Probucol Enhances the Expression of Human Hepatic Scavenger Receptor Class B Type I, Possibly Through a Species-Specific Mechanism

Ken-ichi Hirano, Chiaki Ikegami, Ken-ichi Tsujii, Zhongyan Zhang, Fumihiko Matsuura, Yumiko Nakagawa-Toyama, Masahiro Koseki, Daisaku Masuda, Takao Maruyama, Iichiro Shimomura, Yukihiko Ueda, Shizuya Yamashita

Objective—Scavenger receptor class B type I (SR-BI) is a major receptor for high-density lipoproteins (HDL) in the liver, which is the terminus of reverse cholesterol transport. Overexpression of SR-BI attenuated experimental atherosclerosis in murine models, concomitant with a reduction in plasma HDL-cholesterol levels. Probucol is known to be a potent hypolipidemic drug to regress xanthoma formation and carotid atherosclerosis in conjunction with a marked reduction in HDL-cholesterol levels. The aim of the present study was to know the effect of probucol on the expression of SR-BI and the underlying mechanism.

Methods and Results—We found that probucol increased the expression of SR-BI proteins in vitro in human liver cells and in vivo rabbit model, but not in wild-type C57Bl6 mice. The decay curve of SR-BI protein was markedly retarded in probucol-treated HepG2 cells in the presence of cycloheximide, indicating that probucol may stabilize human SR-BI protein. To determine the underlying mechanism for the observed species-specific effect, we conducted the following host-swap experiments, in which SR-BI was transfected or expressed in heterologous cells or hosts. Probucol did not increase human SR-BI protein in the liver of transgenic mice carrying the entire human SR-BI genome. Although probucol could stabilize even murine SR-BI, when transfected into a human cell line, HepG2, human SR-BI was not stabilized in a mouse hepatoma cell line, Hepa 1-6, treated with probucol.


Key Words: atherosclerosis ● high-density lipoprotein ● probucol ● reverse cholesterol transport ● scavenger receptor class B type I

Reverse cholesterol transport (RCT) is one of the major protective systems against atherosclerosis, in which high-density lipoprotein (HDL) particles play a crucial role as shuttles carrying cholesterol derived from peripheral tissues.1 We have continued to elucidate the molecular mechanism for RCT by analyzing the pathophysiology of patients with abnormal HDL metabolism. It is believed that, in human liver, the terminus of RCT, there are at least 2 distinct pathways for the uptake of HDL-cholesterol. One is the low-density lipoprotein (LDL) receptor pathway where HDL-cholesterol is transferred to LDL by cholesteryl ester transfer protein (CETP), and cholesterol in LDL is taken up by this receptor. The other is an HDL receptor(s)–mediated pathway.2,3

One of the important candidates for hepatic HDL receptor in human is scavenger receptor class B type I (SR-BI).4,5 SR-BI, cloned by Krieger et al., is abundantly expressed in murine liver and mediates the selective uptake of HDL-lipids. Several lines of evidence indicate that this molecule is a physiologically relevant HDL receptor in mice because its hepatic overexpression of this molecule attenuated experimental atherosclerosis in mice, concomitant with a reduction in HDL-C levels and the appearance of smaller sized HDL particles.6-7 Conversely, SR-BI null mice had accelerated atherosclerosis in the apoE-negative background with the appearance of larger sized apo AI-containing particles.8,9

Probucol is a potent hypolipidemic drug, which can reduce Achilles tendon xanthoma in patients with homozygous familial hypercholesterolemia as well as attenuate atherosclerosis in Watanabe heritable hyperlipidemic (WHHL) rabbits.10,11 A unique aspect of this compound is its capability to induce hypoalphalipoproteinemia. We previously reported

Original received February 12, 2005; final version accepted August 18, 2005.
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Arterioscler Thromb Vasc Biol. is available at http://www.atvbaha.org DOI: 10.1161/01.ATV.0000185834.98941.3d

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that probucol treatment induced not only a reduction in HDL-cholesterol levels but also the appearance of smaller-sized HDL particles,12 which very actively promoted cholesterol efflux from the cells.13 We also reported a positive correlation between the reduction in plasma HDL-cholesterol and the reduction rate of Achilles tendon xanthoma.12 However, the mechanism for the reduction of HDL-cholesterol by probucol is not known yet.

The aim of the present study was to determine the effect of probucol on the expression of SR-BI. Because the effect of probucol on atherosclerosis seems to be different among species,14–16 the experimental materials were obtained from different species including human, rabbits, and mice. We found that probucol increased hepatic SR-BI protein in a species-specific fashion. To gain further understanding of the underlying mechanism(s), we conducted the host-swap experiments, in which SR-BI was expressed in heterologous cells or hosts. Results of these experiments indicated that probucol dissolved in ethanol was added at the indicated concentrations to the media. Forty-eight hours after incubation, proteins and mRNA were extracted and subjected to Western blotting and real-time PCR analyses. The effect of probucol on the expression of SR-BI was examined in the cryopreserved hepatocytes obtained from two different donors. Similar results were obtained from both lots of hepatocytes. Upper panels show representative films of Western blot data from a 33-year-old donor (see Materials and Methods). For each experiment, the liver cells with 0 μmol/L probucol values were designated as 100%. Relative abundance of SR-BI mRNA (hatched bars) and protein (solid bars) were graphed from 3 independent experiments (mean±SD), as shown in lower panels. *Statistically significant (at least P<0.05) compared with the value for cells treated without probucol. C, Uptake of HDL-lipids in probucol-treated HepG2 cells. Upper panel, After 48-hour incubation with probucol, Dil-HDL (50 μg/mL) was added. Four hours later, cells were washed by PBS and subjected to the fluorescence microscope (PROVIS AX80TR, OLYMPUS). Lower panel, Association of [3H]CE-HDL3 (solid bars) and 125I-HDL3 (hatched bars) in probucol-treated HepG2 cells were determined as described under Materials and Methods. For each experiment, the HepG2 cells with 0 μmol/L probucol were designated as 100%. Relative radioactivity was plotted from 3 independent experiments (mean±SD). *Statistically significant (at least P<0.05) compared with the values for cells treated without probucol.

Figure 1. In vitro effect of probucol on the expression of SR-BI (A and B) and the uptake of HDL-lipids (C) in human liver cells. A and B, Protein and mRNA levels of SR-BI in HepG2 cells (A) and human cryopreserved hepatocytes (B). Probucol dissolved in ethanol was added at the indicated concentrations to the media. Forty-eight hours after incubation, proteins and mRNA were extracted and subjected to Western blotting and real-time PCR analyses. The effect of probucol on the expression of SR-BI was examined in the cryopreserved hepatocytes obtained from two different donors. Similar results were obtained from both lots of hepatocytes. Upper panels show representative films of Western blot data from a 33-year-old donor (see Materials and Methods). For each experiment, the liver cells with 0 μmol/L probucol values were designated as 100%. Relative abundance of SR-BI mRNA (hatched bars) and protein (solid bars) were graphed from 3 independent experiments (mean±SD), as shown in lower panels. *Statistically significant (at least P<0.05) compared with the value for cells treated without probucol. C, Uptake of HDL-lipids in probucol-treated HepG2 cells. Upper panel, After 48-hour incubation with probucol, Dil-HDL (50 μg/mL) was added. Four hours later, cells were washed by PBS and subjected to the fluorescence microscope (PROVIS AX80TR, OLYMPUS). Lower panel, Association of [3H]CE-HDL3 (solid bars) and 125I-HDL3 (hatched bars) in probucol-treated HepG2 cells were determined as described under Materials and Methods. For each experiment, the HepG2 cells with 0 μmol/L probucol were designated as 100%. Relative radioactivity was plotted from 3 independent experiments (mean±SD). *Statistically significant (at least P<0.05) compared with the values for cells treated without probucol.

**Materials and Methods**

HepG2 and Hepa 1-6 cells were obtained from ATCC and maintained by the standard protocol. Two different lots of cryopreserved hepatocytes were obtained from the hepatocyte bank maintained at In Vitro Technologies (Baltimore, MD). According to information from the company, one donor was a 33-year-old Caucasian male who had died of intracranial hemorrhage. The other was a 23-year-old Hispanic female who died of cerebrovascular accident. The hepatocytes were plated on collagen-coated plates and maintained in a complete medium from In Vitro Technologies. Aprotinin, ALLN, leupeptin, pepstatin, phosphoramidon, lactacystin, and MG125 were purchased from Sigma. The polyclonal antibody against the carboxyterminus of SR-BI was generated, as described previously.17 The antibody against C-terminal linking and modulating proteins (CLAMP) was kindly provided by Dr Hiroyuki Arai (Tokyo University, Tokyo, Japan). Monoclonal antibodies against green fluorescence protein (GFP) (JL8) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (6C5) were purchased from BD Biosciences and Research Diagnostic Inc, respectively.

In vitro treatment with probucol, isolation of lipoproteins and modification of HDL, Western blot analyses, synthesis of cDNA and real time polymerase chain reaction (PCR), lipoprotein cell association assay, generation of transgenic mice expressing human SR-BI, animal protocol, construction of GFP-tagged SR-BI plasmid, transfection of plasmid DNA, cycloheximide experiments, primers used, and statistical analyses are described under Materials and Methods section in the online data supplement available at http://atvb.ahajournals.org.

**Results**

**Probucol Increased SR-BI Protein in Human Hepatoma Cell Line, HepG2, and Human Hepatocytes**

We examined the in vitro effect of probucol on the expression of SR-BI proteins in the human hepatoma cell line, HepG2 (Figure 1A), and cryopreserved hepatocytes (Figure 1B). The addition of probucol to the media increased SR-BI protein in a dose-dependent manner (solid Bars in Figure 1A and 1B). The SR-BI mRNA levels were not apparently changed in either HepG2 cells or cryopreserved hepatocytes (hatched bars in Figure 1A and 1B). Figure 1C shows the effect of probucol on the uptake of Dil-labeled HDL (upper panel) and selective uptake of HDL-lipids (lower panel) in the HepG2 cells. Fluorescent microscopy showed an increased amount of...
uptake of Dil-HDL in cells treated with probucol, whereas the uptake of $^{3}H$ cholesteryl olate was significantly increased by the addition of probucol to the media. In contrast, the amount of cell-associated $^{125}$I-HDL was not changed. These findings suggest that probucol increased the selective uptake of HDL-lipids, which is known to be mediated by SR-B1, and suggest that probucol increased the selective uptake of $^{3}H$ cholesteryl oleate was significantly increased by probucol treatment (15.8 versus 2.3 mg/dL, $P<0.01$), as shown in Figure 2A. The expression of SR-B1 protein was abundant in the liver and adrenal glands and very similar to the patterns of tissue distribution reported Ritsch et al.$^{19}$ (data not shown). Rabbits were kept for 1 month on normal chow diet either with or without 1% probucol. In the animals treated with probucol, plasma HDL-cholesterol levels were significantly reduced during the treatment (15.8 versus 2.3 mg/dL, $P<0.05$ compared with the value of control group). B, Expression of $r$SR-B1 mRNA was determined with Rnase protection assay. C, Expression of mouse SR-B1 (mSR-B1) protein in each group was determined with Western blotting. Pooled cellular protein extracts were made from each group.

Figure 2. In vivo effect of probucol on the expression of SR-B1 in Japanese white rabbits (A and B) and wild-type C57Bl6J mice (C). A, Expression of rabbit SR-B1 ($r$SR-B1) protein in each group determined by Western blotting. Representative images of Western blotting are shown in the upper panel. Relative expression of $r$SR-B1 was plotted (lower panel; n=6, each group). Values are expressed as relative abundance of $r$SR-B1 to that of GAPDH. Bars are expressed as fold of controls (mean±SD of 3 independent experiments). *Statistically significant ($P<0.05$) compared with the value of control group. B, Expression of $r$SR-B1 mRNA was determined with Rnase protection assay. C, Expression of mouse SR-B1 (mSR-B1) protein in each group was determined with Western blotting. Pooled cellular protein extracts were made from each group.

In Vivo Effect of Probucol on the Expression of SR-B1 in Rabbit and Mouse Models

We examined the in vivo effect of probucol on the expression of SR-B1 in Japanese white rabbits and wild type C57Bl6J mice. Before the analyses of rabbit SR-B1 ($r$SR-B1), its tissue distribution was investigated with a Rnase protection assay. mRNA expression of $r$SR-B1 was abundant in the liver and adrenal glands and very similar to the patterns of tissue distribution reported Ritsch et al.$^{19}$ (data not shown). Rabbits were kept for 1 month on normal chow diet either with or without 1% probucol. In the animals treated with probucol, plasma HDL-cholesterol levels were significantly reduced during the treatment (15.8 versus 2.2 mg/dL, $P<0.05$, paired $t$ test). The expression of $r$SR-B1 protein was markedly elevated ($P<0.001$), as shown in Figure 2A. The mRNA expression of $r$SR-B1 tended to increase, but not to a statistically significant extent. (Figure 2B). Next, we tested the in vivo effect of probucol on the expression of SR-B1 in mice. After a 2-week treatment with 5% probucol, the expression levels of murine SR-B1 protein had not changed (Figure 2C), which was consistent with the findings reported by Rinninger et al.$^{20}$

These results indicate that probucol may increase hepatic SR-B1 protein without apparent changes in its mRNA expression levels in humans or rabbits. On the other hand, this effect was not observed in probucol-treated wild-type mice. These effects may therefore be species-specific.

Probucol May Stabilize SR-B1 Protein in HepG2 Cells

We next used HepG2 cells as a model to investigate the underlying mechanism for upregulation of SR-B1 by probucol. Because SR-B1 mRNA levels were not apparently altered in the HepG2 cells, human cryopreserved hepatocytes, or rabbit liver, we focused on the posttranscriptional regulation of SR-B1 by probucol. To determine whether SR-B1 protein is regulated by its proteolytic and proteasomal degradation, we tested the effect of various kinds of protease and proteasome inhibitors on the basal levels of SR-B1 protein in HepG2 cells. As shown in Figure 3A (left panel), some of the inhibitors, including aprotinin, leupeptin, and pepstatin, seemed to increase the protein levels of SR-B1, but N-acetyl-leucyl-leucyl-norleucinal (ALLN) did not increase the SR-B1 protein in the experiment. Proteasome inhibitors such as lactacytin and MG132 reduced SR-B1 protein levels. We confirmed that apoB protein was increased by ALLN (data not shown). On the basis of these findings, we tested the hypothesis that probucol may stabilize the SR-B1 protein by analyzing SR-B1 with immunoblotting in the presence of cycloheximide. A decrease in SR-B1 was apparent at 4 hours, and SR-B1 continued to decay up to 8 hours (Figure 3B, left panel). The treatment with probucol clearly slowed down the rate of SR-B1 degradation (Figure 3B, right panel). It is noted that GAPDH proteins were not decreased up to 8 hours in these experiments.

Figures 1 through 3 demonstrate that probucol increased hepatic SR-B1 protein, which may be species-specific. In the HepG2 cells treated with probucol, the degradation of SR-B1 was apparently delayed, which may account for the increased levels of SR-B1 proteins. This finding led to the question whether or not probucol directly affects the SR-B1 genome or protein itself, or some related genes and proteins, or both. To address this issue, we have conducted the following host-swap experiments, in which SR-B1 was transfected or expressed in heterologous cells or hosts.

Host-Swap Experiment 1

In Vivo Effect of Probucol on Human SR-B1 Expressed in Mice

The first experiment was designed to test the in vivo effect of probucol on human SR-B1 in mice. For that purpose, we generated mouse lines expressing the entire human SR-B1 genome in the murine SR-B1$^{-/-}$ background (Human SR-B1 BAC Tg/mSR-B1$^{-/-}$ mice). In this model, the expression of human SR-B1 was regulated by its own promoter. The mice were treated for 2 weeks with diets containing 5% probucol. As shown in Figure IA (available online at http://atvb.ahajournals.org), human SR-B1 mRNA was clearly detected with the hSR-B1 riboprobe by RNase protection assay and showed no difference of SR-B1 mRNA between the probucol and control groups. Figure IB and IC shows the expression of
human SR-BI protein in whole cell lysates as well as the cytoplasmic and membrane fractions, indicating no apparent difference of SR-BI protein levels between the probucol and control groups. These results indicated that probucol did not increase human SR-BI protein levels in mice, suggesting that the increase in SR-BI protein observed in the probucol-treated HepG2 cells and human cryopreserved hepatocytes may not have been caused by the direct or sole effect of probucol on the human SR-BI genome or the protein itself.

Host-Swap Experiment 2

Cycloheximide Experiments for Human and Mouse SR-BI Expressed in Heterologous Cells

We next examined the in vitro effect of probucol on SR-BI expressed in the heterologous cells. For this experiment, we generated the GFP-tagged constructs for human and murine SR-BI. As shown in Figure IIA (available online at http://atvb.ahajournals.org), we tested the expression of GFP-human SR-BI and GFP-murine SR-BI constructs which were transfected into HepG2 cells. Western blot analyses clearly indicated that GFP-tagged proteins were successfully expressed in HepG2 cells. We confirmed the expression of these constructs in the murine hepatoma cell lines, Hepa 1-6 (data not shown). As shown in Figure IIB, DiI-labeled HDL was significantly taken up by cells expressing GFP-tagged human and murine SR-BI. Figure IIC illustrates the uptake of radiolabeled HDL in cells transfected with GFP-hSR-BI and mSR-BI, showing that the cells expressing GFP-SR-BI chimeric proteins achieved significant selective uptake of HDL-lipids.

We finally tested the degradation of SR-BI proteins expressed in heterologous cells. The human or mouse GFP-SR-BI constructs were transfected into either of HepG2 or Hepa 1-6 cells, the latter is a murine hepatoma cell line, and the cycloheximide experiments were performed in cells treated with or without probucol. GFP-human SR-BI expression was not stabilized in the probucol-treated Hepa 1-6 cells (Figure 4A). On the other hand, when murine SR-BI was expressed in the human hepatoma cell line HepG2, probucol clearly slowed down the decay of endogenous human SR-BI and GFP-murine SR-BI proteins (Figure 4B). We also confirmed that GFP-mouse SR-BI was not stabilized in Hepa 1-6 cells, whereas GFP-human SR-BI was stabilized in Hep G2 cells (data not shown). The results of these 2 host-swap experiments led us to conclude that probucol may stabilize the hepatic SR-BI

Figure 3. Effect of protease and proteasome inhibitors on SR-BI expression (A) and that of probucol on SR-BI degradation in the presence of cycloheximide (B). A, Western blot analyses of SR-BI in HepG2 cells treated with various proteases and proteasome inhibitors. Cells treated with vehicle alone (H2O, ethanol, and DMSO) are also shown. HepG2 cells were incubated in media containing protease inhibitors for 12 hours. Protein levels of SR-BI and GAPDH were analyzed by means of immunoblotting. Aprotinin, leupeptin, and phosphoramidon were dissolved in water, and pepstatin and ALLN in ethanol. Lactacystin and MG125 were dissolved in dimethyl sulfoxide (DMSO). These proteases and proteasome inhibitors were added to media at the following final concentrations: ALLN (10, 50 μmol/L), aprotinin (10 μmol/L), leupeptin (500 μg/mL), pepstatin (20 μmol/L), phosphoramidon (250 μg/mL), lactacystin (10 μmol/L), and MG125 (3 μmol/L). B, HepG2 cells were pretreated for 48 hours with control and probucol-containing media. The cells were incubated in the presence of cycloheximide (CHX; 20 ng/mL) for the indicated times and whole cell lysates were analyzed with immunoblotting. Relative abundance of SR-BI was graphed from 3 independent experiments (mean ± SD). Bars show multiples of SD of 3 independent experiments. *Statistically significant (at least P<0.05) compared with the values of cells with 0 minutes treatment of CHX.

Figure 4. Host swap experiment 2: the expression and degradation of GFP-tagged human and mouse SR-BI proteins in heterologous cells. The cells were transfected with the indicated plasmids using the Nucleofector device and plated onto 12-well dishes. Six hours later, probucol or control medium was added. Forty eight hours after nucleofection, CHX was added and the cells were incubated for the indicated times and whole cell lysates were subjected to Western blotting. These experiments were repeated 3 times with similar results, and the representative data are shown in the figures. A, GFP-human SR-BI was transfected into Hepa 1-6 cells with the Nucleofector device. Neither GFP-human SR-BI nor endogenous mouse SR-BI were stabilized when the cells were treated with probucol. The expression levels of endogenous mouse SR-BI were very low, compared with those of GFP-human SR-BI. The data for endogenous mouse SR-BI were obtained with a longer exposure. B, GFP-mouse SR-BI was transfected into the human hepatoma cell line HepG2. Probucol stabilized both GFP-mouse SR-BI and human endogenous SR-BI.
protein, possibly through host-specific or species-specific mechanism(s). The probucol-induced upregulation did not seem to be caused by the direct effect of probucol on the human SR-BI genome or protein itself, so that it is more likely that probucol may affect some factors existing in human liver cells, which regulate the protein levels of SR-BI. Because it was recently reported that CLAMP/PDZK1 is one of the crucial regulators for the expression of SR-BI, we examined the effect of probucol on the expression of CLAMP/PDZK1 in HepG2 cells, showing no significant changes in either the mRNA or protein levels of this molecule (data not shown).

**Discussion**

The present study for the first time demonstrates that probucol increases the expression of SR-BI proteins in human liver cells and rabbit liver. This effect seems to be species-specific, because probucol did not increase SR-BI protein in wild-type mice. Our data also indicate that the probucol-induced increase in the SR-BI protein may be caused by the slow decay of the protein. Growing evidence has established that overexpression of SR-BI attenuated atherosclerosis in many murine models. It is obvious that SR-BI is an essential molecule that determines plasma HDL-cholesterol levels and atherogenicity in these species. Therefore, this molecule is an important target for the enhancement of reverse cholesterol transport in humans. The present data indicates that the stabilization of SR-BI may be a potentially important strategy to be considered.

The initial part of this study clearly indicated that the upregulation of SR-BI by probucol may occur at posttranscriptional levels. Many literatures reported that the SR-BI protein seems to be tightly regulated at posttranscriptional levels, with an underlying mechanism that seems very complicated. SR-BI protein levels were altered without changes of its mRNA in apoE-knockout mice, vitamin E-fed rodents, and nephrotic rats. CLAMP/PDZK1 was reported to bind with SR-BI and regulate its cell surface expression. Gene targeting of CLAMP/PDZK1 diminished hepatic expression of SR-BI in mice. The small PDZK1-associated protein (SPAP/DD96/MAP17) which binds with CLAMP/PDZK1 was reported to regulate SR-BI protein expression in mice. As mentioned in the text, probucol treatment did not alter the mRNA and protein levels of CLAMP/PDZK1 in the HepG2 cells. Because Kodama and Noguchi et al reported that probucol regulated some proteasome gene and proteins in human endothelial cells, we tested the effect of some proteasome inhibitors on the expression of SR-BI in HepG2 cells. Because the proteasome inhibitors used did not increase the SR-BI protein levels, the upregulation of SR-BI by probucol may be independent of proteasomes. Although we could not clarify the precise mechanism, our study raised the following questions. Does it involve the generation of species-specific biologically active probucol derivatives or the existence of species-specific molecular and/or biochemical targets for probucol? The answers to these questions should be of great importance.

Recently, it has been reported that SR-BI is expressed in foam cells in the human atherosclerotic lesions as well as smooth muscle cells in vitro. Yuhanna et al reported that SR-BI expressed in endothelial cells may play some roles in regulating nitric oxides. We and others reported that SR-BI is expressed in the human central nervous system. It would be of interest to know the effect of probucol on the expression of SR-BI in these kinds of cells and tissues.

Administration of probucol to both humans and animals has been shown to lower HDL-cholesterol levels. However, various different mechanisms could be responsible for probucol-mediated reduction of HDL-cholesterol. It was found that the particle size of HDL particles becomes smaller in patients and animals treated with probucol. Such small HDL particles are very active for cholesterol efflux from the cells, which may lead to the regression of foam cells and xanthomas. On the other hand, Yokoyama and others reported that probucol inhibited apoAI-mediated cholesterol efflux at least in vitro without the alteration of ABCA1 protein levels, which may lead to a reduction in the production of nascent HDL particles in vivo. In the present study, we clearly demonstrated that probucol may increase the expression of SR-BI protein in liver cells, possibly in a species-specific fashion. Recently, we demonstrated that SR-BI is expressed in human hepatic parenchymal cells by means of immunohistochemical analyses. We therefore concluded that the overexpression of SR-BI produced by probucol may at least partially explain the low HDL-cholesterol levels observed in patients treated with this drug.

**Acknowledgments**

This work was partially supported by a research grant from Daiichi Pharmaceutical Co (Tokyo, Japan) for K. Hirano and S. Yamashita. This work was supported by grants-in-aid to S. Yamashita (No.07557074, No. 08671157) from the Ministry of Education, Science, Sports, and Culture of Japan. The authors gratefully thank Dr Yuji Matsuzawa for critical discussions at the early stage of this project, Professor Hiroyuki Arai for providing the antibody against CLAMP, and Chiho Hosono for her skillful technical assistance.

**References**


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Arterioscler Thromb Vasc Biol. 2005;25:2422-2427; originally published online September 8, 2005;
doi: 10.1161/01.ATV.0000185834.98941.3d

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2005/09/12/01.ATV.0000185834.98941.3d.DC1

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Online Figure I
Hirano K, et al. Probucol stabilizes SR-BI
Online Figure II

Hirano K, et al. Probucol stabilizes SR-BI

**A**

![GFP-tagged mSR-BI hSR-BI Mock](image1)

- GFP-tagged mSR-BI
- hSR-BI
- Mock
- Endogenous SR-BI

**B**

<table>
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<tr>
<th>Overlap</th>
<th>GFP</th>
<th>Dil-HDL</th>
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<td><img src="image2" alt="Overlap" /></td>
<td><img src="image3" alt="GFP" /></td>
<td><img src="image4" alt="Dil-HDL" /></td>
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**C**

Cell-associated labeled HDL (% of mock transfected)

- Mock
- GFP-hSRBI
- GFP-mSRBI

Online Figure II
Hirano K, et al. Probucol stabilizes SR-BI
Probucol Enhances the Expression of Human Hepatic Scavenger Receptor Class B Type I, Possibly Through a Species-Specific Mechanism

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Abbreviated Title: Probucol enhances SR-BI expression.
Materials and Methods

In vitro treatment with probucol

Probucol, 4, 4’-(isopropylidenedithio) bis (2, 6-di-tert-butylphenol), was provided from Daiichi Pharmaceutical Co. (Tokyo, Japan). For the in vitro experiment, cells were treated with probucol dissolved in ethanol for 48 h. The cellular contents of probucol were measured by the FPLC methods with minor modifications (1). Treatment with 0, 5, 25, and 50 µM of probucol resulted in the incorporation of probucol in < 0.1, 3.0, 12.3, 24.2 µg/mg of cell protein, respectively, in the HepG2 cells.

Isolation of lipoproteins and modification of HDL

Blood was obtained from normolipidemic volunteers after overnight fasting. HDL3 (1.210 < d < 1.125 g/mL) was separated by preparative ultracentrifugation. \[^3\text{H}]\text{CE-HDL3}: \text{human serum HDL was labeled with }[^3\text{H}]\text{cholesteryl-oleate with the method of Thomas and Rudel (2). }[^3\text{H}]\text{cholesteryl-ether (Amersham) was incubated with }d>1.125 \text{ plasma fraction at 37C for 18-24 h and then labeled HDL3 was purified by ultracentrifugation.}[^{125}\text{I-HDL3}: \text{HDL3 was iodinated by the Pierce iodobead method. The specific radioactivity ranged from 100,000 to 250,000 cpm/µg of protein (3).}[^\text{Dil-HDL3}: \text{HDL3 was labeled with Dil (D-282; 1, 1'-dioctadecyl-3, 3, 3', 3'-tetramethylindocarbocyanine perchlorate) (Molecular probes, Eugene, OR) (4).}

Western blot analyses

SR-BI proteins were analyzed by Western blot, as described previously (5). Briefly, cells and tissues were extracted using a buffer containing 1% Triton
X-100, 0.5% Nonidet P-40 and protease inhibitor cocktails (Nacalai Tesque, Kyoto, Japan). Equal amounts of protein were separated on 4-12% SDS-PAGE and transferred onto a PVDF membrane. Chemiluminescence was used to visualize the proteins according to the manufacturer's instructions (Amersham).

**Synthesis of cDNA and real time quantitative polymerase chain reaction**
cDNA was synthesized using Superscript III First-Strand Synthesis for RT-PCR Kit (Invitrogen) and subjected to real time quantitative PCR. PCR amplification and detection were achieved using 2xDyNAmo HS (Finnzymes, Espoo, Finland) and Opticon 2 (MJ Research).

**Lipoprotein cell association assay**
2.5 x 10^5 cells were seeded in each well of 24 well plates. The next day, the cells were incubated at 37°C with ^125^I-HDL (10 µg of protein/mL) or [^3^H]CE-HDL3 (12 µg/mL) for 4 h in the presence or absence of a 40-fold excess of unlabeled HDL. After incubation, the cells were washed with ice-cold Tris buffer [twice with and once without BSA (2 mg/mL)] and radioactivity and protein determinations were made.

**Generation of transgenic mice expressing human SR-BI bacterial artificial chromosome (BAC) in the murine SR-BI +/- background**
A BAC clone containing the entire human SR-BI genome (SR-BI BAC) was injected into mouse blastocytes of C57Bl6. This BAC clone contained the whole SR-BI genome as well as 70 kb-upstream and 25 kb-downstream regions. The established mice lines were mated with SR-BI knockout mice, which were kindly provided from Rubin E (Berkeley, CA). After several generations, the mice lines expressing entire human SR-BI genome in the mSR-BI +/- background
were selected by PCR.

Animal protocol
Ten-week-old Japanese white rabbits were bred in a room kept at 22°C and equipped with laminar-flow filters (Nihon Bioresearch Inc., Gifu, Japan). They were fed a standard rabbit chow until they were given a diet either with or without 1% probucol. After a one-month treatment with probucol, rabbits were sacrificed.

For the mouse studies, 8-week-old wild type C57Bl6/J mice and human SR-BI BAC Tg/mSR-BI knockout mice were treated with a standard chow either with or without 5% probucol. After a two-week-treatment with probucol, the mice were sacrificed. The mice appeared healthy and their body weight was not affected by the diet containing probucol. Probucol content was \(~100\mu g/g\) of liver tissue of mice fed with the probucol diet.

Construction of GFP-tagged human and murine SR-BI plasmid
Both human and murine SR-BI cDNA were cloned from cDNA libraries by PCR using KOD Plus DNA polymerase (TOYOBO, Tokyo, Japan) and sequenced. The fragments were cloned into the pEGFP-C1 vector (BD Bioscience, Palo Alto, CA).

Transfection of plasmid DNA
The constructs were transfected into HepG2 and Hepa 1-6 cells using the Nucleofector device according to the manufacturer’s protocol (Amaxa Inc., Cologne, Germany). The transfection efficiency was \(~60\%\) and the cell survival rate was \(~70\%\) for HepG2 and Hepa 1-6 cells. For Cos-7 cells, Lipofectamine 2000 reagent (Invitrogen) was used.
**Cycloheximide experiments**

Cells were pre-treated for 48 h either with or without probucol. The cells were then incubated in the presence of cycloheximide (CHX) for the indicated times. Whole cell lysates were extracted and subjected to Western blot analyses.

**Primers used in the present study**

P1: 5'-ctgtgggtgagatcatgtgg-3', human SR-BI (GeneBank Accession No. Z22555)

P2: 5'-gccagaagtcaaccttgctc-3', human SR-BI (GeneBank Accession No. Z22555)

P3: 5'-cctggccaaggtcatccatg-3', human GAPDH (GeneBank Accession No. M33197)

P4: 5'-ggaaggccatgccagtgagc-3', human GAPDH (GeneBank Accession No. M33197)

P5: 5'-gcttcttcctgcgaattgag-3', human CLAMP/PDZK1 (GeneBank Accession No. NM_002614)

P6: 5'-ctttcaagtccacccgtgtt-3', human CLAMP/PDZK1 (GeneBank Accession No. NM_002614)

**Other Procedures**

Protein concentration was determined using Protein Assay CBB Solution (Nacalai Tesque, Kyoto, Japan). Paired Student’s t test or ANOVA was used to obtain statistical comparison of the data. Differences were considered significant at p < 0.05.
References


Figure Legends

Online Figure I
Host swap experiment 1

*In vivo* effect of probucol on the expression of human SR-BI mRNA (A) and protein (B, C) in the liver of human SR-BI BAC Tg/mSR-BI -/- mice

The mice carrying the entire human SR-BI genome were generated as described in the Materials and Methods. They were fed a normal lab chow and probucol-containing diet. After a two-week treatment, the mice were sacrificed and mRNA and protein were extracted from liver.  

**A.** Expression levels of hSR-BI mRNA analyzed with Rnase protection assay.  

**B, C.** Expression levels of hSR-BI in whole cell lysates (B) and cytoplasmic and membranous proteins (C) were determined by means of Western blot analyses.  

Cyt and M denote cytoplasmic and membranous proteins, respectively.

Online Figure II
Expression of GFP-tagged human and mouse SR-BI in HepG2 cells and the uptake of Dil-HDL in COS-7 cells transfected with these constructs

**A.** HepG2 cells were transfected with GFP-tagged human and mouse SR-BI using the Nucleofector device (Amaxa). 24 h after nucleofection, whole cell lysates were obtained and subjected to Western blotting. The PVDF membrane was incubated with either anti-SR-BI antibody (left panel) or anti-GFP antibody (right panel)  

**B.** Cos-7 cells were transfected with GFP-human SR-BI, GFP-mouse SR-BI, or GFP construct by Lipofectaime 2000 reagent (Invitrogen). Twenty-four hours after transfection, Dil-HDL (50 µg/mL) was added. After 4 h incubation with Dil-HDL, the cells were fixed and subjected to laser confocal microscopy.  

**C.** Association of [³H]CE-HDL3 (solid bars) and ¹²⁵I-HDL3 (hatched bars) in transfected Cos-7 cells were determined as
described in Materials and Methods. For each experiment, the mock-transfected cells were designated as 100%. Relative radioactivity was graphed from three independent experiments (mean ± SD). * = statistically significant (at least p < 0.05) compared with the values for cells transfected with mock plasmid.