Restoration of High-Density Lipoprotein Levels by Cholesteryl Ester Transfer Protein Expression in Scavenger Receptor Class B Type I (SR-BI) Knockout Mice Does Not Normalize Pathologies Associated With SR-BI Deficiency

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Objective—Disruption of scavenger receptor class B type I (SR-BI) in mice impairs high-density lipoprotein (HDL)–cholesterol (HDL-C) delivery to the liver and induces susceptibility to atherosclerosis. In this study, it was investigated whether introduction of cholesteryl ester transfer protein (CETP) can normalize HDL-C transport to the liver and reduce atherosclerosis in SR-BI knockout (KO) mice.

Methods and Results—Expression of human CETP in SR-BIKO mice resulted in decreased plasma HDL-C levels, both on chow diet (1.8-fold, \( P<0.001 \)) and on challenge with Western-type diet (1.6-fold, \( P<0.01 \)). Furthermore, the presence of CETP partially normalized the abnormally large HDL particles observed in SR-BIKO mice. Unexpectedly, expression of CETP in SR-BIKO mice did not reduce atherosclerotic lesion development, probably because of consequences of SR-BI deficiency, including the persistence of higher VLDL-cholesterol (VLDL-C) levels, unchanged elevated free cholesterol/total cholesterol ratio, and the increased oxidative status of the animals. In addition, CETP expression did not normalize other characteristics of SR-BI deficiency, including female infertility, reticulocytosis, thrombocytopenia, and impaired platelet aggregation.

Conclusion—CETP restores HDL-C levels in SR-BIKO mice, but it does not change the susceptibility to atherosclerosis and other typical characteristics that are associated with SR-BI disruption. This may indicate that the pathophysiology of SR-BI deficiency is not a direct consequence of changes in the HDL pool. (Arterioscler Thromb Vasc Biol. 2010;30:1439-1445.)

Key Words: atherosclerosis ▪ platelets ▪ cholesteryl ester transfer protein ▪ high-density lipoprotein ▪ scavenger receptor class B type I ▪ oxidation

Several epidemiological studies have shown that high-density lipoprotein (HDL) cholesterol is inversely related to the risk of atherosclerotic lesion development.\(^1\) High levels of HDL in plasma protect against atherosclerosis by facilitating reverse cholesterol transport, a process by which cholesterol is transported from peripheral cells to the liver via HDL.\(^2\) An important player in HDL metabolism is scavenger receptor class B type I (SR-BI), a cell surface receptor that is highly expressed in liver and steroidogenic tissues and mediates the selective uptake of cholesteryl esters (CE) from HDL.\(^3\) Disruption of SR-BI in mice (SR-BIKO) causes the accumulation of cholesterol in HDL, resulting in larger HDL particles.\(^4\) Despite the elevated plasma HDL-cholesterol (HDL-C) levels, these mice are more susceptible to atherosclerotic lesion development.\(^5\)

In the Framingham Heart Study, \( \approx 45\% \) of all clinical events occurred in subjects with normal or elevated HDL-C levels,\(^6\) suggesting that not only the HDL-C levels per se but also other HDL-associated factors are important. The proatherogenic effects of SR-BI deficiency have primarily been attributed to disruption of the flux of cholesterol through the reverse cholesterol transport pathway. However, besides the well-known role of SR-BI in HDL metabolism, SR-BI is also important for facilitating chylomicron\(^7\) and very-low-density lipoprotein (VLDL, \(^8\)) metabolism in wild-type (WT) mice. Furthermore, SR-BI is responsible for clearance of remnants from the circulation in apolipoprotein (apo) E-deficient mice.\(^9\) Thus, the antiatherogenic properties of SR-BI could also be attributed to its role in promoting the clearance of atherogenic apoB-containing lipoproteins. In addition, SR-BI deficiency is associated with a significant increase in oxidative stress in vivo, potentially contributing to the proatherogenic effect of SR-BI deficiency.\(^10\)
Although the function of SR-BI in HDL metabolism and atherosclerosis has been well established in genetically engineered mice, the role of CLA-1 (CD36 and LIMPII analogous-1), the human homologue of SR-BI, in human metabolism is still largely unknown. The role of SR-BI in human HDL metabolism might be influenced by the presence of CE transfer protein (CETP), which transfers CE from HDL to apoB-containing lipoproteins that are subsequently rapidly taken up by the liver.\(^\text{11}\)
CETP is a hydrophobic plasma glycoprotein with a widespread tissue distribution. The highest levels of CETP mRNA are found in liver, spleen, and adipose tissue,\(^\text{12}\) whereas CETP is also locally expressed in the human arterial wall.\(^\text{13}\)

Mice naturally lack CETP.\(^\text{14}\) Transgenic (Tg) mice expressing human CETP have decreased levels of HDL and an increased susceptibility to atherosclerosis, indicating that CETP is a proatherogenic factor.\(^\text{15}\) However, in absence of SR-BI, CETP might offer an alternate route for reverse cholesterol transport, thereby possibly preventing atherosclerosis. In line with this, Harder et al\(^\text{16}\) indeed showed HDL-C lowering and decreased atherosclerotic lesion development in SR-Bi\(^\text{KO}\) mice on expression of CETP under control of the human apoAI promoter. However, in these mice, CETP was solely expressed in the liver and was not regulated in response to high-fat/high-cholesterol diet feeding.

The aim of the current study was to study the influence of CETP plasma activity on HDL cholesterol metabolism and atherosclerotic lesion development in SR-Bi\(^\text{KO}\) mice expressing human CETP under control of its own promoter (SR-Bi\(^\text{KO}\)/CETP\(^\text{Tg}\), 17). Interestingly, despite the decreased plasma HDL-C levels and normalized HDL particle size in SR-Bi\(^\text{KO}\)/CETP\(^\text{Tg}\) animals, CETP activity did not reduce lesion development, nor did it normalize other pathologies associated with SR-BI deficiency.

**Methods**

For detailed methodology, please see the data supplement, available online at http://atvb.ahajournals.org.

In short, SR-Bi\(^\text{KO}\) mice expressing CETP (SR-Bi\(^\text{KO}\)/CETP\(^\text{Tg}\)) were generated by crossing heterozygous SR-Bi\(^\text{KO}\) mice with CETP Tg mice overexpressing human CETP under the control of its own promoter and other major regulatory elements.\(^\text{17}\) To induce atherosclerosis, mice were challenged with a Western-type diet (WTD) for 20 weeks, and atherosclerotic lesion size, serum lipids, and blood cell counts, and oxidation markers (isoprostanes and WTD) for 20 weeks, and atherosclerotic lesion size, serum lipids, and blood cell counts, and oxidation markers (isoprostanes and LCAT), blood cell counts, and oxidation markers (isoprostanes and carboxylic acids) were determined. Furthermore, serum decay and liver uptake of \(^{3}\text{H}\)cholesterol ether–labeled HDL and ex vivo aggregation of platelets were analyzed.

**Results**

The Effects of CETP Expression in Chow-Fed SR-Bi\(^\text{KO}\) Mice on Plasma Lipid Parameters and HDL Kinetics

To investigate the effect of CETP on the clearance of HDL-C in the absence of SR-BI, SR-Bi\(^\text{KO}\) mice were crossed with human CETP\(^\text{Tg}\) mice to generate CETP-expressing SR-Bi knockout (SR-Bi\(^\text{KO}\)/CETP\(^\text{Tg}\)) mice. The CETP activity in these SR-Bi\(^\text{KO}\)/CETP\(^\text{Tg}\) mice was 158±9 nmol/mL per hour in male mice and 241±10 nmol/mL per hour in female mice (Table). In comparison, CETP activity measured in human plasma was 153±16 nmol/mL per hour (n=10). Plasma total cholesterol (TC) levels were decreased 1.7-fold (P<0.001; Table) in male and 1.9-fold (P<0.05) in female SR-Bi\(^\text{KO}\)/CETP\(^\text{Tg}\) mice compared with SR-Bi\(^\text{KO}\) controls. Furthermore, CETP expression reduced free cholesterol (FC) levels 2.3-fold (P<0.001) and 2.1-fold (P<0.05) in males and females, respectively (Table). This decrease in serum cholesterol can be attributed to lower HDL-C concentrations and a smaller HDL particle size, as assessed by fast protein liquid chromatography (Figure 1). Although HDL-C was normalized on expression of CETP in SR-Bi\(^\text{KO}\) female mice, the FC/TC ratio, which is elevated in SR-Bi\(^\text{KO}\) animals (0.35±0.02), remained elevated in SR-Bi\(^\text{KO}\)/CETP\(^\text{Tg}\) mice (0.31±0.02) compared with WT females (0.25±0.01, P<0.05; Table). In male SR-Bi\(^\text{KO}\)/CETP\(^\text{Tg}\) mice, HDL-C and the FC/TC ratio were partially normalized. The FC/TC ratio decreased 1.4-fold (0.38±0.04, P<0.01) compared with SR-Bi\(^\text{KO}\) mice (0.52±0.1) but remained 1.3-fold higher than WT males (0.30±0.02), although this failed to reach statistical significance (Table).

Expression of CETP in SR-Bi\(^\text{KO}\) mice resulted in a normalization of the serum decay of \(^{3}\text{H}\) cholesterol ether (CEt) from HDL in SR-Bi\(^\text{KO}\)/CETP\(^\text{Tg}\) mice (Figure 2A). At 4 hours after injection, 69±3% (n=3) of the injected trace amount of \(^{3}\text{H}\) CEt was cleared from the circulation in SR-Bi\(^\text{KO}\)/CETP\(^\text{Tg}\) mice compared with 65±2% (n=3) in WT animals, whereas in SR-Bi\(^\text{KO}\) mice only 50±8% (n=3) was cleared (P<0.05). On euthanization at 24 hours after injection, the livers were isolated, and the \(^{3}\text{H}\) CEt uptake was measured. In accordance with the normalized clearance, the uptake of \(^{3}\text{H}\) CEt by the liver of SR-Bi\(^\text{KO}\)/CETP\(^\text{Tg}\) mice was enhanced to 34±2% (n=4), compared with only 9±0.4% (n=4) of the injected dose in SR-Bi\(^\text{KO}\) animals (P<0.001; Figure 2B). In comparison, in WT mice expressing functional SR-BI, 46±2% (n=3) of the injected dose was taken up by the liver at 24 hours after injection of \(^{3}\text{H}\) CEt-HDL. At 8 hours after injection, \(^{3}\text{H}\) CEt distribution over the different lipoprotein particles was evaluated (Figure 2C). On injection of the human HDL in the SR-Bi\(^\text{KO}\) mice, a shift to larger HDL was observed, probably reflecting the enrichment of the HDL particles with cholesterol from peripheral tissues. Importantly, the amount of \(^{3}\text{H}\) CEt transported by HDL particles in SR-Bi\(^\text{KO}\)/CETP\(^\text{Tg}\) mice was 2.6-fold lower than that of SR-Bi\(^\text{KO}\) mice SR-Bi\(^\text{KO}\)/CETP\(^\text{Tg}\), whereas a 2.2-fold increase was observed in VLDL and low-density lipoprotein (LDL) particles, confirming that \(^{3}\text{H}\) CEt from HDL is transported to LDL and VLDL particles by CETP in these animals after which the \(^{3}\text{H}\) CEt can be taken up by the liver, probably via the LDL receptor and the LDL receptor–related protein.

The Effect of CETP Activity on Atherosclerosis Susceptibility of SR-Bi\(^\text{KO}\) Mice

Next, we investigated whether the normalized clearance of CE from HDL particles by CETP expression in SR-Bi\(^\text{KO}\)/CETP\(^\text{Tg}\) mice was associated with a reduced atherosclerosis susceptibility in these animals. To this end, male and female SR-Bi\(^\text{KO}\)/CETP\(^\text{Tg}\) mice and their SR-Bi\(^\text{KO}\) littermates were fed a WTD containing 0.25% cholesterol and 15% cacao butter for 20 weeks. On WTD feeding, the CETP activity in plasma increased 3-fold in both male and female SR-Bi\(^\text{KO}\)/CETP\(^\text{Tg}\) animals (Table). The TC levels in plasma increased 2-fold on diet feeding in SR-Bi\(^\text{KO}\)
mice in both male and female animals. In SR-BIko/CETPTg animals, a similar 2-fold increase of plasma TC was seen in males, whereas the levels increased 3-fold in females. Importantly, also under these dietary conditions, CETP expression in SR-BIko mice resulted in reduced plasma TC and FC levels, although not to WT levels (Table). The elevated FC/TC ratio in SR-BIko females (0.44±0.04) compared with WT (0.34±0.01, P<0.01), was partially normalized in SR-BIko/CETPTg (0.37±0.01, P<0.05) female mice. In SR-BIko/CETPTg males the FC/TC ratio remained elevated (0.39±0.02) compared with WT (0.30±0.03, P<0.05) mice and similar to SR-BIko (0.45±0.01) males (Table). In females on WTD, CETP expression in SR-BIko mice increased the endogenous (9.47±0.59 versus 4.45±0.51 nmol CE/h·mL, P<0.001) and the exogenous (97.2±14.9 versus 60.3±6.6 nmol CE/h·mL, P<0.05) LCAT activity, the major enzyme for the esterification of FC. In males, however, no difference on endogenous or exogenous activity was observed (Supplemental Figure I).

Similar to the effects observed on chow diet, plasma cholesterol levels were lower in SR-BIko/CETPTg mice on WTD because of a decrease in HDL-C levels (males: 1.9-fold, P<0.001 and females: 1.6-fold, P<0.001; Table, Figure 1). VLDL-C levels, however, were similarly high in SR-BIko and SR-BIko/CETPTg mice.

To further investigate the effect of CETP expression in SR-BIko mice on the HDL particles, HDL lipid composition...
induced a decrease in the average HDL particle size (12.0±0.5 nm, P<0.01). However, the particles remained larger compared with WT animals (P<0.05, Figure 1B). HDL particles of SR-BiKO mice were composed of 27.6±2.6% FC, 32.0±1.5% cholesterol esters (CE), 39.0±1.8% phospholipids, and 1.5±0.4% triglycerides. CETP expression in SR-BiKO mice reduced the FC content to 17.9±2.3% (P<0.05); however, it remained increased compared with WT animals (7.7±0.4%, P<0.01). CE and phospholipids increased to 35.0±2.7% and 45.0±2.6%, respectively, in SR-BiKO/CETPtg mice, although not significantly (Figure 1B).

After 20 weeks of WTD feeding, the mice were euthanized, and the lesion size in the aortic root was quantified. In all groups, lesions were early cell-rich lesions. Interestingly, in both the male and female groups, no difference in lesion size was observed between SR-BiKO (male, 12.0±0.5×10³ µm² [n=5]; female, 68.6±12.5×10³ µm² [n=5]) and SR-BiKO/CETPtg (male, 11.4±2.8×10³ µm² [n=8]; female, 73.2±13.8×10³ µm² [n=6]) (Figure 3). Thus, remarkably, restoration of the delivery route of HDL-C through the expression of CETP in SR-BiKO mice was not associated with a reduced atherosclerosis susceptibility of these animals.

To gain further insight as to why atherosclerotic lesion development in SR-BiKO/CETPtg mice was not reduced despite restoration of the reverse cholesterol transport pathway, the effect of CETP expression in SR-BiKO mice on the oxidative status of these mice was investigated. To this end, serum, urine, liver, spleen, and the aortic arch were isolated from SR-BiKO and SR-BiKO/CETPtg mice fed WTD for 20 weeks. As previously shown, the SR-BiKO animals displayed a 2.6-fold increase of the isoprostane 8,12-iso-isoprostane F2alpha-VI (iPF2alpha-VI) in plasma, a 1.5-fold increase in urine, and an enhanced accumulation in liver, spleen, and aorta compared with animals with functional SR-Bi fed WTD. Interestingly, CETP expression in SR-BiKO mice did not result in a normalization of the levels of 8,12-iso-iPF2alpha-VI in serum, urine, liver, and the aortic arch in either male or female mice (Figure 4). In fact, in CETP-expressing SR-BiKO mice, a tendency to increased levels of 8,12-iso-iPF2alpha-VI was observed in urine and the different organs analyzed; this increase, however, reached statistical significance only for the spleen. In addition, the levels of carbonyls in serum, spleen, and

and particle size was measured in animals fed WTD for 20 weeks. In agreement with the fast protein liquid chromatography analysis, the average size of HDL particles was larger in SR-BiKO (14.6±0.5 nm, P<0.001) compared with WT (10.8±0.2 nm) animals. Expression of CETP in SR-BiKO mice

Figure 2. Enhanced serum decay and liver uptake of HDL-Cet in SR-BiKO/CETPtg mice. A, The clearance of [³H]HDL from serum was significantly increased in SR-BiKO/CETPtg mice (closed circles) compared with SR-BiKO animals (open circles) and similar to the clearance in WT mice (triangle), within 24 hours postinjection. *P<0.05, **P<0.01, ***P<0.001. B, The uptake of [³H]HDL in the liver of SR-BiKO/CETPtg mice (black bar) was markedly enhanced by 3.7-fold compared with SR-BiKO animals (white bar; ***P<0.001) but remained significantly lower than WT animals (hatched bar; 1.4-fold; #P<0.01). C, The distribution of [³H]HDL-Cet was shifted from HDL to LDL/VLDL particles because of the expression of CETP in SR-BiKO/CETPtg mice (closed circles) compared with SR-BiKO animals (open circles).

Figure 3. CETP expression did not affect atherosclerotic lesion development in SR-BiKO mice on WTD. The mean lesion area (µm²) was determined in oil red O-stained cross-sections of the aortic root at the level of the tricuspid valves. Representative pictures are shown. Magnification ×50. Values represent the mean±SEM from SR-BiKO/CETPtg mice (black bars) (male, n=8; female, n=6) and SR-BiKO mice (white bars) (male, n=5, female n=5). No effect of CETP expression on atherosclerotic lesion development was found in either the male or the female group after 20 weeks of WTD feeding.
The aorta of SR-BIKO mice increased slightly with expression of CETP (Supplemental Figure II).

The Effect of CETP Activity on Female Infertility, Reticulocytosis, and Platelet Abnormalities of SR-BIKO Mice

SR-BI deficiency is associated not only with the accumulation of enlarged HDL particles and an increased susceptibility to atherosclerosis but also with female infertility and a dramatically reduced lifespan of erythrocytes, leading to enhanced erythropoietic activity and the accumulation of reticulocytes. Interestingly, CETP expression could also not overcome the infertility of SR-BIKO female mice, as no litters were obtained over a period of 6 months. As previously described for SR-BIKO mice, female infertility of SR-BIKO/CETPTg mice was restored by feeding breeding pairs a diet containing 0.25% probucol. Regarding reticulocytosis, the presence of CETP decreased the reticulocyte counts from 241 × 10^3/µL (n = 6) to 143 × 10^3/µL (n = 8) (P < 0.05) in male SR-BIKO animals. However, the levels remained substantially higher than those of WT controls (33 ± 3% [n = 6]). In female animals that showed a less dramatic accumulation of reticulocytes in the circulation, no statistically significant reduction in the reticulocyte counts was observed (82 ± 7% [n = 5] in SR-BIKO/CETPTg mice compared with 104 ± 11% [n = 6] in SR-BIKO animals). The enhanced erythrocytosis in SR-BIKO mice is associated with the development of splenomegaly. Consistent with the limited effect of CETP on reticulocyte counts, expression of CETP in SR-BIKO mice did not normalize the relative spleen size (0.65 ± 0.04% of body weight for SR-BIKO/CETPTg mice and 0.73 ± 0.08% body weight for SR-BIKO mice compared with 0.44 ± 0.02% body weight for WT mice; P < 0.01 and P < 0.05 versus SR-BIKO and SR-BIKO/CETPTg, respectively).

In addition to its effects on female fertility and erythrocyte metabolism, SR-BI deficiency is also associated with platelet abnormalities, including abnormally low platelet counts and impaired platelet aggregation. The decreased platelet counts in SR-BIKO mice (males, 257 ± 4 × 10^3/µL, P < 0.001; females, 444 ± 11 × 10^3/µL, P < 0.001) compared with WT animals (males, 1022 ± 81 × 10^3/µL; females, 955 ± 76 × 10^3/µL) was only partially normalized by expression of CETP in male SR-BIKO/CETPTg mice (539 ± 93 × 10^3/µL, P < 0.05) and not affected in females (479 ± 47 × 10^3/µL, Figure 5A). Furthermore, CETP expression did not normalize platelet aggregation induced by PAR-4 peptide that activates the thrombin receptor (Figure 5B).

Discussion

SR-BI is the sole receptor responsible for the delivery of HDL-CE to the liver in mice. In humans, however, CETP facilitates an alternate route for the delivery of HDL-CE to the
liver after transfer to VLDL and LDL and subsequent uptake by the LDL receptor or LDL receptor–related protein.\textsuperscript{23} In this study, we show that expression of CETP in SR-BI\textsuperscript{K0} mice indeed increased the delivery of HDL-CEt to the liver, probably after transfer to VLDL and LDL and partially normalized HDL-C levels and HDL particle size. Strikingly, in spite of these plasma lipid changes, the enhanced susceptibility of these animals to diet-induced atherosclerosis was not influenced. In addition, other pathologies that are associated with SR-BI disruption, including female infertility, reticulocytosis, thrombocytopenia, and impaired platelet aggregation, were not normalized by CETP. These findings indicate that the pathophysiology of SR-BI deficiency is not a direct consequence of the accumulation of the abnormally large HDL particles.

Previously, Harder et al\textsuperscript{16} did find a similar partial normalization of HDL-C in SR-BI\textsuperscript{K0} mice on expression of CETP under control of the human apoAI promoter. However, in contrast to our study, a decrease in atherosclerotic lesion development was observed. In these mice, CETP was exclusively expressed in the liver. In our study, Tg mice were used expressing human CETP under control of its own promoter and other major regulatory elements.\textsuperscript{17} These mice show CETP expression in liver, spleen, small intestine, kidney, adipose tissue, and macrophages.\textsuperscript{17} In addition, as also shown in this study, CETP expression is upregulated in response to high fat/high cholesterol diet feeding.\textsuperscript{17} The difference in expression patterns and regulation of CETP might thus have resulted in the differential outcome of the 2 studies.

SR-BI deficiency in mice results in a delayed clearance of HDL-CE from plasma and a diminished uptake by the liver.\textsuperscript{23} Under normal conditions, FC in HDL is rapidly esterified by HDL-associated LCAT.\textsuperscript{24} The abnormal large HDL particles that accumulate in SR-BI\textsuperscript{K0} mice, however, are poor substrates for LCAT,\textsuperscript{25} leading to an impaired LCAT activity\textsuperscript{26} and a marked increase in the FC/TC ratio in these animals. Expression of CETP in SR-BI\textsuperscript{K0} mice resulted in a partial normalization of HDL particle size by inducing the transfer of CE from HDL to VLDL/LDL. However, the FC/TC ratio remained elevated in the SR-BI\textsuperscript{K0}/CETP\textsuperscript{Tg} animals, and the LCAT activity was significantly increased only in females but not in males, indicating that the increased FC/TC ratio of SR-BI\textsuperscript{K0} mice is not solely the consequence of an impaired LCAT activity. SR-BI is not only essential for the selective delivery of HDL-CE to the liver,\textsuperscript{2,22} but has also been implicated in the bidirectional flux of FC between cells and HDL.\textsuperscript{27} The increased FC/TC ratio in SR-BI\textsuperscript{K0} mice might thus also be a direct consequence of the role of SR-BI in the flux of FC. Excess FC in cells is highly cytotoxic\textsuperscript{28} and FC loading is associated with macrophage death during the progression of atherosclerosis.\textsuperscript{29} The elevated FC/TC ratio might thus be causal for several of the pathologies observed in SR-BI\textsuperscript{K0} mice and not a direct effect of the abnormally large HDL particles. Concurrently, the female infertility was not restored in SR-BI\textsuperscript{K0}/CETP\textsuperscript{Tg} animals, despite normalization of the HDL particle size. Another remarkable phenotypic change in SR-BI\textsuperscript{K2} mice is an enhanced erythropoietic activity, associated with unusual erythrocyte morphology and abnormally high reticulocyte counts in blood. This is probably caused by impaired maturation and enhanced turnover of erythrocytes due to the abnormally high FC levels in the blood circulation, leading to accumulation of excess FC in the membranes of erythrocytes.\textsuperscript{19,20} In addition, SR-BI deficiency in mice leads to splenomegaly, probably because of hematopoietic compensation.\textsuperscript{19} CETP activity only partially normalized the reticulocyte counts in SR-BI\textsuperscript{K0} male mice, whereas in female SR-BI\textsuperscript{K0}/CETP\textsuperscript{Tg} mice, reticulocyte counts were not affected. Furthermore, the increased relative spleen weight of SR-BI\textsuperscript{K0} mice was not normalized by CETP expression. The increased FC/TC ratio in SR-BI\textsuperscript{K0} mice is also correlated with platelet abnormalities,\textsuperscript{21} including decreased platelet counts due to increased platelet clearance rate and impaired platelet aggregation. Expression of CETP in SR-BI\textsuperscript{K0} mice caused an increase in the amount of platelets in blood, although not to normal WT levels, and platelet aggregation was still impaired.

Importantly, the alternate route of HDL-CE delivery to the liver via CETP also did not provide protection against atherosclerosis in the SR-BI\textsuperscript{K0}/CETP\textsuperscript{Tg} mice. SR-BI, however, has also been implicated in several other processes that might have influenced atherosclerotic lesion development in SR-BI\textsuperscript{K0} and SR-BI\textsuperscript{K2}/CETP\textsuperscript{Tg} mice: (1) locally in the arterial wall, macrophage SR-BI induces early lesion development by facilitating the uptake of both modified and native lipoproteins, and it protects against the development of more advanced lesions, probably by stimulating cholesterol efflux\textsuperscript{30,31}; (2) SR-BI in endothelial cells facilitates the activation of endothelial nitric oxide synthase by HDL, leading to an increased bioavailability of the antiatherogenic signaling molecule nitric oxide\textsuperscript{32}; (3) SR-BI in the liver facilitates the removal of the proatherogenic lipoproteins (β)VLDL\textsuperscript{5} and chylomicrons\textsuperscript{2}; and (4) SR-BI protects against oxidative stress.\textsuperscript{10} The high atherosclerosis susceptibility of SR-BI\textsuperscript{K0}/CETP\textsuperscript{Tg} mice might thus simply be a direct consequence of the absence of SR-BI in cells of the arterial wall, including macrophages and endothelial cells. Previously, we have shown that macrophages from CETP\textsuperscript{Tg} mice do not show any alteration in cholesterol efflux to lipid-free apoAI or to HDL compared with those from WT mice.\textsuperscript{33} Therefore, it is unlikely that CETP expression will have affected the cholesterol efflux capacity of SR-BI deficient macrophages. Alternatively, VLDL-C levels and the oxidative status remained elevated and might thus have contributed to the atherosclerosis susceptibility of the SR-BI\textsuperscript{K0}/CETP\textsuperscript{Tg} animals. Interestingly, the oxidative status of SR-BI\textsuperscript{K0}/CETP\textsuperscript{Tg} mice remained increased despite decreased HDL particle size and partial normalization of HDL lipid composition by CETP. Thus, although small, dense HDL particles have a higher antioxidative activity than large HDL,\textsuperscript{34} the elevated oxidative status of SR-BI\textsuperscript{K0} mice appears not to be related to the presence of enlarged HDL particles in these animals. It may be possible that other mechanisms that are associated with SR-BI deficiency, such as impaired removal of oxidized lipids from the circulation\textsuperscript{10} and reduced bioavailability of dietary antioxidants such as beta carotene\textsuperscript{35} and α-tocopherol,\textsuperscript{36} are causative for the increased oxidative stress observed in SR-BI\textsuperscript{K0}/CETP\textsuperscript{Tg} mice.

In conclusion, although CETP activity can restore the transport of HDL-CE to the liver in SR-BI\textsuperscript{K0} mice, it cannot normalize many other processes associated with disruption of SR-BI in mice, nor the susceptibility to atherosclerosis. Importantly, these results indicate that the enhanced atherosclerosis in SR-BI\textsuperscript{K0} mice is not directly correlated with the accumulation of
large HDL in these animals but can merely be attributed to other consequences of SR-BI deficiency, including increased oxidative stress, increased plasma FC/TC ratio, and accumulation of VLDL-C. Furthermore, based on these findings, functional SR-BI mutations in humans who do naturally express CETP are expected to be associated with pathologies similar to those observed in SR-BI-KO mice.

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**Disclosures**

None.

**References**

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Supplemental Material

Supplemental Methods

Mice. Heterozygous SR-BI knockout mice (SR-BI^{KO}, C57Bl/6 N5), kindly provided by M. Krieger and CETP transgenic mice (CETP^{Tg}, strain 5203; C57Bl/6 N10), overexpressing human CETP under the control of its own promoter and other major regulatory elements (1), obtained from The Jackson Laboratory (Bar Harbor, ME, USA), were crossbred at the Gorlaeus Laboratory, Leiden, The Netherlands to generate SR-BI^{KO}/CETP^{Tg} mice. Mice were housed in filter-top cages and given unlimited access to food and water. Male and female mice were maintained on sterilised regular chow, containing 4.3% (w/w) fat and no added cholesterol (RM3, Special Diet Services, Witham, UK), or were fed a semi-synthetic Western-type diet (WTD), containing 15% (w/w) cacao butter, 1% corn oil, 0.25% (w/w) cholesterol, 20% casein, 40.5% sucrose, 5.95% cellulose, 20% cornstarch, 1% choline chloride, vitamins and minerals (Diet W, Special Diet Services, Witham, UK). Animal experiments were performed at the Gorlaeus Laboratory of the Leiden/Amsterdam Center for Drug Research in accordance with the National Laws. All experimental protocols were approved by the Ethics Committee for Animal Experiments of Leiden University.

Histological analysis. To induce the development of atherosclerosis in the aortic root, SR-BI^{KO}/CETP^{Tg} and SR-BI^{KO} mice were fed a WTD for 20 weeks. After sacrifice, the arterial tree was perfused in situ with phosphate-buffered saline (100 mm Hg) for 10 minutes via a cannula in the left ventricular apex. The heart plus aortic root was excised and stored in 3.7% neutral-buffered formalin (Formal-fixx^{®}, Shandon Scientific Ltd., UK). The mean atherosclerotic lesion area of each mouse was quantified from 10 oil red O-stained cryo-sections (10 µm), starting at the appearance of the tricuspid valves up to 300 µm of the ascending aorta using a Leica image analysis system, consisting of a Leica DMRE microscope coupled to a video camera and Leica Qwin Imaging software (Leica Ltd., Cambridge, UK). After perfusion the spleen was also excised and the weight was measured as percentage of bodyweight.

Plasma lipid analyses. After a 4 hour fasting-period, approximately 100 µL blood was drawn by tail bleeding and collected in ethylene diaminetetraacetic acid (EDTA)-coated tubes (Sarstedt, Numbrecht, Germany) from each individual mouse on chow diet and WTD. Free cholesterol concentration in plasma was determined by enzymatic colorimetric assay with 0.025 U/mL cholesterol oxidase (Sigma) and 0.065 U/mL peroxidase (Roche Diagnostics, Mannheim, Germany) in reaction buffer (1.0 KPi buffer, pH=7.7 containing 0.01 M phenol, 1 mM 4-amino-antipyrine, 1% polyoxyethylene-9-lauryl ether, and 7.5% methanol). Total cholesterol content was determined after addition of 0.003 U/mL cholesteryl esterase (Seikagaku Corporation, Japan). Absorbance was read at 490 nm. The distribution of cholesterol over the different lipoprotein particles in plasma was determined by fractionation of 30 µL plasma of each mouse using a Superose 6 column (3.2x300 mm, Smart-system, Pharmacia, Uppsala, Sweden). Total cholesterol content of the effluent was determined as above. VLDL-C, LDL-C, and HDL-C in plasma were calculated from the respective fractions (2-6, 7-11, and 12-22, respectively). Percentual HDL lipid composition was calculated after analysis of the free cholesterol, cholesterol ester,
phospholipid, and triglyceride content of pooled FPLC fractions corresponding to the HDL peak. Phospholipids (Instruchemie, Delfzijl, The Netherlands) and triglycerides (Roche Diagnostics, Mannheim, Germany) were determined by enzymatic colorimetric assays according to manufacturer’s instructions. HDL particle size was measured using a Zetasizer nanoseries (Malvern instrument Ltd, Malvern, United Kingdom).

**CETP and LCAT activity.** CETP activity in plasma was measured as the transfer/exchange of radio-labelled \(^{14}\text{C}\)-cholesteryl oleate (Amersham Biosciences, Piscataway, NJ) between exogenously added human LDL and HDL as described (2). Human HDL (1.063 g/mL < d < 1.21 g/mL) and LDL (1.019 g/mL < d < 1.063 g/mL) was isolated from blood of healthy subjects by differential ultracentrifugation as described by Redgrave et al. (3) and dialyzed against PBS with 1 mM EDTA. Radioactivity in HDL as a measure of transfer activity was determined by liquid scintillation counting. Activity is expressed in \(\mu\text{mol cholesteryl ester transferred/h/mL}\). The exogenous LCAT enzyme activity was measured using apoAI proteoliposomes, as previously reported (4) and the endogenous activity after equilibration of serum with trace amounts of \(^{3}\text{H}\)-cholesterol (5).

**Measurement of isoprostanes and carbonyls.** Urinary, serum, hepatic, splenic, and aortic arch isoprostane 8,12-iso-iPF\(_{2\alpha}\)-VI levels were measured by gas chromatography-mass spectrometry as described previously (6). Urine was collected during a 24 hour period. Blood samples were drawn, centrifuged at 7,000 rpm for 10 min, and serum was separated and stored at –80°C until analysis. Samples were spiked with a known amount of internal standard, extracted and purified by thin-layer chromatography, and analyzed by negative ion chemical ionization gas chromatography-mass spectrometry. The aortic arch, liver, and spleen from each individual mouse was obtained, weighed, minced, and homogenized in PBS containing EDTA (2 mM) and butylated hydroxytoluene (2 mM), pH 7.4, and total lipid was extracted using Folch solution (chloroform-methanol, 2:1, v/v). Next, base hydrolysis was performed using 15% KOH at 45°C for 1 h, and the total levels of 8,12-iso-iPF\(_{2\alpha}\)-VI were processed before analysis as described above. Total protein carbonyls were determined by using the Zenith test kit according to the manufacturer’s instructions (Zenith Technology, Dunedin, New Zealand) (7).

**Serum decay and liver uptake.** Human HDL was labeled with \(^{3}\text{H}\) cholesteryl oleoyl ether (CET) via exchange from donor particles as reported previously (8). Unilamellar liposome donor particles were formed by sonication of egg yolk phosphatidylcholine supplemented with 50 µCi of \(^{3}\text{H}\)CET (9,10). A dose of 215 µg apolipoprotein (± 1.2x10^6 dpm) of \(^{3}\text{H}\)CET-HDL (total volume of 200 µL) was injected into the tail vein of SR-BI KO and SR-BI KO/CETPTg mice. At 5 min after injection, a blood sample was drawn to verify the injected dose. At 1h, 2h, 4h, 6h, 8h, and 24h after injection blood samples were drawn to measure serum decay. Lipoprotein distribution of \(^{3}\text{H}\)CET-HDL was determined at 8h after injection by fractionation of 110 µL serum of a pool of three mice using a Superose 6 column (3.2x300 mm, Smart-system, Pharmacia, Uppsala, Sweden). For analysis of liver association, the liver was excised at 24h after tracer injection, weighed, solubilized, and counted for \(^{3}\text{H}\) radioactivity in a Packard liquid scintillation unit. A correction was made for the radioactivity in the blood present in the liver at the time of sampling as determined by injection of screened \(^{125}\text{I}\)BSA (84.7 µL/g wet weight).

**Blood cell analysis.** Blood was collected in ethylene diaminetetraacetic acid (EDTA)-coated tubes (Sarstedt, Numbrecht, Germany) by tail bleeding of mice after a 4 hour fasting-period. Whole blood samples (85 µL) were measured from mice on chow diet and on WTD, using an automated veterinary hematology analyzer, Sysmex XT-2000iV (Goffin Meyvis, Etten-Leur, the Netherlands) to investigate blood cell counts. For platelet isolation blood was collected into 0.1 volume of 130 mmol/L trisodium citrate by cardiac puncture. Platelet-rich plasma (PRP) was
obtained by centrifugation (300 g, 3 min, 20°C). The plasma and the buffy coat were gently transferred to a new tube, and PRP was concentrated by centrifugation a second time (700 g, 15 sec, 20°C). To obtain washed platelet suspensions, PRP was subsequently centrifuged (2000 g, 2 min, 20°C) in the presence of 0.1 volume of ACD buffer (2.5% (w/v) trisodium citrate, 1.5% (w/v) citric acid, and 2% (w/v) D-glucose in distilled water) and 10 ng/mL prostacyclin (PGI₂) from Cayman Chemical (Ann Arbor, MI). Pellets were resuspended in Hepes-Tyrode buffer (145 mmol/L NaCl, 5 mmol/L KCl, 0.5 mmol/L Na₂HPO₄, 1 mmol/L MgSO₄, 10 mmol/L Hepes, 5 mmol/L D-glucose, pH 6.5) and the washing procedure was repeated once. The platelet pellets were resuspended in Hepes-Tyrode buffer (pH 7.2) and the platelet count was adjusted with Hepes-Tyrode buffer to 2x10¹¹ platelets/L. For the platelet aggregation assay, washed platelet suspensions of WT, SR-BIKO, and SR-BIKO/CETPTg mice were stimulated with 0.5 mmol/L protease-activating receptor-4 (PAR-4) peptide from Bachem (Bubendorf, Switzerland). Optical aggregation was monitored in a Chrono-Log lumiaggregometer (Chrono-Log Corporation, Haverford, PA, USA) at 37°C and a stirring speed of 1000 rpm.

Statistical analyses. Statistical analyses were performed utilising the unpaired Student’s t-test and one-way analysis of variance (ANOVA) after confirmation of gaussian distribution using the test of Golmogorov and Smirnov (Instat GraphPad software, San Diego, USA).
Supplemental Results

Figure I. CETP activity in male SR-BI\textsuperscript{KO} mice did not affect exogenous and endogenous LCAT activity.
Endogenous LCAT activity in SR-BI\textsuperscript{KO} (35.9±4.4 nmol CE/h*\textsuperscript{mL}, white bar) mice did not change by CETP expression in male SR-BI\textsuperscript{KO}/CETP\textsuperscript{Tg} (31.6±0.6 nmolCE/h*\textsuperscript{mL}, black bar) animals. Similar result was observed for exogenous LCAT activity (SR-BI\textsuperscript{KO} 5.52±1.8 nmol CE/h*\textsuperscript{mL} and SR-BI\textsuperscript{KO}/CETP\textsuperscript{Tg} 5.82±1.1 nmol CE/h*\textsuperscript{mL}).
Figure II. The levels of carbonyls, a marker of protein oxidation, was increased in serum, spleen, and aorta of SR-Bi^KO/CETP^Tg mice.
A) In SR-Bi^KO/CETP^Tg (black bar) animals carbonyl levels were significantly increased (**P<0.01) compared to SR-Bi^KO (white bar) mice. B) In the spleen and the aorta the levels of carbonyls increased upon CETP expression in SR-Bi^KO/CETP^Tg mice as compared to SR-Bi^KO mice. In the liver no difference was observed between the two groups. **P<0.01 ***P<0.001.
Supplemental References


