Transport of HDL Cholesterol Esters to the Liver Is Not Diminished by Probucol Treatment in Rats

Brigitte M. Richard, Maria A. Pfeuffer, and Ray C. Pittman

This study examined the relation of decreased high density lipoprotein (HDL) levels in probucol-fed rats and the transport of HDL cholesterol esters (CEs) to the liver. HDLs from both control rats and rats fed 1% probucol for 3 weeks were doubly labeled in their CE and apolipoprotein A-I moieties with intracellularly trapped tracers and then intravenously injected into probucol-fed or control rats for determination of plasma decay kinetics and sites of tracer uptake. Results for HDL from control and probucol-fed rats were not different. The fractional catabolic rate (FCR) of plasma HDL CE was significantly increased by probucol feeding (23%) so that mass transport of HDL CE through the plasma compartment was not significantly different from that in control rats. The plasma FCR for apolipoprotein A-I did not change. Similarly, the FCR for uptake of HDL CE by the liver increased on probucol feeding (29%), resulting in a near-normal rate of HDL CE mass uptake, whereas the FCR for HDL particle uptake (measured by apolipoprotein A-I uptake) did not change. Thus, the maintenance of near-normal HDL CE uptake by the liver was exclusively due to increased selective uptake (32%). To the extent that hepatic uptake of HDL CE mediates reverse cholesterol transport, that process was not significantly compromised in rats fed 1% probucol. (Arteriosclerosis and Thrombosis 1992; 12:862-869)

KEY WORDS • probucol • reverse cholesterol transport • high density lipoproteins • rats

A ccumulation of cholesterol in the arterial wall results from an excess of influx over efflux. Cholesterol influx presumably is mediated largely by the uptake of “atherogenic lipoproteins” containing apolipoprotein (apo) B, while efflux presumably is mediated by processes dependent on high density lipoproteins (HDLs). Based largely on epidemiological evidence, it is expected that reducing the levels of apo B-containing lipoproteins and/or increasing the levels of HDL will retard the atherosclerotic process, an expectation underlying a number of hypocholesterolemic, antiatherosclerotic drugs.

Probucol does not conform to this archetypal model of a lipid-lowering, antiatherosclerotic drug. Although probucol does lower plasma cholesterol levels, it decreases HDL cholesterol levels in the same manner as low density lipoprotein (LDL) cholesterol levels.1 Largely because of this effect on HDL, the drug has not found wide acceptance. Nonetheless, probucol may actually do what an antiatherosclerotic drug should do. Probucol reduces xanthoma size in patients with familial hypercholesterolemia,2,3 suggesting that either cholesterol influx into foam cells of the xanthoma decreases, cholesterol efflux increases, or both. In any case, it is clear that probucol does not critically compromise the efflux of cholesterol. In fact, the degree of xanthoma reduction caused by probucol has been reported to correlate with the degree of HDL cholesterol reduction.2

Results of animal studies are in concert with these results in familial hypercholesterolemia. Probucol inhibits the progression of atherosclerosis in cholesterol-fed monkeys despite lowered HDL cholesterol levels.4 The drug also retards the progression of arterial lesions in LDL receptor-deficient Watanabe heritable hyperlipidemic (WHHL) rabbits5-6; notwithstanding evidence that probucol decreases LDL cholesterol uptake in these animals,6 the drug again did not critically inhibit cellular cholesterol efflux. These results find a parallel in several human HDL deficiency states that are associated with little if any increased atherosclerotic risk.7,8 Thus, under at least some circumstances, reverse cholesterol transport is not critically compromised by low HDL levels that are a consequence of drug treatment or genetic status.

Accepting a central role for HDL in reverse cholesterol transport, the retention of adequate reverse cholesterol transport in probucol-treated animals might be explained in several ways. One possibility is that the cholesterol burden of extrahepatic cells is decreased by probucol, compensating for a decreased capacity for reverse cholesterol transport. Another possibility is that reverse cholesterol transport is relatively insensitive to variations in HDL level; on the face of it, this does not fit well with the strong negative correlation of plasma HDL cholesterol levels with cardiovascular risk that is
Preparation of Double-Labeled HDL

Exclusion chromatography on Sephacryl S-200 (Pharmacia, Uppsala, Sweden) was used because they respond to probucol with decreased HDL levels yet lack plasma CE transfer activity. The absence of transfer activity both eliminates a variable known to be affected by probucol and allows the direct measurement of selective uptake. In rats, HDL CE is taken up by endocytotic and nonendocytotic mechanisms. Endocytotic uptake includes receptor-mediated uptake of particles containing apo E, receptor-independent uptake of HDL particles, and possibly uptake via other receptors. Nonendocytotic uptake is by selective uptake of CE without parallel uptake of HDL particles, a process accounting for >60% of hepatic HDL CE uptake in normal rats. Perturbation of either the endocytotic pathways or the selective uptake pathway could affect the transport of HDL CE to the liver. The present study examined the effects of probucol on both.

Methods

Preparation of Probucol Diet

Probucol (Merrell Dow Pharmaceuticals, Inc., Indianapolis, Ind.) was dissolved in diethyl ether and added to standard rat chow to achieve a probucol content of 1% (by weight). The ether was removed at room temperature. Chow for control rats was treated with ether alone. The rats were fed for 3 weeks.

Lipoprotein Preparations

Rat HDL was isolated from the plasma of overnight-fasted, female Sprague-Dawley rats weighing 230–260 g. HDL was prepared in the density range 1.09–1.21 g/ml or 1.055–1.21 g/ml using sequential preparative ultracentrifugation according to standard techniques. Isolated lipoprotein fractions were dialyzed against phosphate buffered saline containing 0.01% EDTA and 0.02% NaN₃. In some cases, plasma HDL was isolated from female rats weighing 200–220 g and fed for 17 days with chow containing 1% probucol.

Where indicated, HDL particles containing apo E were removed from the HDL preparation by heparin affinity chromatography, as previously described.

Preparation of Double-Labeled HDL

Apo A-I was isolated and labeled as previously described. Briefly, HDL was delipidated using 2.5 ml ethanol/diethyl ether, and apo A-I was separated by gel exclusion chromatography on Sephacryl S-200 (Pharmacia LKB Biotechnology Inc., Piscataway, N.J.). Residual apo E was removed by heparin-agarose affinity chromatography. Purity was assessed by sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE). Apo A-I was labeled by covalent attachment of the intracellularly trapped iodine-125–labeled N-methyl-tyramine cellobiose (125I-NMTC) ligand.

[125I]Cholesteryl oleyl ether ([125I]CEt) was prepared and incorporated into HDL (d = 1.09–1.21 g/ml) from a donor liposomal preparation using partially purified human plasma CE transfer protein, as previously described. The donor liposomes were separated from HDL by ultracentrifugation at a salt density of 1.06 g/ml. [125I]-NMTC-apo A-I was then associated with tritium-labeled HDL by exchange at 37°C for 24 hours. Unbound apolipoprotein was removed by centrifugation at a salt density of 1.21 g/ml. The supernatant fluid containing doubly labeled HDL was dialyzed against phosphate buffered saline at pH 7.4 containing 0.3 mM EDTA and then sterile-filtered (0.45-μm pores) before use.

Experiments to determine the plasma fractional catabolic rates (FCRs) of HDL tracers and their rates of uptake by various tissues were carried out as previously described. Female Sprague-Dawley rats weighing 220–245 g were used for all experiments. Food was removed at 5 PM the night before the turnover studies, which were initiated at 9 AM by the intravenous injection of doubly labeled HDL (about 150 μg protein). Animals were fasted throughout the 24-hour study period but had free access to water.

Periodic blood samples (0.1 ml) were withdrawn from a tail vein for 24 hours after injection of the tracers; samples were taken at 0.08, 0.5, 1, 2, 5, 10, 21, and 24 hours. In no case was a total of >5% of the blood volume removed during the study interval. Plasma samples (0.03 ml) were directly radioassayed for 125I and subsequently radioassayed for 1H after lipid extraction, as previously described.

The animals were anesthetized and exsanguinated 24 hours after tracer injection. The vasculature was perfused using approximately 150 ml phosphate buffered saline. Whole organs, samples of large or diffuse tissues, gut contents, feces, and urine were collected, weighed, homogenized, and radioassayed. In the case of adipose tissue, muscle, and skin, literature values were used to estimate the total tissue weight, as previously described. As in previous studies, tracers in the feces and gut lumen were attributed to primary uptake by the liver; this represents a substantial correction for uptake of the apolipoprotein tracer, but <1% of the [125I]CEt was found in these fractions. The tissue 1H content was assayed by liquid scintillation spectrometry after lipid extraction.

Plasma levels of total and HDL-associated CE were determined for each rat. To do that, 2.5 ml plasma taken at termination of the experiment was ultracentrifuged to prepare fractions of d < 1.05 g/ml and d > 1.05 g/ml. The CE content of the d > 1.05 g/ml fraction was taken as a measure of total HDL CE.

Computer analysis using an iterative curve-peeling program was used to fit a least-squares biexponential curve to each set of plasma decay data and to calculate FCR according to Matthews. The transport of HDL CE through the plasma pool was calculated as plasma HDL CE mass times FCR of [125I]CEt (the HDL CE tracer). Similarly, the transport of HDL CE via the uptake of HDL particles was calculated as plasma HDL...
CE mass times plasma FCR of \(^{125}\text I\)-NMTC–apo A-I (the HDL particle tracer). As a minimum approximation of whole-body selective uptake, the plasma mass transport of HDL CE in excess of that accounted for by the transport of HDL particles was calculated as plasma HDL CE mass times the difference in FCRs for \(^{1\text H}\)CE and \(^{125}\text I\)-NMTC–apo A-I; this does not account for uptake by the kidney of apo A-I that is not associated with HDL particles (about 20% of total apo A-I clearance).^12^

The uptake of HDL components by individual tissues was determined as previously described.\(^{13}\) Selective uptake was calculated as the rate of CE uptake minus the rate of HDL particle uptake (i.e., fractional rate of uptake of \(^{1\text H}\)CE less fractional rate of uptake of \(^{125}\text I\)-NMTC–apo A-I). These values are shown as the FCRs attributable to the whole organ in each animal. In some cases this was converted to a rate of uptake of HDL CE mass by multiplying the fractional rate by the plasma pool of HDL CE. These mass uptakes are shown as uptakes by the entire organ; because these values are dependent on animal size, they are normalized to a theoretical 200-g rat.

### Estimation of Apolipoprotein Amounts

Relative amounts of the apolipoproteins were determined by SDS-PAGE using vertical-slab gel electrophoresis (Hoefer Scientific Instruments, San Francisco, Calif.) run on 10% polyacrylamide gels after delipidation. The sample (25 μg protein of each fractionation) was dissolved in a solution of 2% SDS, 5% β-mercaptoethanol, and 62.5 mM Tris-HCl and then incubated for 3 minutes at 100°C before application to the sample wells. The gels were electrophoresed for 20 hours at 15–17 mA/slab and 15°C. The gels were then fixed in 50% trichloroacetic acid for 1 hour, stained in 0.05% Coomassie brilliant blue R-250 in methanol/acetic acid (25/10 by volume), and destained in methanol/acetic acid (25/10 by volume) for 1–2 days. A calibration curve was constructed from a series of polymerized molecular weight markers (Pharmacia) ranging in mass from 14,000 to 94,000 d. In a few cases, the Phast System (Pharmacia, Uppsala, Sweden)\(^{29}\) was also used to analyze apolipoproteins.

### Pore Limit Electrophoresis

The size of HDL particles was determined using 4–30% acrylamide gradient gels as described by Anderson et al.\(^{30}\) To do this, samples were applied to Pharmacia PAA 4/30 gels (Piscataway, N.J.) and electrophoresed for 3,600 V-hours. The sample volume was adjusted in each case so that 40–60 μg protein was applied in each slot. After electrophoresis, the gels were stained with Coomassie brilliant blue G. Protein standards for estimating molecular dimensions\(^{31}\) were purchased from Pharmacia, and the following values for Stokes' diameter were used: thyroglobulin, 17.0 nm; apoferritin, 12.2 nm; catalase, 10.2 nm; lactate dehydrogenase, 8.1 nm; and serum albumin, 7.1 nm.

### Chemical Analyses

To measure the probucol concentration, 0.5 ml plasma was first extracted using methanol/acetone (3/2 by volume) and then 1 ml heptane was added. The heptane phase was dried and redissolved in acetonitrile/heptane/0.1 M ammonium acetate (92/6/2 by volume) and analyzed by high-performance liquid chromatography on a C-18 column eluted with the same solvent mixture.

To determine the cholesterol and CE levels, samples were lipid-extracted according to Bligh and Dyer\(^{32}\) and redissolved in isopropanol. The amounts of total cholesterol and “free” cholesterol were then measured enzymatically using the reagent kit supplied by Boehringer Mannheim Corp. (Mannheim, FRG); the amount of CE was determined as the difference between the amounts of total cholesterol and free cholesterol. Cholesterol values in extracted samples were corrected for recovery by reference to the concentration of total cholesterol measured directly using a commercial kit or an autoanalyzer apparatus.

For estimation of the phospholipid concentration, phosphorus was quantified by the method of Bartlett\(^{33}\) after extraction of the samples by using the procedure of Bligh and Dyer.\(^{32}\) Phospholipid mass was calculated as 25 times the phosphorus mass. Protein was measured by the method of Lowry et al.\(^{34}\) using bovine serum albumin as the standard.

### Statistical Analysis

Values shown are mean±SD. Statistical significance was determined using Student’s \(^t\) test; where appropriate, comparison was for paired data (e.g., plasma decay of doubly labeled particles). A difference was considered significant at \(p<0.05\). Where percentage changes are shown, these are expressed as the mean percentage change from the average of controls ± the SD of those percentage changes.

### Results

Table 1 shows the plasma and lipoprotein cholesterol and CE levels of rats fed for 3 weeks with regular chow or chow supplemented with 1% probucol. Plasma total cholesterol was 40±8% lower, plasma CE was 35±12% lower, and plasma free cholesterol was 51±16% lower \((p<0.001\) for all) in the rats fed probucol. More than 40% of the decrease in the plasma cholesterol level was due to a reduction in the plasma level of HDL cholesterol, the major lipoprotein carrier of cholesterol in rats. Probucol feeding decreased the HDL CE level by 36±26% \((p<0.01)\). There was no significant correlation between the plasma probucol level and the decrease in either plasma total cholesterol or HDL CE levels. Plasma probucol levels in treated rats were lower than

### Table 1. Effects of Probucol Feeding on Plasma Lipids in Rats

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Control rats Mean±SD</th>
<th>Probucol-fed rats Mean±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol</td>
<td>16 679±124</td>
<td>16 406±55*</td>
</tr>
<tr>
<td>Cholesterol esters</td>
<td>16 471±81</td>
<td>16 307±54*</td>
</tr>
<tr>
<td>Free cholesterol</td>
<td>16 210±67</td>
<td>16 103±35*</td>
</tr>
<tr>
<td>HDL cholesterol esters</td>
<td>12 385±67</td>
<td>13 269±62*</td>
</tr>
<tr>
<td>HDL free cholesterol</td>
<td>8 102±20</td>
<td>8 80±8*</td>
</tr>
</tbody>
</table>

Values are in micrograms per milliliter. Probucol concentration was 2.66±1.43 μg/ml for 16 rats. HDL, high density lipoprotein. *p<0.001, \(p<0.01, \) p<0.02 different from control by Student's \(^t\) test.
levels therapeutically achieved in humans (measured in this laboratory by the same method).

Tracer experiments were conducted to examine the effect of probucol on transport of CE through the HDL pool and on directionality of that transport. To do this, HDL from control rats was labeled with \(^{3}H\)-CE and \(^{125}I\)-NMTC-apo A-I, which are intracellularly trapped tracers. The doubly labeled HDL was intravenously injected into both control and probucol-fed rats. Plasma decay kinetics were followed for 24 hours, at which time the injected tracers were still predominantly associated with the HDL fraction (85±3% of \(^{3}H\)-CE and 79±5% of \(^{125}I\)-NMTC-apo A-I in the 1.05<d<1.21 g/ml fraction). The animals were then killed, and tissues were removed for measurement of the accumulated tracers.

HDL prepared at 1.09<d<1.21 g/ml was used to trace the HDL pool. In support of this, FCR for the CE tracer in the 1.09<d<1.21 g/ml fraction (0.18±0.02 hr\(^{-1}\), n=8) was not significantly different from that in the 1.06<d<1.21 g/ml fraction (0.16±0.02 hr\(^{-1}\), n=9). The respective FCRs for apo A-I in the same experiments were 0.12±0.01 hr\(^{-1}\) (n=8) and 0.14±0.02 hr\(^{-1}\) (n=9), which also were not significantly different.

The plasma FCR for HDL CE was significantly increased by probucol feeding (Table 2), averaging 23±16% higher in the treated animals. In contrast, the FCR for apo A-I in probucol-fed animals was not significantly different from that in control animals. The difference between the FCRs, a minimum estimate of the whole-body fractional rate of selective uptake that does not account for clearance of free apo A-I, increased significantly (52±20%) on probucol feeding.

The mass transport of CE through the plasma HDL pool was calculated for the animals of Table 2. In this set of animals, the transport of HDL CE mass was only 6% less in the probucol-treated animals (not significant) even though plasma HDL CE levels were 25±6% lower in those animals (data not shown). Thus, mass transport of HDL CE through the plasma pool was not significantly decreased by probucol treatment, despite a large decrease in plasma HDL CE levels.

The transport of HDL apo A-I mass through the plasma pool was determined using another set of rats (Table 3) in which probucol treatment reduced HDL total cholesterol levels from 309±79 to 161±35 µg/ml. Levels of HDL-associated apo A-I (d=1.06–1.21 g/ml fraction) were determined in these animals along with the plasma decay kinetics of the CE and apo A-I moieties. HDL apo A-I mass decreased from 574±181 to 358±39 µg/ml (38±7% decrease); apo A-II and apo E masses also decreased significantly, but apo C mass did not. As a consequence of the decrease in apo A-I levels but the unchanged apo A-I FCR (Table 3), there was a significantly lower mass transport of apo A-I in the animals fed probucol, indicating a lower production rate, as previously reported.35

It is possible that the effects of probucol on HDL metabolism were mediated through changes in HDL composition or other properties of the HDL particles. To examine such possibilities, the metabolic fates of HDL from control and probucol-fed rats were compared. Probucol treatment of the rats serving as HDL donors resulted in a 31±11% decrease in HDL CE levels. HDL from both control and probucol-fed rats was doubly labeled with \(^{3}H\)-CE and \(^{125}I\)-NMTC-apo A-I and then injected into parallel sets of control and probucol-fed rats (Table 4). The plasma FCRs were not different for HDL prepared from control and probucol-treated rats. Thus, there was no evidence that changes in HDL due to probucol treatment were responsible for the differing metabolism of HDL in control and probucol-treated rats.

Although there was no evidence for a difference in the plasma FCRs of HDL from control and probucol-fed rats, composition of the two HDLs indeed differed. Table 5 shows the composition of the entire HDL fraction as well as of that fraction depleted of particles containing apo E by heparin-agarose affinity chromatography.19 HDL isolated from probucol-fed rats showed significantly less CE per milligram HDL protein, suggesting smaller HDL particles. This was true of both the whole HDL fraction and the HDL fraction depleted of particles containing apo E. In the case of HDL depleted of apo E, the ratios of cholesterol or phospholipids to protein were also decreased by probucol treatment. The apo E content of the whole HDL fraction decreased on probucol feeding by 34±17% (n=5 for each treatment; data not shown).

The above compositional changes predict that probucol treatment should result in a decreased average HDL particle size, as has been reported.26 This was examined by PAGE on nondenaturing 4–30% gradient gels. HDL (d=1.05–1.21 g/ml) that was depleted of particles containing apo E appeared as a broad peak with a modal Stokes’ radius of 6.08±0.28 nm in control animals (n=19) and of 5.89±0.32 nm in probucol-fed animals (n=20). This difference was marginally significant (p=0.0504).

The tracer studies indicated that mass transport of HDL CE through the plasma was not significantly decreased on probucol treatment, even in the face of a 25% decrease in HDL CE levels. However, this did not
indicate whether reverse cholesterol transport was compromised; such determination depends on mass transport to the liver, not just on transport through the plasma pool. Therefore, we measured the rate of uptake of HDL CE by specific organs of control and probucol-fed rats in terms of the accumulation of intracellularly trapped tracers of HDL CE and HDL apo A-I.23-24 To be assured that 125I-NMTC-apo A-I reasonably traced the hepatic uptake of HDL particles, the uptake of this tracer was compared with that of a nonmobile marker of the HDL particle per se, [14C]sucrose octaoleate.20,23 These two tracers were incorporated into the same synthetic HDL particles along with [3H]CEt, exactly as described previously.20,23 The triply labeled synthetic particles were then injected into rats to determine the plasma decay kinetics and rates of tissue uptake; to ensure that the synthetic particles behaved reasonably like native HDL, parallel animals received rat HDL2 labeled with [3H]CEt and 125I-NMTC-apo A-I only. Rates of hepatic uptake of the various tracers were determined as described in “Methods,” and the results for the liver are shown in Table 6. HDL particle uptake was not different when measured in terms of either 125I-NMTC-apo A-I or [14C]sucrose octaoleate uptake, nor were the ratios of [3H]CEt uptake to uptake of the two particle markers different (all comparisons used paired data). Uptake rates for the tracers in rat HDL2 were much like those for tracers in the synthetic particles, supporting the validity of this system for testing particle tracers.

Table 7 shows the effects of probucol on the fractional rates of uptake of HDL CE tracer by the liver, as well as on the rates of uptake of HDL CE mass. The fractional rate of uptake of HDL CE increased by 20±8% on probucol feeding. This higher fractional rate was due to increased selective uptake, which increased by 32±8% on probucol treatment; the fractional rate of particle uptake, measured in terms of apo A-I uptake, did not change. The uptake of HDL CE mass by the liver was calculated, assuming that the tracers traced the entire HDL pool. HDL CE mass uptake decreased by only 9% on probucol feeding (not significant). The mass uptake of HDL CE by selective uptake was virtually the same in control and probucol-fed rats, while mass uptake of CE by the uptake of HDL particles was significantly less in probucol-fed animals. Thus, probucol-fed animals maintained a nearly normal transport of HDL CE to the liver even in the face of a 25% decrease in plasma HDL CE levels, and this was owing to an increased fractional rate of selective uptake. Consequently, reverse cholesterol transport was not significantly compromised in probucol-treated animals to the extent that the transport of HDL CE mediates that process.

The adrenals and ovaries of rats exhibit high rates of selective uptake.13,20,21 Tables 8 and 9 show the effects of probucol on the uptake of HDL tracers by these steroidogenic tissues. In adrenal gland (Table 8) the fractional rate of uptake of HDL CE in probucol-fed rats was 88±38% higher after probucol feeding; the fractional rate of selective uptake was 93±40% higher, while the fractional rate of uptake of HDL particles (apo A-I) was not significantly changed. There was no significant effect of probucol on HDL CE mass uptake, by either particle uptake or selective uptake.

In ovary (Table 9) the fractional rate of uptake of HDL CE was 69±45% higher in probucol-fed rats, and this was attributable to both increased selective uptake (75±45% increase) and increased uptake of HDL particles (54±19% increase). Ovarian uptake of HDL CE mass was not significantly different in control and probucol-fed rats. Thus, probucol did not decrease the uptake of HDL CE mass by either adrenal gland or ovary.

**Discussion**

The purpose of this study was to determine whether the decrease in HDL levels caused by probucol actually indicates a decrease of HDL CE mass transport to the liver and to measure the effects of probucol on individual pathways for plasma transport and hepatic uptake of HDL CE. Rats were chosen as study subjects because they respond to probucol with lowered HDL CE levels and yet lack plasma CE transfer activity. This lack of transfer activity enabled the direct assessment of plasma decay kinetics without kinetic modeling. More importantly, it allowed determination of the rates and
mechanisms of HDL CE uptake by individual organs. Rats offered the further benefit of precluding another, possibly confounding, effect of probucol—an increase in plasma CE transfer activity.10

Because HDL is a heterogeneous population of particles and because selective uptake (and particle uptake) measured in vitro varies among subfractions separated in terms of density or size,20,37 it might be asked if the HDL tracer used here adequately traced the entire HDL pool. As outlined in "Methods," the FCR of neither the CE nor the apo A-I tracer differed when introduced into either the d = 1.09-1.21 g/ml HDL fraction usually used or into the broader d = 1.06-1.21 g/ml HDL fraction. In work to be reported elsewhere, plasma decay kinetics of the CE and apo A-I tracers in rats were similar for five HDL fractions, and the ratio of CE uptake to apo A-I uptake did not differ significantly in any fraction. Furthermore, the plasma decay kinetics of tracers in HDL from control and probucol-treated rats were alike even though the two HDLs varied in composition. These results, as well as results in other studies,38 indicate that the plasma decay kinetics of CE and apo A-I tracers do not differ substantially regardless of the HDL fraction labeled (at least in the d = 1.06-1.25 g/ml density range, which includes almost all HDL CE). The apparent lack of concordance between in vitro and in vivo results (i.e., metabolic heterogeneity seen in vitro but not apparent in vivo) may be explained by in vivo exchange, transfer, and "normalization" processes that are not available in vitro.

The prominent finding of this study is that mass transport of HDL CE through the plasma, as well as mass transport to the liver, decreased little if any on probucol treatment. This maintenance of normal rates of mass transport occurred in the face of a substantial decrease in the plasma pool of HDL CE. It should be noted that we did not determine the transport of free cholesterol through the HDL pool to the liver, and thus we do not know the contribution of that pathway to reverse cholesterol transport or the effect of probucol on it.

The hepatic uptake of HDL particles, as measured by apo A-I uptake, did not contribute to the maintenance of an about-normal HDL CE mass uptake by the liver. The fractional rate of uptake of apo A-I by the liver did not change on probucol treatment, so that HDL particle uptake decreased about in parallel with the plasma level of HDL CE.

The same pattern was seen in the plasma compartment, as might be expected due to dominance of the liver in whole-body metabolism. Here, too, a decrease in the plasma HDL apo A-I concentration and the lack of an effect on its plasma FCR indicated that probucol decreased the flux of HDL apo A-I through the plasma compartment. Thus, probucol decreases apo A-I production rates, as reported earlier for both rats35 and humans.36,39

The nearly normal transport of HDL CE to the liver of probucol-treated rats was mediated by an increase in the rate of selective uptake. However, the work here does not show the basis for that increase. One possibility is suggested by previous work indicating that selective uptake is upregulated in several tissues of rats treated with 4-aminopyrazolopyrimidine or 17-ethynylestradiol to drastically lower plasma cholesterol levels.40 Although regulation of selective uptake was not observed in the liver in that study, it is possible that the upregulation of hepatic selective uptake seen in the present study was secondary to the lowering of plasma cholesterol levels. To examine this possibility, the rela-

\begin{table}
\centering
\caption{Comparison of \textsuperscript{125}I-NMTC-Apolipoprotein A-I and \textsuperscript{14}C]Sucrose Octaoleate as Tracers for HDL Particles}
\begin{tabular}{|c|c|c|c|c|c|}
\hline
Particle & \textsuperscript{125}ICE & \textsuperscript{14}C]Sucrose octaoleate & \textsuperscript{125}I-NMTC-apo A-I & \textsuperscript{125}ICE & \textsuperscript{14}C]Sucrose octaoleate \\
\hline
Synthetic HDL & 117.2±20.6 & 52.1±14.0 & 47.1±4.1 & 2.26±0.28 & 2.49±0.44 \\
Rat HDL\textsubscript{2} & 128.0±12.5 & 54.6±4.5 & & & \\
\hline
\end{tabular}
\end{table}

\begin{table}
\centering
\caption{Effects of Probucol Feeding on HDL Uptake by Liver in Rats}
\begin{tabular}{|c|c|c|c|c|}
\hline
Parameter & Control rats & Probucol-fed rats & n & Mean±SD \\
\hline
Fractional rate of uptake (10\textsuperscript{-9}hr/liver) & & & & \\
HDL CE uptake & 118±5 & 141±26* & 8 & 8 \\
HDL apo A-I uptake & 46±5 & 46±12 & 8 & 8 \\
Selective uptake & 72±10 & 95±15* & 8 & 8 \\
CE mass uptake (\mu g/liver/hr) & & & & \\
Total HDL CE uptake & 376±74 & 342±50 & 6 & 6 \\
HDL particle uptake & 146±29 & 113±11* & 6 & 6 \\
HDL selective uptake & 230±48 & 229±39 & 6 & 6 \\
\hline
\end{tabular}
\end{table}

\begin{table}
\centering
\caption{Effects of Probucol Feeding on HDL Uptake by Adrenal Gland in Rats}
\begin{tabular}{|c|c|c|c|c|}
\hline
Parameter & Control rats & Probucol-fed rats & n & Mean±SD \\
\hline
Fractional rate of uptake (10\textsuperscript{-9}hr/adrenals) & & & & \\
HDL CE uptake & 3.9±1.3 & 7.4±1.5* & 8 & 8 \\
HDL apolipoprotein A-I uptake & 0.4±0.1 & 0.5±0.1 & 8 & 8 \\
Selective uptake & 3.6±1.2 & 6.9±1.4* & 8 & 8 \\
CE mass uptake (\mu g/adrenals/hr) & & & & \\
Total HDL CE uptake & 13±4 & 18±4 & 6 & 6 \\
HDL particle uptake & 1.1±0.3 & 1.3±0.2 & 6 & 6 \\
HDL selective uptake & 12±4 & 17±4 & 6 & 6 \\
\hline
\end{tabular}
\end{table}
HDL CE mass by the liver caused by probucol, it is also transport mediated by HDL CE. Although the data are not significantly compromise reverse cholesterol decrease in HDL levels resulting from probucol feeding effect in the present study.42 Thus, it seems likely that probucol directly enhances selective uptake resulting from probucol treatment.

fractional rate of selective uptake by the liver to the plasma HDL CE concentration was compared for control and probucol-fed rats. There was a tendency for hepatic selective uptake than control animals at any given level of plasma HDL CE. However, the results of statistical analysis did not allow us to definitely say that the relation differed in control and probucol-fed animals (0.05 < p < 0.1). Thus the data here suggest, but do not prove, that the effects of probucol on HDL CE uptake in rats cannot be wholly explained by modulation of plasma lipoprotein levels.

It is also possible that probucol directly affects the liver so as to increase selective uptake independently of changes in the plasma lipoprotein concentration or composition. To examine this possibility, we measured HDL metabolism in primary cultures of hepatocytes from rats treated or not treated with probucol. While some experiments indicated a greater rate of selective uptake by hepatocytes from probucol-fed rats, this result was not consistent or statistically significant overall. Results obtained using cells from control rats that were exposed to probucol in vitro yielded similar results. Thus, we could not demonstrate an effect of probucol on HDL metabolism by rat hepatocytes. However, Barnhart et al.41 also studied HDL metabolism by primary rat hepatocytes treated with probucol. Those authors found an increase in the cell cholesterol content that was not explained by endogenous synthesis and a decrease in the medium cholesterol content that was not explained by the uptake of HDL particles. These results are consistent with, and supportive of, an increase in the selective uptake of HDL CE as found in the present study in intact animals. Furthermore, studies of Hep G2 human hepatoma cells have consistently shown an increase in selective uptake resulting from probucol treatment.42 Thus, it seems likely that probucol directly enhances selective uptake by rat hepatocytes, even though we were not able to clearly demonstrate such an effect in the present study.

Evidence from the present study indicates that the decreased HDL levels resulting from probucol feeding do not significantly compromise reverse cholesterol transport mediated by HDL CE. Although the data are consistent with a marginal decrease in the uptake of HDL CE mass by the liver caused by probucol, it is also possible that the flux of HDL CE into the liver actually increases relative to cholesterol delivery to extrahepatic tissues owing to the decrease in plasma cholesterol concentrations and a consequent decrease in the uptake of lipoprotein cholesterol by extrahepatic cells.

The data here do not allow us to conclude that the effects of probucol on selective uptake account for the beneficial consequences of probucol treatment in patients with familial hypercholesterolemia23 or in WHHL rabbits4-6; other effects, such as those on lipoprotein uptake, may play important or even dominant roles. Even if an effect of probucol on reverse cholesterol transport were to play a dominant role in these situations, we would not know how to apportion that role between selective uptake and endocytotic uptake after the transfer of CE to more buoyant lipoproteins. Furthermore, it is not known that probucol even has an antiatherosclerotic effect in rats.

Irrespective of the forgoing caveats, the data are consistent with the possibility that changes in selective uptake contribute to, or are required for, the mobilization of cholesterol from human xanthomas and the retardation of lesion development in WHHL rabbits. A recent study from this laboratory indicates that selective uptake plays a role in HDL CE clearance in rabbits and suggests that selective uptake may play a role in humans as well.43 Therefore, it is possible that probucol acts in humans, as it does in rats, to maintain near-normal reverse cholesterol transport by increasing the selective uptake of HDL CE.

During the review of this manuscript, Hayek and coworkers44 reported the effects of probucol treatment on plasma decay kinetics and the transport of HDL apo A-I and CE in mice, a species that also lacks plasma CE transfer activity. Those authors, too, saw that probucol did not change the apo A-I FCR, increased the CE FCR, and decreased the transport of HDL apo A-I through the plasma compartment but did not change the transport of CE. Our results verify those results in a related species and complement them by determining the effects of probucol on uptake by individual organs and the mechanisms of that uptake.

### References

6. Carew TE, Schwentke DC, Steinberg D: Antiatherogenic effect of probucol unrelated to its hypcholesterolemic effect: Evidence that antioxidants 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


29. Phast System Separation Techniques File No. 110. Uppsala, Sweden, Pharmacia, Laboratory Separation Division


Downloaded from http://atvb.ahajournals.org/ by guest on July 7, 2017
Transport of HDL cholesterol esters to the liver is not diminished by probucol treatment in rats.

B M Richard, M A Pfeuffer and R C Pittman

doi: 10.1161/01.ATV.12.7.862
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1992 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/12/7/862

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/