ABC transporters and scavenger receptor BI : important mediators of lipid metabolism and atherosclerosis

Meurs, I.

Chapter 9

Deletion of the high-density lipoprotein receptor SR-BI in mice modulates thrombosis susceptibility and indirectly affects platelet function by elevation of plasma free cholesterol

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Atheroscler Thromb Vasc Biol.
2011, 31:34-42

ABSTRACT

Objective- Scavenger receptor class B type I (SR-BI) is a cell surface receptor that promotes the selective uptake of cholesteryl esters from HDL by the liver. In mice, SR-BI deficiency results in increased plasma HDL cholesterol levels and enhanced susceptibility to atherosclerosis. The aim of this study was to investigate the role of SR-BI deficiency on platelet function.

Methods and Results- SR-BI-deficient mice were thrombocytopenic, and their platelets were abnormally large, probably caused by an increased cholesterol content. The $FeCl_3$ acute injury model to study arterial thrombosis susceptibility showed that SR-BI wild-type mice developed total arterial occlusion after 24±2 min (TTO). In SR-BI-deficient mice, however, the TTO was reduced to 13±1 min (p=0.02). Correspondingly, in SR-BI-deficient mice, platelets circulated in an activated state and showed increased adherence to immobilized fibrinogen. In contrast, platelet-specific disruption of SR-BI by bone marrow transplantation in wild-type mice did not alter plasma cholesterol levels nor affect platelet count, size, cholesterol content, or reactivity, suggesting that changes in plasma cholesterol levels were responsible for the altered responsiveness of platelets in SR-BI-deficient mice.

Conclusion- The function of SR-BI in HDL cholesterol homeostasis and prevention of atherosclerosis is indirectly also essential for maintaining normal platelet function and prevention of thrombosis.

INTRODUCTION

Acute coronary events are not the result of progressive growth of the lesion, but rather of lesion disruption and superimposed thrombus formation. Damage to the endothelial cell layer of the vessel wall results in the exposure of the extracellular matrix to the flowing blood, which triggers platelet activation, platelet plug formation, coagulant activity and the formation of fibrin-containing thrombi that occlude the site of exposed subendothelium.[1] Several lines of evidence suggest that platelet function is modulated by lipoproteins. High levels of pro-atherogenic lipoproteins, including native and oxidized low-density lipoprotein (LDL), are associated with an increased susceptibility to thrombosis by enhancement of platelet responsiveness to aggregation-inducing agents[2,3]. In contrast, several studies, using human platelets, have shown that high density lipoproteins (HDL) inhibit platelet responses, like aggregation and secretion.[4-8] In agreement, platelets of patients with low HDL levels are hyper-responsive to low doses of aggregating agents[9], and high HDL-cholesterol levels in hyperlipidemic and normolipidemic patients are associated with reduced platelet-dependent thrombus formation *ex vivo*.[10]

Scavenger receptor BI (SR-BI) is the first identified cell surface receptor mediating HDL metabolism.[11] It is a multifunctional receptor, capable of binding a wide array of native and modified lipoproteins[12,13] and is abundantly expressed in liver, steroidogenic tissues, and cells within the arterial wall, including endothelial cells, smooth muscle cells, and macrophages.[13-19] It mediates the selective uptake of cholesteryl esters from HDL by the liver and facilitates the efflux of cholesterol from cells in peripheral tissues to HDL.[11,14-16,20] Disruption of SR-BI in mice results in a 3.2-fold increase in free cholesterol levels in the circulation, resulting in an unusually high plasma unesterified-to-total cholesterol ratio (UC:TC).[15,20,21] Much of this excess cholesterol is carried by HDL particles, reflecting impaired delivery to the liver. This is accompanied with a tendency towards slightly increased levels of very low density lipoprotein-cholesterol levels, while LDL-cholesterol levels are not changed.[21] In addition, SR-BI deficiency leads to female infertility[22,23], multiple red blood cell defects[24,25], and an increased susceptibility to diet-induced atherosclerosis.[21,22] Disruption of SR-BI in apoE-deficient mice results in the early onset of occlusive atherosclerotic coronary artery disease, spontaneous myocardial infarctions, and premature death.[26] Coronary arterial lesions in these SR-BI x apoE double knockout mice exhibited extensive fibrin deposition, indicating that SR-BI disruption induces hemorrhage and thrombosis.[22,26] Interestingly, SR-BI is also expressed in megakaryocytes and platelets.[27] In patients with atherosclerosis, a strong inverse correlation exists between the expression of SR-BI, the ability to aggregate and the cholesterol content of the platelets.[27] Correspondingly, Dole *et al.* described that the high UC:TC ratio observed in SR-BI-deficient mice is associated with (i) thrombocytopenia due to high platelet clearance rates, (ii) an increased intracellular cholesterol content, and (iii) abnormal platelet morphologies, including an increase in size, due to high intracellular cholesterol content and the young age of the platelets, and the presence of multi-lamellar structures, probably for accommodation of excess unesterified cholesterol.[28]

In the current study, we studied the effects of increased plasma HDL cholesterol levels in SR-BI-deficient mice on platelet physiology. Disruption of SR-BI in mice results in active circulating platelets, increased adherence to immobilized fibrinogen, and increased

susceptibility to arterial thrombosis *in vivo*. Hence, SR-BI is not only essential for HDL cholesterol homeostasis and atherosclerosis susceptibility, but also for maintaining normal platelet function and prevention of thrombosis.

METHODS

To elucidate the role of SR-BI in platelet function, the susceptibility to *in vivo* arterial thrombosis of wild-type (SR-BI+/+), heterozygous (SR-BI+/–), and homozygous (SR-BI–/–) SR-BI-deficient mice was determined by the $FeCl_3$ acute injury model. Furthermore, the state of activation of circulating platelets was inferred from the level of surface-expressed P-selectin and the conformational state of integrin $\alpha_{IIb}\beta_3$ by flow cytometry, as well as from the level of urinary 2,3-dinor thromboxane B_2 (TxB_2) and 2,3-dinor 6-keto prostaglandin $F_{1\alpha}$ ($PGF_{1\alpha}$) by stable dilution isotope gas chromatography/mass spectrometry assays. Functional consequences of the activation state of the circulating platelets were determined by static adhesion experiments to immobilized fibrinogen as well as aggregation experiments. Cholesterol content of the murine platelets was studied by Filipin III staining of the platelets adhered to fibrinogen. To assess the influence of the plasma UC:TC ratio on platelet function, bone marrow from either SR-BI+/+ or SR-BI–/– mice was transplanted into SR-BI+/+ mice and platelet function was studied. For a full description of these methods, please see supplementary appendix.

RESULTS

Thrombocytopenia and presence of large platelets in SR-BI deficiency

Hematological analysis of SR-BI–/– mice on a regular chow diet (5.7% (w/w) fat and no added cholesterol) showed that SR-BI deficiency caused thrombocytopenia: the platelet number in SR-BI–/– mice ($415\pm97\times10^9$ platelets/L) was reduced by 55% compared to the number of platelets in SR-BI+/+ mice ($921\pm147\times10^9$ platelets/L; $p<0.001$). No statistically significant difference was observed in the platelet count between SR-BI+/+ and SR-BI+/– mice ($874\pm208\times10^9$ platelets/L; $p=0.43$) (Fig 1A). In addition, platelets from SR-BI–/– mice were abnormally large. Their size (7.3±0.4 fL) increased 1.2-fold compared to platelets from SR-BI+/+ or SR-BI+/– mice (both 6.0±0.2 fL; $p<0.001$, Fig 1B).

SR-BI disruption in mice caused a 2.7-fold increase in UC levels and a 1.7-fold increase in TC levels in the circulation (Supplemental Table I), mainly due to a rise in HDL-cholesterol[21]. This led to a 1.6-fold increase of the UC:TC ratio in SR-BI–/– mice as compared to SR-BI+/+ mice ($p<0.001$).

Since lipid exchange between plasma lipoproteins and platelets is a predominantly non-specific process that occurs continuously, we determined the level of unesterified cholesterol in platelets. Hereto, adhered platelets were stained with Filipin III, a fluorescent cholesterol binding dye[29], and visualized microscopically. Fig. 1C clearly illustrates that platelets from SR-BI–/– mice contained a higher level of unesterified cholesterol compared to SR-BI+/+

platelets.

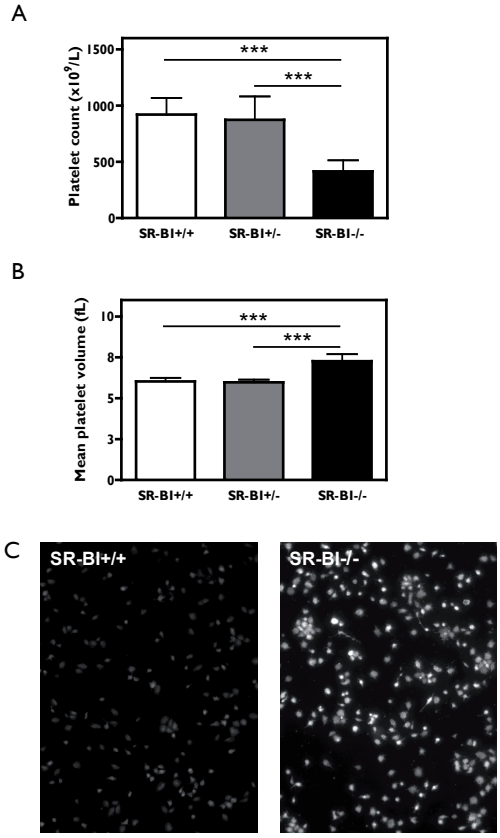

Fig. 1. SR-BI deficiency affects platelet count, size, and cholesterol content.
Platelet count (A) and MPV (B) in whole blood were analyzed using a Sysmex XT-2000iV Hematology Analyzer (Sysmex Europe GMBH, Norderstedt, Germany). The platelet count was corrected for the addition of anti-coagulant. Means±SD, n=13-26, ***p<0.001. C) Washed SR-BI+/+ or SR-BI–/– platelet suspensions (0.5 x 10^{11} platelets/L) were seeded on fibrinogen-coated coverslips and allowed to spread for 60 min. After spreading, platelets were fixed, and unesterified cholesterol in the platelets was stained with Filipin III.

Impaired platelet aggregation by SR-BI deficient platelets

Dole *et al.*[28] showed that aggregation of platelet-rich plasma (PRP) from SR-BI–/– mice was impaired after stimulation by ADP. Similarly, we observed that SR-BI–/– platelet responsiveness in PRP was inhibited by 69% after stimulation with ADP. In addition, aggregation induced by collagen, PAR-4 peptide, and phorbol myristate 13-acetate (PMA) was reduced by 34%, 45%, and 55%, respectively (Fig. 2A-B). Cross-activation experiments in which SR-BI+/+ platelets were resuspended in SR-BI–/– plasma showed that the aggregation response of these platelets after stimulation by the PAR-4 peptide was reduced compared to SR-BI+/+ platelets in autologous plasma (Fig. 2C). Thus, SR-BI+/+ platelets in SR-BI–/– plasma seemed to rapidly acquire features of SR-BI–/– platelets. Since plasma HDL-cholesterol

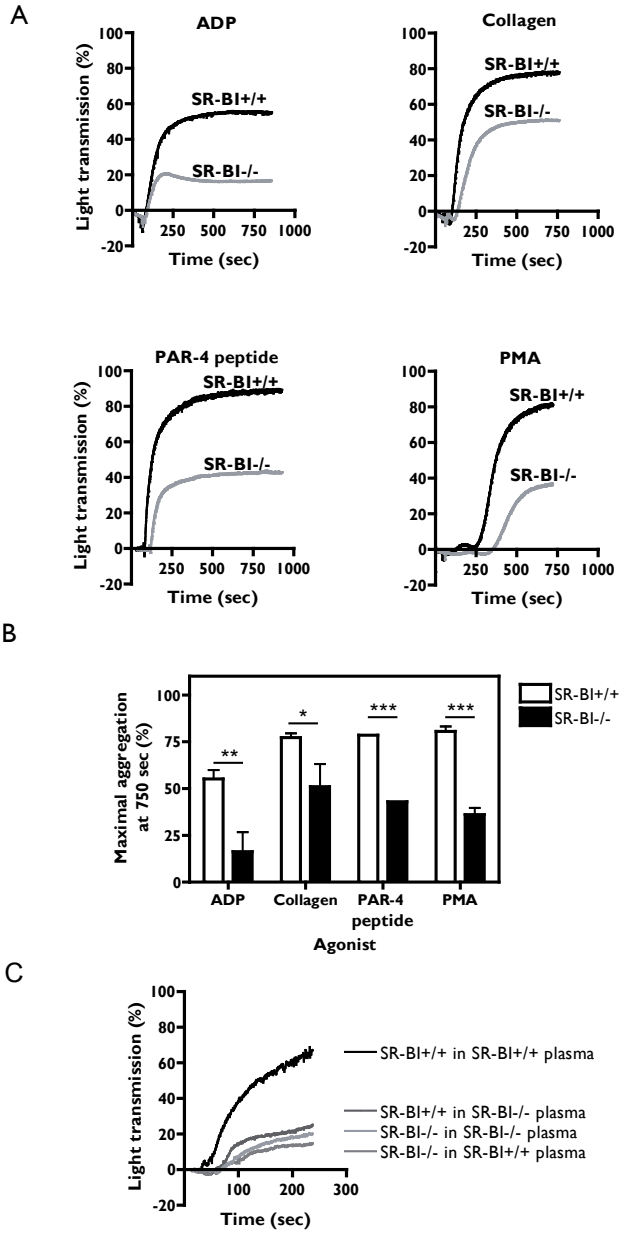

Fig. 2. Impaired aggregation response of SR-BI-/- platelets.
A) PRP was isolated from SR-BI+/+ (black lines) and SR-BI–/– (grey lines) mice, platelet count was adjusted with platelet-poor plasma (PPP) to 2 x 10^{11} platelets/L, and platelets were stimulated with 25 µmol/L ADP, 5 µg/mL collagen, 1 mmol/L PAR-4 peptide, or 0.5 µg/mL PMA. Optical aggregation was measured at 37°C and at a stirring speed of 1000 r.p.m.. Results shown are the mean of 3 independent traces. B) Maximal aggregation at 750 sec after stimulation of SR-BI+/+ (white bars) or SR-BI–/– (black bars) PRP with ADP, collagen, PAR-4 peptide, or PMA. Means±SD, n=3, *p<0.05, **p<0.01, ***p<0.001. C) SR-BI+/+ or SR-BI–/– platelets were incubated in either SR-BI+/+ or SR-BI–/– plasma, platelet count was adjusted with PPP to 2 x 10^{11} platelets/L, and platelet aggregation was induced by 1 mmol/L PAR-4 peptide. Results shown are the mean of 3 independent traces.

levels were increased in SR-BI–/– mice and HDL has been described to inhibit platelet aggregation[4,5], the observed inhibition of aggregation of SR-BI–/– platelets in autologous plasma as well as SR-BI+/+ platelets in SR-BI–/– plasma could well have been caused by the increased plasma HDL level. However, similar results of a reduced aggregation response were found with plasma-free washed platelet suspensions stimulated with ADP, or PMA (Supplemental Fig. I). In addition, incubation of SR-BI–/– platelets in SR-BI+/+ plasma did not improve the aggregation response compared to SR-BI–/– platelets in SR-BI–/– plasma (Fig. 2C). These findings suggest that the absence of SR-BI on platelets is responsible for the reduced platelet aggregation of SR-BI–/– platelets. However, since cholesterol trafficking between cells and plasma (lipoproteins) is accelerated when SR-BI is expressed[30], differences in platelet aggregation might also be explained by differences in cholesterol efflux and thus differences in cholesterol content.

The aggregation response of SR-BI+/– platelets was not different from the aggregation response of SR-BI+/+ platelets (data not shown).

Increased susceptibility to $FeCl_3$-induced thrombosis in mice lacking SR-BI

Platelets play a crucial role in atherothrombosis. We used the $FeCl_3$ acute injury model, in which platelet-rich thrombi are formed that are morphologically similar to those found in humans[31], to investigate the importance of SR-BI in the formation of a pathologic, occlusive thrombus *in vivo*. Hereto, acute arterial injury was induced in the left carotid artery by application of 40% $FeCl_3$ to the adventitial surface of the artery. Thrombus formation was assessed by determining the time to occlusion (TTO) of the artery, which was measured by monitoring the carotid blood flow using an ultrasonic Doppler flow probe. SR-BI+/+ mice developed total arterial occlusion after 24±2 min (Fig. 3). Interestingly, the TTO in SR-BI–/– mice was reduced by 1.8-fold to only 13±1 min ($p<0.001$), indicating that in the absence of SR-BI, mice are more susceptible to thrombosis. In SR-BI+/– mice, arterial occlusion was reached after 18±1 min, which was faster when compared to the TTO observed in SR-BI+/+ mice ($p=0.011$), but significantly slower when compared to the TTO in SR-BI–/– mice ($p=0.015$).

The capacity of the endothelium to generate prostacyclin (PGI_2), a potent platelet inhibitor and vasodilator, might affect thrombosis susceptibility and was measured as the metabolite 2,3-dinor 6-keto prostaglandin $F_{1\alpha}$ ($PGF_{1\alpha}$) in urine. No significant difference was observed between the urinary $PGF_{1\alpha}$ levels of SR-BI+/+ and SR-BI–/– mice (0.5±0.1 ng/mg creatinine vs 0.8±0.2 ng/mg creatinine, respectively; $p=0.245$), indicating a similar platelet inhibition and vasodilation and no additional effect on susceptibility to thrombosis.

SR-BI deficient platelets circulate in an activated state

Interestingly, the enhanced thrombosis susceptibility developed despite the lower *ex vivo* platelet aggregation response observed in SR-BI–/– mice. To determine if the responsiveness of platelets was altered due to the absence of SR-BI, thereby increasing the susceptibility to thrombosis, we studied the activation state of circulating platelets. Upon platelet activation, integrin $\alpha_{IIb}\beta_3$ changes its conformation to a high affinity state in which it is able to bind its ligands. In addition, activated platelets express P-selectin on their surface after fusion of the

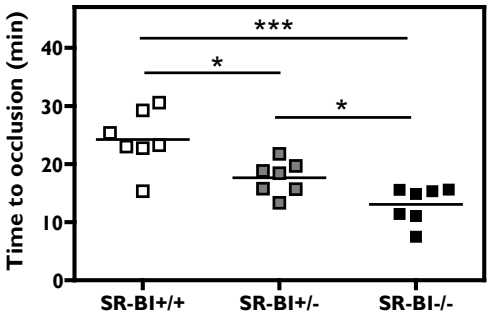

Fig. 3. Increased susceptibility to $FeCl_3$-induced thrombosis in mice lacking SR-BI.
In SR-BI+/+ and SR-BI–/– mice, the left carotid artery was dissected and exposed to a small strip of filter paper saturated with 40% $FeCl_3$ for 3 minutes to initiate the development of a stable completely occlusive thrombus. For analysis of thrombus formation, the carotid blood flow was monitored continuously using an ultrasonic laser doppler flow probe to measure the time between the $FeCl_3$ application and zero blood flow velocity (TTO). Results are expressed as means±SD for groups of 7 mice. *p<0.05, ***p<0.001.

granular membranes of α-granules with the plasma membrane. Hence, both the presence of active integrin $\alpha_{IIb}\beta_3$ as well as surface-expressed P-selectin was determined as a measure of platelet activation. Interestingly, we measured 3.2-fold more integrin $\alpha_{IIb}\beta_3$ in the active high-affinity conformation (relative to the amount of integrin $\alpha_{IIb}\beta_3$ protein) on platelets from SR-BI–/– mice (0.36±0.04 for SR-BI–/– platelets vs 0.11±0.04 for SR-BI+/+ platelets, p<0.001; Fig. 4A) and 4-fold more than on the SR-BI+/– platelets (0.09±0.04, p<0.001). Correspondingly, platelets from SR-BI–/– mice in their basal activation state expressed 1.4-fold more P-selectin (39.0±5.8% for SR-BI–/– platelets vs 27.5±3.0% for SR-BI+/+ platelets, p=0.02, or vs 30.8±2.8% for SR-BI+/– platelets; p=0.05, Fig. 4B). Upon activation, platelets synthesize TxA_2 through the cyclooxygenase-1 (COX-1) pathway.[32,33] TxA_2 is a vasoconstrictor and platelet-aggregating agent, which upon release into the circulation will bind its platelet thromboxane prostanoid α/β (TPα/β) receptor to initiate reinforcing activation pathways. The intake of aspirin, an inhibitor of TxA_2 synthesis, aids in the prevention of arterial thrombosis[34], which implicates TxA_2 as an important factor in cardiovascular disease. The presence of the TxA_2 metabolite 2,3-dinor TxB_2 in urine of mice is thus also a marker for platelet activation. A tendency, although not significant, towards increased levels of 2,3-dinor TxB_2 in the urine of SR-BI–/– mice was observed (17.0±3.6 ng/mg creatinine for SR-BI–/– mice vs 10.8±2.5 ng/mg creatinine for SR-BI+/+ mice, Fig. 4C). Together, these findings indicate that platelets from SR-BI–/– mice circulated in a more activated state *in vivo* as compared to wild-type or SR-BI+/– platelets.

To investigate the functional consequences of the activated state of circulating platelets from SR-BI–/– mice, platelets were allowed to adhere to and spread on immobilized fibrinogen under static conditions during 60 minutes. After 30 min, the first SR-BI+/+ platelets showed signs of spreading. In contrast, the first platelets from SR-BI–/– mice already started to spread after 15 min and this number increased in time (Fig. 4D). Thus, platelets from SR-BI–/– mice spread more easily on immobilized fibrinogen than SR-BI+/+ platelets, which may explain the observed increased susceptibility to $FeCl_3$-induced thrombosis.

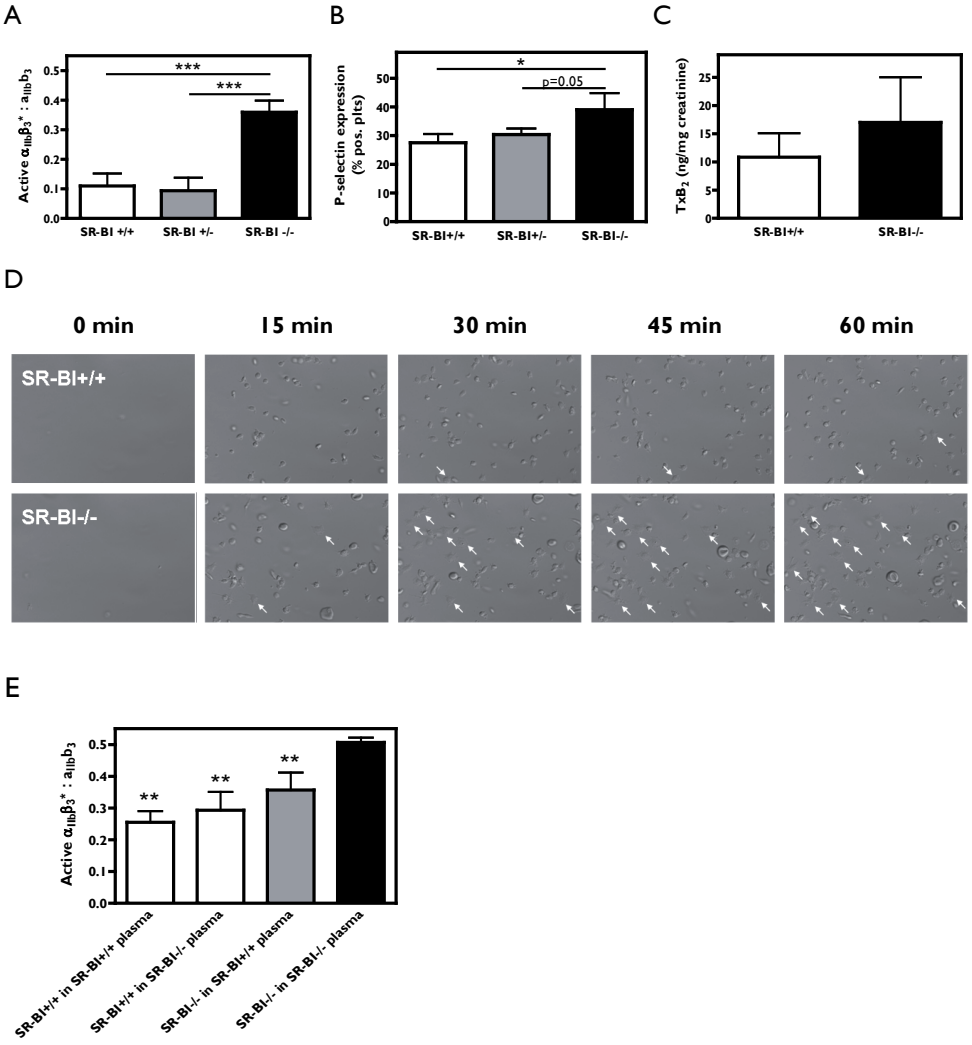

Fig. 4. SR-BI-/- platelets circulate in an activated state *in vivo* and show enhanced adhesion to fibrinogen.

The activity of integrin $\alpha_{IIb}\beta_3$ relative to total integrin $\alpha_{IIb}\beta_3$ protein (A), and surface-expressed P-selectin (B) on circulating SR-BI+/+, SR-BI+/–, or SR-BI–/– platelets were analyzed by flow cytometry. (C) 2,3-dinor TxB_2 levels in the urine of SR-BI+/+ and SR-BI–/– mice were measured by stable dilution isotope gas chromatography/mass spectrometry assays. (D) Washed SR-BI+/+ or SR-BI–/– platelet suspensions (0.5×10^{11} platelets/L) were seeded on fibrinogen-coated coverslips and allowed to spread for 60 min. The arrows indicate fully spread platelets. (E) SR-BI+/+ or SR-BI–/– platelets were incubated in either SR-BI+/+ or SR-BI–/– plasma (90 min, 37°C), and the activity of integrin $\alpha_{IIb}\beta_3$ relative to total integrin $\alpha_{IIb}\beta_3$ protein was analyzed by flow cytometry. Results (A-C and E) are represented as means±SD, n=3-5, *p<0.05, **p<0.01, ***p<0.001.

To investigate whether the increased basal activity of platelets from SR-BI–/– mice was due to the increased UC:TC ratio in SR-BI–/– plasma, the amount of integrin $\alpha_{IIb}\beta_3$ in the active high-affinity conformation was measured after incubation of SR-BI+/+ platelets in SR-BI–/– plasma, and vice versa. We observed a trend towards expression of more active integrin

$\alpha_{IIb}\beta_3$ on SR-BI+/+ platelets in SR-BI–/– plasma compared to SR-BI+/+ platelets in SR-BI+/+ plasma, while expression of active integrin $\alpha_{IIb}\beta_3$ on platelets from SR-BI–/– mice in SR-BI+/+ plasma was significantly reduced compared to SR-BI–/– platelets in autologous plasma (Fig. 4E). These findings suggest that the UC:TC ratio plays a role in platelet responsiveness of SR-BI–/– platelets. The cross-activation experiment did not show a complete reversal of the integrin activity, which may indicate that incubation of SR-BI+/+ platelets in SR-BI–/– plasma, and vice versa, for 90 minutes is not sufficient to completely reverse integrin activity.

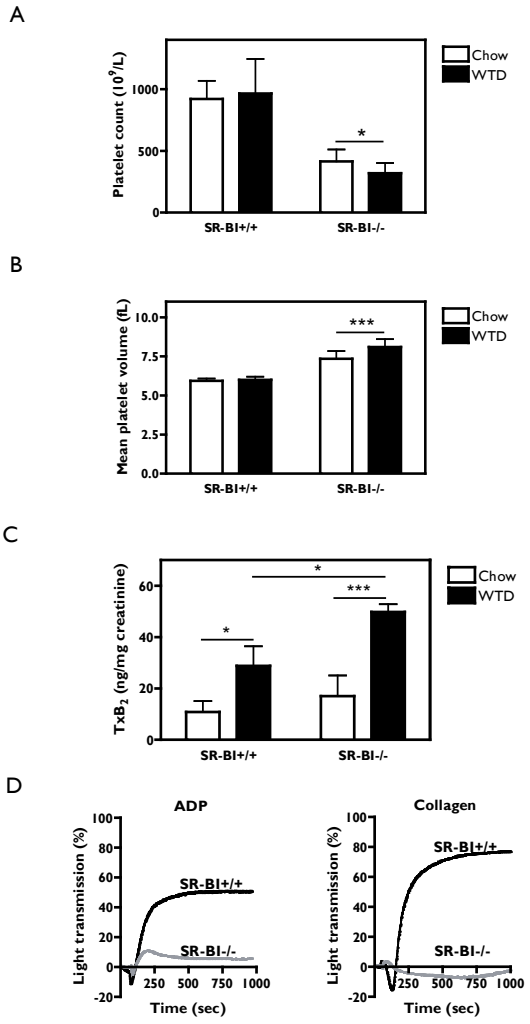

Fig. 5. The effects of SR-BI deficiency on platelet responsiveness are enhanced by a high fat/high cholesterol diet.

SR-BI+/+ and SR-BI–/– mice were fed a high cholesterol Western-type diet for 22 weeks. Platelet count (A), MPV (B), urinary 2,3-dinor TxB_2 levels (C), and aggregation of PRP in response to ADP or collagen (D) were measured as described in the legends to figures 1, 4, and 2, respectively. Results are expressed as means±SD for groups of 3 (TxB_2 levels) or 5 (platelet count and MPV) mice, *p<0.05, ***p<0.001.

A high fat/high cholesterol diet enhances the effects of SR-BI deficiency on platelet responsiveness

Challenging SR-BI+/+ and SR-BI–/– mice with a high fat/high cholesterol Western-type diet, containing 15% (w/w) cacao butter and 0.25% (w/w) cholesterol, induced a dramatic further increase of the UC (2.8-fold) and TC (6.4-fold) levels in the circulation of SR-BI–/– mice (Supplemental Table I), resulting in a further 2.3-fold increase of the UC:TC ratio compared to SR-BI+/+ mice ($p<0.001$). The Western-type diet challenge did not change the platelet count of SR-BI+/+ mice (chow vs WTD: $921\pm147x10^9$ platelets/L vs $965\pm280x10^9$ platelets/L, $p=0.75$), but further decreased the platelet count of SR-BI–/– mice ($415\pm97x10^9$ platelets/L vs $319\pm83x10^9$ platelets/L, $p=0.047$; Fig. 5A). Similarly, the size of platelets from SR-BI–/– mice increased further (from 7.3 ± 0.4 fL to 8.1 ± 0.5 fL, $p<0.001$), whereas no effect was observed regarding the size of SR-BI+/+ platelets (6.0 ± 0.2 fL both on chow and Western-type diet; Fig. 5B). The urinary levels of 2,3-dinor TxB_2 of SR-BI–/– mice were increased 1.7-fold from 28.8 ± 4.4 ng/mg creatinine for SR-BI+/+ mice to $49.8.0\pm1.4$ ng/mg creatinine for SR-BI–/– mice ($p=0.012$; Fig. 5C), indicating that platelets from SR-BI–/– mice in a high cholesterol environment were even more active *in vivo*. As a result, diet-induced hypercholesterolemia in SR-BI–/– mice also further decreased the *ex vivo* platelet aggregation response (Fig. 5D and Fig. 2A). Together, these findings indicate that Western-type diet-induced hypercholesterolemia in SR-BI–/– mice further enhanced the platelet characteristics that were observed while feeding the mice a regular chow diet.

Platelet-specific deletion of SR-BI by bone marrow transplantation does not affect platelet responsiveness

Transplantation of bone marrow offers a unique opportunity to specifically replace genes in cells from haematopoietic origin, including platelets, without affecting plasma cholesterol levels.[35] Therefore, to assess the influence of the UC:TC ratio in plasma on platelet function, bone marrow from either SR-BI+/+ or SR-BI–/– mice was transplanted into SR-BI+/+ mice. Successful reconstitution of recipients with haematopoietic donor cells was established by PCR-assisted amplification. Genomic DNA isolated from the recipient mice, transplanted with bone marrow from SR-BI–/– mice contained a prominent band indicative of the disrupted allele, whereas only a small band was visible representing the SR-BI+/+ allele (Supplemental Fig. II). The control transplanted mice only displayed the SR-BI+/+ band, indicating that the bone marrow transfer was successful. Selective disruption of SR-BI in bone marrow-derived cells by transplantation of SR-BI–/– bone marrow into SR-BI+/+ mice did not alter the plasma UC:TC ratio as compared to control transplanted SR-BI+/+ mice (Supplemental Table II). Moreover, platelet count, size, unesterified cholesterol content determined after Filipin III staining, PAR-4 peptide-induced platelet aggregation, and the basal activation state were also not affected (Fig. 6). Together, these findings indicate that changes in plasma cholesterol levels induced by the absence of SR-BI appear to be responsible for the observed changes in responsiveness of platelets of SR-BI–/– mice.

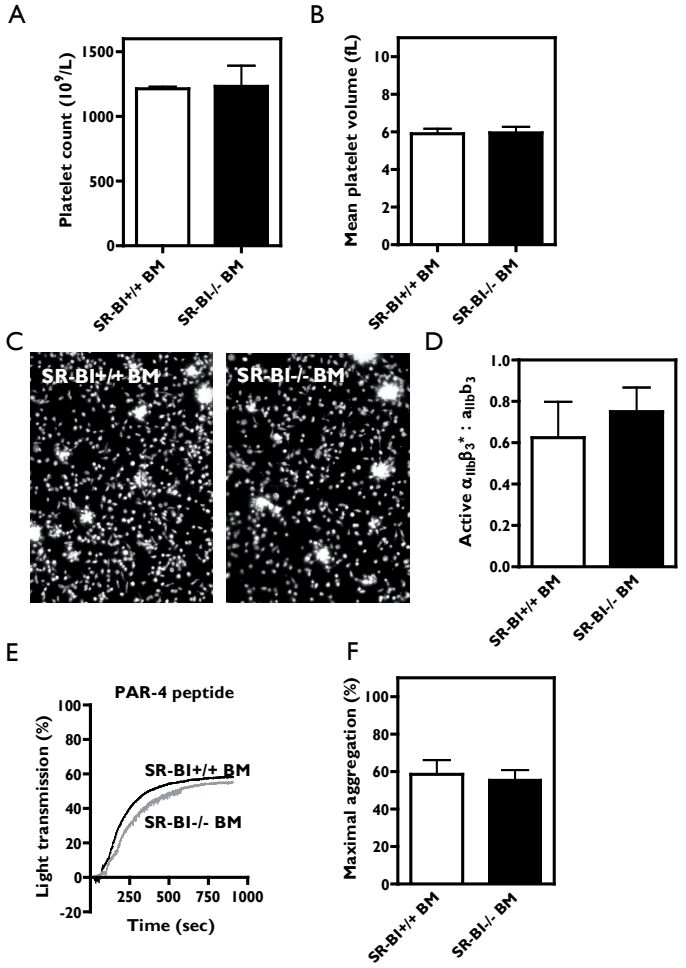

Fig. 6. Effects of platelet-specific SR-BI deficiency on platelet count, platelet size, cholesterol content, and platelet reactivity in normolipidemic conditions of SR-BI+/+ mice.
Platelet count (A) and MPV (B) in whole blood from chimeric SR-BI+/+ mice with SR-BI+/+ or SR-BI–/– bone marrow (BM), adhesion to and spreading of platelets to immobilized fibrinogen (C), the activity of integrin $\alpha_{IIb}\beta_3$ relative to total integrin $\alpha_{IIb}\beta_3$ protein on circulating platelets in whole blood (D), and aggregation of washed platelet suspensions in response to PAR-4 peptide (E and F) were measured as described in the legends to figures 1, 4, and 2, respectively. Results are expressed as means±SD, n=3-5.

DISCUSSION

SR-BI has a distinctive role in HDL metabolism and reverse cholesterol transport *in vivo*.[11,14-16,20] Targeted disruption of SR-BI in mice causes hypercholesterolemia, mainly due to abnormally large HDL particles containing high levels of both unesterified and esterified cholesterol, resulting in an unusually high UC:TC ratio (Supplemental Table). [15,21] The high UC:TC ratio observed in SR-BI-deficient mice was associated with macrothrombocytopenia,

which confirms recent findings[28], showing a 62% reduction in the blood platelet count and a 1.3-fold increase in size when comparing platelets from SR-BI–/– mice to those from SR-BI+/+ or SR-BI+/– mice. The observed macrothrombocytopenia was due to high platelet clearance rates, an increased intracellular cholesterol content, and abnormal platelet morphologies. The latter included an increase in size and the presence of multi-lamellar structures, probably for accommodation of excess unesterified cholesterol. Reciprocal infusion of biotinylated platelets from SR-BI+/+ or SR-BI–/– donor mice into SR-BI+/+ or SR-BI–/– recipient mice, or vice versa, resulted in intracellular unesterified cholesterol levels in the infused platelets that were virtually identical to those of resident platelets.[28] Accumulation of unesterified cholesterol in the SR-BI–/– platelets is thus most likely the result of non-specific lipid exchange from the abnormally UC-rich HDL particles that accumulate in SR-BI–/– mice, and not a direct effect of the absence of SR-BI on the platelets. In addition to these reported effects on platelet morphology, the current study shows the effects of SR-BI deficiency in mice on platelet physiology. Application of $FeCl_3$ to the adventitial surface of the carotid artery causes endothelial denudation, resulting in the formation of occluding platelet-rich thrombi[36] that are very similar to those found in humans.[31] Immediately after injury, resting platelets will adhere to exposed subendothelium and become activated by strong agonists present at the site of injury, including collagen and thrombin. In the $FeCl_3$ acute injury model, the measured time to occlusion in SR-BI–/– mice was reduced compared to the SR-BI+/+ mice, indicating that platelets from SR-BI–/– mice are more susceptible to form occluding thrombi. The increased susceptibility may be explained by the fact that in the absence of SR-BI, platelets circulate in an activated state, as observed by the increase in surface-expressed P-selectin (Supplemental Fig. III), the high level of TxB_2 metabolites in the urine, and the increased expression of active integrin $\alpha_{IIb}\beta_3$, which are all markers for platelet activation. Integrin $\alpha_{IIb}\beta_3$ is a prominent platelet integrin, which upon activation is capable of binding several adhesive proteins, including fibrinogen, to support platelet aggregation. It is also involved in the formation of a network of signaling and structural proteins that strongly interacts with the actin cytoskeleton. This network of proteins is located in cholesterol-rich domains, where they are suggested to be important in the platelet response to stimuli and in stabilizing platelet aggregates.[37] Interestingly, cholesterol depletion affects i.e. platelet aggregation.[38] Furthermore, platelet adhesion to fibrinogen is dependent on the presence of intact integrin $\alpha_{IIb}\beta_3$-containing cholesterol-rich domains at the tips of filopodia[39], indicating that the function of integrin $\alpha_{IIb}\beta_3$ is dependent on the integrity of the cholesterol-rich domains. Hence, an increase in the free cholesterol content of platelets from SR-BI-deficient mice may have an enhancing effect on platelet function due to an increase in cholesterol-rich domains and therefore the activity and functioning of integrin $\alpha_{IIb}\beta_3$. Indeed, in the absence of SR-BI, platelets showed an increased adherence to immobilized fibrinogen *ex vivo*. Fibrinogen binding is known to depend on prior activation of integrin $\alpha_{IIb}\beta_3$, but surface-coated fibrinogen also binds to the closed conformation present on resting platelets.[40] Thus, in addition to binding of immobilized fibrinogen to integrin $\alpha_{IIb}\beta_3$ in the closed conformation, adhesion to and spreading on immobilized fibrinogen by platelets from SR-BI–/– mice might well be facilitated by the increased number of integrin $\alpha_{IIb}\beta_3$ molecules in the high affinity ligand-binding conformation. We also observed a reduction of the TTO in SR-BI+/– mice in the $FeCl_3$ acute injury model compared to the SR-BI+/+ mice, but time to occlusion was significantly

longer when compared to the TTO in SR-BI–/– mice. Surprisingly, SR-BI+/– mice showed no signs of abnormal platelet characteristics and PGI_2 generation was similar in SR-BI+/+ and SR-BI–/– mice, and therefore probably also in SR-BI+/– mice, indicating a similar platelet inhibition and vasodilation. $FeCl_3$-induced acute arterial injury is based on the generation of reactive oxygen species that cause endothelial denudation.[36] In addition to being a key regulator of HDL metabolism, SR-BI is also involved in maintaining endothelial integrity after injury. This process is dependent on signaling via Src kinases, phosphatidylinositol 3-kinase, and p42/44 mitogen-activated protein kinase, leading to the activation of Rac GTP hydrolase and subsequent rearrangement of the actin cytoskeleton, finally resulting in endothelial repair. Maintenance of endothelial integrity is impaired in SR-BI–/– mice[41], and may also be affected in SR-BI+/– mice, resulting in faster platelet adhesion than in control SR-BI+/+ mice. Consequently, the quick formation of occlusive thrombi observed in SR-BI–/– mice may thus be the direct result of the combination of enhanced platelet responsiveness and impaired ability to maintain endothelial integrity.

Imachi *et al.* reported reduced levels of SR-BI on platelets from atherosclerotic disease patients that were inversely correlated with the intracellular cholesterol content of platelets and their ability to aggregate.[27] Moreover, platelets of hypercholesterolemic patients are hyperreactive and show hyperaggregability both *in vivo* and *in vitro* due to an increased cholesterol content[42,43], while cholesterol depletion by cyclodextrin impedes platelet activation.[44] Surprisingly, the aggregation response of platelets from SR-BI–/– mice was inhibited after stimulation by different agonists, despite their increased cholesterol content and the increased thrombosis susceptibility. This discrepancy may be explained by the highly activated state of the murine platelets in the circulation. Upon activation, platelets form and release TxA_2 and secrete active granule contents, including ADP, to initiate the activation of reinforcing activation pathways to aid in the induction of stable platelet activation. [45,46] The magnitude and rate of platelet granule secretion and TxA_2 generation is related to the potency of the stimulus: strong agonists like thrombin and collagen appear to release nearly all granules, whereas the degree of secretion will be less when platelets are stimulated by ADP.[47] Prolonged exposure of platelets to TxA_2 or ADP desensitizes the general platelet response due to desensitization of both the TPα/β receptor for TxA_2 and the P2Y receptors for ADP.[48] Hence, due to the activated state of platelets from SR-BI–/– mice *in vivo*, these platelets may have been subject to prolonged exposure to both ADP and TxA_2, which may have resulted in desensitization of the receptors for TxA_2 and ADP that are essential for positive feedback mechanisms to enhance the platelet response. As a result, *ex vivo* aggregation of platelets from SR-BI–/– mice may be reduced in response to agonist stimulation as compared to SR-BI+/+ platelets that responded normally. This is strengthened by the observation that SR-BI+/+ platelets, after a prolonged incubation in SR-BI–/– plasma, also showed an impaired platelet response. However, this is subject to further investigation, also because differences in cholesterol efflux and therefore differences in intracellular cholesterol content due to the absence or presence of SR-BI[30] may affect the platelet aggregation response.

When feeding mice a high fat/high cholesterol Western-type diet, the plasma UC:TC ratio was induced further as well as the size of SR-BI–/– platelets, probably due to a further increase of the intracellular cholesterol content. Moreover, under the influence of high cholesterol levels, SR-BI–/– platelets produced more TxA_2 indicating that platelets were even

more active. Praticò *et al.*[32] observed an increase in endogenous TxA_2 biosynthesis in mice deficient for either apoE or the LDL receptor and on a Western-type diet, which are murine models for atherosclerosis. The increased TxA_2 generation was the result of increased *in vivo* platelet activation. Under the influence of diet-induced hypercholesterolemia in SR-BI–/– mice, the aggregation response decreased further compared to platelets on a chow diet, which is in accordance with the higher activation state of SR-BI–/– platelets in a high cholesterol environment, pointing at the influence of cholesterol on platelet responsiveness. To investigate the specific influence of plasma cholesterol on platelet responsiveness, SR-BI was specifically disrupted in bone marrow-derived cells from SR-BI+/+ mice by a bone marrow transplantation approach, which did not change the UC:TC ratio in plasma compared to control mice. Platelet-specific deletion of SR-BI did not affect platelet count, size, intracellular cholesterol content, or platelet reactivity compared to control mice, indicating that SR-BI modulates platelet function through regulation of plasma cholesterol levels.

The observation that changes in the plasma UC:TC ratio were responsible for the altered responsiveness of platelets of SR-BI-deficient mice may have important pathophysiological consequences for other disease states that are also characterized by elevated plasma levels of unsterified cholesterol, such as familial Lecithin:Cholesterol Acyltransferase (LCAT) deficiency (FLD). The absence of LCAT, a plasma enzyme that esterifies free cholesterol present in circulating plasma lipoproteins, is associated with elevated levels of unesterified cholesterol and a decreased level of esterified cholesterol, fasting hypertriglyceridemia, and multiple blood cell anomalies.[49] Only a few studies have described the effects of FLD on platelet responsiveness. These studies demonstrated that platelets from FLD patients display decreased platelet adhesiveness in the glass bead filter method of Hellem[50] and a reduced sensitivity to low concentrations of thrombin[51]. Clearly, further research is required to conclusively establish the importance of the elevated unesterified cholesterol levels in FLD on platelet function.

In conclusion, disruption of SR-BI induces a rise in the plasma UC:TC ratio, leading to an increased activation status of circulating platelets and enhanced arterial thrombosis (Supplemental Fig. III), which may contribute to the enhanced atherosclerotic lesion development in SR-BI-deficient mice.[21,22] Hence, the function of SR-BI in maintaining normal cholesterol homeostasis and a low atherosclerosis susceptibility is indirectly also essential for maintaining normal platelet function and prevention of thrombosis.

ACKNOWLEDGEMENTS

This work was supported by grants of the Netherlands Heart Foundation (2006B107 to S.J.A.K., 2008T070 to M.H., 2000D040 to J.K., and 2001T041 and the Established Investigator grant 2007T056 to M.V.E.), the Netherlands Thrombosis Foundation (to J.-W.N.A), and the Netherlands Organization for Scientific Research (VIDI grant 917.66.301 to M.V.E. and I.M.).

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SUPPLEMENTARY APPENDIX

SUPPLEMENTAL MATERIALS AND METHODS

Materials

We obtained adenosine-5'-diphosphate (ADP), arachidonic acid (AA), cholesterol oxidase, and Filipin III from *Streptomyces filipinensis* from Sigma (St.Louis, MO, USA), cholesteryl esterase and peroxidase from Roche Diagnostics (Almere, The Netherlands), Precipath standardized serum from Roche (Woerden, The Netherlands), human fibrinogen and α-thrombin from Kordia Life Sciences (Leiden, The Netherlands), fibrillar equine collagen from Hormon Chemie (München, Germany), prostacyclin (PGI_2) from Cayman Chemical (Ann Arbor, MI), phorbol myristate 13-acetate (PMA) from ICN Biomedicals (Irvine, CA, USA), and protease-activating receptor-4 (PAR-4) peptide from Bachem (Bubendorf, Switzerland). All other chemicals were of analytical grade.

Antibodies

We obtained phycoerythrine (PE)-labeled anti-CD41/CD61 (integrin $\alpha_{IIb}\beta_3$; clone Leo.F2), PE-labeled anti-active CD41/CD61 (active form of integrin $\alpha_{IIb}\beta_3$; clone JON/A), PE-labeled anti-CD62P (P-selectin; clone Wug.E9), and the corresponding PE-labeled negative control from Emfret Analytics GmbH & Co. KG (Eibelstadt, Germany).

Mice

SR-BI-deficient mice were kindly provided by Dr. Monty Krieger[1]. Heterozygous SR-BI-deficient mice were cross-bred to generate wild-type (SR-BI+/+), heterozygous (SR-BI+/–) and homozygous SR-BI-deficient (SR-BI–/–) progeny. The presence of the targeted and wild-type SR-BI alleles was assessed by polymerase chain reaction (PCR) amplification of DNA extracted from tail biopsies (primers 5'-GAT-GGG-ACA-TGG-GAC-ACG-AAG-CCA-TTC-T-3' and 5'-TCT-GTC-TCC-GTC-TCC-TTC-AGG-TCC-TGA-3') as described by Van Eck *et al.*[2]. Female homozygous and heterozygous SR-BI–/– mice (generated on a 129Sv background and back-crossed five times to C57Bl/6) and wild-type littermate controls were maintained on a sterilized regular chow diet, containing 5.7% (w/w) fat and no cholesterol (RM3, Special Diet Services, Witham, UK), or on a semi-synthetic high fat/high cholesterol Western-type diet (WTD), containing 15% (w/w) cacao butter, 0.25% (w/w) cholesterol, and 1% corn oil (Diet W, Abdiets, Woerden, The Netherlands) during 22 weeks. For bone marrow transplantation studies, mice were housed in sterilized filter-top cages and given unlimited access to food and water. Mice were maintained on a sterilized regular chow diet, and drinking water was supplied with antibiotics (83 mg/L ciprofloxacin and 67 mg/L polymixin B sulfate) and 6.5 g/L sucrose for 4 weeks after bone marrow transplantation, which was then substituted for sterile drinking water without antibiotics. Mice were sacrificed 10 weeks after bone marrow transplantation. All experimental protocols were approved by the Ethics Committee for animal experiments of Leiden University. Animal experiments were performed at the Gorlaeus Laboratories of the Leiden/Amsterdam Center for Drug

Research in accordance with the national laws.

Blood Collection

SR-BI+/+, SR-BI+/–, and SR-BI–/– mice were anesthetized by subcutaneous injection of a mixture of xylazine (5 mg/mL), ketamine (40 mg/L) and atropine (0.05 mg/mL), and blood was subsequently collected into 0.1 volume 130 mmol/L trisodium citrate by cardiac puncture. Platelet count and mean platelet volume (MPV) were analyzed in whole blood using a Sysmex XT-2000iV Hematology Analyzer (Sysmex Europe GMBH, Norderstedt, Germany).

Biochemical Analyses

Plasma concentrations of unesterified cholesterol (UC) and total cholesterol (TC) were determined using enzymatic colorimetric assays with 0.025 U/mL cholesterol oxidase, 0.065 U/mL peroxidase,and 15 µg/mL cholesteryl esterase in reaction buffer (1.0 KPi buffer, pH=7.7 containing 0.01 mol/L phenol, 1 mmol/L 4-amino-antipyrine, 1% (v/v) polyoxyethylene-9-laurylether,and 7.5% (v/v) methanol). Absorbance was read at 490 nm. Precipath (standardized serum) was used as internal standard.

Platelet Isolation

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). Platelet-poor plasma (PPP) was prepared by centrifugation of the remainder of the blood sample (12000 *g*, 10 min, 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 PGI_2. Pellets were resuspended in Hepes-Tyrode buffer (145 mmol/L NaCl, 5 mmol/L KCl, 0.5 mmol/L Na_2HPO_4, 1 mmol/L $MgSO_4$, 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), or in PPP from either SR-BI+/+ or SR-BI–/– mice. In PRP and isolated platelet suspensions, the platelet count was adjusted with PPP or Hepes-Tyrode buffer (pH 7.2), respectively, to 2 x 10^{11} platelets/L, unless stated otherwise.

Analysis of platelet aggregation

PRP or washed platelet suspensions of SR-BI+/+, SR-BI+/–, and SR-BI–/– mice (100 µL) were mixed with 150 µL Hepes-Tyrode buffer (pH 7.2) containing 1 mM $CaCl_2$, and stimulated with ADP (25 µmol/L), collagen (5 µg/ml), PMA (0.5 µg/mL), or PAR-4 peptide (1 mmol/L) in the absence or presence (washed platelets stimulated by ADP or PMA) of fibrinogen (0.5 mg/mL). Optical aggregation was monitored in a Chrono-Log lumiaggregometer (Chrono-Log Corporation, Haverford, PA, USA) at 37°C and a stirring speed of 1000 r.p.m.. For cross-activation experiments, SR-BI+/+ or SR-BI–/– platelets were incubated in either SR-BI+/+ or SR-BI–/– plasma for 90 minutes (37°C) to ensure enough time for cholesterol trafficking between plasma and cells, and thus to acquire features of autologous platelets.

Thrombus Formation in Vivo

For *in vivo* thrombosis susceptibility analysis, the left carotid artery from SR-BI+/+, SR-BI+/–, and SR-BI–/– mice was dissected and exposed to a small 1x2 mm strip of filter paper saturated with 40% $FeCl_3$ for 3 minutes. For analysis of thrombus formation, the carotid blood flow was monitored continuously using an ultrasonic Doppler flow probe (Transonic Systems Inc., NY, USA) to measure the time to occlusion (TTO) of the artery. The TTO was determined as the time between the $FeCl_3$ application and zero blood flow velocity.

Analysis of Activation State of Circulating Platelets

To investigate the activation state of circulating platelets, the presence of active integrin $\alpha_{IIb}\beta_3$ relative to the amount of integrin $\alpha_{IIb}\beta_3$ protein as well as surface-expressed P-selectin were determined by flow cytometry in the absence of added agonists. For measurement of active integrin $\alpha_{IIb}\beta_3$ and total integrin $\alpha_{IIb}\beta_3$ protein, 25 µL whole blood was diluted (1:20 (v/v) with Hepes-Tyrode buffer (pH 7.2)), recalcified (1 mM $CaCl_2$), and subsequently incubated with 5 µL PE-conjugated anti-active integrin $\alpha_{IIb}\beta_3$ (5 µL, JON/A) or PE-conjugated anti-integrin $\alpha_{IIb}\beta_3$ (clone Leo. F2), respectively, for 15 min at 20°C. Samples were fixed with 1% formaldehyde in phosphate-buffered saline (PBS) (20°C), and analyzed by flow cytometry. The ratio of active integrin $\alpha_{IIb}\beta_3$*/integrin $\alpha_{IIb}\beta_3$ protein is a marker for platelet activation. A similar experiment was performed after incubation (90 min, 37°C) of SR-BI+/+ platelets in either SR-BI+/+ or SR-BI–/– plasma, and SR-BI–/– in SR-BI+/+ or SR-BI–/– plasma. For measurement of surface-expressed P-selectin, PE-conjugated anti-CD62P (5 µL, Wug.E9) was incubated with 25 µL 1 x 10^6 washed platelets (15 min, 20°C), fixed with 1% formaldehyde in PBS (20°C), and analyzed by flow cytometry (FACScalibur, Becton Dickinson, Mountain View, CA, USA).

In addition, urinary 2,3-dinor thromboxane B_2 (TxB_2) and 2,3-dinor 6-keto prostaglandin $F_{1\alpha}$ ($PGF_{1\alpha}$) levels were determined as a measure for the generation of TxA_2 or prostacyclin (PGI_2). Hereto, TxB_2 and $PGF_{1\alpha}$ were measured in the urine of SR-BI+/+ and SR-BI–/– mice by stable dilution isotope gas chromatography/mass spectrometry assays, as previously described[3]. Briefly, a known amount of each eicosanoid tetradeuterated internal standard was added to the samples. After solid phase extraction, samples were derived and purified by chromatography and analyzed on a Fison MD-800 GC/MS.

Adhesion and Spreading on Fibrinogen under Static Conditions

Coverslips were coated with 100 µg/mL human fibrinogen (1 hr, 20°C) and blocked with 1% human serum albumin in PBS. Washed platelet suspensions were diluted to a concentration of 0.5 x 10^{11} platelets/L in Hepes-Tyrode buffer and prewarmed to 37°C. After seeding on the fibrinogen-coated coverslips, platelets were allowed to spread for 60 min. Platelet spreading was analyzed by differential interface contrast microscopy (Carl Zeiss BV, Göttingen, Germany). Images were taken every 30 sec and analyzed using Axiovision 4.6.3. software (Carl Zeiss MicroImaging GmbH, Göttingen, Germany). Afterwards, coverslips were fixed with 1% paraformaldehyde in PBS for further analysis.

Filipin III staining of adhered platelets

Coverslips from the static adhesion experiments were washed with PBS, incubated in 50 mmol/L NH_4Cl/PBS (10 min, 20°C), washed again and blocked with PBS containing 3% BSA (10 min, 37°C). To detect membrane cholesterol, coverslips were incubated with 50 μg/mL filipin III (2.5 mg/mL stock solution filipin III in dimethylformamide) in PBS/1% BSA (1 h, 20°C)[4]. Finally, coverslips were washed with PBS and mounted in Aqua/Poly Mount. Cells were visualized with a Nikon Eclipse E600 fluorescence microscope (60x Nikon objective) equipped with a CoolSNAP-Pro camera (Media Cybernetics, Inc. Silver Spring, MD), using Image Pro Plus software (Media Cybernetics, Inc.) for analysis.

Bone marrow transplantation

To induce bone marrow (BM) aplasia[5], wild-type C57Bl/6 mice were exposed to a single dose of 9 Gy (0.15 Gy/min, 200 kV, 4 mA) total body irradiation, using an Andrex Smart 225 Röntgen source (XYLON Int., Copenhagen, Denmark) with 6-mm aluminium filter, 1 day before transplantation. Bone marrow was isolated by flushing the femurs and tibias from SR-BI–/– mice or wild-type littermates with PBS. Single-cell suspensions were prepared by passing the cells through a 30-μm nylon gauze. Irradiated recipients received $0.5x10^7$ bone marrow cells by intravenous injection into the tail vein. The haematological assessment of the transplanted mice was determined in genomic DNA from bone marrow cells by PCR. Two oligonucleotides were used for PCR amplification to detect both the wild-type and the null mutant SR-BI gene simultaneously, as described above.

Statistical analysis

All values are reported as means±SD. Statistical significant differences among the means of the different groups of mice were calculated using the two-tailed unpaired Student's t-test (Graphpad Instat Software, San Diego, CA). $P<0.05$ (*) was considered significant.

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Table I. SR-BI deficiency leads to increased plasma HDL cholesterol levels.

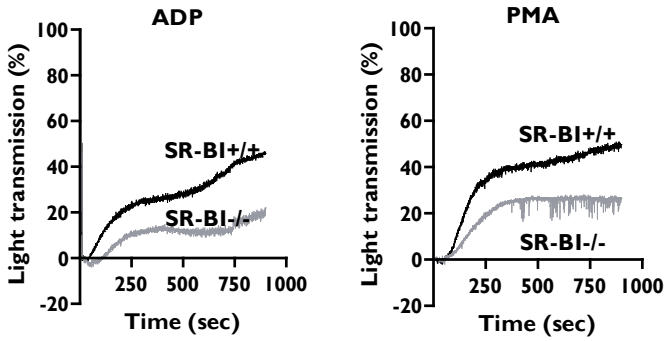
Mice	n	Diet	UC	TC	UC:TC
			mg/dL	*mg/dL*	
SR-BI+/+	8	Chow	23.3±2.7	92.6±8.4	0.25
SR-BI–/–	8	Chow	63.5±3.6***	159.7±5.3***	0.40
SR-BI+/+	5	WTD	28.5±3.1	135.0±2.7	0.21
SR-BI–/–	5	WTD	181.1±8.3***	372.3±10.1***	0.49

Plasma cholesterol levels were measured in SR-BI+/+ and SR-BI–/– mice maintained on a regular chow diet or on a high fat/high cholesterol Western-type diet. Data represent the means±SD, n=8. *** p<0.001

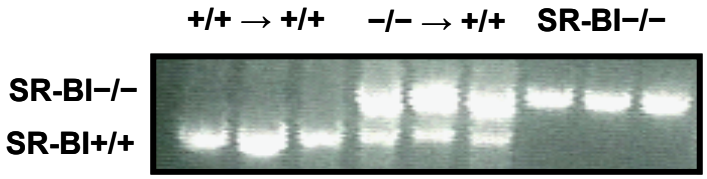
Table II. Plasma lipid levels in wild-type mice after disruption of SR-BI in bone marrow cells.

Mice	n	Diet	UC	TC	UC:TC
			mg/dL	*mg/dL*	
SR-BI+/+ → SR-BI+/+	3	Chow	13.7±2.4	57.7±16.6	0.24
SR-BI–/– → SR-BI+/+	5	Chow	13.5±4.7	52.7±8.8	0.26

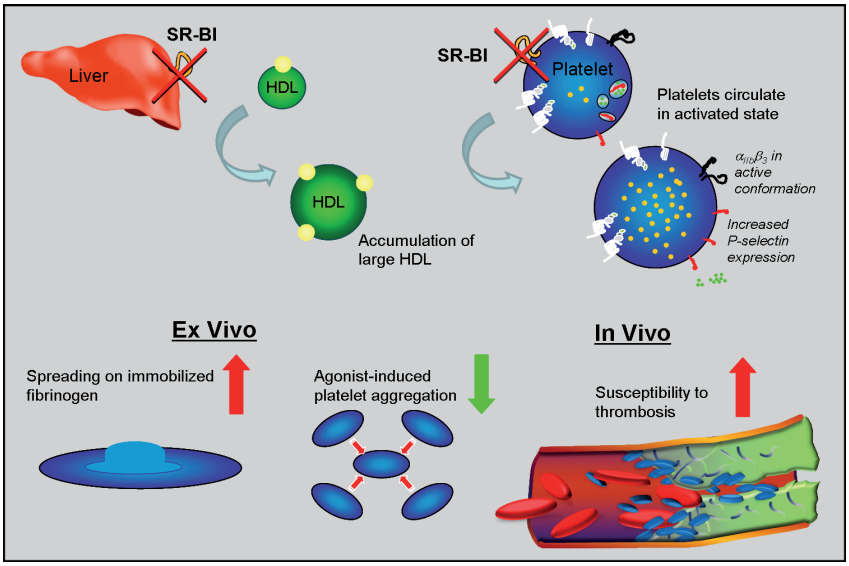
Plasma cholesterol levels were measured in SR-BI+/+ mice 10 weeks after transplantation with SR-BI+/+ or SR-BI–/– bone marrow. Mice were maintained on a regular chow diet. Data represent the means±SD, n=3-5.



Supplemental Fig. I. Impaired aggregation response of plasma-free washed SR-BI-/- platelets. Plasma-free washed platelet suspensions were isolated from SR-BI+/+ (black lines) and SR-BI–/– (grey lines) mice, platelet count was adjusted to 2 x 10^{11} platelets/L, and platelets were stimulated with 25 µmol/L ADP or 0.5 µg/mL PMA. Optical aggregation was measured in a Chrono-Log lumiaggregometer at 37°C and at a stirring speed of 1000 r.p.m.. Traces shown are the mean of 3 independent experiments.



Supplemental Fig. II. Verification of success of bone marrow transplantation. Successful reconstitution of SR-BI+/+ mice with donor haematopoietic cells from either SR-BI+/+ or SR-BI–/– mice was verified by PCR amplification of the wild-type and the null mutant SR-BI gene using genomic DNA isolated from bone marrow of the chimeric SR-BI+/+ mice. PCR amplification of DNA extracted from tail biopsies from SR-BI–/– mice was performed as a control.



Supplemental Fig. III. SR-BI deficiency modulates platelet function and susceptibility to thrombosis. Disruption of SR-BI induces an increase in the plasma UC:TC ratio, mainly due to the accumulation of large cholesterol-rich HDL particles. This leads to the presence of active platelets in the circulation, which is obvious from the increased surface expression of integrin αIIbβ3 in the high affinity open conformation, and P-selectin. The activated SR-BI-deficient platelets show enhanced adherence to immobilized fibrinogen and SR-BI-deficient mice display enhanced arterial thrombosis, which may eventually contribute to the atherosclerosis observed in SR-BI-deficient mice.