Increased Oxidative Stress in Scavenger Receptor BI Knockout Mice With Dysfunctional HDL

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Objective—In the current study the effect of disruption of SR-BI, a prominent regulator of HDL metabolism, on the activity of the HDL-associated antioxidant enzymes PON1 and PAF-AH as well as in vivo oxidative stress were investigated.

Methods and Results—SR-BI deficiency resulted in 1.4-fold ($P<0.001$) and 1.6-fold ($P<0.01$) lower serum paraoxonase and arylesterase activity of PON1, respectively. Furthermore, a trend to slightly lower PAF-AH activity was observed. In vivo oxidative stress was evaluated by measuring isoprostane F2α-VI (iPF2α-VI) and protein carbonyls. Compared with wild-type animals, SR-BI knockout had 1.4-fold ($P<0.05$) higher levels of plasma iPF2α-VI, whereas urinary excretion was increased 2-fold ($P<0.0001$). Plasma carbonyls were 1.5-fold ($P<0.05$) higher in SR-BI knockout animals. Furthermore, iPF2α-VI and carbonyl levels were 2.1-fold ($P<0.01$) and 1.4-fold ($P<0.01$), respectively, increased in livers of SR-BI knockout mice, and in reaction to the increased oxidative stress the expression of several endogenous antioxidant systems was upregulated. On challenging the SR-BI knockout mice with an atherogenic Western-type diet, a further increase in oxidative stress in these animals was observed.

Conclusion—SR-BI deficiency results in a reduced activity of the antioxidant enzyme PON1 and a significant increase in oxidative stress, potentially contributing to the proatherogenic effect of SR-BI deficiency. (Arterioscler Thromb Vasc Biol. 2007;27:2413-2419.)

Key Words: HDL cholesterol ■ antioxidant enzymes ■ oxidative stress ■ isoprostanes ■ mouse models

HDL levels are inversely correlated with the risk for atherosclerosis. An important mechanism by which HDL inhibits the development and progression of atherosclerosis is its facilitating role in reverse cholesterol transport, a process by which excess cholesterol from peripheral tissues is transferred from the plasma to the liver for either recycling or excretion from the body via the bile. However, HDL also has multiple additional endothelial and antithrombotic actions that may provide cardiovascular protection. Furthermore, HDL may protect by inhibiting the oxidative modification of LDL, which plays a central role in the initiation and propagation of atherosclerosis. Several lines of evidence suggest that HDL can act as an antioxidant through the activity of the HDL-associated proteins, including paraoxonase 1 (PON1) and platelet-activating factor acetylhydrolase (PAF-AH).

PON1 protects against LDL oxidation, reverses the biological effects of oxidized LDL, and preserves the function of HDL by inhibiting its oxidation. The protective role of PON1 in atherosclerosis is clearly illustrated by the fact that PON1/apoE double knockout mice develop significantly larger atherosclerotic lesions as compared with apoE knockout mice with functional PON1. PAF-AH attenuates the potent proinflammatory activity of PAF by hydrolyzing its sn-2 ester bond. In addition, it also functions as an antioxidant enzyme by hydrolyzing oxidized phospholipids, such as F2-isoprostanes, formed during the oxidative modification of LDL. Increasing plasma PAF-AH levels in apoE knockout mice reduces injury-induced neointima formation and spontaneous atherosclerotic lesion development.

Although it is generally accepted that HDL protects against the development of atherosclerosis, high levels of HDL, however, are not always protective. In the Framingham Heart Study ≈45% of all clinical events occurred in subjects with normal or elevated HDL cholesterol levels, suggesting that not only the levels but also other HDL-associated factors are important. A prominent regulator of HDL metabolism is the scavenger receptor class B, type I (SR-BI), which mediates the selective uptake of cholesteryl esters from HDL without internalization of the HDL particle. Selective disruption of SR-BI in mice results in highly increased plasma cholesterol levels due to the accumulation of large cholesteryl ester droplets in the liver.
ester-rich HDL and is associated with an increased susceptibility to atherosclerosis.19–21 The proatherogenic effects of SR-BI deficiency have primarily been attributed to disruption of the flux of cholesterol through the reverse cholesterol transport pathway. In the present study, we demonstrate that SR-BI deficiency is also associated with reduced activity of the important endogenous antioxidant enzyme PON1 and a significant increase in oxidative stress in vivo, both of which could contribute to the increased susceptibility to atherosclerosis reported in these mice.

Materials and Methods
For detailed methodology, please see the data supplement, available online at http://atvb.ahajournals.org. Briefly, PAF-AH activity and the arylesterase and paraoxanase activity of PON1 were determined in serum of SR-BI knockout mice using substrate-based assays. PAF-AH and PON1 serum protein levels were determined by Western blotting, whereas the mRNA expressions of PAF-AH and PON1 were determined in spleen and liver respectively, using real-time Quantitative polymerase chain reaction (PCR). Urinary, EDTA-anticoagulated plasma, and tissue levels of the isoprostane iPF2α-VI were measured by gas chromatography-mass spectrometry and total protein carbonyls were determined in plasma and organs by using the Zenith PC test kit (Zenith Technology). The serum decay and liver uptake of oxidized cholesterol esters from HDL was studied after injection of 200 μg [H-ICEOH]-HDL in anesthetized mice.

Results
Decreased PON1 and PAF-AH Activity in SR-BI Knockout Mice
Selective disruption of SR-BI in mice results in the accumulation of large cholesterol ester-rich HDL particles.17,18 The activity of the HDL-associated protein PAF-AH was 599±41 nmol/mL/min in wild-type mice (n=12), whereas in SR-BI knockout mice PAF-AH activity was 508±20 nmol/mL/min (n=12, P=0.11; Figure 1A). Both in SR-BI knockout and in wild-type mice, the peak of the PAF-AH activity was mainly associated with large HDL, whereas there was no difference in HDL-associated PAF-AH activity (supplemental Figure I). PAF-AH protein levels in serum were reduced, probably as a result of reduced PAF-AH mRNA production as evidence by reduced PAF-AH mRNA expression in spleens of SR-BI knockout mice (Figure 1A). Interestingly, the activity of the HDL-associated protein PON1 was significantly reduced in absence of SR-BI (Figure 1B). The paraoxonase activity of PON1 was 1.4-fold (P<0.001) lower in SR-BI knockout mice (71±3 nmol/mL/min; n=12) as compared with wild-type controls (100±6 nmol/mL/min; n=12), whereas the arylesterase activity was 1.6-fold (P<0.01) lower (47±4 μmol/mL/min and 76±7 μmol/mL/min, respectively). In both groups of mice <1% of the PON1 activity was associated with apoB-containing lipoproteins (supplemental Figure I). In wild-type mice, 44% of the activity was associated to small HDL, as compared with only 26% in SR-BI knockouts. Large HDL contained 26% and 24% of the activity in wild-type and SR-BI knockout mice, respectively. Thus, the decrease in PON1 activity was primarily caused by a decrease in the activity of small HDL. Analysis of PON1 protein in serum showed that the reduced activity coincided with lower circulating protein levels (Figure 1B).

Because liver is the primary site for the production of PON1, we determined the effect of SR-BI deficiency on the hepatic mRNA expression of PON1 (Figure 1B). A trend to slightly reduced PON1 mRNA expression was observed, but this difference failed to reach statistical significance (1.5±0.2 and 1.3±0.1 for wild-type and SR-BI knockout mice, respectively).

Increased Lipid and Protein Oxidation in SR-BI Knockout Mice
Next, we investigated the effect of SR-BI deficiency on lipid and protein oxidation by measuring 2 distinct and specific markers, isoprostane F2α (iPF2α) and protein carbonyls, respectively.22,23 As shown in Figure 2A, compared with wild-type mice, SR-BI knockout mice had 1.4-fold higher levels of circulating plasma iPF2α-VI (269±23 pg/mL versus 367±37 pg/mL, P<0.05). Urinary excretion of iPF2α-VI was increased 2-fold in absence of SR-BI (1.95±0.14 versus 0.98±0.08 ng/mg creatinine, P<0.0001; Figure 2A). In addition to increased lipid oxidation, protein oxidation was also increased. Circulating levels of carbonyls in plasma
were 0.247±0.023 nmol/mL ($P<0.05$) in SR-BI knockouts and 0.167±0.019 nmol/mL in wild-type animals.

As shown in Figure 2B, iPF2α-VI levels were 2.1-fold ($P<0.01$) while carbonyls were 1.4-fold ($P<0.01$) higher in livers of SR-BI knockout mice than wild-type animals. In spleen and kidney a 1.4-fold ($P<0.05$) and 1.8-fold ($P<0.01$) increase in iPF2α-VI levels was observed, whereas carbonyls were 1.3-fold ($P<0.01$) and 1.5-fold ($P<0.05$) higher, respectively. In brain and aorta a trend to increased iPF2α-VI levels ($P=0.059$ and 0.051, respectively) was observed, whereas no effect was observed on carbonyl levels. In intestine, iPF2α-VI levels were unaltered, whereas carbonyls were 1.3-fold ($P<0.05$) higher. No significant effect was observed on either iPF2α-VI levels or carbonyls in the ovaries (Figure 2B) or testes (data not shown) of SR-BI knockout mice.

**Effect of SR-BI Deficiency on the Expression of Antioxidant Enzymes in Livers**

Because liver of SR-BI knockout mice showed a significant increase in both lipid and protein oxidation, the antioxidant defense of this organ was evaluated by assessing different antioxidant systems. As shown in Figure 3, the mRNA expression of the glutathione peroxidases GPx1 and GPx4 were 1.7-fold ($P<0.001$) and 1.2-fold ($P<0.05$) higher in livers of SR-BI knockout than wild-type mice. Similarly, superoxide dismutase SOD1 and SOD2 levels were 1.2-fold ($P<0.05$) and 1.3-fold ($P<0.05$) higher in absence of SR-BI. Also the expression of the glutathione S-transferases GSTA2 and GSTA4, which reduce lipid peroxidation products, were 1.7-fold ($P=0.075$) and 1.8-fold ($P<0.05$) higher in mice lacking SR-BI, whereas no effect was observed on GSTA3. Heme oxygenase (HO), which is involved in the removal of free heme (a prooxidant) and the production of bilirubin (an antioxidant), showed a 3-fold increase in SR-BI knockout animals ($P=0.051$). No effect was observed on the expression of catalase.

**Importance of SR-BI for the Removal of HDL-Associated Oxidized Cholesterol Esters by the Liver**

Previous studies have suggested a role for SR-BI in the removal of oxidized cholesterol esters from HDL.24,25 To study the direct role of SR-BI in the removal of oxidized
cholesterol esters from HDL in vivo, the kinetics of the serum decay and liver uptake of \([3H-\text{CEOH}]\) labeled HDL were determined in SR-BI–deficient mice and wild-type littersmates. At 45 minutes after injection 67±2% of the injected trace amount of \([3H-\text{CEOH}]\)-HDL was cleared from the circulation in SR-BI\(^{-/-}\) mice, as compared with 58±4% in SR-BI\(^{+/+}\) mice (Figure 4). In both wild-type and SR-BI–deficient mice the maximum association value for \([3H-\text{CEOH}]\)-HDL to the liver was reached at 20 minutes after injection. At this time point 27±3% of the injected dose of \([3H-\text{CEOH}]\)-HDL was taken up by the liver in wild-type mice, as compared with only 17±1% (\(P<0.05\)) in SR-BI–deficient mice, indicating that SR-BI expression in the liver facilitated the removal of oxidized cholesterol esters from HDL.

Challenging SR-BI Knockout Mice With an Atherogenic Western-Type Diet, Containing 0.25% Cholesterol and 15% Total Fat, Further Increases Oxidative Stress

To analyze the possible relationship between the increased oxidation status in SR-BI knockout mice and their susceptibility to atherosclerosis, the effect of Western-type diet feeding on lipid and protein oxidation was determined after 4 weeks diet feeding. On chow diet monocytes chemoattractant protein 1 (MCP-1) levels, indicative of inflammation were 36±6 pg/mL and 37±5 pg/mL in SR-BI knockout and wild-type mice, respectively, indicating that the observed increased oxidative stress in the SR-BI knockout animals is independent from the
presence of systemic inflammation. Furthermore, no increase in MCP-1 levels was observed in SR-BI knockout animals on Western-type diet (36±6 pg/mL).

Isoprostanes are excreted in the urine, and urine thus provides a global measure of oxidative stress. Western-type diet feeding induced a 1.5-fold ($P<0.05$) increase in urinary iPFP2α-VI in wild-type mice (Figure 5A). Although basal urinary iPFP2α-VI were already higher in SR-BI knockout mice under Chow conditions, Western-type diet feeding induced a 2-fold ($P<0.0001$) increase in urinary iPFP2α-VI levels. Under these conditions urinary excretion of iPFP2α-VI was thus 2.6-fold ($P<0.001$) higher in SR-BI knockout mice as compared with wild-types (4.00±0.15 versus 1.53±0.20 ng/mg creatinine, respectively; Figure 5A). Western-type diet feeding did not further increase iPFP2α-VI and carbonyls in plasma. Of the analyzed organs, especially brain, spleen, kidney, intestine, aorta, and ovary displayed enhanced accumulation of iPFP2α-VI on Western-type diet feeding (Figure 5B). In addition, carbonyls were further increased in liver, kidney, intestine, and aorta.

Western-type diet feeding induced a ≈2.4-fold increase in the paraoxonase activity of PON1 in both SR-BI knockout and wild-type mice. As a result, also under these conditions the paraoxonase activity of PON1 was 1.3-fold ($P<0.01$) lower in SR-BI knockout mice (175±4 nmol/mL/min; n=6) as compared with wild-type controls (234±10 nmol/mL/min; n=6). In addition, the arylesterase activity of PON1 was 1.3-fold ($P<0.01$) lower in SR-BI knockout mice compared with wild-type animals. No significant difference was observed in PAF-AH activity under these conditions.

Discussion

Oxidative stress is the result of an imbalance between increased generation of reactive oxygen species (ROS) and the decreased ability of endogenous antioxidant systems to scavenge them. ROS induce cell, tissue, or organ damage and are involved in the pathogenesis of several diseases, including atherosclerosis and diabetes. In the current study we show for the first time a marked enhancement of oxidative stress in SR-BI knockout mice. SR-BI is a multifunctional receptor capable of binding a wide array of native and oxidatively modified lipoproteins. It is a prominent regulator of HDL metabolism, and selective disruption of SR-BI in mice results in increased plasma cholesterol levels attributable to the accumulation of large cholesteryl ester-rich HDL particles and an increased susceptibility to atherosclerosis.19–21 In addition, SR-BI knockouts develop reticulocytosis,27,28 and female mice lacking SR-BI are infertile because of a defective maturation of oocytes.26 Interestingly, the pathologies observed in SR-BI knockout mice can all be reversed by treatment with the HDL-lowering antioxidant probucol.29,30

To protect the cells and organ systems of the body against ROS, a highly complex antioxidant protection system has evolved, including enzymes such as SOD and catalase, and dietary antioxidants, like α-tocopherol and beta carotene. In the current study, we show that as a reaction to the increased oxidative stress several endogenous antioxidant systems in the SR-BI knockout mice are upregulated, including glutathione peroxidases, superoxide dismutases, and glutathione S-transferases. Interestingly, by microarray expression profiling on livers of SR-BI transgenic animals, lacking HDL, Callow et al also observed an increased expression of glutathione S-transferase.31 This finding supports the hypothesis that in SR-BI knockout mice with dysfunctional HDL and possibly also in SR-BI transgenic mice without any HDL the amount of ROS produced exceeds the capacity of those enzyme systems to counteract them or that the removal system for oxidants from the circulation is hampered. This concept is corroborated by the observation that the activity of the important HDL-associated antioxidant enzyme PON1 was markedly reduced in SR-BI knockout mice. The reduced PON1 activity in SR-BI knockout mice coincided with lower circulating protein levels and a trend to slightly reduced mRNA levels of PON1 in livers of SR-BI knockout mice, indicating that the reduced activity might be partly explained by lower production of PON1. In an elegant report by Deakin et al it was recently shown that oxidation of HDL decreases its ability to remove PON1 from cells and compromises its ability to stabilize the enzyme activity.32 Thus, the increased oxidation status of circulating HDL in SR-BI knockout mice, as evidenced by increased plasma iPFP2α-VI levels, will most likely have attributed to the decreased PON1 protein and activity in the circulation.

Previously, we have shown that feeding SR-BI knockout mice an atherogenic Western-type diet for 20 weeks induces atherosclerotic lesion development, whereas no lesion development was observed in wild-type animals.20 On challenging the SR-BI knockout mice with this Western-type diet, a further increase in oxidative stress in these animals was observed, particularly evidenced by an increased urinary iPFP2α-VI secretion, a global measure of oxidative stress. Interestingly, under these conditions also a dramatic increase in oxidative stress in the aorta was observed. This increased oxidative stress was observed after only 4 weeks Western-type diet feeding. MCP-1 levels were not induced in the SR-BI knockout animals, suggesting that oxidative stress precedes this inflammatory marker in the pathogenesis of atherosclerosis. A significant negative correlation has been demonstrated between HDL-PON1 activity and the levels of lipid hydroperoxides associated with HDL from healthy subjects.33 The physiological significance of this HDL-associated enzyme with antioxidative activity is further emphasized by the association between low plasma PON1 activity and the risk for cardiovascular disease34 and by the fact that overexpression of PON1135 decreases oxidative stress and reduces atherosclerosis in mice.

In addition to the observed reduced PON1 activity also other factors might contribute to the increased oxidative stress in SR-BI knockout mice. Oxidized lipoproteins are known ligands for SR-BI.36 Previously we have shown that the selective uptake of HDL-associated oxidized cholesterol esters could be efficiently blocked by oxidized LDL and phosphatidylserine liposomes to a similar extent as native cholesterol ester uptake, suggesting an important role for SR-BI in the removal of oxidized cholesterol from HDL.24,25 Furthermore, oxidized cholesterol esters were selectively taken up by Chinese hamster ovary cells transfected with
SR-BI. Absence of SR-BI might thus also directly have impaired the removal of oxidized lipids from the circulation. In agreement, we now provide definite proof that SR-BI deficiency results in an impaired uptake of oxidized cholesterol esters from HDL. The effect of SR-BI deficiency on oxidized cholesterol ester clearance from HDL, however, was less dramatic as previously shown for native cholesterol esters, suggesting that in addition to SR-BI additional HDL binding sites on the liver are involved in the removal of oxidized cholesterol esters from HDL. Furthermore, although the impaired clearance of oxidized cholesterol esters from HDL in absence of SR-BI could provide an alternate explanation for the accumulation of oxidized lipids in plasma of these animals, it does not explain the enhanced oxidation status of the liver.

Finally, SR-BI has been implicated in the intestinal absorption of the lipophylic dietary antioxidants beta carotene and alpha-tocopherol. Furthermore, SR-BI mediates the uptake of alpha-tocopherol by tissues. As a result, biliary secretion and the levels of alpha-tocopherol in selected tissues such as brain, lung, and gonads were decreased, whereas circulating alpha-tocopherol levels were increased in SR-BI knockout mice. Thus, reduced bioavailability of these dietary antioxidants might also have contributed to the increased oxidative stress observed in SR-BI knockout mice.

In conclusion, SR-BI deficiency results in a pronounced oxidative imbalance in vivo, which culminates in increased oxidative stress. This increase is, at least in part, the result of a reduced activity of its HDL-associated antioxidant enzyme PON1 and may ultimately contribute to the proatherogenic effect of SR-BI deficiency. Interestingly, in humans coronary heart disease risk associated with a C1050T polymorphism in exon 8 of CLA-1 (C8C8), the human homologue of SR-BI, was confined to a subset of individuals with Gln192Arg and Met55Leu polymorphisms in PON1, indicating that also in humans a clear association exists between SR-BI, PON1, and coronary heart disease.

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Disclosures

None.

References


21. Calvo D, Gomez-Coronado D, Lasuncion MA, Vega MA. CLA-1 is an 85-kD plasma membrane glycoprotein that acts as a high-affinity receptor for both native (HDL, LDL, and VLDL) and modified (OxLDL and AcLDL) lipoproteins. *Arterioscler Thromb Vasc Biol.* 1997;17:2341–2349.


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ONLINE SUPPLEMENTAL METHODS

Mice - Class B, type I scavenger receptor, SR-BI, knockout mice were kindly provided by Dr. M. Krieger. In these mice, the entire coding region of the first coding exon, constituting the N-terminal cytoplasmic domain and a portion of the N-terminal transmembrane domain, were deleted leading to a functionally null allele (1). Heterozygous SR-BI knockout mice were crossed to generate wild-type (SR-BI+/+) and homozygous mutant (SR-BI−/−) progeny. The presence of the targeted and/or wild-type SR-BI alleles was determined by PCR amplification of DNA extracted from tail biopsies. The primers 5′-GAT-GGG-ACA-TGG-GAC-ACG-AAG-CCA-TTC-T-3′ and 5′-TCT-GTC-TCC-TCT-TCC-TTC-AGG-TCC-TGA-3′ were used to detect the presence of the targeted and the wild-type SR-BI allele simultaneously. PCR results in a 1.0-kb band and a 1.5-kb amplification product diagnostic of the wild-type and the targeted allele, respectively. Mice were maintained on regular chow diet containing 4.3% (w/w) fat and no cholesterol (RM3, Special Diet Services, Witham, UK), or were fed a semi-synthetic Western-type diet, containing 15% (w/w) fat and 0.25% (w/w) cholesterol (Diet W, Special Diet Services, Witham, UK). Animal experiments were performed at the Gorlaeus laboratories 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.

Lipid analyses - After an overnight fasting-period, approximately 100 µl blood was drawn from each individual mouse by tail bleeding. The concentrations of free cholesterol in serum were determined by enzymatic colorimetric assays 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% (v/v) polyoxyethylene-9-laurylether, and 7.5% (v/v) methanol). Total cholesterol content was determined after addition of 15 µg/mL cholesteryl esterase (Roche Diagnostics). Absorbance was read at 490 nm.

PAF-AH activity - Hexadecyl PAF (Sigma Chemical Co), dissolved at a concentration of 20 mmol/L in ethanol (80% vol/vol) was mixed with 1-O-hexadecyl-2-[3H-acetyl]-sn-glycero-3-phosphocholine (10 Ci/mmol, DuPont-New England Nuclear), dried under N2, and redissolved in a solution containing fatty acid-free bovine serum albumin (0.25% w/v in saline) to obtain a 50 µmol/L [3H-acetyl]PAF solution. PAF-acetylhydrolase (PAF-AH) activity was measured by the trichloroacetic acid precipitation procedure as previously described (2). The assays were performed in a final volume of 100 µL using 2000-fold diluted serum samples and 10-fold diluted lipoprotein fractions in HEPES-EDTA (2 mmol/L) buffer, pH=7.4 and 10 µL of [3H-acetyl]PAF (50 µmol/L; specific activity ~ 6000 dpm per nmol).

Arylesterase and paraoxanase activity of PON1 - Arylesterase activity was measured using phenylacetate as a substrate (3). Initial rates of hydrolysis were determined spectrophotometrically at 270 nm in a Power Wave 200 microplate scanning spectrophotometer (Bio-Tek Instruments). The assays were performed in a final volume of 250 µL containing 1 mmol/L phenylacetate...
and 2 mmol/L CaCl₂ in 20 mmol/L Tris-HCl buffer, pH 8.0, in the presence of 0.1 µL of mouse serum or 10 µL of lipoprotein fraction for 5 minutes. The extinction coefficient at 270 nm for the reaction was 1307 mol/L⁻¹.cm⁻¹ for 1 µmol of phenyl acetate hydrolysed per minute. The rate of hydrolysis of paraoxon was assessed by determining the liberation of p-nitrophenol at 405 nm at 25°C (4). The assays were performed in a final volume of 250 µL containing 5.5 mmol/L paraoxon and 2 mmol/L CaCl₂ in 100 mmol/L Tris-HCl buffer, pH 8.0, in the presence of 2-4 µL of mouse serum for 4 minutes. The extinction coefficient at 405 nm for the reaction was 17000 mol/L⁻¹.cm⁻¹ for 1 nmol of p-nitrophenol converted per minute.

**Lipoprotein distribution analyses** - The distribution of cholesterol and PAF-AH and PON1 activities over the different lipoproteins in serum was determined by fractionation of 150 µL pooled serum of 4 mice using a Superose 6 column (3.2x300 mm, Smart-system, Pharmacia, Uppsala, Sweden). Total cholesterol content and PAF-AH and PON1 activities of the effluent were determined as above.

**Determination of iPF2α-VI and carbonyl levels** - Urinary, EDTA-anticoagulated plasma, and tissue levels of the isoprostane iPF2α-VI were measured by gas chromatography-mass spectrometry (GC/MS) as described previously (5,6). Urine was collected during a 24 h period. Blood samples were collected, centrifuged at 7,000 rpm for 10 min, and plasma 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. Tissue samples were minced and homogenized, and total lipids were extracted with ice-cold Folch solution (chloroform/methanol; 2:1, v/v). The solution was then vortex-mixed and centrifuged at 800 × g for 15 min at 4 °C. An aliquot of the extracts was used to measure free iPF2α-VI levels. The remaining extracts were subjected to base hydrolysis by adding aqueous 15% KOH and then incubated at 45 °C for 1 h for measurement of total iPF2α-VI by ion chemical ionization GC/MS assay, as previously described (5,6). D4-iPF2α-VI was used as internal standard for the analysis. Total protein carbonyls were determined by using the Zenith PC test kit according to the manufacturer's instructions (Zenith Technology, Dunedin, NZ) (7,8). Briefly, tissue homogenates were first reacted with dinitrophenylhydrazine (DNPH), transferred to a multi-well plate, incubated with blocking reagent, washed and probed with anti-DNPH-biotin antibody solution. After washing, samples were incubated with streptavidin-horseradish peroxidase, washed, and then developed. After 15 min, the reaction was stopped and absorbance immediately read at 450 nm. Oxidized protein standards, internal controls and blanks were always assayed at the same time and in the same way.

**Analysis of gene expression by Real-Time Quantitative PCR** - Livers from SR-BI+/+ and SR-BI−/− mice were isolated after whole-body perfusion using phosphate-buffered saline via a cannula in the left ventricle of the heart, frozen in liquid N₂, and stored at −80°C until RNA isolation. Total RNA was extracted by the acid guanidinium thiocyanate-phenol chloroform extraction method, according to Chomczynski and Sacchi (9). cDNA was synthesized from 0.5-1 µg of total RNA using RevertAid™ M-MuLV reverse transcriptase. Quantitative gene expression analysis was performed on an ABI Prism 7700 sequence detection system (Applied Biosystems, Foster City, CA) using SYBR-green technology, according to manufacturer’s instructions. PCR primers (Table I) were designed using Primer Express 1.7 software with the manufacturer’s default settings (Applied Biosystems).
Table I: Primers for real-time PCR analysis

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<td>CCTCTGGGCGGGGATGCC</td>
<td>TGGAAAATCTCCACCTTCC</td>
<td>70</td>
</tr>
<tr>
<td>SOD1</td>
<td>AH002084</td>
<td>ACCAGTGGACGCTCATTCAA</td>
<td>TCTCCACATGCTTCATTTCA</td>
<td>77</td>
</tr>
<tr>
<td>SOD2</td>
<td>AH004779</td>
<td>CACTTAAACGCCGAGATCAG</td>
<td>CCAGAGCTCTGGATGTCCTCC</td>
<td>131</td>
</tr>
<tr>
<td>β-actin</td>
<td>X03672</td>
<td>AACCAGTGCTGAAAGGATCAG</td>
<td>CACACGCTGGAGGATCCTG</td>
<td>75</td>
</tr>
<tr>
<td>36B4</td>
<td>NM_007475</td>
<td>GACGGCGAGGGACCTCCTTT</td>
<td>GACATCAGGAGGATTTCTGAGT</td>
<td>85</td>
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<tr>
<td>Cyclophilin</td>
<td>AK010338</td>
<td>CCATTTCAAGAAGACGGGTTT</td>
<td>ATTTTGTCATTACTGTTG</td>
<td>131</td>
</tr>
<tr>
<td>GAPDH</td>
<td>NM_008084</td>
<td>TCCATGCAACACTTGTGATTT</td>
<td>TCACGCCACAGTCTTCCA</td>
<td>103</td>
</tr>
<tr>
<td>HPRT</td>
<td>J00423</td>
<td>TTGCTCGAGATGTCATGAGGA</td>
<td>AGCAGTGCAGAAAGAATTATAG</td>
<td>91</td>
</tr>
</tbody>
</table>

The relative gene expression of the target gene was calculated by subtracting the Ct of the target gene from the average Ct of the housekeeping genes (β-actin, 36B4, cyclophilin, GAPDH, and HPRT) and raising 2 to the power of this difference. Ct values are defined as the number of PCR cycles at which the fluorescent signal during the PCR reaches a fixed threshold.

**Immunoblot analysis** - A volume of 1 µl of serum was separated on 10% SDS-PAGE gels under nonreducing conditions and electrophoretically transferred to Protran nitrocellulose membrane (Schleicher&Schnell, Dassel, Germany). Immunolabelling was performed using goat polyclonal anti-PON1 or anti-PAF-AH (PON1 (N-20) and PAF-AH (G-18), respectively, Santa Cruz Biotechnology, USA) as primary antibody and anti-goat IgG (Jackson ImmunoResearch, USA) as secondary antibodies. Finally, immunolabelling was detected by enhanced chemiluminescence (ECL, Amersham Bioscience, UK).

**MCP-1 ELISA** - MCP-1 levels in serum were analysed using a mouse MCP-1 ELISA (eBioscience, San Diego, USA) according to manufacturer’s instructions.

**Serum decay and liver uptake of [3H-CEOH]-HDL** – 3H-cholesteryl-linoleate-hydroxyperoxide [3H-CEOH] was prepared by peroxidation of cholesterol-1,2,6,7-[3H(N)]-linoleate (Amersham, Buckinghamshire, UK) with a lipid soluble peroxyl radical generator 2,2'-azobis-(2,4-dimethyl-valeronitrile) as previously described (10,11). HDL was isolated from the blood of healthy volunteers by differential ultracentrifugation as described by Redgrave et al (12) and labeled with [3H-CEOH] by exchange from donor particles as previously reported (13). The serum decay and liver uptake of [3H-CEOH] from HDL was studied after injection of 200 µg [3H-CEOH]-HDL in anesthetized mice. At the indicated times, blood samples were drawn and liver lobules were tied off and excised. The radioactivity in liver was corrected for serum present at the time of sampling as determined by the distribution of 125I-BSA.

**Statistical analyses** - Statistical significant differences among the means of the different
populations were tested using the Students’ t-test (Graphpad Instat software, San Diego, USA).

REFERENCES


ONLINE SUPPLEMENTAL FIGURE

Supplemental Figure I. Effect of SR-BI deficiency on the cholesterol, PAF-AH, and PON1 distribution over the different lipoproteins. Blood samples were drawn after an overnight fast while feeding regular chow diet. Sera were loaded onto a Superose 6 column and fractions were collected. Fractions 3 to 13 represent apoB-containing lipoproteins; fractions 14 to 18, abnormally large HDL; and fractions 19 to 22 small HDL, respectively. The distribution of cholesterol (top), PAF-AH activity (middle), and PON1 arylesterase activity (bottom) over the different lipoproteins in SR-BI+/+ (○) and SR-BI−/− (●) are shown. Values represent a pool of 4 mice.