months of probucol therapy in both groups and continued to gradually decline during the remainder of the treatment period (Figure 3a). Although measured TBARS initially decreased more rapidly in the 4-tablet group, mean values were nearly comparable by the end of the 6-month treatment period.

When sufficient oxidative modification has occurred to alter the protein moiety of LDL, uptake and degradation in macrophages are greatly increased. In both the 1- and 4-tablet groups, after the first 2 months of therapy there was a decrease in the extent to which LDL could be modified, as evidenced by decreased LDL degradation in the macrophage assay (Figure 3b). This reduction occurred more rapidly in the 4-tablet group than in the 1-tablet group, although the extent of macrophage degradation (i.e., oxidative modification) was similar after 6 months of therapy.

Because assays were performed for each group repeatedly during the study and measures of oxidative susceptibility can vary substantially from assay to assay, an internal standard for each assay was included. In Figure 4, the results for the macrophage degradation assay for each subject after 6 months of therapy are presented as a percentage of the untreated control value for that particular assay. Even when examined this way, the extent of LDL macrophage degradation was reduced to 55% or less of the untreated control values in all samples. In LDL samples from the 4-tablet group, LDL macrophage degradation was reduced to less than 20% of control values.

The relation between LDL probucol levels and macrophage degradation after exposure to oxidant stress at the beginning of the study and after 2 and 6 months of probucol therapy is presented for all subjects in Figure 5. At lower LDL probucol levels, LDL was only marginally protected from oxidative modification. However, when LDL probucol levels were greater than 6 μg/mg LDL, macrophage degradation of either EC- or Cu-oxidized LDL was greatly reduced. Of note, even after 6 months of therapy there were several subjects (all in the 1-tablet group) who had relatively low LDL probucol levels (less than 6 μg/mg LDL) and whose LDLs were only partially protected from oxidation.

The change in LDL susceptibility to oxidative modification that occurred during the study, as assessed by the reduction in the extent of macrophage degradation, was correlated with LDL probucol levels (r=0.65, p=0.005 for EC-modified LDL; r=0.73, p<0.001 for

**Figure 2.** Time course of plasma apolipoprotein (apo) A-I (left panel) and high density lipoprotein (HDL) cholesterol (right panel) levels in plasma of patients treated with 1 tablet per day (•) or 4 tablets per day (○) of probucol. Shown are the mean values for each group at the indicated times.

**Figure 3.** Line plots showing mean levels of thiobarbituric acid–reactive substances (TBARS) (panel a) and macrophage degradation (panel b) for low density lipoprotein (LDL) samples oxidized by exposure to endothelial cells for 16-hour (circles) or 8-hour (squares) exposure to copper sulfate. Mean values ± SD for LDL samples isolated from patients on 1 probucol tablet per day (•, ■) or 4 probucol tablets per day (○, □) are shown. MDA, malondialdehyde.
FIGURE 4. Levels of macrophage degradation for subjects on 1 (•) or 4 (○) tablets of probucol per day. Values are expressed as a percentage of values obtained from control low density lipoprotein (LDL), which was repeatedly isolated from an untreated subject and run simultaneously within each assay. Mean values for each assay are also presented (—). EC, incubation with endothelial cells; CU, oxidation with copper sulfate.

Cu-modified LDL). The decrease in macrophage degradation of probucol-treated LDL also correlated with the decrease in HDL cholesterol \((r=0.58, p=0.02\) for EC-modified LDL; \(r=0.67, p<0.01\) for Cu-modified LDL). LDL probucol levels also correlated with the decrease in HDL cholesterol level \((r=0.6, p=0.01)\).

At the end of 6 months of therapy, 10 of 11 subjects on 1 tablet per day increased their daily probucol dose to 2 tablets per day for a subsequent 3 months. This additional tablet of probucol raised mean LDL probucol levels over twofold higher in the 4-tablet group. The rise in LDL probucol levels occurred slowly, requiring at least 6 months to reach steady-state levels. Probucol lowered HDL cholesterol levels in both groups, by 17.6% in the 1-tablet group and 27.0% in the 4-tablet group. Even in this relatively small study, this difference between probucol dosage groups reached borderline significance.

LDL isolated from subjects in the 4-tablet group was markedly less susceptible to oxidation after only 2 months of therapy, whereas it took nearly 6 months of therapy on 1 tablet per day to attain similar antioxidant protection (Figure 3). There were several subjects in the 1-tablet group (Figure 5) who failed to achieve the maximal protection noted with the full dose. Interestingly, increasing the administered dose to 500 mg/day for 3 additional months in these individuals failed to increase either the concentration of probucol in their LDL or the extent of protection to their LDL from oxidation (data not shown). Although LDL probucol levels tended to be relatively low in these individuals (4–6 μg probucol/mg LDL protein), which could explain their incomplete protection from oxidation, there were other subjects who had similarly low probucol levels, yet their LDL exhibited excellent protection. This suggests that in this range of probucol concentration in LDL, other determinants of LDL oxidation may be important. However, in the 10 subjects with probucol levels of 6 μg/mg LDL or greater, there was uniform reduction in LDL susceptibility to oxidation and modification. The importance of LDL probucol levels in determining susceptibility to oxidation has been shown previously in vitro studies and in rabbits and is now demonstrated in humans as well. A probucol content of 6–12
µg/mg LDL protein is approximately equal to 6–12 moles of probucol per LDL particle. The average LDL particle contains approximately 6 moles of vitamin E.23 Because probucol is approximately seven times more effective an antioxidant than vitamin E on a molar basis,24 at least in a monolayer system, it follows that probucol at these concentrations confers the equivalent protection of 42 moles of vitamin E per LDL particle. It should be mentioned that plasma vitamin E levels were not different in the patients consuming 250 mg or 1 g probucol per day at the end of the study (data not shown).

Although 1 tablet of probucol provided significant antioxidant activity to LDL, it was also associated with a lowering of HDL cholesterol levels. Both the degree of antioxidant activity and the extent of HDL cholesterol lowering were related to LDL probucol levels and were not completely dissociated at the lower probucol dose. It is possible that these two effects are connected and that antioxidant activity contributes to HDL lowering; however, there is evidence to suggest that this is not the case. Other known antioxidants, butylated hydroxytoluene and vitamin E for example, do not lower HDL cholesterol.5-11 Additionally, there is evidence that probucol may lower HDL cholesterol levels by means unrelated to the oxidation process, such as increasing cholesteryl ester transfer protein activity27,28 and decreasing apo A-I production rates.29 The reverse situation, that HDL cholesterol reduction leads to greater resistance of LDL to oxidative modification, also appears unlikely. In fact, it has been previously demonstrated in vitro that the presence of HDL actually reduces LDL susceptibility to oxidation and modification.30 Probucol likely lowers HDL cholesterol levels and reduces LDL susceptibility to oxidative modification through separate mechanisms. The change in HDL cholesterol levels during probucol therapy may serve as a relatively useful clinical marker of LDL probucol content and, thus, antioxidant protection. For example, LDL isolated from participants with changes in HDL cholesterol greater than 10 mg/dl were uniformly resistant to oxidative modification. This may help explain the finding that regression of tendon xanthomas is strongly correlated with decreases in HDL cholesterol levels in familial hypercholesterolemia patients treated with probucol.13

Increasing the daily probucol dose from 1 tablet to 2 tablets further decreased HDL cholesterol and apo A-I levels. However, mean LDL probucol levels only modestly increased for the group as a whole and did not change in the three subjects with the lowest LDL probucol levels. As expected, therefore, there was no significant increase in protection against oxidation and modification. Therefore, for this group as a whole, 2 tablets per day does not appear to offer any advantage over 1 tablet per day.

An important question to ask is whether antioxidant protection in these in vitro systems is a valid estimate of potential in vivo antioxidant protection. There are reasons to believe that currently used in vitro models may provide relevant insight into the benefits of specific antioxidants. It is unlikely that in vivo conditions of oxidative stress are as harsh as those produced by high concentrations of redox metals or overnight conditioning with EGS as used in our in vitro assays. An antioxidant agent that produces 60–80% inhibition in these systems, where LDL does not have access to the continuous supply of antioxidants present in plasma or in the extracellular compartment, will likely add considerably to in vivo antioxidant protection. Probucol, which provides this level of LDL protection in vitro, has been shown to reduce atherosclerosis in animals by an action independent of its cholesterol-lowering ability.7,8,11 However, final demonstration of the relevance of in vitro antioxidant assays awaits clinical corroboration.

In summary, although low-dose probucol therapy provides marked antioxidant activity, it also retains substantial HDL cholesterol-lowering effects. Therefore, probucol may not be the agent of choice for use in an initial clinical trial designed to specifically test the antioxidant hypothesis. Ideally, a similarly potent lipophilic antioxidant without any effects on lipoprotein levels would be preferable. Probucol analogues have been reported to possess antioxidant activity without cholesterol-lowering activity,22 although their effects on HDL cholesterol are unknown. These analogues have not yet been tested in humans. If the antioxidant hypothesis can be established, however, use of low-dose probucol, possibly in conjunction with other interventions such as a diet enriched in monounsaturated fatty acids31 or vitamin E supplementation,32,33 may ultimately prove to be beneficial.

Acknowledgments

The authors thank Daniel Steinberg for advice and support, Haven Webb for assistance in the conduct of these studies, Richard Smith for measurement of apolipoproteins A-I and B, and Ralph Tedeschi of Merrell Dow Research Institute for the probucol tablets.

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doi: 10.1161/01.ATV.12.3.318

_Arteriosclerosis, Thrombosis, and Vascular Biology_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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