Increase in Plasma Cholesteryl Ester Transfer Protein During Probucol Treatment
Relation to Changes in High Density Lipoprotein Composition

Ruth McPherson, Mireille Hogue, Ross W. Milne, Alan R. Tall, and Yves L. Marcel

Probucol is a hypolipidemic agent that causes a marked decrease in high density lipoprotein (HDL) cholesterol. To investigate the mechanism of this effect, two studies were performed in hypercholesterolemic patients who had been stabilized previously on diet and were not receiving other lipid-lowering medication. Plasma cholesteryl ester transfer protein (CETP) concentrations were measured in fasting plasma samples before and after 10 weeks of probucol therapy using a sensitive and specific radioimmunoassay. Plasma total and low density lipoprotein cholesterol concentrations decreased, whereas apolipoprotein (apo) B was unchanged. Plasma apo E concentrations increased markedly. HDL cholesterol and apo A-I decreased in all subjects. These effects of probucol were accompanied by even more striking changes in plasma CETP concentrations, which increased by a mean of 64%. In a second study of six hypercholesterolemic subjects, the time-course effects of probucol on CETP and HDL subspecies were studied. Significant increases in plasma apo E and in CETP occurred after 4 weeks, and CETP, but not apo E, increased further after 16 weeks of treatment. Concomitant and opposite changes occurred in HDL composition, with decreases in HDL cholesterol and lipoprotein containing apo A-I. The increase in plasma CETP concentrations, the decrease in HDL cholesterol, and the increase in plasma apo E concentrations observed during probucol treatment are changes consistent with a postulated increase in reverse cholesterol transport via the remnant pathway.

Methods

Subjects

Study 1. Eleven hypercholesterolemic patients (four men, seven postmenopausal women) not previ-
ously treated with cholesterol-lowering medication were studied before and after 8 weeks of probucol therapy. After dietary treatment, all patients maintained cholesterol levels above the 95th percentile and triglyceride concentrations below the 95th percentile for their age and sex. Four of 11 patients were classified as familial hypercholesterolemic (FH) on the basis of the presence of tendon xanthomatas and appropriate family history. Six patients had been treated with a range of cardioprotective agents including nitrates, β-blockers, and calcium channel antagonists for a period of 12 or more months before the study, and these treatment regimens were not altered during the study. Patients were stabilized on diet (27% fat, polyunsaturated/monounsaturated/saturated fat ratio of 1:1:1, <200 mg/day cholesterol) for 2 months before the initiation of probucol therapy, and dietary stabilization was maintained throughout the study period. All patients were informed of the purpose of the study, which was approved by our Institutional Review Board.

Study 2. To examine in further detail the time-course changes in apo E, CETP, and HDL subspecies during probucol therapy, six additional hypercholesterolemic patients (four women, two men; total cholesterol >95th percentile, triglycerides <95th percentile; five with E3/3, one with E3/2) were studied during a 16-week period after 8 weeks of dietary stabilization as described above. Three of six patients were classified as FH on the basis of the previously mentioned criteria. No patient had been previously treated with a lipid-lowering agent, and other medications were limited to nitrates or calcium channel blockers; these were held constant for 12 months before and throughout the study.

Protocol

Study 1. Baseline EDTA/plasma samples were collected after an overnight fast. Subjects were treated with probucol (500 mg b.i.d. with meals) as a single lipid-lowering agent. Fasting blood samples were again collected at the end of 8–10 weeks of probucol therapy.

Study 2. Subjects were treated with probucol (500 mg b.i.d.) for a 16-week period. Interim blood samples were collected after 4 weeks of therapy.

Determination of Plasma and Lipoprotein Lipid Levels

Plasma cholesterol and triglycerides, HDL-C, and LDL-C were determined by the methods of the Lipid Research Clinics protocol.11 In study 1 in four patients with plasma triglyceride values between 2.5 and 3.0 mM/l, very low density lipoprotein cholesterol (VLDL-C) was measured directly in the plasma by the Friedwald formula.12 In study 2, VLDL (d<1.006 g/ml), LDL plus intermediate density lipoprotein (1.006–1.063 g/ml), HDL (1.063–1.215 g/ml), and HDL₃ (1.215–1.210 g/ml) were determined after sequential density ultracentrifugation in plasma samples from all subjects. Total HDL-C was measured after heparin/manganese precipitation of the apo B–containing lipoproteins in whole plasma.12

Radioimmunoassays

Apo A-I,13 apo E,14 and apo B15 were measured by radioimmunoassay (RIA), that for apo A-I in the presence of Tween-20 as described previously. The RIA for apo A-II was performed using a monoclonal antibody (MAb) that was a generous gift from Linda Curtiss (Scrpps Institute, La Jolla, Calif.). In subjects in study 2, lipoproteins containing apo A-I (Lp A-I) (HDL particles containing only apo A-I)16 and total apo A-I were measured by radioimmunoassays (EIAs),17 and Lp A-I, A-II (HDL particles containing apo A-I and A-II) were calculated by difference. Plasma CETP was measured by solid-phase competitive RIA in the presence of 0.5% Triton using a specific MAb, TP-2, that has been characterized previously.18 In short, plastic wells were coated with partially purified CETP in sodium carbonate buffer, saturated with bovine serum albumin (BSA), and washed. To the coated wells, equal volumes of the unknown antigen (diluted and preincubated in phosphate-buffered saline [PBS]/BSA buffer containing 1% Triton) and of a limiting dilution of purified iodine-125–labeled MAb TP-2, diluted with immunoglobulin G protein in PBS with BSA, were added. The wells were incubated for 90 minutes at 20°C and washed, and the bound radioactivity was determined. All immunoassays were performed in a single run at the end of each study on plasma stored at −70°C.

Statistical Methods

Statistical analyses were performed by Student's t test with correction for unequal variances, Student's t test for paired data, and linear regression using the SAS statistical package.19 Values are reported as mean±SD.

Table 1

<table>
<thead>
<tr>
<th>Variable</th>
<th>Baseline</th>
<th>10 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC (mM/l)</td>
<td>7.25±0.69</td>
<td>6.32±0.84*</td>
</tr>
<tr>
<td>TG (mM/l)</td>
<td>1.94±0.62</td>
<td>1.97±0.75</td>
</tr>
<tr>
<td>VLDL-C (mM/l)</td>
<td>0.84±0.32</td>
<td>0.86±0.33</td>
</tr>
<tr>
<td>LDL-C (mM/l)</td>
<td>5.22±0.72</td>
<td>4.64±0.66*</td>
</tr>
<tr>
<td>HDL-C (mM/l)</td>
<td>1.16±0.22</td>
<td>0.82±0.16*</td>
</tr>
<tr>
<td>Apo A-I (mg/ml)</td>
<td>1.05±0.41</td>
<td>0.75±0.15†</td>
</tr>
<tr>
<td>Apo B (mg/ml)</td>
<td>1.32±0.1</td>
<td>1.39±0.26</td>
</tr>
<tr>
<td>Apo E (mg/ml)</td>
<td>152±7.59</td>
<td>270±84‡</td>
</tr>
<tr>
<td>CETP (mg/ml)</td>
<td>1.84±0.50</td>
<td>3.02±0.87*</td>
</tr>
</tbody>
</table>

Values are mean±SD.

TC, total cholesterol; TG, total triglycerides; VLDL-C, very low density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; Apo, apolipoprotein; CETP, cholesteryl ester transfer protein.

* p<0.001; †p<0.05 vs. baseline.
Results

All subjects satisfactorily completed the treatment in both studies. Side effects were limited to mild gastrointestinal symptoms in two patients that did not necessitate any modification in the protocol.

*Study 1.* Plasma lipoprotein concentrations before and after probucol therapy are shown in Table 1. Total plasma cholesterol and LDL-C levels decreased by 13% and 11%, respectively, while plasma apo B concentrations were unchanged. Consequently, the LDL-C/apo B ratio decreased slightly but not significantly. Plasma apo E levels increased by 67% after probucol treatment.

The effects of probucol on lipoproteins were most consistent with respect to HDL-C and apo A-I levels. In all subjects, there were marked decreases in both HDL-C and apo A-I levels (Figure 1 and Table 1). These effects of probucol on HDL were accompanied by even more striking effects on plasma CETP concentrations, which increased in all subjects (Figure 1) by an average of 64% (Table 1).

Probucol-induced changes in plasma CETP mass did not appear to be related to baseline lipoprotein parameters. Linear regression analyses did not reveal significant relations between changes in CETP concentrations and changes in apos A-I, E, or B or in LDL-C or HDL-C in this small group of subjects.

*Study 2.* In the second group of six subjects, sequential changes in lipoprotein subfractions and apolipoproteins were studied in more detail (Table 2). Total cholesterol decreased by 10% after 4 weeks and by 14.5% after 16 weeks of probucol therapy. VLDL-C decreased significantly after 4 weeks of therapy (−18%) but increased at 16 weeks of treatment to a level that was not significantly different from baseline. A reduction in LDL-C was not evident until 16 weeks of treatment and failed to reach significance for these six subjects. Total HDL-C

<table>
<thead>
<tr>
<th>Variable</th>
<th>Baseline</th>
<th>4 weeks</th>
<th>16 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>TC (mM/l)</td>
<td>8.13±1.41</td>
<td>7.30±1.65*</td>
<td>6.95±1.42*</td>
</tr>
<tr>
<td>TG (mM/l)</td>
<td>1.83±0.81</td>
<td>1.37±0.67*</td>
<td>1.66±0.99*</td>
</tr>
<tr>
<td>VLDL-C (mM/l)</td>
<td>0.72±0.34</td>
<td>0.68±0.24*</td>
<td>0.83±0.57</td>
</tr>
<tr>
<td>LDL-C (mM/l)</td>
<td>5.77±1.31</td>
<td>5.75±1.49</td>
<td>5.10±1.15</td>
</tr>
<tr>
<td>HDL-C (mM/l)</td>
<td>1.63±0.25</td>
<td>1.09±0.08*</td>
<td>1.03±0.10*</td>
</tr>
<tr>
<td>HDL2-C (mM/l)</td>
<td>0.46±0.11</td>
<td>0.38±0.08*</td>
<td>0.33±0.07*</td>
</tr>
<tr>
<td>HDL3-C (mM/l)</td>
<td>1.03±0.21</td>
<td>0.71±0.12</td>
<td>0.71±0.15</td>
</tr>
<tr>
<td>Apo A-I (mg/ml)</td>
<td>1.81±0.18</td>
<td>1.21±0.20*</td>
<td>1.28±0.26*</td>
</tr>
<tr>
<td>Apo A-II (mg/ml)</td>
<td>0.23±0.08</td>
<td>0.24±0.07</td>
<td>0.26±0.06</td>
</tr>
<tr>
<td>Lp A-I (mg/ml)</td>
<td>0.53±0.09</td>
<td>0.41±0.11*</td>
<td>0.35±0.12*</td>
</tr>
<tr>
<td>Lp A-I/A-II (mg/ml)</td>
<td>1.46±0.19</td>
<td>1.29±0.16*</td>
<td>1.30±0.17*</td>
</tr>
<tr>
<td>Lp A-I/Lp A-I/A-II</td>
<td>0.36±0.04</td>
<td>0.32±0.05</td>
<td>0.28±0.09*</td>
</tr>
<tr>
<td>Apo E (µg/ml)</td>
<td>181±58</td>
<td>231±104*</td>
<td>237±50*</td>
</tr>
<tr>
<td>CETP (µg/ml)</td>
<td>2.33±0.74</td>
<td>2.72±0.72*</td>
<td>3.06±0.79*</td>
</tr>
</tbody>
</table>

Values are mean±SD. TC, total cholesterol; TG, total triglycerides; VLDL-C, very low density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; HDL-C, high density lipoprotein cholesterol; Apo, apolipoprotein; Lp, lipoprotein; CETP, cholesteryl ester transfer protein.

*p<0.05 vs. baseline.
decreased by 33% after 4 weeks and by 37% after 16 weeks of therapy (p<0.05). HDL-C decreased by 17% at 4 weeks and by 28% after 16 weeks, whereas the decrease in HDL-C (−31%) did not differ at 4 and 16 weeks of treatment. Apo A-I, measured by RIA, decreased by 33% after only 4 weeks of treatment, and this decrease was maintained throughout the study. Apo A-II levels, also measured by RIA, did not change during probucol treatment.

Levels of Lp A-I and Lp A-I, A-II protein measured by EIA decreased significantly and promptly, but the decrease in Lp A-I (−32%) was greater than the decrease in Lp A-I, A-II (−11%). Although the Lp A-I/Lp A-I, A-II ratio was significantly reduced by the end of 16 weeks of treatment (−22%), this result must be interpreted with caution because the level of Lp A-I, A-II was calculated by difference. In addition, the levels of apo A-I measured by RIA and by EIA should not be compared directly since the RIA assay but not the EIA assay is performed in the presence of detergent, which causes the dissociation of apo A-I from the lipoproteins and alters its immunoreactivity. Further studies involving larger numbers of patients are required to confirm these findings. In addition, special attention must be given to the fact that alteration in lipoprotein size after probucol treatment may also affect the immunoreactivity of apo A-I in the EIA system. Apo E increased by 27% after 4 weeks of treatment and by 31% (p<0.05) after 16 weeks of therapy.

Plasma concentrations of CETP were somewhat higher at baseline in the second group of subjects, which differed from the first in having higher baseline cholesterol levels. CETP levels increased by 17% after 4 weeks of therapy and by 31% after 16 weeks of treatment (p<0.05). As in the previous study, the percentage increases in apo E and in CETP were almost identical.

Comparison of the plasma CETP responses to probucol for patients with or without FH revealed no significant differences. Plasma CETP increased after 10 or 16 weeks or probucol treatment by 0.82±0.74 μg/ml in seven FH patients and by 0.81±0.62 μg/ml in 10 non-FH patients.

Discussion

Probucol therapy increases the plasma concentration of CETP, which is of functional significance in cholesterol transport. CETP transfers cholesterol ester from apo A-I-containing lipoproteins to apo B-containing lipoproteins, mainly VLDL and VLDL remnants, which, as apo E–rich VLDL remnants, become an ultimate vehicle for reverse cholesterol transport. Other mechanisms for the delivery of HDL-cholesterol to the liver include the interaction of apo E–rich HDL with the hepatic apo E receptor and the selective uptake of cholesterol ester from HDL without particle endocytosis. It is of interest that the apo E–rich subtraction of HDL increases markedly in CETP-deficient subjects. This may represent an adaptive response to compensate for a malfunctioning CETP pathway.

Plasma concentrations of HDL-C represent a balance between the factors facilitating accumulation of HDL-C ester (secretion of nascent HDL, its interaction with peripheral cellular binding sites for HDL, and the activity of lecithin:cholesterol acyl transferase) and factors promoting the delivery of HDL-C ester to the liver via one of the routes described above, including the CETP-mediated pathway. Thus, steady-state levels of HDL-C are not an appropriate index of the efficiency of the reverse cholesterol transport system in all circumstances. Certain metabolic situations associated with very low levels of HDL-C, such as Tangier's disease and other apo A-I deficiency states, are not always associated with accelerated atherosclerosis. Therefore, the low HDL-C levels associated with probucol therapy are not a priori evidence of increased atherosclerosis risk.

The mechanisms by which probucol decreases HDL-C have not been clarified. Kinetic studies in a small number of subjects have demonstrated that apo A-I synthesis is decreased during probucol treatment, but this effect does not explain the significant decrease in HDL particle size that occurs during probucol therapy. Previous reports have shown that probucol increases the rate of cholesterol ester transfer from HDL to VLDL in both hyperalphalipoproteinemic patients and hypercholesterolemic subjects. However, transfer activity measured in vitro reflects not only the mass of CETP in plasma but also the composition of both donor (mostly apo A-I–containing) and acceptor (mostly apo B–containing) lipoproteins, both of which are altered by probucol therapy. Alterations in transfer activity may also be secondary to effects on a postulated inhibitor of CETP.

The mechanisms by which probucol increases HDL-C mass are not clear. Changes may occur in CETP synthesis, CETP metabolism, or both. CETP is synthesized in the liver, intestine, and spleen as well as by cultured monocytes/macrophages, but adipose tissue and muscle appear to be the major sites of CETP synthesis in humans. We suggest, based on a
number of laboratory observations, that CETP may share regulatory factors with apo E since the plasma concentrations of these two proteins appear to respond similarly to a number of metabolic, dietary, or pharmacological factors. CETP mRNA level in rabbit liver is enhanced by cholesterol feeding, which is also accompanied by increases in plasma apo E. In other studies in progress by this group, both apo E and CETP concentrations are also increased in normal subjects after cholesterol feeding. Similarly, we have demonstrated that CETP levels are higher than normal in patients with elevations in plasma apo E (type III dysbetalipoproteinemia and type V chylomicronemia; R. McPherson, unpublished observations). We have also observed a significant relation between plasma CETP and apo E concentrations in a population of 50 normal subjects (r=0.40) and in 53 hyperlipidemic subjects (r=0.44). The present studies demonstrate that during probucol therapy, there is a significant increase in plasma apo E concentration that precedes the increase in CETP. After 4 weeks of probucol treatment, the increase in apo E was much greater than the increase in CETP, but between 4 and 16 weeks of therapy, there was no further change in apo E concentrations, whereas there was a marked increase in CETP levels. Probucol treatment also results in increased levels of apo E mRNA in rabbit splenic cells, although not in liver. On the basis of these observations, we suggest that probucol first increases peripheral apo E synthesis, which may facilitate the delivery of VLDL remnant cholesteryl ester to the liver. There is a secondary effect of probucol on CETP synthesis that is likely to represent increased production of CETP in muscle and adipose tissue, the latter being a major site of probucol storage. Further studies are in progress to determine the effect of probucol on the levels of CETP mRNA in adipose tissue in humans.

We do not believe that assays for CETP or apo E are affected by changes in protein conformation induced by probucol since the CETP assay is performed using high concentrations of Triton. Additionally, CETP is associated almost exclusively with the most dense Lp A-I particles or is free in plasma and, accordingly, should be affected minimally by alterations in the lipid/protein ratios of HDL. Similarly, the apo E assay is performed in the presence of Tween and is not influenced by the lipid composition of lipoprotein particles.

Probucol has effects on the composition of most classes of lipoproteins that are consistent with increased CETP activity. The LDL-C/apo B ratio decreases, and other more prolonged studies have demonstrated an increase in the LDL triglyceride/LDL-C ratio. This may reflect increased CETP activity since LDL may also act as a donor particle for cholesteryl ester transfer to VLDL remnants.

We observed marked changes in HDL composition during probucol treatment including a reduction in both HDL-C and apo A-I and a decrease in both Lp A-I and Lp A-I,a-II. Most, but not all, previous studies have demonstrated that the probucol-induced reduction in HDL-C is predominately due to reductions in the HDL2 subfraction. It is of interest that we have observed a greater effect of probucol treatment on Lp A-I, the HDL subspecies that is the more effective particle in promoting cholesteryl efflux from peripheral cells; however, this finding, observed in a small number of subjects, must be confirmed.

In the plasma of fasting normal subjects, we have demonstrated that CETP is most concentrated in the HDL3 and VLDL fractions. Cheung et al. have reported that the majority of CETP activity in plasma is associated with Lp A-I particles. Thus, the changes noted in HDL composition would be an anticipated result of increased CETP mass and activity. Additional studies are in progress using gradient gel electrophoresis and immunoblotting techniques to further characterize changes in HDL particle size and in CETP distribution during probucol therapy. It is unlikely that changes in CETP are secondary to changes in HDL concentrations or composition since in hyperlipidemic subjects, there is no relation between any HDL variable and CETP levels.

In summary, probucol has important effects on plasma CETP concentrations that may provide an explanation for the well-documented effects of this drug on HDL-C levels. Whether this effect is a primary action of probucol or is secondary to changes in the regulation of apo E metabolism or in the composition and availability of donor or acceptor lipoproteins for cholesterol transfer remains to be established. The ultimate effect of an increase in CETP on atherosclerosis is likely to depend on the efficacy with which the recipient particles for cholesteryl ester transfer are cleared by the liver. Further studies are in progress to define the regulation of CETP metabolism and the significance of this protein in reverse cholesterol transport.

References


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**KEY WORDS**: probucol • cholesteryl ester transfer protein • high density lipoproteins • apolipoprotein A-I • apolipoprotein E
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doi: 10.1161/01.ATV.11.3.476
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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