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Hoekstra, M.; Sluis, R.J. van der; Hildebrand, R.B.; Lammers, B.; Zhao, Y.; Pratico, D.; ... ; Eck, M. van

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BASIC SCIENCES

Disruption of Phospholipid Transfer Protein–Mediated High-Density Lipoprotein Maturation Reduces Scavenger Receptor BI Deficiency–Driven Atherosclerosis Susceptibility Despite Unexpected Metabolic Complications

Menno Hoekstra,* Ronald J. van der Sluis,* Reeni B. Hildebrand, Bart Lammers, Ying Zhao, Domenico Praticò, Theo J.C. van Berkel, Patrick C.N. Rensen, Sander Kooijman, Matti Jauhiainen, Miranda van Eck

OBJECTIVE: We tested the hypothesis that enlarged, dysfunctional HDL (high-density lipoprotein) particles contribute to the augmented atherosclerosis susceptibility associated with SR-BI (scavenger receptor BI) deficiency in mice.

APPROACH AND RESULTS: We eliminated the ability of HDL particles to fully mature by targeting PLTP (phospholipid transfer protein) functionality. Particle size of the HDL population was almost fully normalized in male and female SR-BI×PLTP double knockout mice. In contrast, the plasma unesterified cholesterol to cholesteryl ester ratio remained elevated. The PLTP deficiency-induced reduction in HDL size in SR-BI knockout mice resulted in a normalized aortic tissue oxidative stress status on Western-type diet. Atherosclerosis susceptibility was—however—only partially reversed in double knockout mice, which can likely be attributed to the fact that they developed a metabolic syndrome-like phenotype characterized by obesity, hypertriglyceridemia, and a reduced glucose tolerance. Mechanistic studies in chow diet–fed mice revealed that the diminished glucose tolerance was probably secondary to the exaggerated postprandial triglyceride response. The absence of PLTP did not affect LPL (lipoprotein lipase)-mediated triglyceride lipolysis but rather modified the ability of VLDL (very low-density lipoprotein)/chylomicron remnants to be cleared from the circulation by the liver through receptors other than SR-BI. As a result, livers of double knockout mice only cleared 26% of the fractional dose of [^{14}C]cholesteryl oleate after intravenous VLDL-like particle injection.

CONCLUSIONS: We have shown that disruption of PLTP-mediated HDL maturation reduces SR-BI deficiency-driven atherosclerosis susceptibility in mice despite the induction of proatherogenic metabolic complications in the double knockout mice.

VISUAL OVERVIEW: An online visual overview is available for this article.

Key Words: atherosclerosis ■ diet, high fat ■ hypertriglyceridemia ■ mice ■ obesity

HDLs (high-density lipoproteins) mediate reverse cholesterol transport, that is, the mobilization of excess cholesterol from peripheral cells for subsequent excretion via the liver. This process is considered as one of the major atheroprotective functions of HDL. SR-BI (scavenger receptor class B type I) is an important player in the reverse cholesterol transport pathways as it facilitates the selective removal of cholesteryl esters from HDL particles by a wide variety of cells, including hepatocytes, arterial wall macrophages,

Correspondence to: Menno Hoekstra, Division of BioTherapeutics, Leiden Academic Centre for Drug Research, Gorlaeus Laboratories, Einsteinweg 55, 2333CC Leiden, The Netherlands. Email hoekstra@lacdr.leidenuniv.nl

*These authors contributed equally to this article.

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Nonstandard Abbreviations and Acronyms

apoA1	apolipoprotein A1
apoE	apolipoprotein E
BPI	bactericidal/permeability increasing protein
CETP	cholesteryl ester transfer protein
DKO	double knockout
HDL	high-density lipoprotein
HL	hepatic lipase
LBP	lipopolysaccharide-binding protein
LPL	lipoprotein lipase
LRP1	LDL receptor-related protein 1
PLTP	phospholipid transfer protein
SR-BI KO	SR-BI knockout
SR-BI	scavenger receptor BI
VLDL	very low-density lipoprotein

and adrenocortical cells.[1,2] SR-BI deficiency is therefore associated with the accumulation of relatively large, cholesterol-enriched HDL particles in the blood circulation[3,4] and a diminished availability of cholesterol for flux into the bile compartment[5] and the production of glucocorticoids by the adrenals.[4,6–9] SR-BI knockout (SR-BI KO) mice are also characterized by markedly increased plasma levels of unesterified cholesterol, which is associated with splenomegaly, anemia, reticulocytosis, thrombocytopenia, and an altered platelet reactivity.[10–13] While wild-type mice are virtually resistant to the development of atherosclerosis in response to a Western-type diet challenge, a SR-BI KO mice do develop atherosclerotic lesions after 20 weeks on Western-type diet.[14] Notably, thus far, the exact cause of the SR-BI deficiency-associated predisposition to atherosclerosis remains unknown but is likely related to the impairment of the reverse cholesterol transport pathway. We have previously shown that the antioxidant capacity of HDL particles is diminished in SR-BI KO mice, which translates into a higher (tissue) oxidative stress status on Western-type diet.[15] Based upon this latter finding, we hypothesize that the increased susceptibility to atherosclerosis is secondary to the presence of circulating large (dysfunctional) HDL particles.

PLTP (phospholipid transfer protein) is a ubiquitously expressed monomeric protein of 81 kDa that belongs to the family of lipid transfer/lipopolysaccharide-binding proteins that includes CETP (cholesteryl ester transfer protein), LBP (lipopolysaccharide-binding protein), and BPI (bactericidal/permeability increasing protein).[16,17] Within the plasma compartment, PLTP can be associated with apoA1 (apolipoprotein A1)-containing lipoproteins, that is, HDL, as well as apoE (apolipoprotein E)-containing lipoproteins such as the triglyceride-rich

Highlights

- Genetic lack of PLTP (phospholipid transfer protein) reverses SR-BI (scavenger receptor BI) deficiency-associated HDL (high-density lipoprotein) particle enlargement.
- PLTP deficiency does not fully overcome atherogenesis in SR-BI KO mice.
- SR-BI×PLTP double knockout mice on a high-fat diet develop metabolic complications.
- PLTP deficiency is associated with a higher postprandial triglyceride response in mice.

VLDL (very-low-density lipoproteins) and chylomicrons.[18,19] PLTP-mediated transfer of phospholipids, liberated during lipolysis of triglycerides in the core of these particles, to HDL is a crucial step in the maturation of HDL particles.[20] To verify the causal association of the enlarged HDL particles to the SR-BI deficiency-associated atherosclerotic phenotype, we therefore determined the impact of attenuating HDL maturation by deletion of PLTP function on atherosclerosis susceptibility in SR-BI KO mice.

MATERIALS AND METHODS

Animals

All animal work was performed at the Gorlaeus Laboratories of the Leiden Academic Centre for Drug Research in compliance with Dutch government guidelines and the Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. The Dutch Ethics Committee and regulatory authority at Leiden University approved all experiments. Mice were given unlimited access to food and water and subjected to a controlled 12-hour light/dark cycle. SR-BI KO mice, originally generated by the laboratory of Dr Monty Krieger,[3] and PLTP KO mice (both C57BL/6J background) were crossbred to generate SR-BI×PLTP double knockout (DKO) mice. To study atherosclerosis susceptibility, 14-week-old male and female C57BL/6J wild-type (WT), SR-BI KO, and DKO mice were challenged with a Western-type diet containing 0.25% (w/w) cholesterol, 15% (w/w) cacao butter, and 1% (w/w) corn oil (Diet W, Special Diet Services, Witham, United Kingdom) for 20 weeks. After sacrifice, the mice were perfused in situ with PBS (100 mm Hg) for 10 minutes via a cannula in the left ventricular apex. The heart plus aortic root were excised and stored in 3.7% neutral-buffered formalin (Formal-fixx, Shandon Scientific, Ltd, United Kingdom) to analyze the degree of atherosclerosis. In a second study focused on the development of the metabolic syndrome-type stage, WT, SR-BI KO, PLTP KO, and DKO mice were maintained on a normal laboratory diet (Diet RM3 [E] DU; Special Diet Services, Witham, United Kingdom) until their sacrifice at 34 weeks of age. In both studies, a selection of other organs such as the liver, spleen, adrenals, and white and brown adipose tissue were also excised after perfusion, weighed, and stored in formalin or at −20°C for further analysis.

Radiolabeled HDL-Cholesteryl Ester Flux Analysis

The kinetics of cholesteryl ether labeled HDL ([^{3}H]CEt-HDL) were measured in male WT, SR-BI, KO, and DKO mice essentially as described previously.[1] HDL (1.063<d<1.21 g/mL) was isolated from healthy human subjects and labeled with [^{3}H]CEt via exchange from donor particles with a density of 1.03 g/mL (mass ratio of HDL protein/particle phospholipid 8:1) in the presence of human lipoprotein-deficient serum as the CETP source. Radiolabeled HDL was then isolated by density gradient ultracentrifugation. A dose of 215 µg apolipoprotein (≈1.2×10^6 dpm) of [^{3}H]CEt-HDL (total volume of 200 µL) was injected into the tail vein of mice. At 5 minutes after injection, a blood sample was drawn to verify the injected dose. At 1, 2, 4, 6, 8, and 24 hours after injection, blood samples were drawn to measure plasma decay. The liver and adrenals were excised at 24 hours after injection for radioactivity measurements.

Plasma Lipid Analysis

After 4 hours of fasting, ≈100 µL blood was drawn from each individual mouse by tail bleeding and collected in ethylenediaminetetraacetic acid-coated tubes (Sarstedt, Numbrecht, Germany). Unesterified cholesterol and cholesteryl ester concentrations in plasma as well as the distribution of lipids over the different lipoproteins were determined by an enzymatic colorimetric assay as previously described.[21] Phospholipids (Instruchemie, Delfzijl, The Netherlands) and triglycerides (Roche Diagnostics, Mannheim, Germany) were measured by enzymatic colorimetric assays according to manufacturers' instructions. HDL and VLDL lipid compositions were calculated after analysis of the unesterified cholesterol, cholesteryl ester, phospholipid, and triglyceride content of elution fractions 3 (VLDL) and 16 (HDL) derived from fast performance liquid chromatography (FPLC)–based lipoprotein fractionation.

Oral Glucose and Fat Tolerance Tests

Mice were weighed and fasted 4 hours before oral administration of the glucose bolus of 2 g/kg or 400 µL olive oil (Sigma). Blood glucose levels were measured before and at 15, 30, 45, 60, 90, 120, and 180 minutes after administration of the glucose bolus with a Roche Accu-Chek system. Plasma triglycerides were measured at 1, 2, 3, and 4 hours after the olive oil load using the colorimetric assay from Roche.

Blood Cell Analysis

Blood was collected in ethylenediaminetetraacetic acid-coated tubes (Sarstedt, Numbrecht, Germany) by orbital bleeding of mice after a 4-hour fasting period. Blood concentrations of the major leukocyte classes, that is, neutrophils, monocytes, lymphocytes, and eosinophils were determined using an automated veterinary hematology analyzer (Sysmex XT-2000iV; Goffin Meyvis, Etten-Leur, the Netherlands).

Histological Analysis

The mean atherosclerotic lesion area of each mouse was quantified taking the guidelines for experimental studies described in the AHA statement[22] into account. Ten Oil red O-stained cryosections (10 µm), starting at the appearance of the tricuspid valves up to 300 µm of the ascending aorta, were analyzed 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, United Kingdom).

Analysis of Gene Expression by Real-Time Quantitative PCR

Quantitative gene expression analysis was performed as previously described.[23] In short, total RNA was isolated using a standard phenol/chloroform extraction method and reverse transcribed using RevertAid Reverse Transcriptase. Gene expression analysis was performed using SYBR-Green technology (Eurogentec). GAPDH and ribosomal protein lateral stalk subunit P0 (36B4) were used as the housekeeping genes.

Measurement of Isoprostanes and Carbonyls

Plasma and aortic arch isoprostane 8,12-iso-iPF2α-VI levels were measured by gas chromatography-mass spectrometry as described previously.[24] In short, plasma 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. Tissues from each individual mouse were weighed, minced, and homogenized in PBS containing ethylenediaminetetraacetic acid (2 mmol/L) and butylated hydroxytoluene (2 mmol/L), 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 hour and the total levels of 8,12-iso-iPF2α-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).[25]

Radiolabeled Triglyceride-Rich Lipoprotein Flux Analysis

Triglyceride-rich lipoprotein-mimicking particles of ethylenediaminetetraacetic acid 80 nm were prepared according to the sonication and ultracentrifugation procedure from Redgrave and Maranhao[26] from 100 mg total lipid at a weight ratio triolein/egg yolk phosphatidylcholine/lysophosphatidylcholine/cholesteryl oleate/cholesterol of 70:22.7:2.3:3.0:2.0, as described.[27] [^{3}H]triolein (TO) (100 µCi) and [^{14}C]CO (10 µCi) was added to the emulsions to trace the in vivo fate of the particle-associated triglycerides and cholesteryl esters. Mice were fasted for 4 hours and injected intravenously with 200 µL of particle emulsion. Blood samples were taken from the tail vein at 2, 5, 10, and 15 minutes after injection to determine the plasma decay of [^{3}H]TO and [^{14}C]CO. Subsequently, mice were euthanized by cervical dislocation and perfused with ice-cold PBS. Uptake of [^{3}H]TO- and [^{14}C]CO-derived radioactivity by organs was expressed per gram wet tissue weight. Plasma [^{3}H]TO and [^{14}C]CO clearance rates (K) were derived through one phase exponential decay-based curve fitting using Instat GraphPad software (San Diego).

Statistical Analysis

Data from individual experimental groups were tested for outliers using the Grubbs test supplied in Graphpad Quickcalcs

online software (http://www.graphpad.com). A 2-way ANOVA was performed using Graphpad Prism software to uncover the contributions of sex and the different genotypes to the overall variation in the data from the atherosclerosis study. A Bonferroni post-test was used to identify statistical significant differences between the different genotype groups. A 2-way ANOVA with Bonferroni post-test was also used to analyze the data from the metabolic study with the aim to identify genotype-associated effects. For all data, Gaussian distributions were confirmed using the test of Golmogorov and Smirnov (Instat GraphPad software, San Diego). Probability values <0.05 were considered significant.

RESULTS

To study the effect of attenuating HDL maturation in SR-BI deficiency on atherosclerosis susceptibility, we generated SR-BI×PLTP DKO mice through crossbreeding of SR-BI KO mice with PLTP KO mice. Ablation of PLTP functionality in SR-BI KO mice was associated with a sex-independent decrease in both plasma unesterified cholesterol and cholesteryl ester concentrations. Unesterified cholesterol levels in normal laboratory diet-fed DKO mice were lower than those in age- and sex-matched SR-BI KO but remained significantly higher than those found in the WT mice that were included as atherosclerosis negative controls (Figure 1A). In contrast, the SR-BI deficiency-associated cholesteryl ester accumulation in the plasma compartment was completely reversed as a result the PTLP function disruption (Figure 1A). The plasma unesterified cholesterol to cholesteryl ester ratio was therefore not different between SR-BI KO and DKO mice and significantly higher than that in WT mice (Figure 1B). The PLTP deficiency-associated plasma cholesteryl ester lowering in DKO mice could indeed be attributed to the defective generation of large (cholesteryl ester-enriched) HDL particles that normally are present in SR-BI KO mice, as can be appreciated from the FPLC profiles in Figure 1C. Accordingly, plasma HDL-cholesterol levels were lowered to WT values in DKO mice (Figure 1D). Radiolabeled HDL flux studies using human HDL verified that the virtual normalization of HDL particle size in DKO mice as compared with SR-BI KO mice was not due to an improved plasma clearance and tissue uptake of HDL-cholesteryl esters (Figure I in the online-only Data Supplement). Notably, plasma VLDL-cholesterol levels were >3-fold higher in both male and female DKO mice as compared with their WT and SR-BI KO counterparts under normal laboratory diet conditions, while LDL-cholesterol levels generally also tended to be higher as a result of the combined absence of PLTP and SR-BI (Figure 1D). The failure to generate mature HDL particles in DKO mice was thus apparently paralleled by a change in the metabolism of apolipoprotein B/E-containing lipoproteins.

Previous studies have suggested that the SR-BI deficiency-associated change in plasma (HDL-)cholesterol

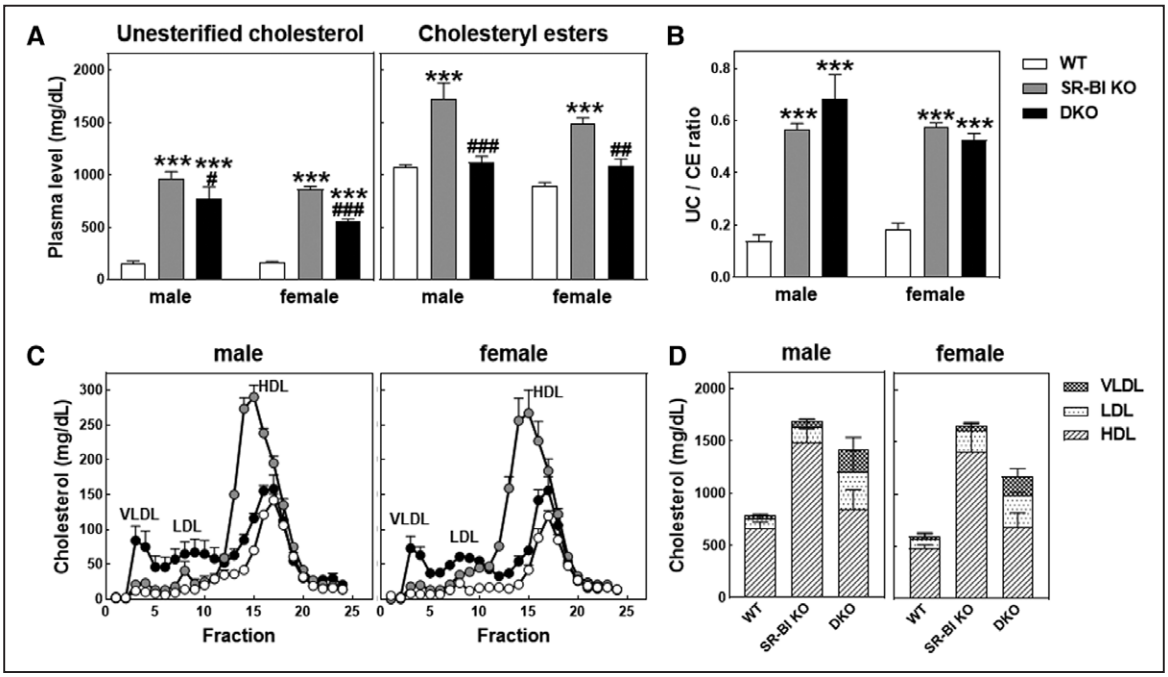

Figure 1. Plasma unesterified cholesterol (UC) and cholesteryl ester (CE) levels (A), the plasma UC/CE ratio (B), and fast performance liquid chromatography (FPLC) profiles for cholesterol distribution over the different lipoproteins (C) in normal laboratory diet–fed male and female WT (white bars/circles), SR-BI (scavenger receptor BI) knockout (KO; gray bars/circles), and double knockout (DKO; black bars/circles) mice. A quantification of the cholesterol amounts within the different FPLC lipoprotein subsets is provided in (D).

Data in all panels represent mean±SEM of 5 to 8 mice per group. ***$P<0.001$ vs WT. #$P<0.05$, ##$P<0.01$, ###$P<0.001$ vs SR-BI KO. HDL indicates high-density lipoprotein; and VLDL, very low-density lipoprotein.

levels underlies the appearance of the distinct red blood cell and platelet phenotypes in SR-BI KO mice.[10–13] The SR-BI KO mice in our present study were—as expected—slightly anemic (ie, a relatively low erythrocyte count and increased mean cellular volume), displayed marked reticulocytosis, and exhibited lowered platelet numbers and a higher mean platelet volume as compared with their respective WT controls (Figure 2). Most of these hematologic parameters were not normalized in DKO mice. More specifically, the number and size of platelets as well as the number of erythrocytes were still significantly affected in DKO mice (Figure 2). Although the concentration of reticulocytes in the blood was significantly reduced in DKO mice as compared with SR-BI KO mice, it remained markedly higher than in WT mice (Figure 2). The mean cellular volume of erythrocytes was, however, completely normalized in DKO mice (Figure 2). Of interest, blood leukocyte levels were higher in DKO mice as compared with WT mice. When comparing the hematologic profile to the abovedescribed blood lipid findings, it can be suggested that the platelet phenotype of SR-BI KO mice is probably related to the higher plasma unesterified cholesterol to cholesteryl ester ratio, while the reticulocyte and erythrocyte volume phenotypes rather seem to be causally related to, respectively, the plasma total cholesterol and plasma HDL-cholesterol levels.

To ascertain the presence of atherosclerotic lesions in our SR-BI KO mice, we used a dietary trigger that has previously been shown to reproducibly result in plaque development in the aortic root of these mice but not in WT animals.[11,14] All experimental groups were therefore fed a Western-type diet containing 0.25% cholesterol and 16% fat for 20 weeks. Similarly, as observed under normal laboratory diet feeding conditions, Western-type diet-fed DKO mice exhibited intermediate plasma unesterified cholesterol levels as compared with the WT and SR-BI KO groups (Figure 3A) and plasma cholesteryl ester concentrations within the WT range (Figure 3A). FPLC-based lipoprotein fractionation showed that under these dietary conditions genetic disruption of PLTP resulted in only a partial reversal of the SR-BI deficiency-associated HDL enlargement (Figure 3B). In line with the overall higher plasma unesterified cholesterol to cholesteryl ester ratio in SR-BI KO and DKO mice, their FPLC-derived HDL fractions were equally enriched in unesterified cholesterol (Figure 3C). Interestingly, both male and female DKO mice also exhibited higher plasma triglyceride levels as compared with WT mice (Figure 3A). DKO VLDL fractions, in particular of female mice, were therefore not only enriched in unesterified cholesterol but also contained relatively high amounts of triglycerides (Figure 3D). As can be appreciated from Figure 3E, the SR-BI deficiency-associated increase in the plasma level of the lipid peroxidation marker F2-isoprostane was also not reversed by additional PLTP deletion. Plasma F2-isoprostane levels in DKO mice (331±50 pg/mL for males and 318±22 pg/mL for females) were nearly as high as those detected in SR-BI KO mice (505±56 and 415±65 pg/mL in SR-BI KO males and females versus 78±17 and 153±7 pg/mL in WT controls). It can thus be concluded that ablation of the PLTP function did not fully correct either the HDL particle composition or HDL's antioxidant activity and induced hypertriglyceridemia in the SR-BI deficiency setting.

As judged from the reduced aortic F2-isoprostane levels (Figure 4A) and levels of protein carbonyls (Figure 4B) in DKO mice as compared with SR-BI KO mice,

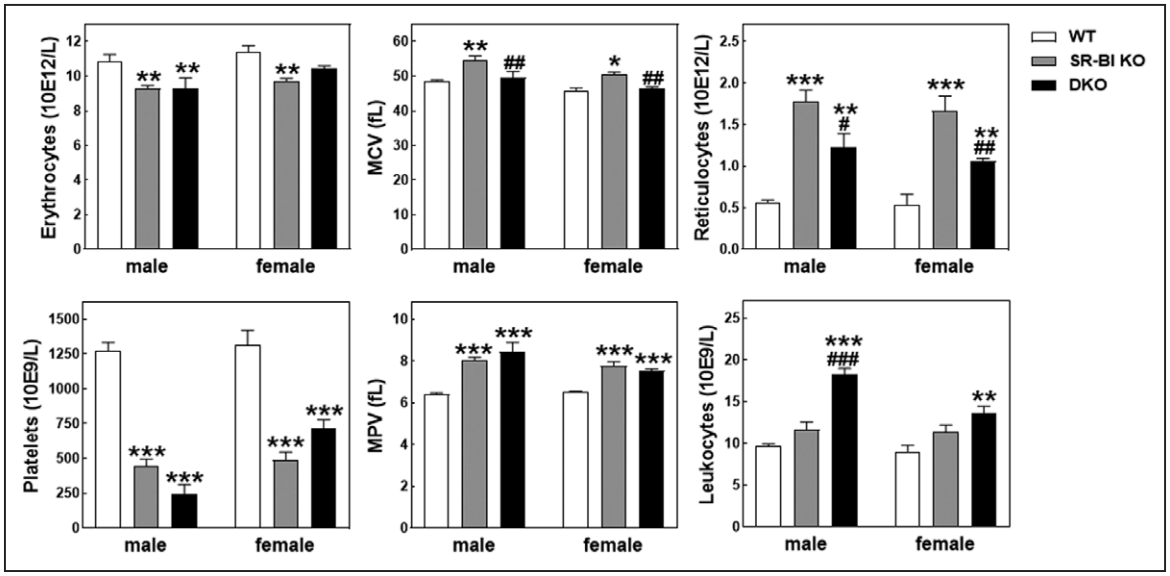

Figure 2. Key hematologic parameters as measured in blood of normal laboratory diet-fed wild-type (WT; white bars), SR-BI (scavenger receptor BI) knockout (KO; gray bars), and double knockout (DKO; black bars) mice.
Data represent mean±SEM of 5 to 8 (DKO) mice per group. *P<0.05, **P<0.01, ***P<0.001 vs WT. #P<0.05, ##P<0.01, ###P<0.001 vs SR-BI KO.

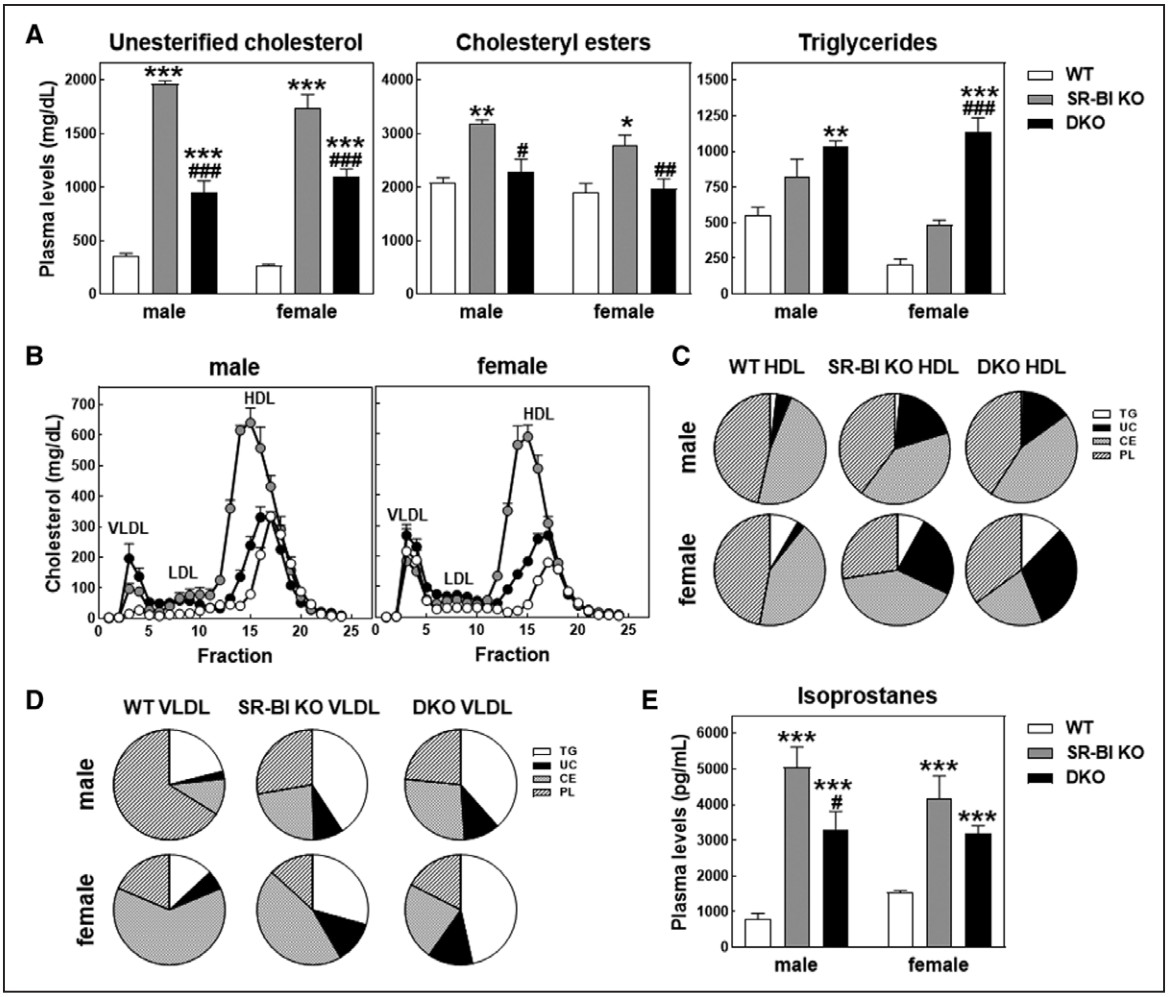

Figure 3. Plasma unesterified cholesterol, cholesteryl ester, and triglyceride levels (A), fast performance liquid chromatography profiles for cholesterol distribution over the different lipoproteins (B), the weight distribution (mass %) of the 4 main lipid species carried in the HDL (high-density lipoprotein; C) and VLDL (very low-density lipoprotein; D) particles, and plasma isoprostane levels (E) in Western-type diet-fed wild-type (WT; white bars), SR-BI KO (gray bars), and double knockout (DKO; black bars) mice. Data in all panels represent mean±SEM of 5 to 8 mice per group. *P<0.05, **P<0.01, ***P<0.001 vs WT. #P<0.05, ##P<0.01, ###P<0.001 vs scavenger receptor BI knockout (SR-BI KO).

the SR-BI deficiency-associated relatively high tissue oxidative stress levels were still reduced to normal WT levels by the additional PLTP deficiency. Strikingly, the normalization of the aortic oxidation status in DKO mice did not translate into a parallel reversal of the SR-BI deficiency-associated susceptibility for the development of atherosclerotic lesions within the aortic root (Figure 4C and 4D). Similarly as observed before,[14] female SR-BI KO mice exhibited a markedly higher atherosclerotic lesion extent as compared with their male counterparts after the 20-week Western-type diet challenge (2-way ANOVA P<0.01 for sex). As such, as opposed to the significant 103-fold increase (P<0.001) in lesion size in SR-BI KO females as compared with WT females, the 28-fold higher lesion size in male SR-BI KO mice failed to reach significance. Atherosclerosis lesion sizes in male DKO mice were therefore also not significantly different from those in male SR-BI KO and WT mice, although lesions appeared to be somewhat reduced in DKO mice as compared with SR-BI KO mice. In contrast, female DKO mice exhibited a clear intermediate atherosclerosis susceptibility phenotype, with an aortic root lesion size that was 46% lower than in female SR-BI KO mice (P<0.05) but 55-fold higher than in female WT controls (P<0.05). Although PLTP deletion did translate into an overall reduction in lesion size in both sex types, PLTP deficiency was thus not able to fully reverse the atherosclerosis susceptibility of SR-BI KO mice.

An unexpected finding during the Western-type diet challenge was the remarkable difference in body weight gain between the individual mouse types (2-way ANOVA: P<0.001 for genotype effect). No genotype-associated difference in starting body weight was noted (Figure 5A). However, both male and female DKO mice rapidly gained more weight than their WT and SR-BI KO counterparts in response to the Western-type diet

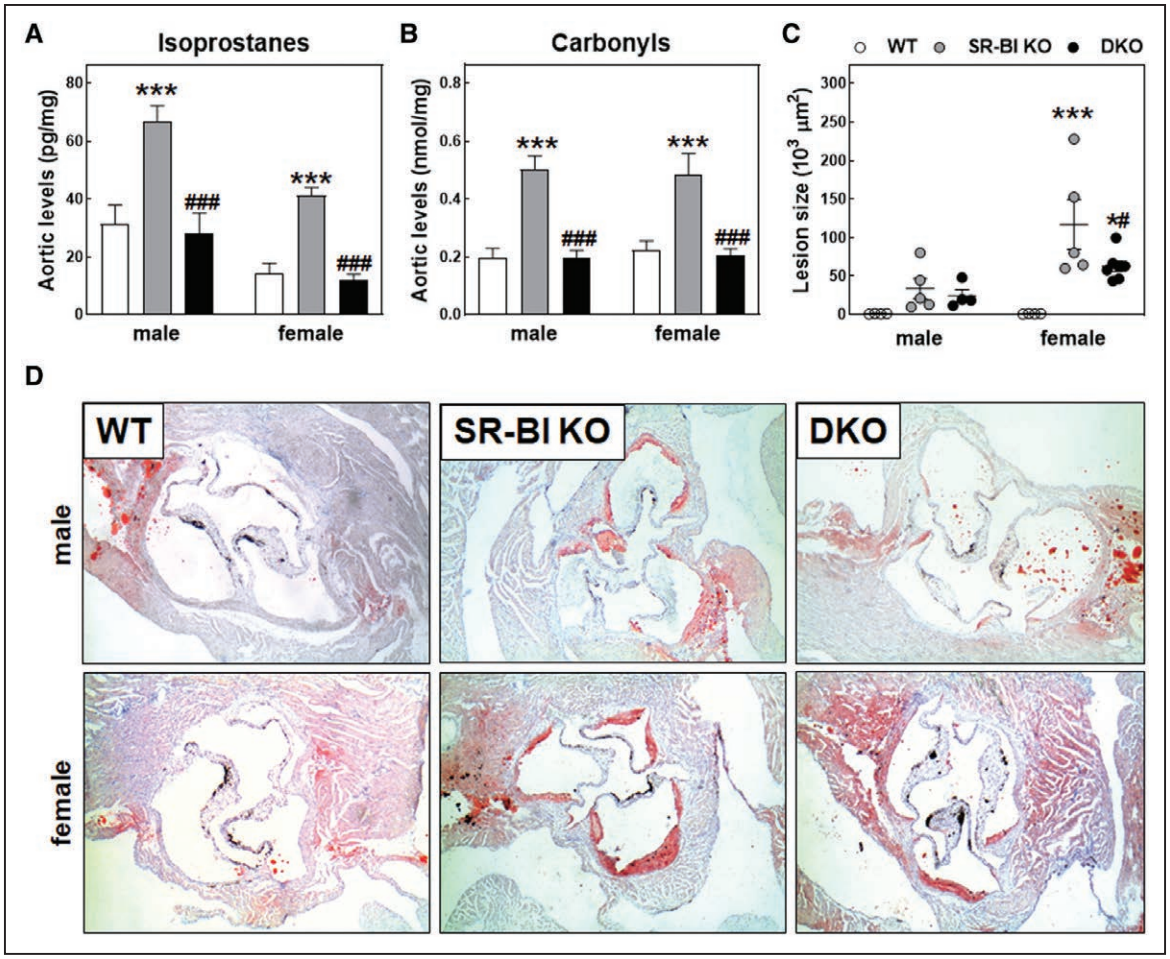

Figure 4. Aortic arch isoprostane (A) and carbonyl (B) concentrations and aortic root atherosclerotic lesion sizes (C) in Western-type diet-fed wild-type (WT; white bars), scavenger receptor BI knockout (SR-BI KO; gray bars), and double knockout (DKO; black bars) mice. D, Representative images of Oil red O–stained aortic root sections.
Data represent mean±SEM of 5 to 8 mice per group. *P<0.05, ***P<0.001 vs WT. #P<0.05, ###P<0.01 vs SR-BI KO.

challenge (Figure 5A). As a result, body weights of DKO mice were significantly higher for both sexes already after 10 weeks of Western-type diet feeding (P<0.01 versus WT; P<0.001 versus SR-BI KO; Figure 5A). The accelerated body weight gain led to an obese phenotype in both male and female DKO mice, with average body weights of ≥40 grams after 20 weeks of diet feeding (Figures 5B). SR-BI KO mice and, to a lesser extent, DKO mice displayed splenomegaly (Figure 5C), which can highly likely be attributed to their higher plasma unesterified cholesterol to cholesteryl ester ratio. In line with the notion that the increase in body weight was driven by increased adiposity, the gonadal white adipose tissue pads were 2- to 4-fold larger in DKO mice as compared with WT and SR-BI KO mice, depending on the sex (Figure 5D). An essentially similar weight profile was detected for the visceral white adipose tissue depots (Figure 5E). However, no consistent genotype effect on liver weight was noted (Figure 5F).

Obesity and hypertriglyceridemia are considered risk factors for the development of atherosclerosis but also glucose intolerance and type II diabetes mellitus. In accordance, both male and female DKO mice displayed a diminished glucose tolerance as compared with SR-BI KO an WT mice in response to an oral glucose challenge (Figure 5G). Blood glucose levels peaked after 15 to 30 minutes in male WT mice, while they already declined again in female WT mice from 15 minutes onward, ultimately returning to baseline levels at 180 minutes in both sex types. The blood glucose profiles in SR-BI KO mice were essentially the same as those in WT mice of the same sex. In contrast, blood glucose levels remained markedly higher in the DKO animals as compared with WT and SR-BI KO mice. More specifically, in female DKO mice, blood glucose levels remained at the relatively high peak level until 60 minutes after the oral dosing, before eventually also declining—but rather slowly—back to almost basal levels. In male DKO mice, glucose levels had not even returned to baseline levels at 180 minutes after the oral glucose dosing with blood glucose levels at t=180 minutes of 18.3±6.1 mmol/L for male DKO mice versus 10.1±1.3 and 9.4±3.0 mmol/L for male WT and

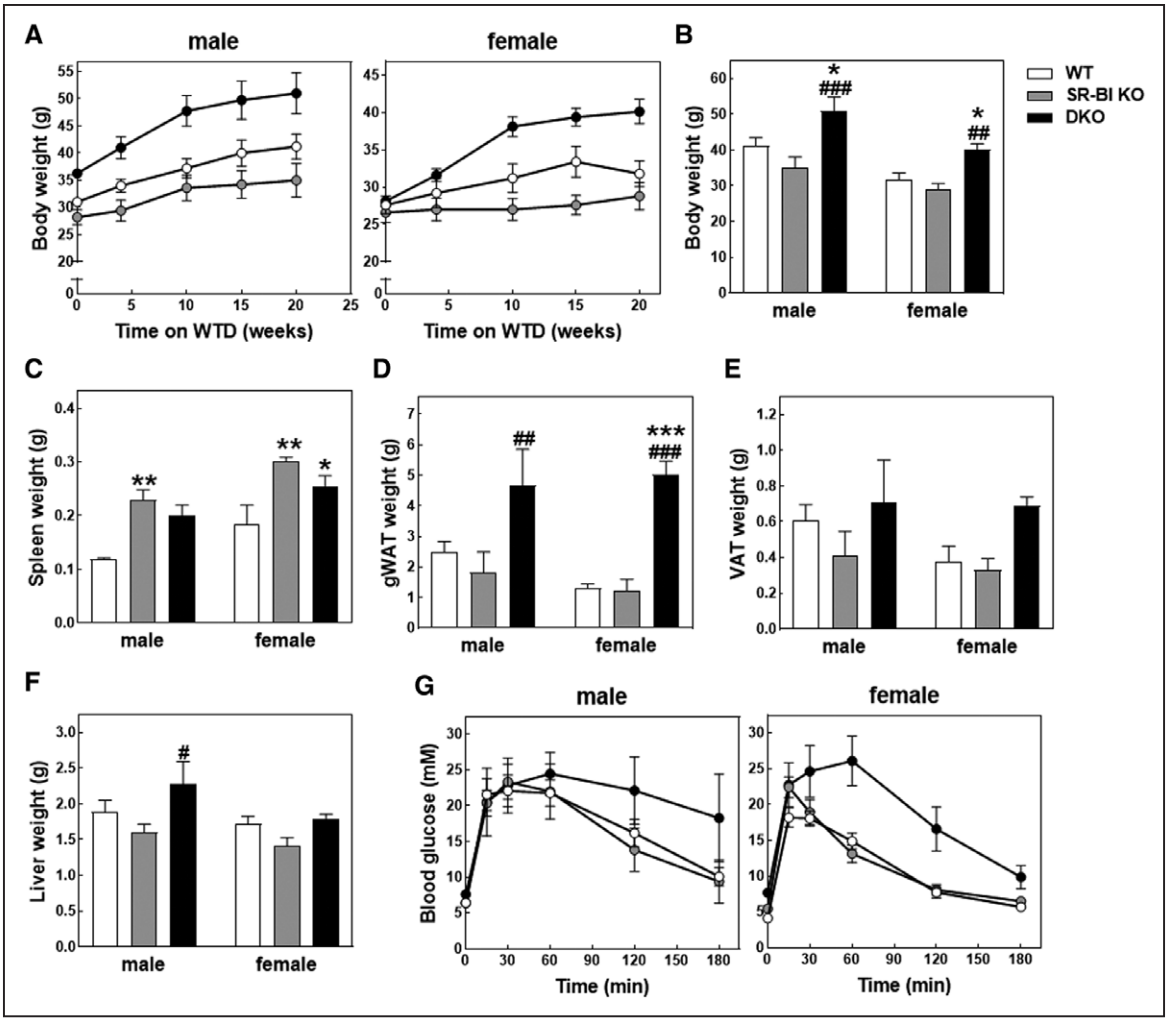

Figure 5. A, Body weight development in response to Western-type diet feeding in wild-type (WT; white circles), scavenger receptor BI knockout (SR-BI KO; gray circles), and double knockout (DKO; black circles) mice. Sacrifice total body weights (B), spleen weights (C), gonadal white adipose tissue (gWAT) weights (D), visceral white adipose tissue (VAT) weights (E), liver weights (F), and the blood glucose responses to an oral glucose challenge (G) in Western-type diet-fed WT (white bars/circles), SR-BI KO (gray bars/circles), and DKO (black bars/circles) mice.

Data represent mean±SEM of 5 to 8 mice per group. **P*<0.05, ***P*<0.01, ****P*<0.001 vs WT. #*P*<0.05, ##*P*<0.01, ###*P*<0.001 vs SR-BI KO.

SR-BI KO mice, respectively. As a result, the area under the curve was markedly higher in DKO mice (3852±696 mmol/L per minute for males and 3415±488 mmol/L per minute from females) as compared with both SR-BI KO (2975±504 and 2069±124 mmol/L per minute) and WT (3118±288 and 2011±145 mmol/L per minute) mice. However, the difference between genotypes in these latter values failed to reach statistical significance due the large intragroup variations.

To uncover the reason behind the unanticipated occurrence of the metabolic phenotype in DKO mice, we performed follow-up mechanistic studies in age-matched mice continuously fed a normal laboratory diet. Atherosclerosis-resistant PLTP KO mice were also included in these experiments to be able to discriminate between potential genotype-dependent primary effects of PLTP or SR-BI deficiency on metabolism and secondary effects related to a difference in fat mass. No genotype-associated difference in food consumption was noted between the different genotypes in male or female mice (data not shown). As can be appreciated from Figure 6A and 6B, also no significant differences were found in body weight or glucose tolerance under these normal laboratory diet conditions. If anything, SR-BI KO and PLTP KO mice, but not DKO mice, managed somewhat better in their glucose handling than their wild-type counterparts. As such, this suggests that the glucose intolerance phenotype observed on Western-type diet was probably secondary to the presence of (morbid) obesity. In further support, no genotype-associated change was detected in fasting plasma triglyceride levels (t=0; Figure 6C). In marked contrast, the plasma postprandial triglyceride responses were significantly different in the 4 types of mice (Figure 6C). WT mice were able to maintain their plasma triglyceride levels within the basal range after an oral olive oil load. In accordance with our previous

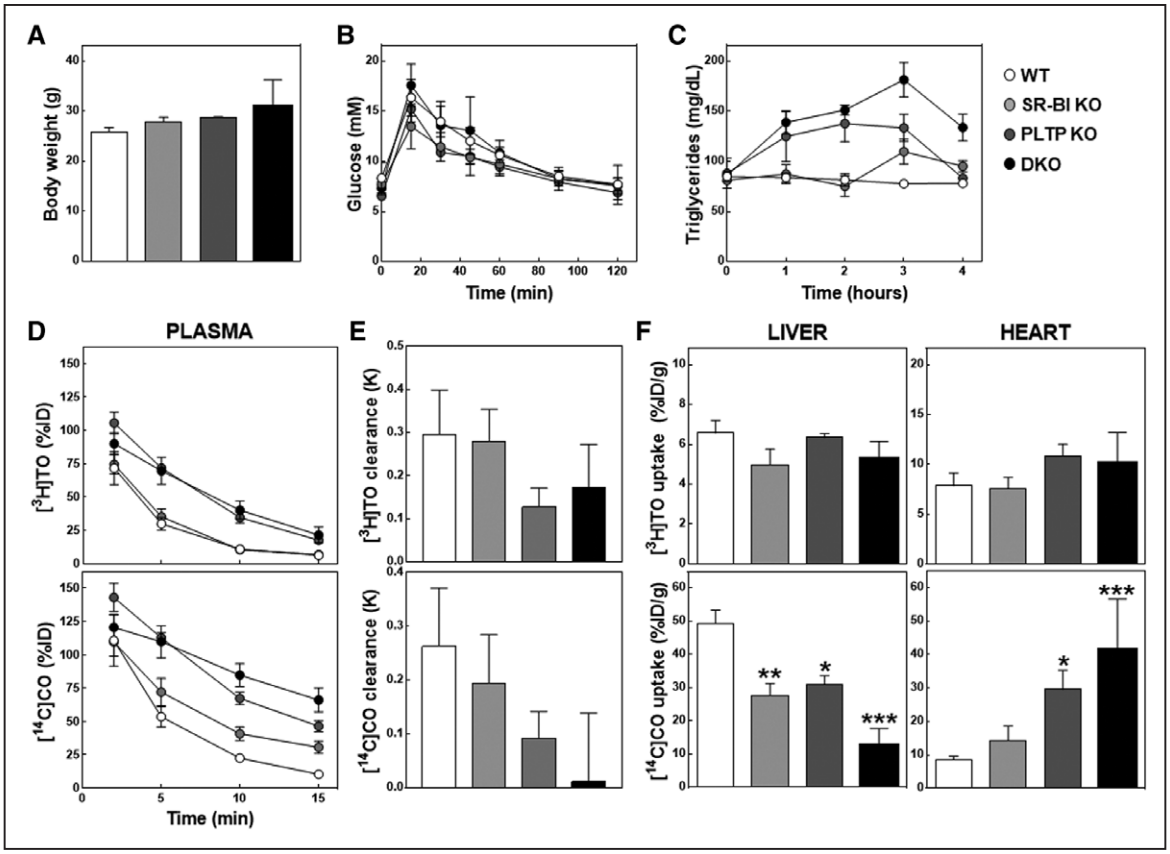

Figure 6. Sacrifice body weights (A), blood glucose responses to an oral glucose challenge (B), and plasma triglyceride responses to an oral olive oil load (C) in normal laboratory diet–fed female wild-type (WT; white bars/circles; N=8), scavenger receptor BI knockout (SR-BI KO; light gray bars/circles; N=5), PLTP (phospholipid transfer protein), KO (dark gray bars/circles; N=4), and double knockout (DKO; black bars/circles; N=7) mice. Plasma decay (D) and tissue uptake (F) of radiolabeled triglyceride-rich lipoprotein-associated fatty acids ([^{3}H]TO-derived activity) and cholesteryl esters ([^{14}C]CO) in chow diet–fed male mice. The calculated plasma clearance rates are shown in (E).
*P<0.05, **P<0.01, ***P<0.001 vs WT.

observation that SR-BI KO mice exhibit a higher postprandial response,[28] plasma triglyceride levels tended to be higher in SR-BI KO mice versus WT mice at 3 hours after the oral dosing but did return to WT levels at the 4-hour time point. Strikingly, plasma triglyceride levels were markedly higher in PLTP KO mice as compared with WT mice at the 1, 2, and 3 hour time points, while they, similarly to WT and SR-BI KO mice, returned to basal levels at the 4-hour time point (Figure 6C). The effect of the double genetic deficiency on the plasma triglyceride profile after oral olive oil gavage matched that of the combined individual effects detected in PLTP KO and SR-BI KO mice. As a result, the area under the curve of the triglyceride response was significantly higher in DKO mice as compared with WT mice (582±43 mg.h/dL for DKO versus 324±43 mg.h/dL for WT, respectively; P<0.001). The level of triglycerides measured in plasma after an oral olive oil bolus is the net effect of absorption of triglycerides in the intestine, lipolysis of the triglycerides in chylomicrons once in the circulation, and removal of the triglyceride-poor but cholesterol-enriched remnant particles. Triglyceride-rich VLDL-like particles, radiolabeled with glycerol [^{3}H]oleate ([^{3}H]TO) and [^{14}C]cholesteryl oleate ([^{14}C]CO), were injected intravenously allowing investigation of the metabolism of these particles and tracing of their core lipids in time. [^{3}H]TO clearance from the plasma compartment was somewhat slower in PLTP KO and DKO mice as compared with both SR-BI KO and WT mice with average plasma half-lives of 5.4 and 4.0 minutes versus 2.5 and 2.3 minutes, respectively (Figure 6D). The associated [^{3}H]TO elimination rate constants (K) were 0.30±0.10 for WT mice, 0.28±0.07 for SR-BI KO mice, 0.13±0.04 for PLTP KO mice, and 0.17±0.10 for DKO mice (Figure 6E). This observation is in line with our previous findings that the plasma LPL (lipoprotein lipase) and HL (hepatic lipase) activity is similar in SR-BI KO and wild-type mice.[29] Given the general short plasma half-life of [^{3}H]TO, it is not surprising that upon sacrifice, that is, at 15 minutes after particle injection, no difference was found among the different genotype groups in the uptake of [^{3}H]TO by the primary target organs heart and liver (Figure 6F). In accordance with the notion that in WT mice VLDL/chylomicron-remnants are rapidly cleared from the circulation, the plasma half-life of the [^{14}C]CO

tracer (2.6 minutes; K: 0.26±0.11) was almost similar to that of the [^{3}H]TO tracer (2.3 minutes: K: 0.30±0.10; Figure 6D and 6E). In contrast, [^{14}C]CO plasma clearance was slower than that of [^{3}H]TO in SR-BI KO mice (average half-life: 3.6 minutes; K: 0.19±0.0.09 for [^{14}C]CO versus 2.5 minutes; K: 0.28±0.07 for [^{3}H]TO; Figure 6D and 6E). In further support of the suggested impact of SR-BI deficiency on the uptake of chylomicron- and VLDL-remnants by hepatocytes,[28,29] a significantly lower amount of [^{14}C]CO was recovered in SR-BI KO livers as compared with WT livers at 15 minutes after administration of the triglyceride-rich particles (−44%; $P<0.001$; Figure 6F). The plasma clearance of [^{14}C]CO was also markedly delayed in PLTP KO mice (average plasma half-life: 7.5 minutes; K: 0.09±0.05; Figure 6D and 6E), and this was paralleled by a reduced hepatic remnant particle uptake at 15 minutes after injection (−37%; $P<0.001$). Strikingly, remnant particle clearance was even further delayed in DKO mice with an average [^{14}C]CO plasma half-life of 58 minutes (K: 0.01±0.13; Figure 6D and 6E). As a result, livers of DKO mice had taken up only 26% of the fractional dose of [^{14}C]CO that could be recovered in WT livers at 15 minutes after intravenous particle injection ($P<0.001$; Figure 6F). In contrast, a relatively high level of [^{14}C]CO tracer was detected in hearts of PLTP KO and DKO mice (Figure 6F). Given that we have previously found that SR-BI deficiency does not alter hepatic expression levels of other receptors involved in lipoprotein remnant uptake, that is, the LDL receptor and LRP1 (LDL receptor-related protein 1),[29] it can be suggested that in response to the PLTP deficiency a VLDL-remnant particle is generated that is highly dependent on SR-BI for its subsequent clearance by hepatocytes and therefore ultimately accumulates in alternative tissues in a SR-BI deficiency setting.

DISCUSSION

In the current study, we aimed to verify a possible causal contribution of the accumulation of enlarged HDL particles in the circulation of SR-BI KO mice and the atherosclerosis-susceptible phenotype of these animals. Hereto, we have eliminated PLTP-mediated HDL maturation in SR-BI KO mice by cross-breeding with PLTP KO mice. In line with our hypothesis that PLTP is required for the enlargement of the HDL particles in SR-BI KO mice, HDL size was nearly normalized in PLTP lacking DKO mice. Atherosclerosis susceptibility was attenuated but was not fully reversed in the DKO mice. However, it should be acknowledged that the PLTP deficiency–associated reduction in atherosclerotic lesion size occurred in the context of the fact that DKO mice exhibited typical symptoms of the metabolic syndrome, that is, they became (morbidly) obese, displayed hypertriglyceridemia, and were rather glucose intolerant when challenged with the Western-type diet.

Although PLTP deficiency did not fully correct the abnormal HDL lipid composition, that is, the unesterified cholesterol accumulation, or reduced plasma isoprostane levels on a Western-type diet, we noted that aortic oxidative stress markers in DKO mice were comparable to those in WT mice. As such, this suggests that the HDL functionality in terms of lowering tissue oxidative stress may still be effectively restored to normal in DKO mice. Dedicated functional in vitro studies with isolated individual HDL fractions from mice on Western-type diet to investigate the protective effects on cellular oxidation processes are of interest in follow-up of our findings.

The fact that DKO mice still displayed a significant extent of atherosclerosis despite the effective lowering of aortic oxidative stress levels implies that other mechanisms than the increase in tissue oxidation status may also underlie the enhanced atherosclerosis susceptibility in SR-BI KO mice. The SR-BI deficiency-associated accumulation of plasma unesterified cholesterol, anemia, and thrombocytopenia were all maintained in the DKO setting. The presence of anemia in mice has been associated with a reduction in atherosclerosis susceptibility,[30] thereby eliminating the anemic phenotype as an underlying cause of the remaining residual atherosclerosis. In contrast, platelets are well known to execute a variety of effects that impact the pathogenesis of atherosclerosis and can also be relevant in our experimental setting.[31] Furthermore, the potentially atheroprotective effect of SR-BI located in (aortic) endothelial cells, that is, promotion of nitric oxide production,[32] is also still impaired due to the continued genetic lack of a functional SR-BI protein in the DKO mice. PLTP deficiency also impacted on triglyceride and glucose metabolism in the SR-BI deficient mice and the mice developed features of metabolic syndrome. Metabolic syndrome, in the human context, substantially increases the risk for the development of atherosclerotic cardiovascular disease and type II diabetes mellitus.[33–35] Therefore, our impression is that the anticipated reversal of the atherosclerosis susceptibility in DKO mice has been partially nullified by the development of this pathological metabolic condition.

In light of previous findings from Jiang et al[36] that PLTP deficiency in mice is generally associated with a lower hepatic secretion of apoB-containing (triglyceride-rich) lipoproteins, the fact that fasting plasma triglyceride levels were higher in Western-type diet-fed PLTP-deficient SR-BI KO mice than in SR-BI KO mice expressing PLTP may, at first, be conceived as surprising. However, a primary conclusion of our mechanistic studies was that, in absence of PLTP, a VLDL/chylomicron-remnant particle is generated that is primarily cleared by SR-BI. Inability of the liver to effectively remove remnant particles from the circulation thereby explains the overt hypertriglyceridemia found in DKO mice on a Western-type diet. The [^{3}H]TO flux data suggest that the effect on particle clearance is not driven by differences in LPL-facilitated

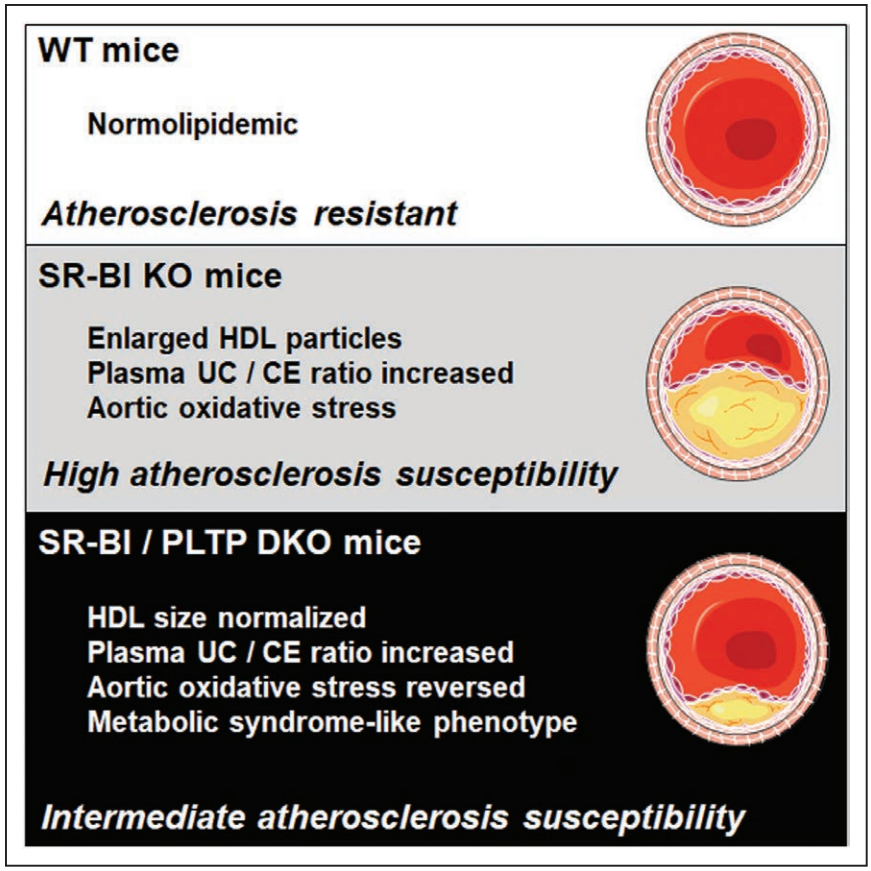

Figure 7. Schematic overview of the key findings of our study.
The increased atherosclerosis susceptibility related to the high aortic oxidative stress level present in scavenger receptor BI knockout (SR-BI KO) mice is not completely reversed in scavenger receptor BI×phospholipid transfer protein double knockout (DKO) mice because of the development of a proatherogenic metabolic syndrome-like phenotype.

VLDL-triglyceride hydrolysis. This is in agreement with the general notion that PLTP acts as a passive bystander in the lipolytic process and transfers phospholipids from apoB/apoE-containing lipoproteins to HDL after lipolysis has already taken place. In support of this assumption, VLDL and LDL particles of PLTP KO mice are enriched in phospholipids, while total triglyceride levels in plasma in single PLTP KO mice are not higher than those found in WT mice.[20] Furthermore, previous studies by Kawano et al[37] also did not detect an increase in plasma triglyceride levels in PLTP KO as compared with WT mice (with functional SR-BI) on either normal laboratory diet or Western-type diet. The exact mechanistic explanation behind the finding that the VLDL-remnant particles in PLTP KO mice are highly dependent on SR-BI for their removal from the blood circulation by the liver remains to be determined. However, our findings regarding the relative importance of SR-BI in clearing lipoprotein particles from PLTP KO mice do nicely fit with the observation from Kawano et al that blocking SR-BI function through neutralizing antibody treatment impairs the cellular influx of unesterified cholesterol and phospholipids from vesicles derived from PLTP-deficient mice.[37] In previous studies, we have shown that apoE-containing larger lipoprotein species (such as chylomicron remnants[28] and β-VLDL[29] serve as a good substrate for SR-BI-mediated whole particle uptake. The current studies highlight that this route becomes more important in the absence of PLTP. Ishikawa et al[38] have observed that during lipolysis, the VLDL particle becomes more accessible for binding of apoE. Given that PLTP can theoretically also modify the distribution/accessibility of individual VLDL-associated proteins, we anticipate that the amount and distribution of apoE between lipoprotein classes may be different in PLTP KO mice, thereby shifting the preference for remnant particle uptake from the primary remnant receptors located on hepatocytes, that is, the LDL receptor and LRP1, toward SR-BI. More detailed mechanistic studies are clearly warranted to validate this interesting hypothesis.

In conclusion, we demonstrated that disruption of PLTP activity reduces atherosclerosis susceptibility of SR-BI–deficient mice, despite impairment of the metabolism of triglyceride-rich lipoproteins and the induction of phenotypic features of metabolic syndrome (see graphical summary in Figure 7). Our studies highlight that the presence of an active PLTP protein in the plasma compartment facilitates the uptake of triglyceride-rich lipoproteins from the blood circulation by the liver probably by affecting the process where nascent triglyceride-containing lipoproteins are efficiently converted into remnant particles that can be rapidly cleared by hepatocytes.

ARTICLE INFORMATION

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Affiliations

From the Division of BioTherapeutics, Leiden Academic Centre for Drug Research, The Netherlands (M.H., R.J.v.d.S., R.B.H., B.L., Y.Z., T.J.C.v.B., M.v.E.); Alzheimer's Center at Temple, Department of Pharmacology, Philadelphia, PA (D.P.); Division of Endocrinology, Department of Medicine (P.C.N.R., S.K.) and Einthoven Laboratory for Experimental Vascular and Regenerative Medicine, Leiden University Medical Center, The Netherlands (P.C.N.R., S.K); and Minerva Foundation Institute for Medical Research, Biomedicum, Helsinki, Finland (M.J.).

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Disclosures

None.

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