The Effects of Diet on Occlusive Coronary Artery Atherosclerosis and Myocardial Infarction in Scavenger Receptor Class B, Type 1/Low-Density Lipoprotein Receptor Double Knockout Mice

Mark Fuller, Omid Dadoo, Viktoria Serkis, Dina Abutouk, Melissa MacDonald, Neel Dhingani, Joseph Macri, Suleiman A. Igdoura, Bernardo L. Trigatti

Objective—Deficiency of the high-density lipoprotein receptor, scavenger receptor class B, type I (SR-BI), in apolipoprotein E knockout or hypomorphic mice, respectively, results in spontaneous or diet-inducible occlusive coronary artery (CA) atherosclerosis, myocardial infarction, and early death. Here, we examine effects of SR-BI deficiency on cardiovascular phenotypes in low-density lipoprotein receptor (LDLR) knockout mice fed different atherogenic diets.

Approach and Results—SR-BI/LDLR double knockout and control LDLR knockout mice were fed atherogenic diets containing different amounts of fat, cholesterol, and sodium cholate. Double knockout mice fed atherogenic diets high in cholesterol exhibited significantly reduced survival compared with LDLR knockout mice fed the same diets. In addition to increased diet-accelerated aortic sinus atherosclerosis, we observed significant diet-induced CA atherosclerosis in double knockout mice and diet-dependent accumulation of platelets in CA atherosclerotic plaques. This was accompanied by substantial myocardial fibrosis in double knockout mice fed high cholesterol diets. Atherogenic diet fed double knockout mice also exhibited higher circulating cytokine levels, monocytosis with increased proportions of Ly6C<sup>hi</sup> and Ly6C<sup>int</sup> monocytes, and higher adhesion molecule expression in CA endothelial cells compared with control LDLR knockout mice.

Conclusions—Diet-accelerated atherosclerosis and occlusive, platelet-rich CA disease in SR-BI/LDLR double knockout mice is affected by amounts of cholesterol and cholate in atherogenic diets and is accompanied by increased expression of vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 in CAs and increased Ly6C<sup>hi</sup> and Ly6C<sup>int</sup> monocytes in circulation. The increased vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 in CA endothelial cells in SR-BI-deficient mice likely explains their increased susceptibility to atherosclerosis in CAs. (Arterioscler Thromb Vasc Biol. 2014;34:2394-2403.)

Key Words: atherosclerosis ■ HDL ■ receptors, lipoprotein ■ thrombosis

Atherosclerosis is a chronic inflammatory disease driven by complex interactions between circulating lipoproteins, immune cells, and the cells of the artery wall. Atherosclerotic coronary artery disease (CAD) is a leading cause of death worldwide, and hypercholesterolemia is a major risk factor. High levels of cholesterol circulating in low and very low-density lipoproteins (LDL and VLDL, respectively) are major risk factors for atherosclerosis, whereas high levels of high-density lipoproteins (HDLs) are considered to be atheroprotective. Traditional murine models of atherosclerosis are genetically predisposed to hypercholesterolemia and include the apolipoprotein E (apoE) knockout mouse and the fat-fed LDL receptor (LDLR) knockout mouse. The aortic sinus, the lesser curvature of the aortic arch, and other regions of large arteries where blood flow is nonlaminar are robustly susceptible to the development of atherosclerotic plaques in these mouse models. However, coronary arteries (CAs), the arteries that supply blood to the heart, remain largely resistant to atherosclerosis. These mice, therefore, do not present with many of the clinical complications of atherosclerosis experienced by human CAD patients, including myocardial infarction and early death.

See cover image

The HDL receptor, scavenger receptor class B type I (SR-BI), is expressed on the surface of multiple cell types and has been shown to mediate both HDL-dependent atheroprotective
Atherosclerosis in the Aortic Sinus

Figure 1E to 1L shows images representing aortic sinus plaque burden in control LDLR knockout mice (Figure 1E–1H) and dKO mice (Figure 1I–1L) fed each of the atherogenic diets. Aortic sinus plaque size was analyzed after 12 weeks of feeding the HF or HC diets and at 10 weeks of feeding the HFC diet or 3.5 weeks of feeding the HFCC diet. These times were chosen to correspond to the length of time for ~50% survival of the dKO mice on the respective diets. Although longer feeding periods generally resulted in larger plaques, aortic sinus plaque area was dramatically and significantly increased in dKO mice fed any of the 4 diets that we tested compared with control LDLR knockout mice fed the same diet for the same length of time (Figure 1M–1P). dKO mice fed a normal chow diet and age-matched to HF- and HC-fed mice (22 weeks old) also developed substantially more atherosclerosis in their aortic sinuses compared with age-matched LDLR knockout mice (Figure 1A–1C in the online-only Data Supplement). The extent of atherosclerosis in normal chow–fed dKO mice was much less than similarly aged dKO mice that had been fed either the HF or HC diet for 12 weeks (compare Figure 1B and 1C in the online-only Data Supplement). The extent of atherosclerosis in the aortas of SR-BI–deficient mice was not assessed.15

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

Survival of SR-BI/LDLR dKO Mice Fed Different Atherogenic Diets

We studied the effects of 4 different atherogenic diets with varying levels of fat, cholesterol, and sodium cholate on the progression of atherosclerosis in SR-BI/LDLR dKO and control LDLR knockout mice. Mice were fed a HF diet (Western-type diet; 22% fat, 0.15% cholesterol), a HC diet (2% cholesterol), a HFC diet (15.8% fat, 1.25% cholesterol), or the same diet containing sodium cholate (high fat, high cholesterol, cholate [HFCC] diet, commonly referred to as the Paigen diet).16 As controls, dKO and LDLR knockout mice were also fed a normal chow diet. The dKO mice fed the HFCC, HFC, and HC diets but not those fed the HF diet ≤12 weeks exhibited reduced survival compared with LDLR knockout mice fed the same diets (Figure 1A–1D). Average survival for dKO mice fed the HFCC, HFC, and HC diets was 3.5, 9.4, and 11.4 weeks, respectively. No dKO mice fed the HFCC diet survived >4.5 weeks of feeding, whereas ≈7% of mice fed the HFC diet and ≈64% of mice fed the HC diet lived the full 12 weeks. Neither dKO mice nor LDLR knockout control mice fed normal chow diets exhibited any reductions in survival during the course of our studies. These survival trends are similar to those reported by Nakagawa-Toyama et al16 in SR-BI knockout/apoE hypomorphic mice when fed the HFCC, HFC, and HF diets.

Atherosclerosis in the Aortic Sinus

The dKO mouse is, therefore, a novel model of diet-inducible CA atherothrombosis.

Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>apoE</td>
<td>apolipoprotein E</td>
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<tr>
<td>CA</td>
<td>coronary artery</td>
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<tr>
<td>CAD</td>
<td>coronary artery disease</td>
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<td>HC</td>
<td>high cholesterol</td>
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<td>HDL</td>
<td>high-density lipoprotein</td>
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<td>HFC</td>
<td>high fat, high cholesterol</td>
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<tr>
<td>HFCC</td>
<td>high fat, high cholesterol, cholate</td>
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<tr>
<td>ICAM-1</td>
<td>intercellular adhesion molecule-1</td>
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<tr>
<td>IL-6</td>
<td>interleukin-6</td>
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<td>LDL</td>
<td>low-density lipoprotein</td>
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<td>LDLR</td>
<td>low-density lipoprotein receptor</td>
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<tr>
<td>SR-BI</td>
<td>scavenger receptor class B, type I</td>
</tr>
<tr>
<td>TNFα</td>
<td>tumor necrosis factor-α</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>vascular cell adhesion molecule-1</td>
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<td>VLDL</td>
<td>very low-density lipoprotein</td>
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Atherosclerosis in CAs

CA atherosclerosis was measured by scoring all CAs observed in at least 3 oil red O–stained cross-sections into 5 categories based on the level of occlusion. Representative images of CAs that were plaque-free, which contained fatty streaks, or which were <50% occluded, >50% occluded, and 100% occluded with raised atherosclerotic plaques are shown in Figure 2A to 2E. All SR-BI/LDLR dKO mice developed occlusive CA atherosclerosis when fed any of the 4 atherogenic diets (Figure 2F–2I). SR-BI/LDLR dKO mice had significantly greater proportions of CAs with all categories of atherosclerotic plaques and correspondingly significantly smaller proportions of CAs that were plaque-free compared with control LDLR knockout mice fed the same diets for similar time periods. In fact, >95% of CAs observed in all LDLR knockout control groups, regardless of atherogenic diet, were either plaque-free or contained only fatty streaks. In contrast, <60% of CAs from SR-BI/LDLR dKO mice were either plaque-free or contained only fatty streaks, regardless of the atherogenic diet. SR-BI/LDLR dKO mice fed the HF diet for 12 weeks exhibited the lowest proportion of completely occluded CAs (18%), whereas the SR-BI/LDLR dKO mice fed the HC (12 weeks), HFC (10 weeks), or HFCC diet (3.5 weeks) had similarly high proportions with ≥30% of CAs completely occluded. Interestingly, the CA atherosclerosis burden did not seem to correlate directly with the atherosclerotic plaque sizes in the aortic sinus in SR-BI/LDLR dKO mice fed the 4 atherogenic diets. In particular, dKO mice fed the HFCC diet for 3.5 weeks had the lowest burden of aortic sinus atherosclerosis (Figure 1P) but among the highest burden of CA atherosclerosis (Figure 2I). In contrast, chow-fed dKO mice developed comparatively little CA atherosclerosis by 22 weeks of age (Figure IE in the online-only Data Supplement).

An important feature of human coronary heart disease is acute thrombosis on top of atherosclerotic plaques in CAs. We, therefore, looked for molecular evidence of thrombosis in dKO mice fed atherogenic diets. Thrombosis was detected in cryosections of CAs by immunofluorescence for CD41, a surface protein expressed by platelets. Figure 2J to 2Q show representative images of CD41-stained (red) CAs in mice fed each of the 4 diets. No CAs from LDLR knockout mice stained positively for platelets, consistent with the general

![Figure 1. Scavenger receptor class B, type I (SR-BI)/low-density lipoprotein receptor (LDLR) double knockout (dKO) mice exhibit diet-dependent reduced survival and develop larger atherosclerotic plaques in their aortic sinuses compared with similarly treated LDLR KO control mice. Female SR-BI/LDLR dKO and control LDLR KO mice aged 10 to 12 wk were fed atherogenic diets as indicated on the left and monitored until they reached cardiac end point for ≥12 weeks. A to D, Kaplan–Meier survival curves for SR-BI/LDLR dKO mice compared with control LDLR KO mice fed each diet. SR-BI/LDLR dKO mice were harvested and analyzed at cardiac end point or 12 wk of feeding; the arrows indicate the feeding times for LDLR KO control mice used for all other comparisons. *P<0.05 by Kaplan–Meier survival analysis vs control LDLR KO mice fed the same diet, †high fat, high cholesterol, cholate (HFCC)–fed dKO mice, §high cholesterol (HC)–fed dKO mice, ¶high fat (HF)–fed dKO mice. E to L, Representative oil red O–stained cryosections of aortic sinuses from control LDLR KO (I to L) and SR-BI/LDLR dKO (I to L) mice fed different atherogenic diets for the time periods indicated in A to D. M to P, Quantification of aortic sinus atherosclerosis in SR-BI/LDLR dKO and control LDLR KO mice fed the indicated diets for time periods shown in A. *P<0.1 by Student t test between dKO and control mice.](http://atvb.ahajournals.org/)

Figure 1. Scavenger receptor class B, type I (SR-BI)/low-density lipoprotein receptor (LDLR) double knockout (dKO) mice exhibit diet-dependent reduced survival and develop larger atherosclerotic plaques in their aortic sinuses compared with similarly treated LDLR KO control mice. Female SR-BI/LDLR dKO and control LDLR KO mice aged 10 to 12 wk were fed atherogenic diets as indicated on the left and monitored until they reached cardiac end point for ≥12 weeks. A to D, Kaplan–Meier survival curves for SR-BI/LDLR dKO mice compared with control LDLR KO mice fed each diet. SR-BI/LDLR dKO mice were harvested and analyzed at cardiac end point or 12 wk of feeding; the arrows indicate the feeding times for LDLR KO control mice used for all other comparisons. *P<0.05 by Kaplan–Meier survival analysis vs control LDLR KO mice fed the same diet, †high fat, high cholesterol, cholate (HFCC)–fed dKO mice, §high cholesterol (HC)–fed dKO mice, ¶high fat (HF)–fed dKO mice. E to L, Representative oil red O–stained cryosections of aortic sinuses from control LDLR KO (I to L) and SR-BI/LDLR dKO (I to L) mice fed different atherogenic diets for the time periods indicated in A to D. M to P, Quantification of aortic sinus atherosclerosis in SR-BI/LDLR dKO and control LDLR KO mice fed the indicated diets for time periods shown in A. *P<0.1 by Student t test between dKO and control mice.
absence of atherosclerotic plaques. On the contrary, platelet CD41 was detected in CA plaques from dKO mice fed each of the 4 atherogenic diets (Figure 2R–2U). Interestingly, both the abundance of platelet staining (Figure 2N–2Q) and the number of CAs per section that stained positively for platelet CD41 (Figure 2R–2U) were substantially higher in the SR-BI/LDLR dKO mice fed the HFCC diet than for those fed other atherogenic diets.

**Myocardial Infarction**

Frozen cross-sections of myocardial tissue were stained with Masson trichrome to detect collagen-rich fibrotic areas. Representative images are shown in Figure 3A to 3H. SR-BI/LDLR dKO mice fed the HFCC, HFC, and HC diets had similar levels of myocardial fibrosis, which were substantially and significantly higher than levels observed in HF-fed dKO mice (Figure 3I–3L). No fibrosis was detected in any LDLR knockout control mice regardless of diet (Figure 3I–3L) or in dKO mice fed a normal chow diet ≤22 weeks of age (Figure IF and IG in the online-only Data Supplement). Similarly, we observed no differences in heart to body weight ratio between dKO and LDLR knockout mice fed the normal chow diets. Heart to body weight ratios were significantly increased to a similar extent in all atherogenic diet–fed dKO groups compared with LDLR knockout control mice fed the same diets for similar periods of time (Figure 3M–3P), suggesting that...
increased heart size is likely not a direct response to myocardial fibrosis.

Plasma Lipoproteins and Cytokines
To investigate potential mechanisms underlying the increased susceptibility to atherosclerosis in the aortic sinus and CAs observed in dKO mice compared with control LDLR knockout mice, we analyzed plasma lipid levels and lipoprotein cholesterol profiles from mice fed the 4 atherogenic diets and the normal chow diet. Consistent with previous reports, plasma total cholesterol was 1.5-fold higher in the chow-fed LDLR knockout mice across all 4 atherogenic diets, such that the ratios of plasma total cholesterol levels to free total cholesterol were consistently 2- to 3-fold higher compared with the LDLR knockout mice fed the control diet and each of the 4 atherogenic diets, such that the ratios of free to total cholesterol were consistently 2- to 3-fold higher in dKO mice fed each of the diets. We also noted that plasma levels of each cytokine were consistently higher in dKO than the LDLR knockout control mice across all 4 atherogenic diets, such that the ratios of plasma levels of each cytokine were consistently higher in dKO than the LDLR knockout control mice across all 4 atherogenic diets.

Figure 3. Large myocardial infarctions are observed in scavenger receptor class B, type I (SR-BI)/low-density lipoprotein receptor (LDLR) double knockout (dKO) mice fed diets that are very high in cholesterol. A to H, Representative images of heart cryosections from SR-BI/LDLR dKO and control LDLR KO mice fed the indicated atherogenic diets stained with Masson trichrome. I to L, Quantification of average infarct size in control LDLR KO and SR-BI/LDLR dKO mice fed each of the diets. M to P, Heart weights normalized to body weights for each group of mice. *P<0.05 by Student t test. HC indicates high cholesterol; HF, high fat; HFC, high fat, high cholesterol; and HFCC, high fat, high cholesterol, cholate.

Because inflammation is also a major driver of atherosclerosis, we measured interleukin-6 (IL-6) and tumor necrosis factor-α (TNFα) in plasma from these mice. IL-6 and TNFα were undetectable in LDLR knockout or dKO mice fed normal chow ≤22 weeks of age (data not shown). Each of the 4 atherogenic diets resulted in significantly increased levels of both IL-6 (Figure 4E–4H) and TNF-α (Figure 4I–4L) in both the LDLR knockout and the dKO mice; however, plasma levels of each cytokine were consistently higher in dKO than in LDLR knockout mice fed the same diet. We also noted that plasma levels of IL-6 and TNFα were higher in dKO mice fed the HFCC, HFC, and HC diets compared with dKO mice fed the HF diet, suggesting that they may have been influenced by dietary cholesterol and cholate.
Blood Cells

Because immune cells are major components of atherosclerotic plaques and can also influence levels of inflammatory cytokines in the plasma, we ran hematology profiles, including monocyte and lymphocyte counts, on the blood of HFCC-fed mice. Circulating monocytes and lymphocytes were both significantly elevated in dKO mice compared with control LDLR knockout mice (Table II in the online-only Data Supplement). HFCC-fed dKO mice also exhibited substantially and significantly larger spleens than control LDLR knockout mice, which were further increased in size on atherogenic diet feeding (Table II in the online-only Data Supplement). HFCC-fed dKO mice also exhibited substantially and significantly larger spleens than control LDLR knockout mice, which were further increased in size on atherogenic diet feeding (Table II in the online-only Data Supplement).

CA Endothelial Cell Activation

To further investigate the robust susceptibility of SR-BI/LDLR dKO mice to CA atherosclerosis, we investigated...
the expression of VCAM-1 and ICAM-1 in the CAs of both control LDLR knockout and dKO mice by immunofluorescence. Figure 6A to 6D depicts representative images of VCAM-1 and ICAM-1 staining in nonatherosclerotic CAs from HFCC-fed LDLR knockout and dKO mice. Quantification of VCAM-1 and ICAM-1 immunostaining in nonatherosclerotic CAs from mice fed each of the 4 atherogenic diets revealed that atherogenic diet–fed dKO mice had significantly greater proportions of nonatherosclerotic CAs exhibiting immunodetectable VCAM-1 (Figure 6E–6H) and ICAM-1 (Figure 6I–6L) compared with control LDLR knockout mice fed the same diets for similar times. In contrast, no differences were seen in VCAM-1 or ICAM-1 levels in LDLR knockout mice fed either the atherogenic diets or fed a normal diet ≤22 weeks of age, and no differences were seen between the chow-fed 22-week-old dKO and LDLR knockout mice (Figure IM–IR in the online-only Data Supplement). These data demonstrate that each of the 4 atherogenic diets tested induces significant VCAM-1 and ICAM-1 expression in nonatherosclerotic CAs only in the dKO mice.

Discussion

In this study, we demonstrated that SR-BI/LDLR dKO mice develop diet-accelerated occlusive CA atherosclerosis and myocardial infarction, in which the severity of the disease can be modulated by altering different components of the diet. We have previously shown that dKO mice develop increased aortic atherosclerosis compared with control LDLR knockout mice when fed a HF diet; however, there was no difference in survival, and the study did not examine atherosclerosis in CAs. Consistent with our previous findings, dKO mice fed the HF diet for 12 weeks did not exhibit reduced survival but had significantly increased aortic sinus atherosclerosis compared with LDLR knockout controls (Figure 1). Occlusive CA atherosclerosis was observed in these mice (Figure 2); however, only small myocardial infarctions were detected (Figure 3). In contrast, higher levels of occlusive CA atherosclerosis developed in dKO mice fed diets much higher in cholesterol (Figure 2). This was accompanied by large myocardial infarctions (Figure 3) and significantly reduced survival (Figure 1) compared with similarly fed LDLR knockout mice. Control LDLR knockout mice fed any of the 4 atherogenic diets that

![Figure 6](http://atvb.ahajournals.org/)

**Figure 6.** Larger proportions of coronary arteries (CAs) stain positively for endothelial activation markers vascular cell adhesion molecule-1 (VCAM-1) and intercellular adhesion molecule-1 (ICAM-1) in scavenger receptor class B, type I (SR-BI)/low-density lipoprotein receptor (LDLR) double knockout (dKO) mice compared with control LDLR KO mice fed each of the atherogenic diets. VCAM-1 and ICAM-1 expression in CAs was detected by immunofluorescence in cryosections. A to D, Images of CAs stained for VCAM-1 (A and B) or ICAM-1 (C and D), represented in red, from high fat, high cholesterol, cholate (HFCC)–fed LDLR KO (A and C) and SR-BI/LDLR dKO (B and D) mice. Autofluorescence of the artery wall is shown in green; DAPI-counterstained nuclei are shown in blue. Lower panels represent higher magnification views of the boxed regions. VCAM-1– and ICAM-1–activated CAs, as defined in the Materials and Methods, were counted and expressed as a proportion of the total arteries counted (E to L). Overall, *P<0.001 for dKO vs LDLR KO for both markers by 2-way ANOVA. *P<0.05 by Holm–Sidak post hoc test. HC indicates high cholesterol; HF, high fat; and HFC, high fat, high cholesterol.
we tested developed virtually no CAD ≤12 weeks of feeding. We also showed that aggregated platelets are present in a subset of occluded CAs in the dKO mice, and that platelet staining is most prominent in CAs of dKO mice fed the HFCC diet (Figure 2), which led to the most rapid development of myocardial infarction (Figure 3) and the earliest death of dKO mice (Figure 1). These data suggest that the nature of the atherogenic diet affects not only the extent and time course of development of CA atherosclerosis but also affects the severity of platelet accumulation within plaques, possibly reflecting plaque rupture and thrombosis.

Deficiency of SR-BI increases cholesterol associated with enlarged HDL particles in otherwise wild-type mice and in LDLR knockout mice fed normal chow (Figure ID in the online-only Data Supplement), and in VLDL and intermediate density lipoprotein/LDL in apoE knockout mice fed normal chow and apoE hypomorphic mice fed the HFCC diet. In contrast, SR-BI/LDLR dKO mice have lower cholesterol levels associated with VLDL and LDL compared with LDLR knockout mice on all 4 of the atherogenic diets. This is consistent with our previous reports of reduced cholesterol associated with VLDL and reduced plasma apoB in SR-BI/LDLR dKO mice compared with LDLR knockout mice when both were fed the HF diet, and with the reduced apoB levels observed in SR-BI/apoE dKO mice compared with apoE single knockout controls. In the HF or HC-fed SR-BI/LDLR dKO mice, this seems to be a consequence of reduced VLDL production as compared with similarly fed LDLR knockout control mice (Figure II in the online-only Data Supplement). The mechanism of reduced VLDL production in dKO mice is not clear; however, SR-BI overexpression has been shown to enhance apoB trafficking and apoB secretion in Caco-2 cells, whereas loss of SR-BI function and knockdown of SR-BI blunts apoB trafficking and secretion, respectively. SR-BI may have an equivalent role in hepatocytes. A similar reduction in VLDL levels has also been reported in LDLR knockout mice that lack apolipoprotein A1, the major apolipoprotein of HDL. Additionally, apolipoprotein A1 deficiency has been linked with reduced hepatic VLDL production in mice, suggesting that interaction between SR-BI and its major ligand may be responsible for influencing the production of VLDL; further experiments are required to confirm this. It is possible that this effect of a lack SR-BI on VLDL production is an antiatherogenic effect that is masked by more severe proatherogenic forces, yet it is also possible that this represents as a consequence of a fundamental disruption in VLDL metabolism, resulting in promotion of atherosclerosis. Nevertheless, in this context, increased atherosclerosis is associated with lower overall and VLDL cholesterol levels, and this observation suggests that the susceptibility of SR-BI/LDLR dKO mice to CAD cannot be explained by exacerbated hypercholesterolemia, although the disproportionately large ratio of free cholesterol to total cholesterol (Table I in the online-only Data Supplement) indicates there is likely a major effect on lipoprotein composition, which could, in turn, affect CAD susceptibility. The influence of a lack of SR-BI on the composition and function of different lipoproteins in these mice requires further investigation.

There is mounting evidence that SR-BI may play an atheroprotective role in immune cells. Bone marrow–specific deficiency of SR-BI in both apoE knockout and LDLR knockout mice increases atherosclerosis in the aorta. Conversely, we have recently shown that restoring SR-BI expression in the bone marrow of SR-BI knockout/apoE hypomorphic mice reduces diet-induced occlusive CA atherosclerosis, whereas others have shown that transplanting SR-BI/apoE dKO mice with wild-type bone marrow has a similar effect. SR-BI in macrophages reduces the inflammatory response to lipopolysaccharide treatment in vitro, and SR-BI deficiency is associated with higher serum cytokine levels in naïve, septic, and lipopolysaccharide-challenged mice.

Results from the current study show that SR-BI/LDLR dKO mice challenged with atherogenic diets have increased levels of both IL-6 and TNFα in plasma, accompanied by increased numbers of both monocytes and lymphocytes in blood compared with LDLR knockout mice fed the same diets. We have previously demonstrated that monocyte recruitment into atherosclerotic plaques is attenuated by restoration of SR-BI expression in the bone marrow of SR-BI knockout/apoE hypomorphic mice, and that SR-BI–deficient monocytes bind VCAM-1 and ICAM-1 more readily than SR-BI–expressing monocytes. Monocytes can be divided into subsets based on their level of Ly6C expression. Ly6Cmonocytes are considered to be more inflammatory, adhere more efficiently to activated endothelium, migrate more efficiently into established atherosclerotic plaques, and selectively accumulate in atherosclerotic plaques compared with Ly6Cmonocytes. We observed a large shift in the monocyte populations with HFCC-fed dKO mice exhibiting significantly higher proportions of Ly6Cmonocytes and Ly6Cmonocytes and correspondingly lower proportions of Ly6Cmonocytes compared with LDLR knockout control mice fed similar diets.

Although increased inflammation and altered lipoprotein metabolism may explain an increase in susceptibility to atherosclerosis in general, the robust dichotomy between the SR-BI/LDLR dKO mice and the LDLR knockout controls in terms of CA atherosclerosis suggests that SR-BI may influence the susceptibility of the vessels themselves. A major factor that dictates the regions of the vascular system that are prone to atherosclerosis is the activation of endothelial cells. Endothelial cells from arterial regions that experience laminar flow conditions express very low levels of VCAM-1 and ICAM-1, and these arteries tend to be resistant to atherosclerosis. Alternatively, endothelial cells from arterial regions that experience nonlaminar blood flow and low shear stress exhibit reduced endothelial nitric oxide synthase expression and activation and increased expression of adhesion molecules VCAM-1 and ICAM-1. This provides favorable sites for monocyte adhesion and initiation of atherosclerosis. SR-BI mediates HDL-induced upregulation and activation of eNOS in vivo and concomitant suppression of VCAM-1 and ICAM-1 expression by endothelial cells in vitro. Our results provide in vivo evidence that endothelial cells in nonatherosclerotic CAs of dKO mice fed atherogenic diets express more VCAM-1 and ICAM-1 compared with CA endothelial cells in LDLR knockout control mice. This may at least in
part explain why SR-BI deficiency in LDLR knockout mice gives rise to diet-induced CA atherosclerosis, whereas LDLR single knockout mice seem to be largely resistant. A limitation of study is that it is difficult to determine if the Ly6C\(^{hi}\) monocytes, inflammation, and upregulated VCAM-1 and ICAM-1 expression in CA endothelial cells precede development of CAD, or if they are consequences of enhanced lesion development. Further investigation will be required to conclude that these are causative factors of CAD in these mice.

In summary, SR-BI/LDLR dKO mice are a robust and flexible mouse model of diet-accelerated occlusive CA atherothrombosis and myocardial infarction, which could prove to be a useful tool in understanding the mechanisms and exploring new treatments for human coronary heart disease. We think that mouse CAD resulting from SR-BI deficiency is a multifactorial phenomenon that may be influenced by a lack of SR-BI function in multiple cell types, including hepatocytes, immune cells, and endothelial cells. In particular, the increased expression of endothelial cell adhesion molecules in CAs, together with the increased proportions of circulating Ly6C\(^{hi}\) monocytes, may conspire to trigger diet-induced occlusive CA atherosclerosis in these mice, which, depending on the atherogenic diet used, leads to substantial platelet accumulation in occluded CAs, myocardial infarction, and early death. The importance of specific protective roles of SR-BI in each of these cell types and characterization of the molecular pathways involved requires further investigation and may lead to the identification of new therapeutic targets in the treatments of cardiovascular diseases.

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Disclosures

None.

References

Significance

Scavenger receptor class B, type I is an high-density lipoprotein receptor expressed on the surface of multiple cell types and plays critical roles in facilitating selective cholesterol transport between high-density lipoprotein and cells and mediating high-density lipoprotein–induced signaling. Here we show that a lack of scavenger receptor class B, type I in low-density lipoprotein receptor knockout mice leads to diet-accelerated occlusive coronary artery atherosclerosis with evidence of platelet accumulation, myocardial infarction, and spontaneous death. This phenotype is associated with leukocytosis, increased inflammation, and elevated expression of adhesion molecules in the coronary endothelium. This work highlights the importance of the protective roles of scavenger receptor class B, type I in nonhepatic tissues and describes a novel small animal model of diet-accelerated coronary heart disease.
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Supplementary Figure I

A. LDLR KO
B. SR-BI/LDLR dKO
C. Plaque Area
D. Total Cholesterol (mM)
E. Percent of Arteries
F. LDLR KO
G. SR-BI/LDLR dKO
H. Monocytes (Fold Change)
I. Lymphocytes (Fold Change)
J. Ly6C Low Cells (%)
K. Ly6C Int Cells (%)
L. Ly6C High Cells (%)
M. VCAM
N. ICAM
O. VCAM
P. ICAM
Q. % of VCAM activated arteries
R. % of ICAM activated arteries
Supplementary Figure I. Atherosclerosis and associated phenotypes in normal chow fed SR-BI/LDLR dKO and control LDLR KO mice. Female mice were fed a normal chow diet and were analyzed at 22 weeks of age to match the longest period of atherogenic diet feeding. (A,B) Representative oil red O-stained sections of aortic sinuses from LDLR KO and SR-BI/LDLR dKO mice, respectively. (C) Quantification of average plaque area in the aortic sinuses of each group. (D) Representative FPLC lipoprotein cholesterol profiles for each group. (E) Quantification of coronary artery atherosclerosis in each group. (F,G) Representative Masson's Trichrome stained sections of myocardium illustrating absence of fibrosis in both groups of mice. (H,I) Relative monocyte and lymphocyte levels in each group as measured on a Hemavet Multi Species Hematology System. (J-L) Proportions of circulating Ly6C$^{hi}$, Ly6C$^{int}$ and Ly6C$^{lo}$ monocytes in each group as measured by flow cytometry. (M-P) Representative immunofluorescent images of coronary arteries stained for VCAM-1 (M,N) and ICAM-1 (O,P), represented in red, from each group of mice. (Q,R) Quantification of VCAM-1 (Q) and ICAM-1 (R) activated coronary arteries as outlined in the materials and methods.
Supplementary Figure II. Hepatic VLDL triglyceride secretion is reduced in SR-BI/LDLR dKO mice compared to LDLR KO control mice fed HF and HFCC diets. 10 week old mice were fed HF or HFCC diet for 2 weeks. Mice were fasted overnight and lipoprotein lipase was inhibited by a single injection of tyloxapol (A) or poloxamer (B) at time 0. Blood was collected hourly for 4 (A) or 3 (B) hours. (A) HF diet fed mice. (B) HFCC diet fed mice. *P<0.05 vs. LDLR KO for a given time point by student's T-test.
### Supplementary Table I. Plasma lipid parameters in LDLR KO and SR-BI/LDLR dKO mice fed different diets.

Total cholesterol, free cholesterol, and triglycerides were measured using commercial assays. Cholesterol ester levels were calculated as total cholesterol - free cholesterol. Results are presented as mean ± SEM for each measurement. Sample sizes are indicated in parentheses in the genotype column.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Genotype</th>
<th>Total Cholesterol (TC) (mM)</th>
<th>Free Cholesterol (FC) (mM)</th>
<th>FC:TC Ratio (%)</th>
<th>Cholesterol Ester (mM)</th>
<th>Triglycerides (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chow</td>
<td>LDLR KO (7)</td>
<td>12.2 ± 1.0</td>
<td>3.2 ± 0.3</td>
<td>26.3 ± 0.9%</td>
<td>9.0 ± 0.5</td>
<td>2.3 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>SR-BI/LDLR dKO (11)</td>
<td>17.8 ± 0.6*</td>
<td>9.0 ± 0.5*</td>
<td>50.4 ± 1.6%*</td>
<td>8.8 ± 0.4</td>
<td>2.9 ± 0.3</td>
</tr>
<tr>
<td>HF</td>
<td>LDLR KO (7)</td>
<td>36.0 ± 3.2</td>
<td>11.1 ± 1.2</td>
<td>30.9 ± 2.2%</td>
<td>24.9 ± 2.4</td>
<td>2.6 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>SR-BI/LDLR dKO (9)</td>
<td>27.0 ± 2.0*</td>
<td>19.7 ± 1.4*</td>
<td>74.1 ± 4.1%*</td>
<td>7.3 ± 1.3*</td>
<td>1.7 ± 0.3*</td>
</tr>
<tr>
<td>HC</td>
<td>LDLR KO (8)</td>
<td>57.1 ± 5.4</td>
<td>15.1 ± 1.7</td>
<td>27.1 ± 2.3%</td>
<td>42.0 ± 4.5</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>SR-BI/LDLR dKO (8)</td>
<td>32.7 ± 3.3*</td>
<td>17.8 ± 2.1</td>
<td>55.0 ± 4.2%*</td>
<td>15.0 ± 2.3*</td>
<td>1.6 ± 0.4</td>
</tr>
<tr>
<td>HFC</td>
<td>LDLR KO (12)</td>
<td>50.0 ± 3.8</td>
<td>13.5 ± 0.9</td>
<td>27.6 ± 1.1%</td>
<td>36.5 ± 3.3</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>SR-BI/LDLR dKO (10)</td>
<td>38.7 ± 3.7</td>
<td>26.5 ± 2.0*</td>
<td>70.3 ± 2.9%*</td>
<td>12.2 ± 2.0*</td>
<td>1.2 ± 0.1*</td>
</tr>
<tr>
<td>HFCC</td>
<td>LDLR KO (14)</td>
<td>106.0 ± 5.2</td>
<td>27.1 ± 1.4</td>
<td>25.7 ± 0.8%</td>
<td>78.9 ± 4.1</td>
<td>2.1 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>SR-BI/LDLR dKO (8)</td>
<td>49.9 ± 5.0*</td>
<td>41.5 ± 3.5*</td>
<td>81.3 ± 5.4%*</td>
<td>10.1 ± 2.9*</td>
<td>1.3 ± 0.3</td>
</tr>
</tbody>
</table>

### Supplementary Table II. Spleen weights. ND: Not determined. *P<0.01 by student’s T-test. Sample sizes are indicated in parentheses in the genotype column.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Genotype</th>
<th>Spleen Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chow</td>
<td>LDLR KO (3)</td>
<td>0.11 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>SR-BI/LDLR dKO (5)</td>
<td>0.31 ± 0.04*</td>
</tr>
<tr>
<td>HF</td>
<td>LDLR KO (10)</td>
<td>0.19 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>SR-BI/LDLR dKO (7)</td>
<td>0.95 ± 0.05*</td>
</tr>
<tr>
<td>HC</td>
<td>LDLR KO (3)</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>SR-BI/LDLR dKO (3)</td>
<td>1.09 ± 0.21</td>
</tr>
<tr>
<td>HFC</td>
<td>LDLR KO (12)</td>
<td>0.12 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>SR-BI/LDLR dKO (6)</td>
<td>1.03 ± 0.05*</td>
</tr>
<tr>
<td>HFCC</td>
<td>LDLR KO (7)</td>
<td>0.13 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>SR-BI/LDLR dKO (7)</td>
<td>1.63 ± 0.07*</td>
</tr>
</tbody>
</table>
Supplementary Table III. Hematology profiles of Chow- and HFCC-fed SR-BI/LDLR dKO and control LDLR KO mice. Whole blood was collected from tail or submandibular veins of 22 week old chow-fed mice or 12 week old mice fed the HFCC diet for 2 weeks. Hematology profiles were generated using a Hemavet Multi-Species Hematology System (Drew Scientific). Results shown are mean ± SEM for each output. Sample sizes are indicated in parentheses in the genotype column. *P<0.01 vs LDLR KO.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Genotype</th>
<th>RBC Count (M/μl)</th>
<th>Hematocrit (%)</th>
<th>Mean RBC Volume (fL)</th>
<th>RBC Dist. Width (%)</th>
<th>Platelet Count (Fold Change)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chow</td>
<td>LDLR KO (6)</td>
<td>9.42 ± 0.50</td>
<td>51.9 ± 2.8</td>
<td>55.0 ± 0.2</td>
<td>14.1 ± 0.1</td>
<td>1 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>SR-BI/LDLR dKO (5)</td>
<td>7.66 ± 0.62</td>
<td>49.5 ± 4.1</td>
<td>64.1 ± 1.6*</td>
<td>22.7 ± 1.0*</td>
<td>0.82 ± 0.10</td>
</tr>
<tr>
<td>HFCC</td>
<td>LDLR KO (5)</td>
<td>10.42 ± 0.27</td>
<td>59.6 ± 1.6</td>
<td>57.2 ± 0.6</td>
<td>21.0 ± 0.9</td>
<td>1 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>SR-BI/LDLR dKO (6)</td>
<td>5.24 ± 0.21*</td>
<td>45.3 ± 1.4*</td>
<td>86.6 ± 1.1*</td>
<td>28.2 ± 0.4*</td>
<td>0.88 ± 0.05</td>
</tr>
</tbody>
</table>
Materials and Methods

Animals

All procedures involving animals were approved by the Animal Research Ethics Board of McMaster University and are in accordance with the guidelines of the Canadian Council on Animal Care. All mice are on mixed C57BL/6:129 backgrounds. SR-BI^{+/−}/LDLR KO mice were bred together to generate littermate SR-BI/LDLR dKO mice and LDLR KO control mice. Only female mice were used in this study.

Diet Induction of Atherosclerosis

The following atherogenic diets were used in this study: The high fat, high cholesterol, cholate containing (HFCC or “Paigen”) diet (Harlan Teklad TD88051) contains 15% fat (7.5% from cocoa butter), 1.25% cholesterol and 0.5% sodium cholate. The high fat, high cholesterol (HFC) diet (Harlan Teklad TD90221) is identical to the HFCC diet except that it lacks sodium cholate. The high cholesterol (HC) diet is a normal chow diet supplemented with 2% cholesterol (Harlan Teklad TD01383). The high fat (HF) or Western type diet contains 21% butter fat and 0.15% cholesterol (Dyets Inc. 112286). SR-BI/LDLR dKO and control LDLR KO mice were fed one of the above diets starting at 10-12 weeks of age and monitored daily. Mice were fed until they reached cardiac endpoint or for up to twelve weeks. Cardiac endpoint was identified as exhibiting one or more of the following symptoms: hunched posture, laboured breathing, unsteady gate and ruffled coat. For HFCC and HFC fed groups, separate control LDLR KO groups in which the length of feeding equaled the mean survival of the corresponding SR-BI/LDLR dKO groups were generated. Separate cohorts of control dKO and LDLR KO mice were also fed the normal chow diet until they were 22 weeks of age (age matched to the 12 week atherogenic diet fed mice).
Histology

Hearts were perfused in situ through the left ventricle with 10 U/mL heparinised saline followed by 10% formalin. Hearts were then excised, fixed overnight in 10% formalin, cryoprotected in 30% sucrose for 24 hours and embedded in OCT. 10µm transverse cryosections were cut from the middle of the heart to the aortic sinus in 0.5mm intervals; the aortic sinus was sampled in 0.1mm intervals from the base of the valve leaflets to the coronary ostia.

Atherosclerosis was detected in the aortic sinus and CA’s by oil red O staining. Atherosclerotic plaque cross-sectional area in the aortic sinus was measured manually using ImageJ software in the section that best represented three full and intact valve leaflets. CA atherosclerosis was assessed in transverse sections from the middle of the heart up to the sinus in 0.5mm intervals. CA’s were scored as either lacking atherosclerosis (no plaque), containing fatty streaks, or which were <50% occluded, >50% occluded or 100% occluded by atherosclerotic plaques.

Myocardial fibrosis was detected by Masson’s trichrome stain (Sigma), which stains collagen-rich fibrotic tissue blue, and healthy myocardium red. Total infarcted area was measured manually using the outline function in ImageJ software in images taken with a 10X objective lens on an Axiovert 200M microscope (Zeiss). Fibrotic area was measured in non-overlapping images of two transverse sections from each mouse near the top of the heart spaced 0.5mm apart and expressed as average cross-sectional area per section.

Immunofluorescence

Platelets in CA’s were detected using a rat anti-mouse CD41 antibody (BD Pharmingen, 553847, Mississauga, Canada). CA’s staining positive for platelets were counted and normalized to the total number of sections stained. Endothelial adhesion molecule expression was detected using cell-culture supernatants from rat B-lymphocyte hybridoma cells that
produce antibodies against mouse VCAM-1 or ICAM-1 (CRL-1909 and CRL 1878, respectively, ATCC, Manassas, VA, USA). We defined an ICAM-1-activated artery as containing a continuous line of at least 4 cells staining positive for ICAM-1, while VCAM-1 activated arteries were defined as arteries with detectable VCAM-1 expression. Using these criteria, all CA’s observed in at least 4 sections per mouse were counted and classified as either activated or not activated for each marker. All antigens were visualized using a goat anti-rat IgG secondary antibody conjugated to AlexaFluor 594 (Molecular Probes, Burlington, ON, Canada).

Plasma lipid and lipoprotein analysis
Plasma was prepared from blood collected via cardiac puncture at the time of harvest. Total cholesterol, free cholesterol and triglyceride were measured by enzymatic assays following manufacturers’ protocols (total cholesterol: Cholesterol Infinity, Thermo Scientific, Ottawa, ON, Canada, free cholesterol: Free Cholesterol E, Wako Diagnostics, Mountain View, CA, USA, triglyceride: L-Type Triglyceride M, Wako Chemicals, Richmond, VA, USA). Plasma lipoproteins were fractionated by size exclusion chromatography over a Superose 6 column using an AKTA Fast Protein Liquid Chromatography System (GE Biosciences, Baie d’Urfe, QC, Canada). Total cholesterol in the resulting fractions was measured by enzymatic assay as described above.

Triglyceride production
10 week old mice were fed the HF or HFCC diet for 2 weeks. Mice were fasted overnight before lipoprotein lipase (LPL) was inhibited by intraperitoneal poloxamer 500mg/kg (HF) or intravenous tyloxapol 500mg/kg (HFCC). Blood was collected hourly following LPL inhibition, and triglyceride concentration in plasma was measured as described above.

Plasma Cytokine Measurements
Interleukin 6 and tumor necrosis factor (TNF-α) levels in plasma were measured by ELISA (BioLegend, San Diego, CA) following the manufacturer's instructions.

Blood Cell Analysis

Blood was collected via the tail vein from a subset of HFCC or control diet fed mice prior to harvest. Blood cells were counted using a Hemavet Multi-Species Hematology System (Drew Scientific). Ly6C expression was assessed by flow cytometry on a BD LSR II flow cytometer. Briefly, red blood cells and dead cells were excluded from analysis based on forward scatter and side scatter plots. Monocytes were defined as blood cells expressing both CD11b and CD115 and detected with FITC- and PE- conjugated antibodies, respectively (BD Pharmingen). Ly6C expression was then analyzed in this sub-population using an APC-conjugated antibody (BD Pharmingen).

Statistical Analysis

For comparisons between two groups, data was first analyzed for Normality using the Shapiro-Wilk test. Data that passed this test for Normality were analyzed using Student’s t-test and considered significant if P<0.05. Comparisons between multiple groups were made using one-way or two-way ANOVA where appropriate coupled with Dunn’s and Holm-Sidak post-hoc tests, respectively.
