Probucol Enhances Selective Uptake of HDL-Associated Cholesteryl Esters In Vitro by a Scavenger Receptor B-I–Dependent Mechanism

Franz Rinninger, Nan Wang, Rajasekhar Ramakrishnan, Xian Cheng Jiang, Alan R. Tall

Abstract—Recently, the class B, type I scavenger receptor (SR-BI) has been shown to mediate the selective uptake of high density lipoprotein (HDL) cholesteryl esters (CEs), ie, lipid uptake independent of HDL holoparticle uptake. In vivo, this selective uptake delivers CEs to the liver for excretion and to steroidogenic tissues for hormone synthesis. Probucol, a hydrophobic antioxidant drug, lowers plasma cholesterol in humans and rodents and may inhibit progression of atherosclerosis and postangioplasty restenosis. In this study, the effect of probucol on HDL selective CE uptake was investigated in mice and in cells expressing SR-BI. Probucol feeding lowered plasma HDL cholesterol and markedly increased selective CE uptake from HDL in the liver and adrenal glands. However, probucol did not alter SR-BI protein levels in membranes from these organs. When incubated with control Chinese hamster ovary (CHO) cells, HDL isolated from probucol-treated mice (P-HDL) and HDL from control mice (C-HDL) showed similar low selective uptake of CEs. However, when incubated with SR-BI–transfected CHO cells, P-HDL showed a 2-fold increase in selective uptake compared with C-HDL. In an adrenal cell line (Y1-BS1), which expresses SR-BI in an adrenocorticotropic hormone–inducible manner, P-HDL showed significantly greater selective CE uptake than did C-HDL, and the differential response was amplified by adrenocorticotropic hormone treatment. In contrast to P-HDL, incorporation of this compound into HDL in vitro did not result in stimulation of selective CE uptake by SR-BI–transfected CHO cells, even though a significant mass of probucol could be detected in the HDL preparation. The specific interaction of P-HDL with SR-BI in cell culture could be observed after only 24 hours of probucol feeding, when there were minimal changes in HDL size and composition. Thus, probucol or one of its metabolites increases selective CE uptake in vivo by modifying HDL in a way that causes enhanced interaction with SR-BI. The increased interaction of P-HDL with SR-BI in the liver and arterial wall may be partly responsible for the effects of probucol on atherosclerosis and restenosis. **(*Arterioscler Thromb Vasc Biol*. 1999;19:1325-1332.)**

Key Words: HDL ■ selective uptake ■ scavenger receptor BI ■ probucol

Plasma HDL plays a critical role in cholesterol metabolism in vivo. HDL removes cholesterol from cells in culture and from peripheral tissues.[1,2] After cholesterol esterification, HDL-associated cholesteryl esters (CEs) can be transferred to other lipoprotein fractions by cholesteryl ester transfer protein (CETP) in some species[3] or directly delivered to tissues.[1] In rats, HDL-associated CEs can be taken up by the liver and steroidogenic tissues without parallel uptake of HDL apolipoproteins, and this metabolic pathway has been designated the selective CE uptake pathway.[4] This selective uptake delivers CEs to steroidogenic tissues for hormone synthesis and to the liver, where HDL-derived cholesterol is either secreted into the bile, used for bile acid synthesis, or secreted in newly synthesized lipoproteins.[5] The HDL-mediated transport of cholesterol from extrahepatic tissues to the liver, designated reverse cholesterol transport, is believed to play a critical role in whole-body cholesterol homeostasis.[1]

The class B, type I scavenger receptor (SR-BI) is a cell surface HDL receptor that mediates selective lipid uptake.[6] In cultured cells, murine SR-BI, an 82-kDa glycoprotein that appears to be clustered in caveolae,[7] binds HDL and mediates selective CE uptake from this lipoprotein fraction.[6] In mice and rats, SR-BI is most abundantly expressed in the liver and steroidogenic tissues, which are the most active sites of HDL selective CE uptake.[4,6,8,9] SR-BI expression in rodent steroidogenic tissues is coordinately regulated with steroid hormone synthesis by trophic hormones.[10,11] Hepatic overexpression of murine SR-BI in mice substantially reduces plasma HDL and increases biliary cholesterol.[12] Moreover, mice with induced mutations that attenuate expression of SR-BI show increased HDL levels and proportionately reduced tissue-selective uptake of HDL CEs.[13,14] In summary, these studies show that SR-BI is a physiologically regulated HDL receptor that modifies HDL levels and mediates selective uptake of HDL lipids in vivo.

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Probucol is a nonpolar antioxidant that substantially lowers plasma cholesterol.[15,16] Besides LDL cholesterol, this drug reduces HDL cholesterol in humans and rodents.[17,18] In parallel with a decrease in plasma HDL cholesterol, probucol increases the selective CE uptake from HDL by the liver in rats and to some extent in hepatic cells in culture.[19,20] The antioxidant activity of probucol may prevent cellular lipid loading and the formation of foam cells in atherosclerotic lesions by limiting oxidative modification of LDL, thus minimizing macrophage uptake of cholesterol-rich lipoproteins.[21–23] Interest in probucol was raised substantially by the recent demonstration that this compound significantly reduces the rate of coronary artery restenosis after coronary balloon angioplasty in humans.[24]

The hypothesis of the present study was that the increased selective uptake of HDL-associated CEs due to probucol treatment of mice would be mediated by an increase in SR-BI expression. When no change in SR-BI expression was found, we further explored the potential mechanism(s) by examining the interaction of HDL from probucol-treated animals (P-HDL) with cells expressing increased levels of SR-BI. These studies revealed that P-HDL enhances selective CE uptake by facilitating the interaction of HDL with SR-BI. These findings illuminate an important biological property of probucol that may account for some of its novel effects on restenosis and atherosclerosis.

Methods

Animals

Adult female mice (C57BL/6, Taconic Farms, Germantown, NY) of 20 to 25 g body weight were used in all experiments. These animals were caged with alternating 12-hour light (7 AM to 7 PM) and 12-hour dark cycles. The mice had free access to food and water. In most cases, the animals were fed for 1 day or for 14 days a regular powdered mouse chow diet with no addition (control) or a chow diet containing 0.2% probucol (wt/wt, Sigma Chemical Co).[18] All experiments with mice were approved by the Institutional Animal Care and Use Committee of Columbia University.

Lipoprotein Preparations

Mice were fasted for at least 4 hours before blood drawing. Murine HDL was prepared in the density range $d=1.063$ to 1.21 g/mL from anticoagulated (EDTA) plasma of control mice by sequential preparative ultracentrifugation according to standard procedures.[25] HDL was finally dialyzed against phosphate-buffered saline (PBS) (pH 7.4) containing EDTA (0.3 mmol/L) and NaN_3 (0.02%). In some cases, blood was obtained in parallel from control and probucol-treated mice. Before blood harvest, the animals were fed for 24 hours a chow diet supplemented or not with probucol (0.2% wt/wt).[18] Compared with control mice, total plasma cholesterol was at least 51% lower in mice fed for 24 hours a diet containing probucol.

Preparation of Doubly Radiolabeled HDL

In brief, HDL protein was initially labeled by covalent attachment of the intracellularly trapped ^{125}I-*N*-methyl tyramine cellobiose (^{125}I-NMTC) ligand.[26] Thereafter, ^{125}I-NMTC-HDL was labeled with [^{3}H]cholesteryl oleyl ether ([^{3}H]CEt, Amersham) to trace the CE moiety.[5,27] [^{3}H]CEt was introduced in a liposomal preparation and exchanged (6 hours, 37°C) into ^{125}I-NMTC-HDL by using highly purified recombinant human plasma CETP. The donor liposomes were separated from labeled HDL by ultracentrifugation at $d=1.063$ g/mL, and then HDL was reisolated by another spin at $d=1.21$ g/mL to remove CETP. Thereafter the HDL preparation was exhaustively dialyzed against PBS (pH 7.4) containing EDTA (0.3 mmol/L) and sterile-filtered (0.45 μm). P-HDL was radiolabeled exactly as described above for C-HDL, and this procedure was done in strict parallel.

In some cases, probucol was incorporated into HDL in vitro after preparation. In brief, in one approach, probucol was introduced in an ethanolic solution. In this case, doubly radiolabeled HDL (1 mg) was incubated with (10 μmol/L) or without probucol in 26 mL of Dulbecco's modified Eagle's medium (DMEM) containing BSA (5 mg/mL), ethanol (1% vol/vol), and NaN_3 (0.02%).[20,28] After incubation (4 hours, 37°C), HDL was subsequently reisolated by ultracentrifugation and dialyzed (PBS). Alternatively, a lipid microemulsion with or without probucol and [^{3}H]CEt was prepared as outlined by Tsujita and Yokoyama.[29] This lipid emulsion was incubated (37°C) in PBS containing ^{125}I-NMTC-HDL and CETP similarly as described above for double radiolabeling of HDL.[5]

Metabolism of Doubly Radiolabeled HDL in Mice

Experiments to investigate the plasma decay (fractional catabolic rate, or FCR) of both HDL tracers and their tissue sites of uptake were carried out as previously described.[4,8,31] In brief, mice were fasted for 12 hours before tracer injection and throughout the 24-hour study period but had free access to water. Doubly radiolabeled HDL was injected intravenously (iliac vein) at 10 AM into the mice. Thereafter periodic blood samples were drawn from a tail vein at 0.16, 0.5, 2.0, 5.0, 10.0, and 24.0 hours. Plasma samples were directly radioassayed for ^{125}I and analyzed for ^{3}H after lipid extraction.[32]

Twenty-four hours after tracer injection, the animals were anesthetized with isoflurane and exsanguinated. Then the vasculature was perfused with saline (50 mL per animal). Organs and gut contents were collected, weighed, homogenized, and radioassayed. The tissue content of ^{125}I radioactivity was directly assayed and that of ^{3}H was analyzed by liquid scintillation spectrometry after lipid extraction.[4,32] As in previous studies, tracers in the gut contents were attributed to primary uptake by the liver.[4,26]

Calculations for the Experiments Performed With Mice

FCRs for both HDL-associated tracers (^{125}I-NMTC and [^{3}H]CEt) in mice were calculated by fitting the data from each animal separately by two 2-pool models, 1 for each tracer. Each 2-pool model had a circulating pool in equilibrium with a noncirculating pool.[33] In each case, it was confirmed that 2 pools fit the data significantly better than did a 1-pool model; a third pool was not required for any study.

Uptake of both HDL tracers by murine tissues was expressed in terms of organ FCRs.[4] These organ FCRs were obtained by multiplying the plasma FCR of a given HDL tracer with the fraction of the tracer recovered in a specific tissue. The fraction of tracer uptake attributed to an organ was calculated as the radioactivity recovered in that organ divided by the total radioactivity recovered from all extravascular sources, including all tissues and gut contents.[4] Thus, the organ FCRs represent the fraction of the plasma pool of the traced HDL component cleared per hour by a specific organ. ^{125}I-NMTC, ie, the tracer of the protein moiety, represents HDL particle metabolism.[5] Selective CE uptake was obtained as the rate of CE tracer uptake minus that due to HDL particle uptake (ie, ^{125}I-NMTC).[4,31]

Detection of SR-BI Protein and mRNA

Anti–SR-BI antisera were prepared by immunization of rabbits with a recombinant murine SR-BI fragment (amino acids 315 to 412) that was prepared in a bacterial expression system and purified.[10] After murine tissue harvest, membranes were prepared by ultracentrifugation from the liver or from 6 pooled adrenal glands.[10] Tissues were homogenized in PBS in the presence of protease inhibitors (0.5 μg/mL leupeptin, 1 μg/mL aprotinin, 1 μg/mL pepstatin A, 0.2 mmol/L PMSF, and 1 mmol/L EDTA).

Immunoblotting was performed with liver and adrenal membrane preparations.[10] Routinely, 50 μg of liver membrane protein or 20 μg of adrenal membrane protein was loaded per lane. Membranes were subjected to 7.5% SDS–polyacrylamide gel electrophoresis (PAGE) under reducing conditions. SR-BI protein immunoreactivity was identified at its authentic molecular size (82 kDa).[6] SR-BI expression

levels were determined by chemiluminescence detection (Amersham). SR-BI mRNA abundance in the adrenal glands and liver was determined by RNAse protection assay as previously described[10] using β-actin as an internal standard.

Cell Culture

Control Chinese hamster ovary (CHO) cells or CHO cells stably transfected with a murine SR-BI cDNA (overexpressing SR-BI CHO cells) were obtained and maintained as previously described.[34] Y1-BS1 cells[5,11,35] were maintained in Ham's F-10 medium (Gibco) supplemented with heat-inactivated horse serum (12.5% vol/vol, Gibco), heat-inactivated FBS (2.5% vol/vol, Gibco), L-glutamine (2 mmol/L), penicillin (100 U/mL), and streptomycin (100 μg/mL). Human Hep 3B hepatoma cells were cultured in DMEM supplemented with FBS (10% vol/vol) and antibiotics.[27]

Uptake Assay for Doubly Radiolabeled HDL by Cells in Culture

When the cells had reached near-confluence, the culture medium was removed and cells were washed twice with PBS. Thereafter the cells were preincubated (37°C) for 16 hours in the respective serum-free medium (Ham's F-12, Ham's F-10, or DMEM) supplemented with 5 mg/mL BSA (Sigma) and antibiotics. In some experiments with Y1-BS1 cells, adrenocorticotropic hormone (ACTH, 100 nmol/L, Sigma) was present in this incubation medium as well. After this 16-hour preincubation period, the medium was removed and the cells were washed twice with PBS. Uptake of HDL tracers was then initiated by incubation (4 hours, 37°C) of the cells in the respective medium containing BSA (5 mg/mL) and doubly radiolabeled HDL (10 μg HDL protein per mL).[27] Thereafter the medium was aspirated and cells were washed twice with PBS. Cells were then released from the wells by incubation (5 minutes, 37°C) in saline containing trypsin/EDTA (0.05% wt/vol trypsin and 0.53 mmol/L EDTA, total volume of 1 mL; Gibco). Trypsin activity was quenched by the addition of 1 mL PBS containing 50 mg/mL BSA. The cell suspension was transferred to tubes by rinsing the wells with PBS and pelleted by low-speed centrifugation. After aspiration of the supernatant, the cell pellets were washed with 5 mL PBS. Finally, the cell pellets were dissolved in 0.1N NaOH solution, followed by sonication. Aliquots of the cell suspensions were directly assayed for ^{125}I in a gamma spectrometer. [^{3}H]CEt was extracted[32] from the cell suspensions, followed by liquid scintillation spectrometry, and protein was analyzed as described.[36]

Calculations for Uptake of HDL-Associated Tracers by Cells in Culture

In experiments with cultured cells, the cellular uptake of each lipoprotein tracer is shown in terms of apparent HDL particle uptake expressed in terms of HDL protein.[5] This allows direct comparison of uptake of both tracers on a common basis. HDL selective CE uptake is calculated as the difference in [^{3}H]CEt uptake in excess of ^{125}I-NMTC, assuming that ^{125}I-NMTC uptake traces HDL particle uptake.[5,27]

TABLE 1. Effects of Probucol Feeding of Mice on Plasma Total and HDL Cholesterol Levels

Mice	Total Cholesterol	HDL Cholesterol
Control	52.2±2.6	37.0±4.0
Probucol	9.1±0.3	6.8±0.8

Values are means±SEM in mg per 100 mL plasma. In each group, n=6 samples were determined. These samples originated from n=3 mice in each group. An independent set of n=4 mice in each group yielded qualitatively identical results. See Methods for details on diets and lipid analyses.

Nondenaturing Gradient PAGE

Mouse HDL and mouse plasma were analyzed by gradient PAGE performed under nondenaturing conditions[37] (Lipogel, Zaxis). Lipoprotein size was estimated by comparison with standard proteins (high-molecular-weight calibration kit, Pharmacia). Finally, the gels were stained with the lipoprotein-specific stain Sudan black B.

Other Techniques

Protein was determined according to the method described by Lowry and coworkers.[36] For analytical purposes, HDL was isolated by ultracentrifugation.[25] Total cholesterol, unesterified cholesterol, phospholipid, and triglycerides were measured using commercial kits (Wako). Esterified cholesterol represents the difference between total and unesterified cholesterol. SDS-PAGE followed the procedure described by Laemmli[38] and coworkers. From HDL, after extraction with chloroform/methanol, probucol was analyzed by high-performance liquid chromatography.

Statistical Analysis

Values shown are mean±SEM. Statistical significance was determined by two-tailed Student's *t* test for unpaired data.

Results

Mice were fed for 14 days a chow diet supplemented with probucol (0.2% wt/wt) or control chow.[18] After this period, probucol decreased total plasma cholesterol by 83% and plasma HDL cholesterol by 82% compared with corresponding values in control mice (Table 1).

To explore the mechanism of the probucol-induced decrease in HDL cholesterol, C-HDL isolated from control mice was doubly radiolabeled in the protein and CE moieties with ^{125}I-NMTC and [^{3}H]CEt, respectively.[5,26] These HDL tracers are intracellularly trapped at their sites of tissue uptake.[5,26] This labeled HDL was injected intravenously into both control and probucol-fed mice. Previous experiments suggested a rapid equilibration of control HDL with HDL in probucol-treated mice.[19]

In control mice, the plasma FCR for HDL-associated [^{3}H]CEt was higher compared with ^{125}I-NMTC, ie, the protein tracer that represents the metabolism of holo-HDL particles

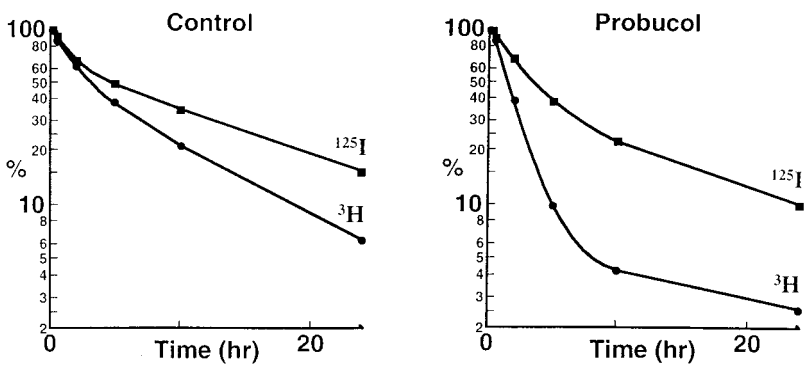

Figure 1. Effects of probucol feeding on plasma decay kinetics of doubly radiolabeled HDL in mice. ^{125}I-NMTC/[^{3}H]CEt-HDL was prepared from control animals as described in Methods. This preparation was injected into control (left) and probucol-fed (right) mice after 14 days of probucol feeding. After injection during a 24-hour interval, periodic blood samples were drawn and plasma was analyzed for ^{125}I (squares) and ^{3}H (circles). Plasma FCRs for both HDL tracers were calculated as described in Methods. Shown is a typical experiment for each group of n=4 mice.

TABLE 2. Effects of Probucol Feeding on Plasma Decay Kinetics (FCRs) of Doubly Radiolabeled HDL in Mice

Mice	^{125}I-NMTC-Protein	[^{3}H]CEt	^{3}H−^{125}I
Control	0.112±0.012*	0.164±0.007†	0.052±0.006‡
Probucol	0.227±0.04*	0.552±0.084†	0.325±0.044‡

Values are mean±SEM in pools per hour. The specific radioactivity of this doubly labeled HDL preparation was 59 cpm/ng HDL protein for ^{125}I; for ^{3}H, the respective value was 185; see Methods for details. Control female mice, n=4, and probucol-treated female mice, n=4.

Student's 2-tailed *t* test for unpaired data: *$P<0.05$, †$P<0.005$, ‡$P<0.001$.

(Figure 1 and Table 2). The difference between both FCRs represents lipid catabolism independent of holoparticle catabolism, ie, selective CE uptake from HDL by the whole body.[8] Probucol treatment of mice increased the plasma FCR for HDL-associated [^{3}H]CEt ≈3-fold compared with control mice (Figure 1 and Table 2). This drug also enhanced the plasma FCR of the protein tracer ^{125}I-NMTC by ≈2-fold (Table 2). The fractional increase in FCR due to probucol was thus higher for [^{3}H]CEt than for ^{125}I-NMTC. The difference in catabolic rates between the 2 tracers ([^{3}H]CEt minus ^{125}I-NMTC), ie, whole-body selective lipid uptake, increased ≈6-fold in probucol-treated mice (Table 2). These results show that probucol increases the selective catabolism of plasma HDL CEs in mice.

Twenty-four hours after HDL injection, the tissue sites of tracer uptake were analyzed.[4,31] For the liver of control mice, the FCR for HDL-associated [^{3}H]CEt was substantially higher than that of ^{125}I-NMTC (Figure 2, left). Previous studies established that the organ FCR for ^{125}I-NMTC represents HDL holoparticle metabolism.[5] Therefore, the higher rate of hepatic [^{3}H]CEt clearance indicates that this organ selectively takes up CEs from HDL in control mice.[4,8] Probucol treatment of mice increased the hepatic FCR for HDL-associated [^{3}H]CEt by ≈4-fold, and this compound stimulated uptake of ^{125}I-NMTC by ≈2.5-fold (Figure 2, left). Again, the difference between [^{3}H]CEt and ^{125}I-NMTC represents selective CE uptake from HDL by the liver, and this metabolic parameter increased 4-fold as a result of probucol treatment.

Uptake of HDL-associated tracers by mouse adrenal glands in vivo is shown in Figure 2 (right). In control mice, the adrenal FCR for HDL-associated [^{3}H]CEt was ≈5-fold in excess of that of ^{125}I-NMTC, indicating selective CE uptake from HDL (Figure 2, right). Probucol treatment of mice increased the adrenal FCRs for [^{3}H]CEt and ^{125}I-NMTC by ≈9- and 5-fold, respectively. As a result of these changes, selective CE uptake from HDL, ie, the difference between [^{3}H]CEt and ^{125}I-NMTC, by adrenal glands increased ≈10-fold in probucol-treated mice compared with control animals (Figure 2, right). Changes in uptake rates were minor in the kidneys, lungs, heart, spleen, muscle, adipose tissue, and carcass (data not shown). In summary, in mice, probucol induced a significant decrease in plasma HDL cholesterol and markedly increased the selective CE uptake from HDL in the liver and adrenal glands.

SR-BI plays an essential role in selective CE uptake from HDL. To see whether the expression of this membrane protein was altered in probucol-fed mice, Western blotting was performed on membranes prepared from the liver and adrenal glands by using an SR-BI–specific antiserum (Figure 3). There was no detectable effect of probucol treatment on SR-BI expression levels. Similar data were obtained when SR-BI mRNA was quantified in these organs by RNAse protection analysis (data not shown).[10] Thus, even though the liver and adrenal glands demonstrated a substantial increase in HDL selective CE uptake in response to probucol, this increment was not the result of an increase in SR-BI expression.

To further explore the mechanism of the probucol-induced increase in HDL selective CE uptake, HDL was prepared from mice fed probucol for 24 hours (ie, P-HDL) or from control mice (ie, C-HDL) and doubly labeled with ^{125}I-NMTC and [^{3}H]CEt as described above. In a typical preparation, doubly labeled P-HDL contained 14.8 μmol/L probucol per mg HDL protein (determined by high-performance liquid chromatography), whereas in C-HDL no probucol could be detected. Doubly labeled P-HDL and C-HDL were incubated with control CHO cells or CHO cells stably transfected with an SR-BI cDNA (Figure 4).[34] Uptake of both tracers by cells was determined, and the data were expressed in terms of apparent HDL particle uptake.[5,27] Previous studies established that ^{125}I-NMTC traces HDL holoparticle metabolism, and the difference in apparent HDL particle uptake between [^{3}H]CEt and ^{125}I-NMTC represents selective CE uptake.[5] For

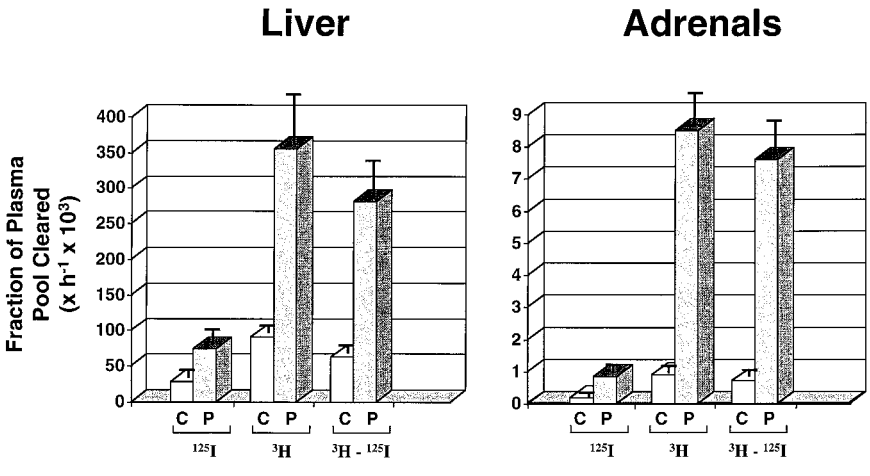

Figure 2. Organ FCRs for doubly radiolabeled HDL by the liver and adrenal glands in control and probucol-treated mice. Mice were fed a control or a probucol-containing chow diet for 14 days. HDL was prepared from control animals and labeled with ^{125}I-NMTC and [^{3}H]CEt as outlined in Methods. Doubly radiolabeled HDL was injected into control (C) and probucol-fed (P) mice. During the subsequent 24-hour interval, plasma was harvested to determine decay of both tracers. Twenty-four hours after tracer injection, the animals were humanely killed, and the tissues were harvested and analyzed for the content of each tracer. Organ FCRs for ^{125}I-NMTC (^{125}I) and [^{3}H]CEt (^{3}H) were calculated as described in Methods, and selective CE uptake represents the difference between the 2 tracers (^{3}H minus ^{125}I). Values are mean±SEM of n=4 mice in each group.

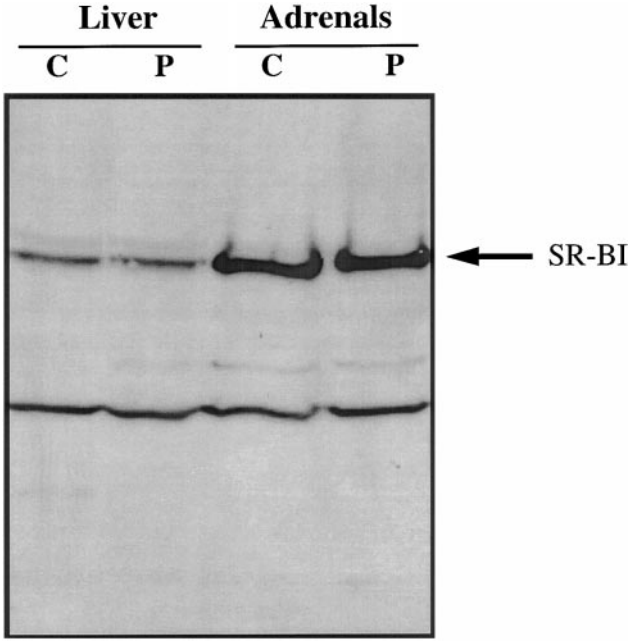

Figure 3. Immunoblot analysis of SR-BI expression in liver and adrenal glands of control and probucol-treated mice. Mice were fed for 14 days a diet that was supplemented or not with probucol (0.2% wt/wt). The liver and adrenal glands were harvested, and plasma membranes were isolated as described in Methods. Membrane proteins (50 μg of liver, 20 μg of adrenal gland) were separated on an SDS-PAGE gel (7.5%) under reducing conditions, transferred to a nitrocellulose membrane, and immunoblotted with anti–SR-BI antiserum. Eight independent experiments yielded qualitatively identical results.

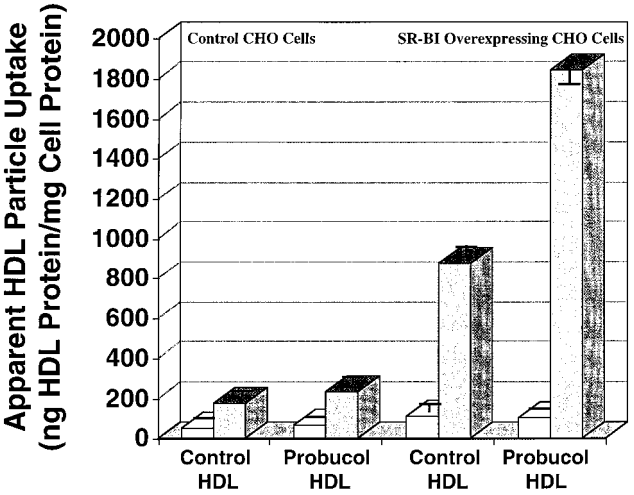

Figure 4. Uptake of doubly radiolabeled C-HDL and P-HDL by CHO cells with low and high SR-BI expression. CHO cells with low (left) or high (right) SR-BI expression were cultured in parallel as described in Methods. Thereafter both lines of cells were incubated (4 hours, 37°C) in medium that contained doubly radiolabeled HDL (10 μg HDL protein per mL) isolated from control or probucol-treated mice (24 hours of probucol feeding; see Methods). After this incubation, cells were harvested, cellular tracer content was determined, and apparent HDL particle uptake was calculated as described in Methods. ^{125}I (open bars) represents apparent HDL particle uptake according to ^{125}I-NMTC; ^{3}H minus ^{125}I (hatched bars) represents the difference in apparent HDL particle uptake between both tracers, ie, apparent selective CE uptake. Values are mean±SEM of n=3 independent incubations. Five independent experiments yielded qualitatively identical results.

labeled C-HDL, in both lines of CHO cells, apparent HDL particle uptake according to [^{3}H]CEt was in excess of that due to ^{125}I-NMTC. Thus, both types of CHO cells selectively took up HDL-associated CEs ([^{3}H]CEt minus ^{125}I-NMTC). As expected, in CHO cells with high SR-BI expression, the rate of apparent selective CE uptake from HDL was increased ≈5-fold compared with control cells with low SR-BI expression (Figure 4).

CHO cells with low SR-BI expression were incubated in medium containing labeled P-HDL (Figure 4). In this case, apparent HDL particle uptake (^{125}I-NMTC) and apparent HDL selective CE uptake ([^{3}H]CEt minus ^{125}I-NMTC) were only marginally different between P-HDL and C-HDL. However, for cells with high SR-BI expression, there was an ≈9-fold increase in [^{3}H]CEt uptake from P-HDL. Compared with C-HDL, this represented a 2.1-fold increment in apparent HDL selective CE uptake. In contrast, P-HDL and C-HDL yielded a similar rate for particle uptake (^{125}I-NMTC) in cells with high SR-BI expression (Figure 4). This experiment indicates that P-HDL demonstrates enhanced selective CE uptake as a result of its interaction with SR-BI.

To further evaluate the possibility that SR-BI and P-HDL synergistically increase the rate of HDL selective CE uptake by cells, selective uptake from C-HDL and P-HDL was evaluated in murine Y1-BS1 adrenocortical tumor cells.[5,11,35] These cells have a relatively high basal expression of SR-BI and show induction of SR-BI protein and mRNA after treatment with ACTH[11,34]; parallel effects were also observed for selective CE uptake.[5] In these cells, P-HDL displayed somewhat higher (1.3-fold) selective uptake than did C-HDL under basal conditions (Figure 5). However, after ACTH treatment, the increment in selective uptake was more pronounced for P-HDL than for C-HDL. Again, there was no difference in P-HDL and C-HDL particle uptake, as assessed by the protein label (^{125}I-NMTC). In parallel experiments, ACTH treatment resulted in a 2-fold induction of SR-BI protein levels as determined by Western blots (data not shown) and as reported previously.[10,11] Also, this experiment suggests that P-HDL interacts with SR-BI to enhance selective CE uptake from HDL.

In the experiments described above, probucol was introduced biologically into HDL. However, this drug can be incorporated in lipoproteins in vitro.[20,28,29] This was done by exchange from a liposomal donor particle with CETP (see Methods). In a typical preparation, the final doubly labeled P-HDL contained 76.2 μmol probucol per mg HDL protein, whereas no probucol could be detected in C-HDL. Control CHO cells or CHO cells overexpressing SR-BI were incubated (37°C, 4 hours) with C-HDL (10 μg protein per mL) or P-HDL (10 μg HDL protein per mL) under experimental conditions (data not shown) similar to those in Figure 4.[29] However, in this case, there was no enhancement of selective CE uptake for P-HDL compared with C-HDL in either cell type. An identical result was obtained when probucol was incorporated into HDL by ethanol injection (data not shown).[20] In this case, in a typical preparation, the final doubly labeled P-HDL preparation contained 240 μmol probucol per mg HDL protein, whereas no probucol could be detected in C-HDL. These results indicate that in vivo, probucol modifies HDL to enhance its interaction with

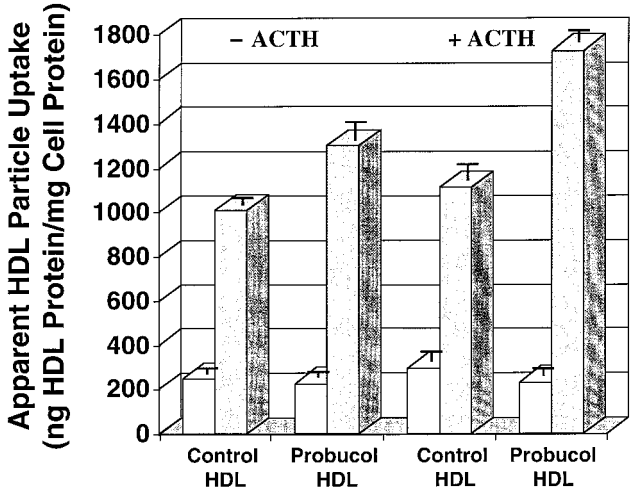

Figure 5. Uptake of doubly radiolabeled C-HDL and P-HDL by murine Y1-BS1 adrenocortical tumor cells and the effect of ACTH. Murine Y1-BS1 adrenocortical tumor cells were preincubated (37°C) for 16 hours in Ham's F-10 medium that was supplemented with 5 mg/mL BSA and antibiotics; ACTH (100 nmol/L) was absent (left) or present (right) as indicated. Thereafter followed an incubation (37°C, 4 hours) in medium that contained BSA, ^{125}I-NMTC/[^{3}H]CEt C-HDL (10 μg protein per mL), or ^{125}I-NMTC/[^{3}H]CEt P-HDL (10 μg protein per mL), and again ACTH (100 nmol/L) was absent or present. After this incubation the cells were harvested, cellular tracer content was determined, and apparent HDL particle uptake was calculated as described in Methods. ^{125}I (open bars) represents apparent HDL particle uptake according to ^{125}I-NMTC; ^{3}H minus ^{125}I (hatched bars) represents the difference in apparent HDL particle uptake between the 2 tracers, ie, apparent selective CE uptake. Values are mean±SEM of n=4 independent incubations.

SR-BI, but not in vitro. To better understand this observation, P-HDL was analyzed and compared with C-HDL.

The chemical composition of doubly radiolabeled C-HDL or P-HDL (in vivo feeding, 24 hours) was analyzed. Compared with C-HDL, triglycerides were increased by 43% in P-HDL (data not shown). However, protein, esterified and unesterified cholesterol, and phospholipid differed only marginally between both HDL preparations. The possibility was also considered that probucol modifies the apolipoprotein composition of HDL. Therefore, C-HDL and P-HDL were analyzed by SDS-PAGE under reducing conditions. Probucol had no effect on HDL apolipoprotein composition compared with C-HDL (data not shown). Moreover, native PAGE showed no change in HDL size in mice treated for 24 hours with probucol compared with C-HDL (data not shown). In contrast to these findings, HDL from mice fed probucol for 2 weeks showed a decrease in overall particle size as assessed by native PAGE (data not shown), consistent with previous reports.[39]

Discussion

The recent discovery that SR-BI mediates selective uptake of HDL CEs by murine tissues suggested that this molecule might be responsible for the increased selective uptake previously described in probucol-treated animals[18,19] and confirmed herein. Surprisingly, there was no effect of this drug on SR-BI expression level. However, P-HDL displayed increased selective CE uptake when incubated with cells in an SR-BI–dependent fashion. This effect could not be reproduced by direct addition of probucol to HDL in vitro, even though these HDL preparations contained substantially more probucol than did P-HDL. Thus, it appears that an in vivo modification of HDL substantially improves the SR-BI–mediated selective uptake process. Such an enhancement of the interaction between plasma lipoproteins and SR-BI may in part be responsible for the antiatherogenic properties of probucol observed in many animal models.[22,40,41]

Recent evidence obtained in cell culture and in genetically manipulated mice indicates that SR-BI is able to mediate the selective uptake of HDL CEs and is likely to be the major molecule responsible for this process in the liver and adrenal gland.[6,10–12,14] Mice with attenuated expression of SR-BI show a proportional reduction of HDL selective uptake in the liver,[14] and an SR-BI antibody largely blocks selective uptake in cultured adrenal cells.[42] These results suggest that the effects of probucol on selective uptake are likely to involve SR-BI. This protein is upregulated in the adrenal gland and other steroidogenic tissues in response to hormonal stimulation and alterations in cellular cholesterol stores.[9–11] Thus, we had anticipated that SR-BI might be upregulated by probucol treatment. Unexpectedly, there was no evidence for regulation of SR-BI in the liver or adrenal gland, even though selective CE uptake by these organs was substantially increased. These observations suggested that mechanisms independent of the regulation of SR-BI expression must contribute to the probucol-induced increase in HDL selective CE uptake.

A highly plausible explanation was provided by cell culture experiments that showed an enhancement of selective uptake for P-HDL compared with C-HDL. This enhancement was observed only in cells with relatively high levels of SR-BI expression, ie, SR-BI–transfected CHO cells and Y1-BS1 adrenocortical tumor cells.[11,34] In the latter cells, the enhancement of selective uptake from P-HDL was increased by ACTH treatment, in parallel with increased SR-BI expression levels.[11,34] By contrast, in cells with very low levels of SR-BI expression (control CHO cells), P-HDL and C-HDL showed similar low levels of selective uptake. These findings suggest a specific enhancement of SR-BI–mediated selective uptake by P-HDL. The mechanism of such an effect is uncertain, but it is interesting to note that P-HDL and C-HDL demonstrated similar uptake of the nondegradable protein label ^{125}I-NMTC in SR-BI–transfected CHO cells and in ACTH-treated Y1-BS1 cells, suggesting that probucol does not increase binding or uptake of HDL protein.

Interestingly, only P-HDL showed enhanced interaction with SR-BI–expressing cells; this effect was not seen when probucol was added to HDL in vitro, even though substantial amounts of probucol could be detected in the final, labeled HDL preparations.[20,29] This suggested either that HDL was modified in vivo as an indirect result of probucol administration or that P-HDL carries an active metabolite formed in vivo. Although prolonged probucol administration results in changes in HDL size and composition,[18,39] murine P-HDL isolated after only a 24-hour treatment was identical in size and apolipoprotein composition to C-HDL and showed only a minor enrichment in triglycerides. Nonetheless, P-HDL displayed an enhancement of selective uptake in SR-BI–expressing cells. Although triglycerides have a fluidizing effect on lipoprotein core lipids,[43] it seems unlikely that these minor compositional changes would be responsible for the

enhanced interaction with SR-BI. It is known that the bulk of probucol transported in the blood is found in lipoproteins, along with several probucol metabolites.[44] Some hydrophobic probucol metabolites have been shown to act as potent fluidizing agents when mixed with CEs.[16] Thus, our results could indicate that a probucol metabolite carried in P-HDL may be responsible for enhanced interaction with cellular SR-BI, leading to increased selective uptake of HDL CEs.

It appears likely that the markedly increased (5-fold in the liver and 10-fold in the adrenal gland) selective uptake of HDL CEs accounts for a major part of the HDL cholesterol–lowering effect of probucol. The cell culture results suggest that these in vivo effects may in part reflect the stimulation of selective uptake by SR-BI in the liver. However, the magnitude of the effect in vivo was larger than that seen in cell culture (2-fold). This could be, because the turnover studies were conducted after 2 weeks of probucol treatment, whereas P-HDL for cell culture experiments had to be used 1 day after initiating probucol treatment. Alternatively, there could be additional mechanisms operating in vivo.

Although the current studies deal with HDL, probucol also lowers LDL cholesterol and stimulates catabolism of LDL CEs by the liver.[45,46] SR-BI is known to bind LDL as well as HDL.[47] Recent studies in SR-BI–transgenic mice show a marked reduction in LDL cholesterol and apo B levels compared with those in controls.[48] A mechanism similar to that observed here involving interaction of LDL and the hepatic SR-BI could be responsible for the LDL cholesterol–lowering effects of probucol, which can occur in the absence of LDL receptors.[45,46]

In many animal models, probucol shows marked antiatherogenic effects.[22,40,41] Probucol also causes regression of xanthomas and xanthelasmas in familial hypercholesterolemic and non–familial hypercholesterolemic subjects.[49] However, the effects of probucol on atherosclerosis are complex. In the human Probucol Quantitative Regression Swedish Trial (PQRST), probucol did not influence femoral artery atherosclerosis as assessed by angiography.[50] Paradoxically, probucol actually increased the extent and developmental stage of atherosclerosis in apo E–knockout mice by an apo A-I–independent mechanism.[51] In primates, rabbits, and mice, probucol appears to have distinctive effects on the cellularity of lesions by decreasing the population of macrophage foam cells while increasing the prominence of smooth muscle cells and extracellular matrix deposition.[52,53] In numerous studies of rabbits, probucol reduced the extent of atherosclerosis and appeared to be superior to other antioxidants such as vitamin E or probucol analogues.[22,40,54,55] This result suggested either that the effects of antioxidants on atherosclerosis need to surpass a threshold level and that probucol is more effective than other antioxidants or that there are distinct biological effects of probucol.[44,55] The present study describes a novel biological effect of probucol and thus supports the latter explanation. Enhanced reverse cholesterol transport by the SR-BI–dependent mechanism or as a result of increased levels of plasma CETP[56] may explain the more potent effects of probucol on atherosclerosis. SR-BI increases the transport of HDL cholesterol across the liver into bile.[12] Moreover, SR-BI appears to be expressed in foam cells in atheromatous lesions,[34] and SR-BI facilitates the HDL-mediated efflux of cholesterol from cells.[34] HDL from probucol-treated humans shows enhanced ability to promote removal of cholesterol from macrophage foam cells.[39] Thus, the enhanced interaction of P-HDL with SR-BI may favor cholesterol removal from macrophage foam cells, as well as increased cholesterol transport across the liver. The enhanced interaction between P-HDL and SR-BI may result in improved reverse cholesterol transport and may underlie the antiatherogenic properties of probucol.

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