Lower Plasma Levels and Accelerated Clearance of High Density Lipoprotein (HDL) and Non-HDL Cholesterol in Scavenger Receptor Class B Type I Transgenic Mice*

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Yukihiko Ueda‡, Lori Royer§, Elaine Gong‡, Junli Zhang‡, Philip N. Cooper‡, Omar Francone§, and Edward M. Rubin§

From the ‡Lawrence Berkeley National Laboratory, Berkeley, California 94720 and the §Department of Cardiovascular and Metabolic Diseases, Pfizer, Inc., Groton, Connecticut 06340

Recent studies have indicated that the scavenger receptor class B type I (SR-BI) may play an important role in the uptake of high density lipoprotein (HDL) cholesterol ester in liver and steroidogenic tissues. To investigate the in vivo effects of liver-specific SR-BI overexpression on lipid metabolism, we created several lines of SR-BI transgenic mice with an SR-BI genomic construct where the SR-BI promoter region had been replaced by the apolipoprotein (apo)A-I promoter. The effect of constitutively increased SR-BI expression on plasma HDL and non-HDL lipoproteins and apolipoproteins was characterized. There was an inverse correlation between SR-BI expression and apoA-I and HDL cholesterol levels in transgenic mice fed either mouse chow or a diet high in fat and cholesterol. An unexpected finding in the SR-BI transgenic mice was the dramatic impact of the SR-BI transgene on non-HDL cholesterol and apoB whose levels were also inversely correlated with SR-BI expression. Consistent with the decrease in plasma HDL and non-HDL cholesterol was an accelerated clearance of HDL, non-HDL, and their major associated apolipoproteins in the transgens compared with control animals. These in vivo studies of the effect of SR-BI overexpression on plasma lipoproteins support the previously proposed hypothesis that SR-BI accelerates the metabolism of HDL and also highlight the capacity of this receptor to participate in the metabolism of non-HDL lipoproteins.

The risk of developing coronary artery disease is directly related to plasma levels of low density lipoprotein (LDL) cholesterol and inversely associated with the concentration of high density lipoprotein cholesterol (HDL) (1, 2). Although numerous studies have revealed many steps involved in LDL metabolism, significantly less is known about HDL metabolism. Novel insights concerning the metabolism of HDL have come from a recent series of experiments suggesting that the scavenger receptor class B type I (SR-BI) is an HDL receptor (3–9). SR-BI, a member of the CD36 superfamily, was originally cloned as a receptor for modified LDL (10–12). Transfected cells expressing SR-BI show high affinity binding with HDL and take up cholesterol ester from the particle by a selective uptake pathway markedly distinct from the LDL receptor pathway. Further support for SR-BI being an HDL receptor comes from the observations that SR-BI is expressed in liver and non-placental steroidogenic tissues, long hypothesized sites of HDL cholesterol delivery (3–6).

The expression of SR-BI in tissues appears to be responsive to changes in hormonal status and HDL cholesteryl ester supply (13–16). In the adrenal glands of knockout mice deficient in apoA-I (13), hepatic lipase (13), or lecithin-cholesterol acyltransferase (14), the expression of SR-BI is increased markedly. Moreover, the expression of SR-BI is up-regulated in the adrenal gland and down-regulated in the liver of rats after high dose estrogen treatment (15). The estrogen-induced increase of SR-BI is accompanied by enhanced uptake of lipid from HDL. Similarly, the administration of human chorionic gonadotropin also causes an increase in SR-BI expression in the steroidogenic Lydig cells of rat testis (15). These observations suggest that the SR-BI expression may be regulated in response to the supply and demand for cholesterol by some tissues.

Some of the most convincing studies suggesting a role for SR-BI in HDL metabolism come from the characterization of mice with genetically manipulated SR-BI expression. Two studies have examined mice with marked reductions in SR-BI expression caused by gene targeting in embryonic stem cells (8, 9). Increased HDL cholesterol levels in animals deficient in SR-BI suggest the importance of SR-BI in the clearance of HDL cholesterol. The in vivo effect of transient increases in SR-BI expression was studied in mice after adenovirus-mediated SR-BI gene transfer (7). This was associated with a marked decrease in plasma HDL cholesterol and an increase in biliary cholesterol. The adenoviral study, although supporting the hypothesis that increased SR-BI expression results in accelerated clearance of HDL cholesterol, is complicated by the massive short term increase in the expression of SR-BI and the hepatotoxicity that accompanies murine adenoviral infections.

To investigate the effect of steady-state increases in SR-BI expression on HDL metabolism we created several lines of SR-BI transgenic mice. One of the transgenics was a mouse BAC clone containing the SR-BI gene with the promoter region replaced by human liver-specific apoA-I promoter. With this
transgene two lines of SR-BI transgenic animals were studied, one with a 10-fold increase in SR-BI transcripts compared with control mice and a second line with a 2-fold increase. To determine the effect of SR-BI on lipoprotein metabolism and plasma cholesterol homeostasis we examined the impact of the increased levels of SR-BI on HDL and non-HDL lipoproteins and associated apolipoproteins with regard to plasma levels and clearance rates.

**MATERIALS AND METHODS**

**Generation of SR-BI Construct and Transgenic Mice—** A mouse BAC clone, BACM16, containing the full length of the SR-BI gene, was obtained from Genome Systems (St. Louis). The multiple steps involved in creating the apoA-I promoter SR-BI construct are outlined in Fig. 1a.

The human apoA-I promoter region from nucleotides -1613 to +72 of the transcription start site was amplified from genomic DNA by PCR. The forward primer contained a KpnI site at the 5′-end, and the reverse primer contained SR-BI cDNA sequence +1 to +50. The PCR product was cloned into the vector pCR2.1, using a TA Cloning kit (Invitrogen, San Diego) to construct pCR_AISRBI5. The 4-kb EcoRI fragment of BACM16 containing the first exon of the SR-BI gene was subcloned into pBluescript SK+ (Strategene, La Jolla, CA) to construct pSSRBI5. The SR-BI promoter-replacing vector pBSAISRBI5 cm was constructed by sequential insertion into pBluescript SK (Molecular Cloning, 2nd ed. 1995). The SR-BI promoter-replacing vector pBSAISRBI5 cm was digested with EcoRI and ligated into the 65-kb EcoRI RARE fragment of BACM16. The construct, pBSAISRBI5 cm, was screened by positive selection on LB plates supplemented with ampicillin and chloramphenicol. Panel b, 10 μg each of total RNA isolated from various tissues of high SR-BI or wild type FVB was hybridized with radiolabeled riboprobe complementary to the 5′-end of the SR-BI gene and was digested with RNase A/T1 mix. The protected fragments were separated on 5% polyacrylamide and 8 M urea gel and detected by autoradiography. The expression of the endogenous gene and the transgene was shown by 248- and 176-base protected bands, respectively.

**Fig. 1.** Design of SR-BI construct and tissue distribution of the expression of the transgene. Panel a, a BAC clone, BACM16, containing the full length of the SR-BI gene was fragmented at EcoRI sites in the first intron of the gene and in the chloramphenicol gene in the vector sequence by RARE cleavage. The fragments were separated by pulse field gel electrophoresis. The SR-BI promoter-replacing vector, pBSAISRBI5 cm, was constructed with a 1.7-kb EcoRI-KpnI fragment of pCRAISRBI5, a 3.2-kb EcoRI-BssHII fragment of the 5′-end of the SR-BI gene, and a 0.5-kb NotI-EcoRI fragment of the chloramphenicol gene in pBluescript SK(+) . pBSAISRBI5 cm was digested with EcoRI and ligated into the 65-kb EcoRI RARE fragment of BACM16. The construct, pBSAISRBI5 cm, was cloned into the vector pCR2.1, using a TA Cloning kit (Invitrogen, La Jolla, CA) to construct pBSAISRBI5 cm. The SR-BI promoter-replacing vector, pBSAISRBI5 cm, was digested with EcoRI and ligated into the 65-kb EcoRI RARE fragment of BACM16 separated by pulse field gel electrophoresis. The BAC clone pBACAISRBI5 cm was screened by positive selection on LB plates supplemented with ampicillin and chloramphenicol and then by PCR with the forward primer within the apoA-I promoter sequence (apoA-I-620f) and the reverse primer within the first exon of SR-BI (SR-BI r 176).

BAC DNAs were prepared for microinjection by modified alkaline lysis methods followed by phenol-chloroform extraction, and then an overnight dialysis against the injection buffer (10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 100 mM NaCl) through Millipore type VS membrane (0.025-μm pore size; Millipore, Bedford, MA). The SR-BI transgenic mice in the inbred FVB background were generated by standard microinjection methods (18). Mice were screened by PCR with a primer set, apoA-I-620f and SR-BI r 176.

**Diets and Apolipoprotein and Lipoprotein Analyses—** Mice were fed Purina mouse chow (no. 5001) until 6 weeks of age when blood samples were collected from the tail vein after an overnight fast. The animals were then fed an atherogenic diet high in fat and cholesterol (1.25% cholesterol, 0.5% cholic acid, and 15% fat) (19). Blood samples were collected again at 4 weeks after initiation of the atherogenic diet. Total cholesterol was determined by the standard enzyme method (Wako, Osaka, Japan). HDL cholesterol was measured after selective precipitation of non-HDL lipoproteins by dextran sulfate and magnesium chloride.

Plasma levels of mouse apoA-I and apoB were determined by enzyme-linked immunosorbent assays as described previously (20). Mice apoB levels were determined by enzyme-linked immunosorbent assay using a rabbit polyclonal antibody prepared from an immunized animal.
injected with mouse LDL. Antiserum was purified by ligand immunoaffinity chromatography to isolate those antibodies reactive against apoB. Antibodies were purified on a HiTrap protein A affinity column and biotinylated.

Plasma samples of five to seven animals of each group were combined and subjected to FLPLC fractionation analysis with two tandem Superose 6 columns (Amersham Pharmacia Biotech) as described previously (21).

The size distribution of the d ≤ 1.21 g/ml lipoprotein fractions were obtained by nondenaturing polyacrylamide gradient gel electrophoresis essentially as described by Nichols et al. (22). Lipoprotein fractions were isolated by ultracentrifugation of plasma pooled from five to seven animals of each group.

**Gene Expression Analyses**—Total RNA was isolated from brain, lungs, heart, liver, spleen, kidney, small intestine, adrenal glands, and ovary or testis of the animals using RNA Stat 60 (Teltest Inc., Friendswood, TX).

RNAse protection assay was run by RPAII kit (Ambion, Austin, TX). Briefly, total RNA (10 μg) was hybridized with labeled probes overnight at 45 °C and then digested with RNAse A/T1 mix. The protected fragments were separated on a 5% polyacrylamide and 8 M urea gel and were detected by autoradiography.

To identify the transgene expression from that of the endogenous gene we designed a riboprobe on the 5′-end of the SR-BI gene expanding from the 5′-untranslated region to the first exon; the probe on the 3′-end of the gene recognized both the transgene and endogenous gene. Primer sets were generated to amplify the probes for SR-BI and a probe for mouse LDL receptor from wild type FVB mouse liver cDNA as a template by PCR with T7 promoter sequence supplemented on the 5′-upstream of the reverse primers. The probes were radiolabeled with [α-32P]UTP (Amersham Pharmacia Biotech) using a MAXiscript kit (Ambion).

Immunoblot analysis was carried out with liver membrane proteins solubilized with Triton X-100. The indicated amounts of reduced protein samples were electrophoresed on pre-cast SDS-polyacrylamide gels (4–20% acrylamide; Novex, San Diego) and transferred to nitrocellulose. Mouse SR-BI was detected by rabbit anti-mouse SR-BI antiserum directed to residues 405–509 for mouse SR-BI after blocking with 3% dry milk in phosphate-buffered saline. The primary antibody was detected using a peroxidase-conjugated goat anti-rabbit IgG (Sigma) followed by ECL Western blotting detection reagents (Amersham Pharmacia Biotech).

**In Vivo Turnover Studies of Lipoproteins**—In vivo turnover studies were carried out as described previously (7). LDL (d = 1.019–1.063 g/ml) was isolated from human plasma and HDL (d = 1.063–1.210 g/ml) from FVB mouse plasma by sequential ultracentrifugation and then subjected to iodination with 125I using IODO-BEADS (Pierce). The labeled lipoproteins (10–15 μg of protein, 50–100 cpm/ng) were injected into the tail vein of each animal. The injected lipoprotein was less than 0.5% of the mouse LDL or HDL pool size. Blood (50 μl) was withdrawn from the retroorbital plexus into heparinized capillary tubes at the indicated time points after injection. The apolipoprotein retention was measured by the radioactivity in the trichloroacetic acid precipitate of the plasma. The radioactivity of the precipitate at 2 min after injection was defined as 100%.

**RESULTS**

**Production of SR-BI Transgenic Mice**—A 120-kilobase pair BAC clone, BACM16, containing the entire SR-BI gene was injected into FVB mouse embryos as was a second DNA preparation containing a construct engineered from this clone, BACAISRBI, whose SR-BI promoter was replaced with the human apoA-I promoter (Fig. 1a). Two BACM16 transgenic founder mice and seven transgenic founder mice containing BACAISRBI were created (Table I). No detectable effect on plasma HDL levels was observed in the two founder mice with the BACM16, whereas four of the seven founder mice of BACAISRBI transgenics had significantly lower HDL cholesterol levels. Among the BACAISRBI transgenic lines B2611 (referred to as low SR-BI) and B2614 (referred to as high SR-BI) with moderate and profound decreases in HDL cholesterol levels were identified by autoradiography.
SR-BI Increases HDL and LDL Clearance

SR-BI Transcript and Protein Assays—To examine tissue specificity of expression of the transgene and to differentiate it from that of the endogenous SR-BI gene, we performed RNase protection assays. The riboprobe at the 5’-end of the SR-BI cDNA used for these studies distinguishes the two SR-BI transcripts in the transgenic mice generating a 176-base pair band for the transgene and a 248-base pair band for the endogenous SR-BI gene. The expression of the transgene is prominent in the liver of transgenic mice as expected from previous studies (23) (Fig. 1a). In agreement with previous studies in mice, the endogenous SR-BI expression though detectable in the liver, ovary, and lung, was highest in the adrenal gland (3). The liver expression of the SR-BI transgene did not alter the pattern or levels of the endogenous SR-BI gene. Analyses of SR-BI expression in these mice indicate that the expression levels of SR-BI in the liver of low and high SR-BI transgenic mouse lines are 2-fold and >10-fold higher than those of control FVB mice, respectively (Fig. 2a).

To examine the SR-BI protein product in the liver of transgenic mice, cell membrane proteins were collected by ultracentrifugation, separated by SDS-polyacrylamide gel electrophoresis, and immunoblotted using an antibody against mouse SR-BI. In both transgenic lines studied, the SR-BI protein levels reflected the results of the RNase protection studies (Fig. 2b) and were about 2- and 10-fold higher than endogenous levels in the low and high SR-BI transgensics, respectively.

Plasma Lipids and Lipoprotein Analysis—The increases in SR-BI expression levels observed in the low and high SR-BI transgenic mice livers correlated inversely with plasma cholesterol concentrations. The total cholesterol levels of the transgenics were 5.9% of control FVB in the high SR-BI Tg line and 58.5% of control FVB in the low SR-BI Tg line (Fig. 3a). The HDL cholesterol levels of the high and low SR-BI transgenics were 4.0 and 61.6% of control FVB mice, respectively (Fig. 3b). To determine whether the decrease in HDL cholesterol is accompanied by a parallel decrease in apoA-I concentration, murine apoA-I levels were determined by enzyme-linked immunosorbent assay. The concentration of apoA-I was 1.0 and 69.2% of non-transgenic controls in high SR-BI transgenics and low SR-BI transgenics, respectively (Fig. 3d), suggesting that increased expression of SR-BI decreases not only HDL cholesterol levels but also HDL particle number.

An unexpected result in the analysis of the SR-BI transgenics was that the decrease in plasma cholesterol was not exclusively in the HDL fraction but was also present in non-HDL fractions. The decrease in non-HDL cholesterol, though not as great as the decrease in HDL cholesterol, was significant in the high and low SR-BI transgenics, 90 and 45% lower, respectively, than that of the non-transgenic mice (Fig. 3c). Plasma apoB levels were also decreased in mice with elevated hepatic SR-BI expression (Fig. 3e). Compared with non-transgenic controls, apoB was decreased by 35 and 26% in the high and low transgenic mice, respectively. FPLC gel filtration analysis using pooled plasma confirmed that there were dose-dependent decreases in HDL and non-HDL cholesterol in mice expressing SR-BI, compared with non-transgenic controls (Fig. 4a).

To examine the impact of SR-BI on the regulation of plasma cholesterol levels of mice fed an atherogenic diet, we determined HDL cholesterol, apoA-I, non-HDL cholesterol, and apoB levels in SR-BI transgenics and non-transgenic littermates fed this diet for 4 weeks. Total plasma cholesterol was increased significantly in all genotypes (Fig. 5a), and these changes were primarily the result of increases in non-HDL cholesterol (Fig. 5c). Comparisons of the transgenics and controls demonstrate that the level of SR-BI expression was roughly associated with a dose-dependent decrease in total cholesterol and non-HDL cholesterol. Plasma apoB levels of high and low SR-BI transgenics were 36 and 19% lower, respectively, than those of non-transgenic controls (Fig. 5c). As a consequence, the markedly decreased ratio of non-HDL cholesterol to apoB (98.9 ± 54 in high SR-BI, 647 ± 85 in low SR-BI, and 778.3 ± 248 in FVB) could be used as an indication of a decreased particle size in mice overexpressing SR-BI. Taken
together, these findings indicate that moderate increases in SR-BI levels are associated with significant changes in lipoprotein particle size and suggest that SR-BI is also able to promote selective cholesteryl ester uptake from non-HDL as well as HDL particles.

**SR-BI and LDLr Gene Expression Studies**—The effects of diet and the SR-BI transgene on the expression of LDL receptor and endogenous SR-BI gene were studied. RNase protection assays of total liver RNA isolated from mice before and after high fat diet treatment were performed and qualitatively compared (Fig. 2c). Expression of the SR-BI transgene and the SR-BI endogenous gene was not influenced by the diet. In contrast, LDLr was down-regulated after high fat diet treatment in the control and low SR-BI transgenic mice, whereas in the high SR-BI transgenics LDLr expression was not affected by diet.

**Lipoprotein Particle Size Distribution**—The lipoprotein size distribution of control and SR-BI transgenic mice is shown in Fig. 4, b and c. In agreement with previous studies (24), HDL isolated from wild type mice is shown as a monodisperse population of particles with a peak diameter of 10.2 nm. HDL isolated from low SR-BI mice also consisted of a unimodal distribution, but its peak diameter is decreased markedly, suggesting a denser, less lipid-rich particle (Fig. 4b). Surprisingly, the same phenomenon was observed in apoB-containing lipoprotein fractions (Fig. 4c). The peak diameter of the VLDL fraction in high SR-BI shows a striking decrease, 35.5 nm compared with 37.3 nm in FVB mice. The peak size of the LDL/IDL fraction also shows a remarkable and dose-dependent reduction in SR-BI transgenics: 27.4 nm in high SR-BI, 29.4 nm in low SR-BI, and 30.8 nm in FVB mice. These observations support the hypothesis that SR-BI promotes the selective uptake of cholesteryl ester from apoB-containing lipoproteins leading to the formation of smaller particles.

**In Vivo Turnover Studies of Lipoproteins**—To evaluate the effect of SR-BI overexpression on the metabolism of lipoproteins in vivo, 125I-labeled human LDL and mouse HDL were injected into mice of different genotypes. The disappearance of radioactivity from plasma was followed for 24 h. The plasma clearance rate of labeled HDL was increased significantly in
The transgenic mice created for these studies with increased hepatic levels of SR-BI have enabled us to determine the effect of steady-state overexpression of this gene on lipoprotein metabolism. The inverse correlation between SR-BI expression and HDL turnover rates suggests that the increased expression of SR-BI and its mediated clearance of HDL result in lower plasma concentrations of this lipoprotein. These findings are consistent with prior in vitro as well as in vivo characterizations of SR-BI (3–9) and add further support to the hypothesis that SR-BI is an important participant in HDL metabolism.

HDL cholesterol uptake by the adrenal gland and liver is believed to occur in part by the selective uptake pathway, a process whereby cholesteryl esters are selectively removed from the particles. This selective removal of cholesteryl esters would be predicted to result in changes in HDL composition and particle size. The presence of the smaller denser HDL particles in the SR-BI transgenics, demonstrated by nondenaturing polyacrylamide gel electrophoresis, is thus consistent with increased selective uptake of HDL cholesterol in the SR-BI-overexpressing mice. The accelerated clearance of apoA-I in the SR-BI transgenics may in part be a result of the changes in the HDL particle size accelerating the metabolism of this apoA-I-containing particle by the kidney (25), as has been noted previously in mice with increased SR-BI expression caused by adenoviral gene transfer (7).

The LDL turnover results, although not as dramatic as the HDL turnover results, indicate faster clearance of labeled LDL in SR-BI transgenics and suggest the involvement of SR-BI in LDL metabolism. The decreased size of non-HDL lipoproteins coupled with their reduced concentrations in mice overexpressing SR-BI suggests the possibility that the selective uptake of cholesteryl esters via SR-BI is not restricted to HDL but also includes non-HDL lipoproteins. Another possible explanation for the reduced concentrations of non-HDL lipoproteins in the SR-BI transgenics is that SR-BI overexpression may impact on the production and secretion of VLDL in the liver or the uptake of cholesterol-rich non-HDL particles. Although stimulated VLDL secretion was observed in the recovery phase after adenovirus-mediated short term overexpression of SR-BI (7), the persistent overexpression of SR-BI obtained in the present study would be predicted to suppress this secretion, which is regulated by pooled intracellular cholesterol.

The sensitive and quantitative RNase protection assays enabled assessment of the effect of SR-BI activity and diet on the expression of the endogenous murine SR-BI and LDLr. Although prior studies demonstrated that adrenal SR-BI expression is affected by apoA-I (13), hepatic lipase (13), and lecithin-cholesterol acyltransferase deficiency (14), the expression of the endogenous hepatic SR-BI gene was not affected by SR-BI transgene activity or diet in the present study. LDLr expression was unchanged in the SR-BI transgenics when the animals were fed mouse chow; however, the expression of LDLr was diminished in the low SR-BI transgenics and nontransgenic control mice when fed the high fat high cholesterol diet. This effect of diet on LDLr expression was not detected in high SR-BI high expressors, possibly suggesting that intracellular cholesterol pools and their effect on LDLr expression may be dependent upon the route by which cholesterol enters the cell. In summary, although it is not clear how the mechanism of SR-BI overexpression impacts on LDLr expression, our studies clearly indicate that the regulation of SR-BI and LDLr are in many ways distinctly different.

An important but yet unanswered question concerning SR-BI is its effect on an organism’s susceptibility to diet-induced atherogenesis. The predicted acceleration of reverse cho-
lesterol transport, a process whereby excess cholesterol accumulates in the peripheral tissues such as the vasculature is mobilized, in the SR-BI overexpression transgensics would at first suggest that increasing the activity of SR-BI should have an anti-atherogenic effect. An alternative perspective also consistent with the results of the present study would suggest that increased expression of SR-BI should increase an organism’s susceptibility to atherosclerosis by decreasing both apoA-I and HDL plasma concentration. This relates to the multiple in vitro and in vivo studies that have attributed antiatherogenic properties to the HDL particle itself, such as acting as an antioxidant and preventing LDL modification (26, 27), effects that would be diminished at reduced plasma concentrations of this lipoprotein. The eventual analysis of atherosclerosis susceptibility in the SR-BI transgensics, created in the inbred genetic background described in this study, may offer an important vista into the role of SR-BI as a potentially manipulable atherosclerosis susceptibility factor.

The present characterization of SR-BI-overexpressing mice has revealed several sites where SR-BI may be involved in lipoprotein metabolism. This includes its previously studied role in HDL metabolism as well as a largely uncharacterized role in the metabolism of non-HDL lipoproteins. This latter property might be viewed as having been predicted by the fact that SR-BI was first identified by its ability to bind native LDL as well as modified LDL (10). The major findings of the present study that increased SR-BI expression in transgenic mice dramatically lowers plasma levels and accelerates clearance of both HDL and non-HDL cholesterol, highlights the potential major role of this molecule in lipoprotein metabolism in vivo.

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