FXR Deficiency Causes Reduced Atherosclerosis in \textit{Ldlr}^{−/−} Mice

Yanqiao Zhang, Xuping Wang, Charisse Vales, Florence Ying Lee, Hans Lee, Aldons J. Lusis, Peter A. Edwards

Objective—Based on the observation that \textit{Fxr}^{−/−} mice exhibit a proatherogenic lipoprotein profile, we investigated the role of FXR in the development of atherosclerosis.

Methods and Results—Administration of a western diet to \textit{Fxr}^{−/−} mice or wild-type mice does not result in the development of significant atherosclerotic lesions. Consequently we generated \textit{Fxr}^{−/−}\textit{Ldlr}^{−/−} (DKO) mice and compared lesion development with \textit{Ldlr}^{−/−} mice. After 16 weeks on a Western diet, \textit{en face} analysis of the aorta indicated that the male DKO mice had reduced atherosclerotic lesions as compared with \textit{Ldlr}^{−/−} mice. Plasma low-density lipoprotein cholesterol and high-density lipoprotein cholesterol levels were reduced by 40% to 50%, whereas triglyceride levels increased 4-fold in the male DKO mice. Finally, peritoneal macrophages freshly isolated from male DKO mice had reduced expression of CD36 mRNA and decreased neutral lipid accumulation, as compared with \textit{Ldlr}^{−/−} mice.

Conclusions—FXR deficiency in male, but not female, \textit{Ldlr}^{−/−} mice results in a reduction in the size of atherosclerotic lesions in the aorta. The reduction in atherosclerosis may result from a decrease in plasma low-density lipoprotein cholesterol, coupled with reduced expression of CD36 in macrophages of DKO mice. (\textit{Arterioscler Thromb Vasc Biol}. 2006;26:000-000.)

Key Words: atherosclerosis ■ cholesterol ■ FXR ■ LDLR ■ nuclear receptor

Atherosclerosis is a chronic inflammatory disease, characterized by accumulation of lipids and fibrous elements in the walls of large arteries.\textsuperscript{1} It is well-established that high-plasma low-density lipoprotein cholesterol (LDL-C) level is an independent risk factor for the development of atherosclerosis. High levels of LDL-C are thought to result in the generation of oxidized (ox) and aggregated LDL in the subendothelial areas of the arterial wall. Ox-LDL contains bioactive lipids that promote the entry of monocytes into the subendothelial space, where they differentiate into macrophages. These macrophages then take up ox-LDL and/or aggregated LDL via scavenger receptors (CD36 or SR-A) or phagocytosis. The result is the generation of lipid-engorged macrophages (also called foam cells) that are found in the early fatty streak as well as more complex advanced lesions.\textsuperscript{1-3}

The role of CD36 in atherosclerotic lesion development has become controversial. Original studies showed that a null mutation of CD36 in mice impaired the conversion of peritoneal macrophages to foam cells.\textsuperscript{3,5} Consistent with these in vitro results, loss of function studies using \textit{Cd36}^{−/−}\textit{Apoe}^{−/−} mice demonstrated that atherosclerotic lesions were reduced in the double knockout mice as compared with \textit{Apoe}^{−/−} mice.\textsuperscript{6} In contrast, Moore et al recently reported that CD36 or SR-A deficiency did not reduce atherosclerotic lesion size in their studies with either \textit{Cd36}^{−/−}\textit{Apoe}^{−/−} or \textit{Sr-A}^{−/−}\textit{Apoe}^{−/−} and \textit{Apoe}^{−/−} mice.\textsuperscript{4} Thus the role of these scavenger receptors in atherogenesis remains unclear.

Farnesoid X receptor (FXR) is a member of the nuclear receptor superfamily that is highly expressed in liver, intestine, kidney, and adrenal gland,\textsuperscript{7} with very low levels (\textasciitilde 10%) being detected in white adipose tissue.\textsuperscript{8} FXR is activated by specific bile acids that include chenodeoxycholic acid (CDCA) and cholic acid,\textsuperscript{9-11} and also structurally unrelated synthetic compounds, such as GW44462 and fexaramine.\textsuperscript{13} FXR plays an important role in maintaining bile acid, cholesterol, triglyceride, and glucose homeostasis.\textsuperscript{12,14-18} Importantly, plasma cholesterol and triglyceride levels are increased in \textit{Fxr}^{−/−} mice.\textsuperscript{14} Consistent with these data, activation of FXR, after treatment with bile acids or a synthetic FXR agonist, or hepatic expression of constitutively active FXR, significantly lowers plasma triglyceride, cholesterol, and glucose levels.\textsuperscript{12,16,17,19} FXR activation also significantly improves insulin sensitivity in diabetic mice.\textsuperscript{16,18}

Both hypercholesterolemia and insulin intolerance contribute to atherosclerosis.\textsuperscript{20} Based on the demonstrated roles of FXR in controlling lipid and glucose metabolism, we hypothesized that FXR deficiency would result in accelerated...
FXR<sup>−/−</sup> Mice Display a Proatherogenic Lipid Profile in Plasma

<table>
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<tr>
<th>Group</th>
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<th>Triglyceride</th>
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<th>HDL-C</th>
<th>HDL-C:TC</th>
<th>UC</th>
<th>FFA</th>
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<td>FXR&lt;sup&gt;−/−&lt;/sup&gt; Chow</td>
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<td>0.19±0.01</td>
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<td>203±15†</td>
<td>160±10†</td>
<td>0.79±0.02</td>
<td>56±4†</td>
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<tr>
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<td>156±9</td>
<td>127±8</td>
<td>0.81±0.01</td>
<td>25±2</td>
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<tr>
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<td>328±23*</td>
<td>186±15*</td>
<td>0.57±0.04*</td>
<td>84±8*</td>
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</table>

Wild-type or FXR<sup>−/−</sup> mice were fed a chow diet or Western diet for 3 months (n=8 mice per group). Plasma lipid profiles were measured after a 5-hour fast. TC indicates total cholesterol; UC, unesterified cholesterol; FFA, free fatty acids. HDL-C:TC (column 6) indicates the ratio of HDL-C to TC. *P<0.001; †P<0.01. All P values are vs wild-type mice on the corresponding chow or Western diet.

En Face Analysis of Aortas

The aorta, including the ascending arch, thoracic, and abdominal segments, was dissected, gently cleaned of the adventitia, and stained with Sudan IV. The surface lesion area was quantified with commercially available software (Image-Pro Plus, Media Cybernetics) as previously described.

Statistical Analysis

Statistical significance was analyzed using Mann–Whitney test or unpaired Student t test for unequal variance. All values are expressed as mean±SE. Differences were considered statistically significant at P<0.05.

Results

FXR Null Mice Displayed Proatherogenic Plasma Lipid Profile

FXR<sup>−/−</sup> mice, originally generated by Sinal et al, were on a mixed genetic background. These mice were backcrossed to C57BL/6J mice for seven additional generations before being used in the current study. Nonetheless, consistent with the initial report by Sinal et al, compared with their wild-type littermates, FXR<sup>−/−</sup> mice had increased plasma levels of triglyceride, total cholesterol (TC), HDL-C, and unesterified cholesterol (UC) when fed a chow diet (Table). After administration of a Western diet for 3 months, FXR<sup>−/−</sup> mice had significantly higher plasma levels of triglyceride, cholesterol, HDL-C, UC, and free fatty acids (FFA) than wild-type mice on the same diet (Table). The increase in plasma HDL-C levels of FXR<sup>−/−</sup> mice was consistent with decreased hepatic expression of SR-B1 (Figure 1E), the scavenger receptor that facilitates HDL-C clearance from blood.

Despite the finding that FXR<sup>−/−</sup> mice had increased plasma HDL-C (Table, column 5), the ratio of HDL-C to total cholesterol was unchanged in chow-fed animals (wild-type 79% versus FXR<sup>−/−</sup> 79%, column 6). However, when fed a Western diet, the ratio of HDL-C to TC significantly declined in the FXR<sup>−/−</sup> mice (wild-type 81% versus FXR<sup>−/−</sup> 57%, P<0.001). Collectively, the data suggest that FXR<sup>−/−</sup> mice fed a western diet have a more pronounced proatherogenic lipoprotein profile.

FXR Null Mice Do Not Develop Atherosclerosis

The proatherogenic lipid profile of FXR<sup>−/−</sup> mice led us to study the role of FXR in atherosclerosis. However, after 16 weeks on a Western diet, no significant levels of atherosclerotic lesions were detected in the aortic roots or aortic arches of FXR<sup>−/−</sup> mice or wild-type littermates (data not shown).
These data suggest that FXR deficiency alone is not sufficient to promote the development of atherosclerosis.

**Generation and Characterization of Fxr−/− Ldlr−/− Double Knockout Mice**

Fxr−/− Ldlr−/− (DKO) mice on a C57BL/6 background were generated as described in Methods. We report here the initial studies to characterize the DKO mice. Compared with Ldlr−/− mice fed a chow diet, the DKO mice had increased plasma triglyceride and cholesterol levels (Figure 1A and B), but unchanged plasma HDL-C levels (Figure 1C). Consistent with the unchanged plasma HDL-C levels, hepatic SR-BI expression in the DKO and Ldlr−/− mice was not significantly different (Figure 1E). FPLC analysis showed that the DKO mice had increased plasma levels of very-low-density lipoprotein cholesterol (VLDL-C) and LDL-C, whereas HDL-C levels were unchanged (Figure 1D). Despite the increased levels of plasma cholesterol, no significant levels of atherosclerotic lesions were detected in the aortas of 4-month-old DKO or Ldlr−/− mice fed a chow diet (data not shown).

**Reduced Atherosclerosis in Male DKO Mice Fed a Western Diet**

To investigate the potential role of FXR in atherosclerosis, both Ldlr−/− and DKO mice (male, n=13 mice/group; female, n=7 to 8 mice/group) were fed a Western diet for 16 weeks. The food intake and survival rate were not different between these two genotypes (data not shown). Both male and female DKO mice showed decreased body weights, compared with Ldlr−/− mice (Figure 2A). The mechanism underlying the differences in body weight between the 2 genotypes remains unclear at this time.

En face analysis of the aortas showed that male DKO mice had a 66% reduction in aortic lesion area, as compared with lesions in Ldlr−/− mice (Figure 2B and 2C; P<0.001). The data of Figure 2B show that lesions of Ldlr−/− female mice fed the western diet were reduced as compared with male Ldlr−/− mice (24% versus 42%; P<0.05). However, lesions of female Ldlr−/− and DKO mice fed the Western diet were not significantly different (Figure 2B). Thus, loss of FXR reduced lesion size in Ldlr−/− male, but not female mice. Previous reports have shown that estrogen treatment inhibits atherosclerotic lesion initiation and progression. Consequently, additional studies will be required to determine whether the difference between male and female mice reported in the current study is a result of difference in endogenous estrogen levels.

**Altered Plasma Lipoprotein Levels in Fxr−/− Ldlr−/− Mice**

In an attempt to identify the mechanism that might explain the reduced atherosclerotic lesion size in male DKO mice, we
analyzed the plasma lipid profiles. As shown in Figure 3A and 3D, plasma triglyceride (A), cholesterol (B), HDL-C (C), and free fatty acids (FFA) (D) were analyzed after a 16-hour fast. *P<0.05, **P<0.01.

Figure 3. Plasma lipid profile in Ldlr−/− vs DKO mice fed a Western diet. Male or female Ldlr−/− or DKO mice were fed a Western diet for 16 weeks. Plasma triglyceride (A), cholesterol (B), HDL-C (C), and free fatty acids (FFA) (D) were analyzed after a 16-hour fast. *P<0.05, **P<0.01.

Figure 4. Plasma cholesterol distribution in Ldlr−/− vs DKO mice fed a Western diet. Male and female Ldlr−/− or DKO mice were fed a Western diet for 16 weeks (male, n = 13 mice/group. Female, n = 7 to 8 mice/group). Pooled plasma (50 μL), obtained from male (A) or female (B) mice after a 16-hour fast, was analyzed using FPLC. C, Plasma LDL-C concentrations.

Reduced CD36 mRNA and Neutral Lipid Levels in Peritoneal Macrophages Isolated From DKO Mice

Atherosclerosis is an inflammatory disease.1,20 To determine whether inflammation is reduced in male DKO mice, we analyzed the hepatic expression of selected genes that have been proposed to be involved in the development of atherosclerosis and/or inflammation. The data, shown in Figure 5A, indicated that tumor necrosis factor-α, intercellular adhesion molecule-1, P-selectin, vascular cell adhesion molecule-1, serum amyloid A2, and tissue plasminogen activator were all induced in the livers of male DKO mice (Figure 3C). Consequently, we conclude that the reduction in atherosclerotic lesions noted in male DKO mice is not a result of decreased hepatic expression of inflammatory genes.

To better distinguish the changes in plasma lipoproteins, we performed the FPLC analysis shown in Figure 4. The data show that male DKO mice fed a Western diet had increased plasma levels of VLDL-C, and reduced levels of LDL-C and HDL-C, as compared with male Ldlr−/− mice (Figure 4A and 4C). Thus, in male DKO mice, the decrease in LDL-C and HDL-C was paralleled by an increase in VLDL-C (Figure 4). As a result, there was little change in total plasma cholesterol levels (Figure 3B). In contrast, female DKO mice had elevated LDL-C and unchanged LDL-C and HDL-C levels (Figure 4B), with the result that total plasma cholesterol levels increased by 37% (Figure 3B). Figure 4C shows that plasma LDL-C levels decreased by 50% in male DKO mice to levels that are seen in both female Ldlr−/− and DKO mice. Because LDL-C is an independent risk factor for atherosclerosis, we hypothesize that the reduced atherosclerosis noted in male DKO mice (Figure 2) results from the reduced levels of plasma LDL-C (Figure 4C). Consistent with this proposal, male DKO and female Ldlr−/− and DKO mice have both similar plasma concentrations of LDL-C (Figure 4C) and similar levels of atherosclerotic lesions (Figure 2B).
and Ldlr/− mice 4 days after thioglycollate treatment and allowed the cells to adhere to cover slips for four hours. Figure 5B shows the results obtained when the freshly isolated DKO and Ldlr/− macrophages were stained with oil red O to assess neutral lipid levels; the data shows that the neutral lipid levels were decreased in the DKO cells.

Interestingly, CD36 mRNA levels were decreased significantly in the peritoneal macrophages freshly isolated from DKO mice (Figure 5C). In contrast, the levels of mRNAs encoding SR-A, SR-BI, L-1β2, II-6, tumor necrosis factor-α, Cox2, and apoE (Figure 5C) and genes involved in fatty acid synthesis (SREBP-1c, FAS, and SCD-1) (data not shown) were similar in macrophages derived from Ldlr/− and DKO mice. Thus, the change in CD36 gene expression is consistent with decreased oil red O staining of macrophages (Figure 5B). Importantly, FXR is not detectable in wild-type26 or Ldlr/− macrophages (see Ct values of relevant genes in supplemental Table II), suggesting that changes in macrophage gene expression and function are a result of an altered environment.

We also investigated whether PPARγ and PPARδ pathways were altered in DKO macrophages. The mRNA levels of LXRα, a PPARγ target gene,27 were unchanged in DKO macrophages (Figure 5C). However, ADRP (adipose differentiation-related protein), a PPARδ target gene,28 was significantly reduced in DKO, as compared with Ldlr/− macrophages (Figure 5C). ADRP has been proposed to promote lipid droplet formation in macrophages.29,30 Taken together, these data suggest that the altered gene expression in Ldlr/− macrophages, especially the decrease in CD36 mRNA expression, may contribute to the reduced atherosclerosis noted in male DKO mice.

**Discussion**

The proatherogenic plasma lipid profile in Fxr/− mice14 (Table) led us to hypothesize that FXR deficiency would contribute to and possibly accelerate atherosclerosis. The results reported here are both surprising and quite unexpected; using a Fxr/− Ldlr/− (DKO) mouse model, we demonstrate that FXR deficiency in male Ldlr/− mice results in a reduction in atherosclerotic lesion size (Figure 3). We further demonstrate that the male DKO mice had reduced plasma LDL-C levels (Figure 4). Because LDL-C is well-established as an independent risk factor for the development of atherosclerosis, we hypothesize that the decreased plasma LDL-C levels accounts, at least in part, for the reduced atherosclerosis noted in the aortas of the male DKO mice.

The decreased atherosclerosis noted in the current study with male Fxr/− Ldlr/− mice contrasts with the data recently reported by Hanniman et al using Fxr/− Apoe/− mice.31 In the latter study, Hanniman et al demonstrated that administration of a high-fat/high-cholesterol diet to male Fxr/− Apoe/− mice resulted in increased plasma lipids and atherosclerosis, as demonstrated by en face analysis of the aorta.31 They also showed that Fxr/− Apoe/− had a 33% reduction in survival rate, as compared with Apoe/− mice (100% survival).31 In contrast, our studies show that 100% male or female Fxr/− Ldlr/− mice survived when fed a Western diet for 16 weeks (data not shown). These contrasting results and conclusions may be a result of the difference in the genetic background of the mice (Ldlr/− versus Apoe/−) and the changes in plasma lipids that were observed (decreased LDL-C in the current study, versus increased LDL-C in the study by Hanniman et al).31 Because apoE mRNA levels were unchanged in macrophages isolated from Fxr/− Ldlr/− and Ldlr/− mice fed a Western diet (Figure 5C), we conclude that the decrease in atherosclerotic lesions in male Fxr/− Ldlr/− mice (Figure 2) is not a result of altered macrophage expression of this apolipoprotein.

Several lines of evidence have demonstrated that CD36 deficiency in macrophages is associated with a reduction in foam cell formation and atherosclerosis.5,6,32 The findings in the current study that showed that freshly isolated DKO peritoneal macrophages exhibited decreased neutral lipid staining, decreased CD36 mRNA levels and that the DKO mice had decreased atherosclerosis are consistent with these previous studies. However, additional studies will be necessary as the link between CD36 expression and atherosclerosis has recently been challenged by Moore et al; these authors reported that Cd36/− Apoe/− mice exhibited increased aortic...
sinus lesions, even though the DKO peritoneal macrophages exhibited reduced lipid accumulation in vitro.4

In addition to CD36, ADRP mRNA levels were also significantly decreased in the DKO macrophages (Figure 5C). ADRP has been proposed to play a role in the formation of lipid droplet in macrophages.26 However, the role of ADRP and PPARδ pathway in atherosclerosis in the Fxr−/−Ldlr−/− mice remains to be determined. Because FXR is not expressed in macrophages (supplemental Table II),26 we propose that the alterations in CD36 and ADRP mRNA expression in DKO macrophages are likely a secondary effect resulting from changes in the levels of plasma bile acids, lipids and inflammation.

In the current study we noted that administration of the western diet for 16 weeks resulted in a greater increase in body weight of Ldlr−/− mice, as compared with Fxr−/−Ldlr−/− mice, despite similar food intake (Figure 2A and data not shown). When challenged with a high-fat/high-cholesterol diet, Fxr−/−Apoe−/− mice also gained less body weight than Apoe−/− mice.31 Together, these data suggest that a null mutation of FXR provides protection against diet-induced obesity. However, the mechanism that leads to this protection is unknown at this time.

FXR activation has been shown to reduce plasma triglyceride, cholesterol, and glucose levels.12,14–18 Because hypertriglyceridemia, hypercholesterolemia, and insulin resistance are all risk factors for atherosclerosis,20,21 the hypolipidemic and hypoglycemic effects that follow FXR activation suggest that FXR agonists may prove to be useful. Thus the finding that loss of FXR function, at least in male Ldlr−/− mice, results in decreased atherosclerosis is surprising. However, additional studies will be necessary to assess whether FXR antagonists will be beneficial in the treatment of atherosclerosis. Other studies have shown that nuclear receptors, that include FXR, LXR, and PPAR, are bound to responsive elements on target genes as a complex with corepressors and that this complex inhibits transcription.33 Agonists have been shown to promote dissociation of the corepressors and recruitment of coactivators; the result is increased transcription of target genes.33 Nonetheless, deletion of specific nuclear receptors has been shown to have diverse effects on the expression of different target genes; some genes are repressed, some are unchanged, and some are induced.33 Thus, the final physiological effect will be a composite of all transcriptional changes. Consequently, additional studies will be necessary to determine the role of FXR antagonists (and/or agonists) on the development of atherosclerosis in hyperlipidemic mouse models.

Acknowledgments

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### Supplementary Table I

**Supplementary Table I.** Mouse primer sequences utilized in qRT-PCR

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<th>qPCR primer sequence</th>
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Peritoneal macrophages from \textit{Ldlr}^{-/-} mice (n=5) were isolated as described in Methods. qRT-PCR was performed and the relative Ct values were obtained after normalization to cyclophilin. The lower Ct values correspond to higher gene expression levels. FXR is not expressed in macrophages since its Ct value is more than 35.