Liver ABCA1 Deletion in LDLrKO Mice Does Not Impair Macrophage Reverse Cholesterol Transport or Exacerbate Atherogenesis

Xin Bi, Xuewei Zhu, MyNgan Duong, Elena Y. Boudyguina, Martha D. Wilson, Abraham K. Gebre, John S. Parks

Objective—Hepatic ATP binding cassette transporter A1 (ABCA1) expression is critical for maintaining plasma high-density lipoprotein (HDL) concentrations, but its role in macrophage reverse cholesterol transport and atherosclerosis is not fully understood. We investigated atherosclerosis development and reverse cholesterol transport in hepatocyte-specific ABCA1 knockout (HSKO) mice in the low-density lipoprotein (LDL) receptor KO (LDLrKO) C57BL/6 background.

Approach and Results—Male and female LDLrKO and HSKO/LDLrKO mice were switched from chow at 8 weeks of age to an atherogenic diet (10% palm oil, 0.2% cholesterol) for 16 weeks. Chow-fed HSKO/LDLrKO mice had HDL concentrations 10% to 20% of LDLrKO mice, but similar very low-density lipoprotein and LDL concentrations. Surprisingly, HSKO/LDLrKO mice fed the atherogenic diet had significantly lower (40% to 60%) very low-density lipoprotein, LDL, and HDL concentrations (50%) compared with LDLrKO mice. Aortic surface lesion area and cholesterol content were similar for both genotypes of mice, but aortic root intimal area was significantly lower (20% to 40%) in HSKO/LDLrKO mice. Although macrophage 

Conclusions—The markedly reduced plasma HDL pool in HSKO/LDLrKO mice is sufficient to maintain macrophage reverse cholesterol transport, which, along with reduced plasma very-low-density lipoprotein and LDL concentrations, prevented the expected increase in atherosclerosis. (Arterioscler Thromb Vasc Biol. 2013;33:2288-2296.)

Key Words: atherosclerosis cardiovascular diseases cholesterol lipids lipoproteins

Atherosclerosis-associated cardiovascular disease (CVD) is the leading cause of death worldwide. The inverse relationship between plasma high-density lipoprotein (HDL) levels and CVD risk has made raising HDL levels a popular potential therapeutic target for CVD prevention. ATP binding cassette transporter A1 (ABCA1) belongs to a large family of the ATP binding cassette transporters. ABCA1 mediates cellular free cholesterol (FC) and phospholipid efflux to apolipoprotein A-I (apoA-I), resulting in the formation of nascent HDL that undergoes subsequent maturation to become plasma HDL. The critical role of ABCA1 in HDL formation was established when it was found to be the genetic defect in Tangier disease, a disorder in which HDL levels are <5% of normal. Studies with animal models have also documented the essential function of ABCA1 in maintaining plasma HDL levels.

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Despite the well-established role of ABCA1 in HDL formation, its effect on atherosogenesis is less clear. Premature atherosclerosis has been reported in some, but not all, people with Tangier disease. Common genetic variants in ABCA1 have been reported to influence the risk and severity of CVD; however, low HDL caused by loss-of-function mutations in ABCA1 does not contribute to increased risk of CVD in the general population. Controversial results also exist in studies with mouse models. Overexpression of human ABCA1 (hABCA1) in the liver and macrophages of B6 mice resulted in an antiatherogenic lipid profile and lower aortic atherosclerosis, whereas in apoE knockout (KO) mice, overexpressing hABCA1 increased atherosclerosis with minimal effect on plasma lipids. Physiological overexpression of a
full-length hABCA1 containing bacterial artificial chromosome in apoE KO and low-density lipoprotein (LDL) receptor KO (LDLrKO) mice both revealed an atheroprotective role of ABCA1.15,16 Significantly larger lesions occurred in ApoE or LDLrKO mice transplanted with bone marrow from mice with total body ABCA1 deficiency.17–20 In contrast, total body ABCA1 deficiency in apoE KO or LDLrKO mice did not result in increased atherosclerosis compared with control mice.18 The complex relationship between global ABCA1 expression and atherosclerosis susceptibility observed in humans and mouse models of atherosclerosis was at least partially attributed to reduction in atherogenic lipoproteins concomitant with ABCA1 deficiency, or to the use of different promoters for transgenic overexpression.

Subsequent studies with hepatocyte-specific ABCA1 KO (HSKO) mice suggested a major role for the liver in maintaining systemic HDL levels, leading to investigation of the role of hepatic ABCA1 in atherogenesis.6 Joyce et al21 found that liver-specific overexpression of ABCA1 in LDLrKO mice led to increased atherosclerosis, presumably because of increased plasma concentrations of apoB-containing lipoproteins (apoB Lp) concomitant with a significant increase in plasma HDL. A more recent study of hepatic ABCA1 deletion in chow-fed apoE KO mice showed a significant increase in early-stage atherosclerosis.18 However, several issues were not addressed in that study. First, only early atherosclerosis was examined; mice consumed a chow diet for 12 weeks, and total plasma cholesterol (TPC) concentrations were relatively low (250–400 mg/dL). Thus, the effect of hepatic ABCA1 deletion on more advanced atherosclerosis is unknown. Second, HDL cholesterol (HDL-C) concentrations are quite low in apoE KO mice (≈80% lower than wild-type mice).6,23 Fast protein liquid chromatography (FPLC) fractionation of plasma lipoproteins showed that chow-fed HSKO/LDLrKO mice had significant reductions in HDL-C (1.74 versus 0.21 mmol/L in males; 67.3 versus 8.1 mg/dL in males, P<0.0001; 1.50 versus 0.28 mmol/L in females; 58.0 versus 10.9 mg/dL in females, P<0.0001), contributing to the lower TPC levels in HSKO/LDLrKO versus LDLrKO mice (4.48 versus 2.68 mmol/L in males; 173.1 versus 103.6 mg/dL in males, P<0.0001; 5.17 versus 3.44 mmol/L in females; 199.7 versus 133 mg/dL in females, P=0.0004). VLDL-C and LDL-C concentrations were similar between genotypes (Figure 1C). The less pronounced hyperlipidemia in atherogenic diet–fed HSKO/LDLrKO mice was mainly attributed to lower VLDL-C (14.43 versus 6.08 mmol/L in males; 557.9 versus 235.0 mg/dL in males, P<0.0050; 16.49 versus 10.05 mmol/L in females; 637.7 versus 388.7 mg/dL in females, P=0.0283) and LDL-C levels (16.19 versus 11.65 mmol/L in males; 626.2 versus 450.5 mg/dL in males, P=0.0221; 11.80 versus 10.32 mmol/L in females; 456.3 versus 399.0 mg/dL in females, P=0.3441), whereas HDLC concentrations remained significantly different between genotypes (2.0 versus 1.05 mmol/L in males; 77.4 versus 40.5 mg/dL in males, P=0.0007; 0.98 versus 0.36 mmol/L in females; 37.9 versus 14.0 mg/dL in females, P<0.0001; Figure 1D). These data document the critical role of hepatocyte ABCA1 in maintaining the plasma HDL-C pool in hyperlipidemic mice and demonstrate a potential role for hepatic ABCA1 in regulating plasma apoB-containing lipoprotein concentrations during atherogenesis.

Materials and Methods

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

Results

Hepatocyte ABCA1 Deletion Reduces Plasma Lipids

To investigate the influence of hepatic ABCA1 expression on RCT and development of more advanced atherosclerotic lesions in LDLrKO mice. Our results suggest a minimal impact of hepatic ABCA1 deletion on in vivo macrophage RCT and atherogenesis development in atherogenic diet–fed LDLrKO mice, although plasma HDL concentrations were markedly reduced in HSKO/LDLrKO mice compared with LDLrKO mice. This surprising outcome likely resulted from the significant reduction in atherogenic lipoproteins (ie, very low-density lipoprotein [VLDL] and LDL) observed in diet-fed HSKO/LDLrKO mice, as well as sufficient HDL to mediate RCT.

VLDL Catabolism Is Increased in HSKO/LDLrKO Mice

To determine the explanation for reduced VLDL and LDL concentrations in atherogenic diet–fed HSKO/LDLrKO mice, we investigated VLDL production and catabolism. VLDL TG production was measured after in vivo inhibition of TG lipolysis with intravenous Triton administration to fasted mice. The rate of hepatic VLDL TG mass accumulation during Triton block of lipolysis was similar for both genotypes of mice (Figure 1A and 1B). We previously reported that HDL cholesterol (HDL-C) levels in chow-fed HSKO mice were ≈80% lower than wild-type mice.6,23 Fast protein liquid chromatography (FPLC) fractionation of plasma lipoproteins showed that chow-fed HSKO/LDLrKO mice had significant reductions in HDL-C (1.74 versus 0.21 mmol/L in males; 67.3 versus 8.1 mg/dL in males, P<0.0001; 1.50 versus 0.28 mmol/L in females; 58.0 versus 10.9 mg/dL in females, P<0.0001), contributing to the lower TPC levels in HSKO/LDLrKO versus LDLrKO mice (4.48 versus 2.68 mmol/L in males; 173.1 versus 103.6 mg/dL in males, P<0.0001; 5.17 versus 3.44 mmol/L in females; 199.7 versus 133 mg/dL in females, P=0.0004). VLDL-C and LDL-C concentrations were similar between genotypes (Figure 1C). The less pronounced hyperlipidemia in atherogenic diet–fed HSKO/LDLrKO mice was mainly attributed to lower VLDL-C (14.43 versus 6.08 mmol/L in males; 557.9 versus 235.0 mg/dL in males, P<0.0050; 16.49 versus 10.05 mmol/L in females; 637.7 versus 388.7 mg/dL in females, P=0.0283) and LDL-C levels (16.19 versus 11.65 mmol/L in males; 626.2 versus 450.5 mg/dL in males, P=0.0221; 11.80 versus 10.32 mmol/L in females; 456.3 versus 399.0 mg/dL in females, P=0.3441), whereas HDL-C concentrations remained significantly different between genotypes (2.0 versus 1.05 mmol/L in males; 77.4 versus 40.5 mg/dL in males, P=0.0007; 0.98 versus 0.36 mmol/L in females; 37.9 versus 14.0 mg/dL in females, P<0.0001; Figure 1D). These data document the critical role of hepatocyte ABCA1 in maintaining the plasma HDL-C pool in hyperlipidemic mice and demonstrate a potential role for hepatic ABCA1 in regulating plasma apoB-containing lipoprotein concentrations during atherogenesis.
LDLrKO mice were attributable to increased VLDL particle catabolism. Expression of hepatic genes involved in VLDL catabolism was similar for HSKO/LDLrKO and LDLrKO mice (Figure IIC in the online-only Data Supplement). However, plasma apoE levels were lower in atherogenic diet–fed HSKO/LDLrKO mice in addition to the anticipated reduction in apoA-I levels attributable to low plasma HDL concentrations (Figure IID in the online-only Data Supplement). Furthermore, most of the plasma apoE as well as apoA-I migrated in the HDL size range (8–10 nm) on nondenaturing gradient gels (Figure IIE and IIF in the online-only Data Supplement). Given these results, we speculate that lower VLDL-C levels in HSKO/LDLrKO mice were likely a result of decreased competition by apoE-containing plasma HDL for hepatic VLDL uptake, resulting in increased removal of VLDL particles from plasma in HSKO/LDLrKO versus LDLrKO mice.

Effect of Hepatocyte ABCA1 Deletion on Hepatic and Biliary Lipids
To address whether hepatocyte ABCA1 ablation impacts liver lipid metabolism, we measured hepatic and biliary lipid levels. Unlike our previous study in which similar hepatic lipid content was observed for chow-fed HSKO versus wild-type mice,²³ atherogenic diet–fed HSKO/LDLrKO mice had lower (significant in female mice) hepatic total cholesterol (TC), FC, and cholesterol ester concentrations relative to LDLrKO mice (Figure 2A and 2B). Hepatic TG and phospholipid concentrations were similar between the 2 genotypes (Figure 2A and 2B). However, there was no significant difference in biliary TC, phospholipid, and bile acid concentrations or molar percentage composition between the 2 genotypes (Figure IIIA–IID in the online-only Data Supplement), and fecal cholesterol excretion was similar (Figure IIIE in the online-only Data Supplement). To investigate whether the decreased liver cholesterol content was associated with transcriptional regulation concomitant with ABCA1 ablation, we measured expression of genes involved in hepatic lipid metabolism. SREBP1c was significantly downregulated, and several other genes (HMGCoA synthase, ACC1) showed downward trends in expression in HSKO/LDLrKO mouse liver, suggesting decreased de novo lipogenesis (Figure 2C). Liver expression of several LXR target genes was similar in HSKO/LDLrKO and LDLrKO mice (Figure 2C).

Impact of Hepatocyte ABCA1 on Atherosclerosis Development
To investigate the impact of hepatocyte ABCA1 deficiency on atherosclerosis development in LDLrKO mice, 3
measurements of atherosclerosis were made. En face aortic surface lesion area (Figure 3A and 3C) and aortic cholesterol content (Figure 3B) were similar for HSKO/LDLrKO and LDLrKO mice, although there was a trend toward reduced aortic cholesterol content in HSKO/LDLrKO mice. Furthermore, cross-sectional analysis of Oil red O–stained aortic root sections revealed significantly smaller lesions in both female (0.51 versus 0.40 mm²; \( P = 0.0141 \)) and male (0.48 versus 0.29 mm²; \( P = 0.0341 \)) HSKO/LDLrKO mice versus their LDLrKO counterparts (Figure 3D and 3E), suggesting that deletion of hepatocyte ABCA1 expression may actually protect against more advanced atherosclerotic lesion development in the aortic root. Additional support for this concept was obtained with additional analysis of lesion complexity; aortic root sections stained with Masson’s trichrome showed less necrosis, acute inflammation, and adventitial inflammation in lesions of HSKO/LDLrKO versus LDLrKO mice fed the atherogenic diet for 16 weeks (Figure IV A in the online-only Data Supplement). In a separate experiment to evaluate very early stages of aortic atherosclerosis (ie, 5 weeks atherogenic diet feeding), we observed similar aortic cholesterol content and aortic root lesion area between genotypes (Figure IVB and IVC in the online-only Data Supplement). Taken together, unlike previous findings in apoE KO mice,16 the absence of hepatic ABCA1 did not accelerate early-stage atherogenesis in LDLrKO mice and seemed to protect against late-stage, more advanced atherosclerosis.

**Figure 2.** Hepatic lipid content and gene expression. Lipid content was determined using detergent-based enzymatic assays of hepatic lipid extracts from 4-hour–fasted male (A) or female (B) mice after 16 weeks of atherogenic diet consumption (n=7–12). C, Hepatic gene expression in male mice (n=7). Data were expressed in mean±SEM. *\( P < 0.05 \). CE indicates cholesterol ester; FC, free cholesterol; LDLrKO, low-density lipoprotein receptor KO; TC, total cholesterol; and TG, triglyceride.

**Figure 3.** Atherosclerosis evaluation. A, Aorta surface lesion area was expressed as the percentage of total aorta surface area. B, Aortas were lipid extracted for quantification of total cholesterol (TC) and free cholesterol (FC) content using gas-liquid chromatography. Cholesterol ester (CE) content was calculated as (TC–FC)×1.67. C, Representative en face aorta from a low-density lipoprotein receptor KO (LDLrKO; left) and hepatocyte-specific ABCA1 knockout (HSKO)/LDLrKO (right) mouse. D, Representative LDLrKO (left) and HSKO/LDLrKO (right) mouse aortic root sections stained with Oil Red O. E, Aortic root lesion area. Each point represents the average lesion area of 3 sections per mouse. Horizontal lines denote the mean±SEM for each genotype. *\( P < 0.05 \).

**Role of Hepatocyte ABCA1 in Macrophage RCT In Vivo**

One atheroprotective mechanism proposed for HDL is the transport of excess macrophage cholesterol to the liver for excretion (ie, RCT).26 To determine whether the large reduction of plasma HDL in HSKO/LDLrKO mice diminished RCT, we performed in vivo macrophage RCT assays. \(^3\)H-cholesterol–radiolabeled J774 macrophages were injected into the peritoneal cavity of HSKO/LDLrKO or LDLrKO mice after 5 weeks of atherogenic diet feeding. The plasma \(^3\)H–cholesterol closely tracked with lipoprotein cholesterol mass (Figure 4A and 4B). The amount of \(^3\)H tracer in plasma 48 hours after injection was significantly lower in HSKO/LDLrKO mice, likely reflecting the lower levels of plasma lipoproteins in these mice (Figure 4C). However, the tracer levels in the liver, bile, and feces were similar between the 2 groups (Figure 4D–4F), suggesting that in vivo macrophage RCT was not impaired in HSKO/LDLrKO mice, despite the much lower HDL-C in these mice. A similar outcome was obtained using radiolabeled bone marrow–derived macrophages injected into mice fed the atherogenic diet for 16 weeks (Figure V in the online-only Data Supplement).
also measured cholesterol content of resident peritoneal macrophages in atherogenic diet–fed mice and observed no significant difference between the 2 genotypes, although there was a trend toward decreased cholesterol in HSKO/LDLrKO mice (Figure VI in the online-only Data Supplement). Collectively, these data suggest that in vivo macrophage RCT is not impaired in HSKO/LDLrKO mice, despite significantly lower plasma steady-state HDL-C levels.

**Plasma Cholesterol Efflux Capacity**

In vivo RCT results suggested that the plasma HDL pool in HSKO/LDLrKO mice was sufficient to maintain normal cholesterol efflux from macrophages for ultimate excretion into feces. One possible explanation for this outcome could be a fraction of mouse plasma HDL that is highly efficient at effluxing macrophage FC, compensating for low plasma HDL levels in RCT in HSKO/LDLrKO mice. For example, preβ1 seems to be the preferred acceptor for ABCA1-mediated FC efflux in human plasma, but its concentration is typically <10% of total HDL. To examine this possibility, we measured the ability of apoB lipoprotein–depleted plasma from atherogenic diet–fed HSKO/LDLrKO and LDLrKO mice to efflux 3H-cholesterol from cholesterol-loaded macrophages and observed a significant reduction (24%) in HSKO/LDLrKO versus LDLrKO plasma (10.14% versus 7.75%, P=0.0067; Figure 5A). However, analysis of individual animal HDL-C concentrations versus percentage of efflux values (Figure 5B) demonstrated <2-fold variation in percentage efflux values compared with a 10-fold variation in plasma HDL-C concentrations, suggesting that HDL-C concentration, per se, is not a primary determinant of plasma efflux capacity.

After the cholesterol efflux experiment, we fractionated a subset of individual plasma-containing efflux media using an FPLC column capable of separating plasma HDL particles, preβ1 and lipid-free apoA-I from one another (Figure VII in the online-only Data Supplement). The distribution of 3H-cholesterol between the main HDL peak (fractions 41–50) and preβ1 HDL elution region (fractions 51–55) did not reveal...
a disproportionate amount of $^3$H-FC in the preβ 1 peak in HSKO/LDLrKO versus LDLrKO plasma (Figure 5C), and the percentage of $^3$H-FC in the preβ 1 peak relative to the entire HDL elution region (ie, fractions 41–55) was 20.9±3.4% and 20.7±5.2%, respectively (n=6 genotype). In addition, apoA-I Western blot analysis of plasma separated by agarose gel electrophoresis did not show an increase in preβ 1 HDL for HSKO/LDLrKO compared with LDLrKO mice (Figure VII D in the online-only Data Supplement). Taken together, these results suggest that HSKO/LDLrKO mice do not compensate for reduced plasma HDL levels with an increase in amount or cholesterol efflux efficiency of preβ 1 HDL to maintain in vivo RCT at levels comparable with LDLrKO mice.

**Discussion**

Hepatocyte ABCA1 plays a crucial role in HDL biogenesis, but its role in atherogenesis is less clear. In the current study, we addressed this gap in knowledge by performing atherosclerosis and in vivo macrophage RCT studies in atherogenic diet–fed HSKO mice crossed into the LDLrKO background. Compared with LDLrKO (control) mice, HSKO/LDLrKO mice had reduced TPC, primarily because of a 40% to 50% reduction in VLDL and LDL, and similar or reduced atherosclerosis. Furthermore, in vivo macrophage RCT to feces was similar for both genotypes of mice, although efflux of macrophage $^3$H-FC was significantly reduced in apoB lipoprotein–deficient mice as compared to LDLrKO mice (Figure VI D in the online-only Data Supplement). Together, these results suggest that HSKO/LDLrKO mice do not compensate for reduced plasma HDL levels with an increase in amount or cholesterol efflux efficiency of preβ 1 HDL to maintain in vivo RCT at levels comparable with LDLrKO mice.

In support of the concept that HDL function may be more important than HDL-C in determining CHD risk, we show that HDL-C and maintains endothelial function, all of which are atheroprotective. However, recent studies have challenged the assumption that raising HDL-C levels will uniformly translate into reductions in CHD. Other studies have suggested the role of HDL particle number and size (ie, subfraction distribution) are better predictors of CHD risk than HDL-C and that HDL function may be more important in preventing CHD than HDL-C. Animal studies have shown a more consistent association between increased atherosclerosis and decreased macrophage RCT than with reduced HDL-C. In support of the concept that HDL function may be more important than HDL-C in determining CHD risk, we show that a substantial reduction of plasma HDL-C in atherogenic diet–fed HSKO/LDLrKO mice did not significantly affect aortic atherosclerosis, in vivo macrophage RCT, or fecal cholesterol excretion. These results are compatible with the idea of a small, dynamic HDL pool that efficiently removes cholesterol from arterial macrophage foam cells and rapidly transports it to the liver for excretion without a detectable increase in plasma HDL-C.

Pre-β 1 HDL is a preferential acceptor for macrophage cholesterol efflux via ABCA1 and may function accordingly, although it is generally <10% of total HDL mass. However, a large HDL pool would not be necessary for this mechanism to be operational because aortic cholesterol ester mass in atherogenic diet–fed HSKO/LDLrKO mice is <1% of the steady-state plasma cholesterol pool. Overall, these observations support the concept that HDL quality or function may be a better predictor of atheroprotection than HDL-C.
were similar between groups, suggesting the involvement of other potential pathways, such as heparan sulfate proteoglycans. However, expression of hepatic lipoprotein catabolic genes, such as SR-BI, syndecan 1, hepatic lipase, lipoprotein lipase, and apoC3, were similar between genotypes (Figure 2B). Van Eck et al have shown that HDL can effectively compete for hepatic uptake of βVLDL particles via SR-BI dependent- and independent-mediated mechanisms. In the absence of functional LDLr, VLDL residence time in plasma is significantly prolonged, allowing alternate hepatic VLDL particle uptake pathways to predominate. We show that the pool of apoE-enriched HDL, a likely competitor for alternate hepatic VLDL particle uptake pathways, is diminished in atherogenic diet–fed HSKO/LDLrKO mice (Figure II in the online-only Data Supplement). Based on our combined results and the previous studies by Van Eck et al, we propose that the increased VLDL particle catabolism in HSKO/LDLrKO mice is mediated through decreased competition for hepatic uptake of VLDL by apoE-containing HDL. Regardless of the exact mechanism, hepatic ABCA1 expression seems to be an important regulator of plasma apoB Lp as well as HDL levels, both of which modulate atherosclerosis progression.

In conclusion, we investigated the impact of hepatocyte ABCA1 deletion on relatively advanced atherosclerosis and macrophage RCT during disease progression. Unexpectedly, we found that hepatocyte ABCA1 deletion did not exacerbate lesion development in atherogenic diet–fed LDLrKO mice, and was atheroprotective in the aortic root, likely because of reduced apoB Lp levels and maintenance of macrophage RCT, despite a large reduction in plasma HDL-C. Our results are also compatible with the idea proposed by Yamamoto et al that hepatic ABCA1 may normally recycle a significant amount of plasma HDL-C removed by the liver back into plasma to maintain the plasma HDL-C pool. If true, these findings would result in a paradigm shift because decreased hepatic ABCA1 expression, resulting in lower plasma HDL-C, may actually increase RCT and reduce CHD risk by reducing the recycling of hepatic cholesterol back into plasma through nHDL formation by ABCA1.

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Disclosures
None.

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Hepatocyte ATP binding cassette transporter A1 (ABCA1) plays a pivotal role in maintaining plasma high-density lipoprotein (HDL) levels, but its impact on macrophage reverse cholesterol transport and atherogenesis is less clear. In this study, we show the importance of hepatic ABCA1 in regulating both plasma HDL and apoB lipoprotein metabolism under hyperlipidemic conditions. Despite a 50% reduction in plasma HDL cholesterol in the absence of hepatic ABCA1, atherosclerosis was not worsened, likely because of the maintenance of in vivo macrophage reverse cholesterol transport and the concomitant paradoxical 40% to 50% reduction in plasma very low-density lipoprotein and low-density lipoprotein levels. In addition, macrophage cholesterol efflux to apoB lipoprotein–depleted plasma varied <2-fold compared with a 10-fold variation in plasma HDL cholesterol concentrations, supporting the concept that steady-state HDL cholesterol concentration is not the primary determinant of plasma cholesterol efflux capacity. Therapeutic interventions targeting hepatic ABCA1 expression to alleviate cardiovascular burden should take into consideration that paradoxical effects on apoB lipoprotein metabolism may oppose atheroprotection.
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SUPPLEMENTAL MATERIAL

Supplemental Table I. VLDL/IDL chemical composition and size

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<th>Genotype</th>
<th>% of total mass</th>
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<td></td>
<td>FC</td>
<td>CE</td>
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<tr>
<td>LDLrKO</td>
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<tr>
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VLDL/IDL were isolated from atherogenic diet-fed mice by ultracentrifugation at d=1.019 g/ml. Chemical composition was analyzed by enzymatic or chemical assays. VLDL/IDL particle size was determined by dynamic light scatter. Mean ± SEM, n=4. IDL, intermediate density lipoprotein.
Supplemental Figure I. VLDL secretion was evaluated after *in vivo* inhibition of lipolysis with triton. Plasma TG levels were measured by enzymatic assay before (0 min) and after (30, 60, 90, 180 min) intravenous Triton injection. (A) Plasma TG concentration was plotted for each genotype of mice (mean ± SEM; n=4-6), and the line of best fit was determined by linear regression analysis. (B) TG secretion rate during the 3h Triton block experiment was determined for each animal as the slope of the regression line of plasma TG vs. time. Results were then plotted for both genotypes of mice as mean ± SEM, n=4-6.
Supplemental Figure II. Plasma VLDL was isolated, radiolabeled with $^{125}$I, and injected intravenously into fasted atherogenic diet-fed mice (n=5/genotype). Periodic blood samples were taken after tracer injection, and whole plasma (A) and apoB (B) radiolabel remaining in plasma was determined. Turnover curves were plotted as percentage of injected tracer remaining in plasma (A) or percentage of $^{125}$I-apoB remaining in plasma (B) relative to the injected dose vs. time. Mean ± SEM, n=5. (C) Hepatic expression of genes involved in VLDL catabolism (n=7 male mice). Data was expressed in mean ± SEM. * $P<0.05$. (D) Plasma samples (1µl) were separated using 12% SDS-PAGE followed by Western blot analysis for mouse apoA-I and apoE. From left to right: atherogenic diet-fed LDLrKO vs. HSKO/LDLrKO mouse plasma (n=4/group). (E-F) Plasma samples (1µl) from atherogenic diet-fed mice (n=4/group) were subjected to 4-30% non-denaturing gradient gel electrophoresis (NDGGE) and Western blotted for mouse apoA-I (E) or apoE (F). Plasma from apoE knockout (apoE$^{-/-}$) and apoA-I knockout (apoA-I$^{-/-}$) mice was used as negative controls.
Supplemental Figure III. Gallbladder bile total cholesterol (TC), phospholipid (PL), and bile acid (BA) concentrations (A,B) and molar percentage (C,D) were determined for male (A, C) and female (B, D) mice (n=6-9). E. Two-day fecal collections were assayed for cholesterol content by gas liquid chromatography and the data were normalized to mg cholesterol/day/100g body weight (n=3-5). Data are expressed as mean ± SEM.
Supplemental Figure IV. (A) Representative Masson’s trichrome-stained aortic root sections from 16wk atherogenic diet fed LDLrKO (left) and HSKO/LDLrKO (right) mice. (B) Aortas were isolated from mice fed the atherogenic diet for 5 wks. Aortic lipid was extracted for quantification of total cholesterol (TC) and free cholesterol (FC) content using gas-liquid chromatography. Cholesterol ester (CE) content was calculated as (TC-FC) x1.67. (C) Aortic root lesion area. Each point represents the average lesion area of 3 sections per mouse. Data are expressed as mean ± SEM, n=8 HSKO/LDLrKO, 7 LDLrKO.
Supplemental Figure V. $^3$H-cholesterol radiolabeled, cholesterol-loaded bone marrow-derived macrophages from LDLrKO mice were injected into the peritoneal cavity of mice fed an atherogenic diet for 16 wks. Forty-eight hr after macrophage injection, plasma $^3$H-cholesterol radiolabel (A) and cholesterol mass (B) distribution was determined after FPLC fractionation of plasma lipoproteins. $^3$H-cholesterol tracer was measured in plasma at different time points (C), and in liver (D), bile (E) and feces (F) 48 hr after macrophage injection. Data are expressed as mean ± SEM (n=3).
Supplemental Figure VI.

Resident macrophages were harvested by peritoneal lavage from mice fed an atherogenic diet for 16 wk. Cells were cultured for 2h in RPMI medium containing 1% Nutridoma and non-adherent cells were removed by washing 3 times with PBS. The adherent macrophages were lipid extracted and the extract was assayed for total cholesterol (TC) and free cholesterol (FC) content. Data are normalized for cellular protein and expressed as mean ± SEM (n=3-5).
Supplemental Figure VII. FPLC fractionation of plasma HDL and pre-β 1 HDL. $^{125}$I-apoA-I alone or plasma from an atherogenic-diet fed LDLrKO mouse with added $^{125}$I-apoA-I on ice was fractionated by FPLC (1 X 30 cm Superdex HR200, flow rate at 0.3 ml/min, 300 µl/fraction) and individual fractions were collected and counted for $^{125}$I (A) or assayed for cholesterol
concentration (B). Media containing apoB-depleted plasma used for 4h macrophage FC efflux (Figure 5) study was fractionated by FPLC and $^3$H-radiolabel was quantified for each fraction by liquid scintillation spectroscopy (C). Vertical lines in panels A-C denote elution positions of VLDL/LDL, HDL, pre-β 1 HDL and apoA-I. (D) Agarose electrophoresis of plasma. Plasma samples (1 µl) were subjected to 0.7% agarose gel electrophoresis followed by Western blot analysis for mouse apoA-I. From left to right: chow-fed C57Bl/6 mouse plasma, n=1, atherogenic diet-fed LDLrKO mouse plasma, n=5, atherogenic diet-fed HSKO/LDLrKO mouse plasma, n=5. Alpha and pre-β HDL migration positions are shown for reference.
MATERIALS AND METHODS

Animals and Diet

HSKO mice in the C57BL/6 background (>99%) were generated as described previously \(^1,2\) and crossed with LDLrKO mice in the C57Bl/6 background (Jackson Laboratories) to generate heterozygous HSKO/LDLrKO. Mice used for atherosclerosis studies were generated by crosses of heterozygous HSKO/LDLrKO mice; genotypes of offspring were determined by PCR\(^1\). Female and male HSKO/LDLrKO and LDLrKO littermate control mice were fed a chow diet until 7-9 wks of age, and then switched to an atherogenic diet containing 10% palm oil and 0.2% cholesterol for 16 wks in most experiments, unless otherwise indicated. Diet composition has been published previously \(^3\). Mice were maintained on a 12h light/dark cycle. All protocols and procedures were approved by the Institutional Animal Care and Use Committee.

Lipid and lipoprotein analysis

Plasma was collected by tail bleeding or cardiac puncture from mice fasted for 4 hr. TPC, free cholesterol (FC), and triglycerides (TG) concentrations were determined by enzymatic assays using commercial kits\(^4\). Cholesterol distribution among lipoproteins was determined after fractionation of plasma by gel filtration chromatography using a Superose 6 10/300 GL column (GE Healthcare). An aliquot of plasma containing approximately 15 µg total cholesterol was injected onto the column and eluted with 0.9% saline containing 0.01% EDTA and 0.01% sodium azide at a flow rate of 0.4 ml/min. The column effluent was mixed with a commercially available enzymatic total cholesterol reagent (Pointe Scientific, Inc., Canton, MI) delivered at 0.125 ml/min. After passing through a knitted reaction coil maintained at 37°C, the absorbance of the reaction mixture was read at 500 nm using a UV-VIS detector. The area under the VLDL, LDL, and HDL peaks was calculated using Chrom perfect Spirit (Justice Laboratory Software) chromatography software. To calculate the cholesterol concentration in each lipoprotein fraction, the ratio of the respective peak area to total peak area was multiplied by the total plasma cholesterol. Liver lipid analysis was performed by enzymatic assay of detergent-extracted liver\(^5\).

Analysis of atherosclerotic lesions

Mice were sacrificed after 16 wks of atherogenic diet consumption. First they were anesthetized with ketamine/xylazine, and the vasculature then was perfused with cold PBS. Aortas were isolated and fixed in 10% buffered formalin. After fixation, aortas were cleaned of adventitial fat and pinned open for measurement of surface lesion areas. Images of en face aortas were analyzed using WCIF Image J software. Aortas were then lipid extracted for quantification of total and FC content using gas-liquid chromatography, as described previously \(^3\). Aortic roots were embedded in Optimal Cutting Temperature (Tissuetek) media in a plastic mold, frozen, and cut at 8 µm intervals. Sections were collected from the aortic region moving toward the apex of the heart and sequentially placed on 8 slides, such that each slide had sections 64 µm apart. The sections were fixed in 10% buffered formalin, stained in 0.5% Oil Red O for 25 minutes and counterstained with hematoxylin. Stained sections were photographed and Image-Pro software (Media Cybernetics Inc., Rockville, Md.) was used to quantify lesion area. The lesion areas of three sections representing different regions were averaged for each mouse.

In vivo Macrophage RCT

Macrophage RCT assays were conducted as described by Rader and colleagues \(^6\) with minor modifications \(^3\). J774 mouse macrophages or bone marrow-derived macrophages (BMM) from LDLrKO mice were radiolabeled and cholesterol-loaded with \(^3\)H-cholesterol and acetylated LDL,
respectively. Cells were then injected into the peritoneal cavity of recipient mice fed the atherogenic diet. Plasma samples were collected at 6h, 24h, and 48h after injection. Feces were collected throughout the 48h study. At necropsy, tissues were harvested and radiolabel levels in plasma, liver, bile, and feces were then quantified after lipid extraction and liquid scintillation counting. Aliquots of plasma were also fractionated by FPLC and cholesterol mass, and radiolabel distribution among lipoprotein fractions was quantified.

**Real-time PCR Analysis**

At sacrifice, tissues were harvested and snap frozen in liquid nitrogen. Total RNA was isolated from livers of male mice using TRIzol (Invitrogen), and real-time RCR was performed as reported previously. Primer sequences were the same as described previously. GAPDH was used as the endogenous control.

**Cellular cholesterol efflux**

Cholesterol efflux from J774 macrophages to plasma of mice fed the atherogenic diet was measured as previously reported, with minor modifications. Briefly, 350,000 J774 cells were plated into each well of a 24-well plate and incubated in labeling medium (RPMI 1640 medium containing 1% FBS and 2 µCi 3H-cholesterol) for 24 hours. Cells were then washed once and incubated with efflux medium (MEM-HEPES media containing 2.8% apoB lipoprotein-depleted plasma). Four hours later, medium was harvested and cellular lipid was extracted with isopropanol. A 100 µl aliquot of medium and cellular lipid extract was taken for scintillation counting to determine percentage cholesterol efflux during incubation. Aliquots of efflux medium were fractionated on a Superdex 200HR column (1X30 cm), and radioactivity of each fraction was determined by scintillation counting.

**Biliary lipids**

A measured volume of gallbladder bile collected from mice (n=5-10) was subjected to neutral lipid extraction. 5-α cholestane (5 µg) was added to each extraction tube as an internal standard. Aliquots of the bottom organic phase were used to determine total cholesterol (TC) content by gas-liquid chromatography and phospholipid (PL) content by enzymatic assay. Bile acid (BA) was measured as reported previously using the top phase of the lipid extract.

**Fecal cholesterol excretion**

Two-day fecal collections were subjected to lipid extraction, and cholesterol content was measured as described previously.

**Macrophage cholesterol content**

Peritoneal cells were harvested by lavage from 4h-fasted mice. Cells were suspended in RPMI-1640 medium containing 1% Nutridoma-SP (Roche Applied Science), 100 units/ml penicillin, 100 µg/ml streptomycin, and 2 mM L-glutamine and cultured at 37°C for 2 hours. Then, non-adherent cells were removed by washing with PBS and adherent macrophages were extracted with isopropanol at room temperature overnight. TC and FC content was determined by gas-liquid chromatography and cellular protein was measured by Lowry protein assay after NaOH digestion, as previously reported.
In vivo VLDL TG secretion rate determination

Tyloxapol (500 mg/kg body weight) was injected intravenously into 4h-fasted, anesthetized, atherogenic diet-fed mice (n=4-6). Blood was collected before (0 min), 30, 60, and 90 min after injection for measurement of plasma triglyceride (TG) concentration by enzymatic assay. VLDL TG secretion rate was determined by calculating the slope of the time vs. plasma TG concentration plot for each animal using linear regression analysis.

VLDL/IDL composition and size analysis

Plasma was collected from LDLrKO mice (n=4/group) fed an atherogenic diet for 16 wk after they were fasted for 4h. VLDL/IDL were isolated by ultracentrifugation at d=1.019 g/ml (100,000 rpm for 4h, Beckman Coulter TLA100.2 rotor) and chemical composition was determined by enzymatic assay and chemical assays. Lipid and protein content were expressed as percentage of total mass. Aliquots of VLDL/IDL were used to measure particle size using a Zetasizer Nano S dynamic light scattering instrument (Malvern Instruments Ltd., Worcestershire, UK). Particle sizes are reported as the major peak mean by volume analysis.

In vivo VLDL turnover

VLDL were separated from plasma of fasted LDLrKO mice fed an atherogenic diet for 16 wk by ultracentrifugation at d=1.006 g/ml. The VLDL preparation was refloated at d=1.006 gm/ml and radiolabeled with $^{125}$I using the iodine monochloride method. Ninety-seven percent of the radioactivity was trichloroacetic acid-precipitable. VLDL tracer (0.25 x 10$^6$ cpm) was diluted to 200 µl with saline for retro-orbital injection into 4h-fasted, anesthetized recipient mice (n=5/genotype) fed an atherogenic diet for 16 wk. Blood samples were collected at 2 and 30 min and 1, 3, 5, 8 and 24 h after injection and $^{125}$I radiolabel in plasma was determined by gamma radiation counting. ApoB was precipitated from plasma using isopropyl alcohol and $^{125}$I radiolabel in apoB was quantified by gamma radiation counting. Turnover curves were plotted as the percentage of injected tracer remaining in plasma or as the percentage of $^{125}$I-apoB remaining in plasma relative to the injected dose vs. time.

Gel electrophoresis

Plasma (1 µl) from LDLrKO or HSKO/LDLrKO mice fed an atherogenic diet for 19 wk was fractionated by 0.7% agarose gel electrophoresis. Following a 2h capillary transfer of the fractionated plasma from the agarose gel to a 0.2 µm nitrocellulose membrane, Western blot analysis was performed using anti-mouse apoA-I (Meridian Life Science, Inc. K23600G). SDS-PAGE and 4-30% non-denaturing gradient gel electrophoresis and Western blot analysis was performed as previously described.

Statistical analysis

Results are reported as mean ± standard error of the mean. Data were analyzed using two-tailed Student’s t test (with Welch’s correction in case of unequal variance) using Graphpad Prism software. P <0.05 was considered statistically significant.
References


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