FXR Deficiency Causes Reduced Atherosclerosis in 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 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 Fxr−/− mice or wild-type mice does not result in the development of significant atherosclerotic lesions. Consequently we generated Fxr−/−Ldlr−/− (DKO) mice and compared lesion development with Ldlr−/− mice. After 16 weeks on a Western diet, en face analysis of the aorta indicated that the male DKO mice had reduced atherosclerotic lesions as compared with 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 Ldlr−/− mice.

Conclusions—FXR deficiency in male, but not female, 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. (Arterioscler Thromb Vasc Biol. 2006;26:2316-2321.)

Key Words: atherosclerosis ■ cholesterol ■ FXR ■ LDLR ■ nuclear receptor
atherosclerosis. To test this hypothesis, we generated Fxr−/−–Ldlr−/− double knockout (DKO) mice and compared lesion development with Ldlr−/− mice after administration of a diet enriched in fat and cholesterol. Unexpectedly, these studies demonstrate that FXR deficiency in male DKO mice leads to reduced atherosclerotic lesions as compared with lesion size in male Ldlr−/− mice.

Methods

Animals and Diets

Ldlr−/− mice on a C57BL/6J background were purchased from Jackson Laboratory (Bar Harbor, Me). Fxr−/− mice14 were backcrossed to C57BL/6J mice for a total of 7 generations before being crossed with Ldlr−/− mice to generate Fxr−/−–Ldlr−/− (DKO) mice. All mice were fed a standard chow diet unless otherwise indicated. At 8 to 10 weeks of age, wild-type, Fxr−/−, Ldlr−/−, or DKO mice were fed a high-fat/high-cholesterol diet (Western diet) (Research Diets, #D12108, containing 21% fat [w/w], 1.25% cholesterol [w/w]) for 12 to 16 weeks, as indicated in the figure legends. All procedures were conducted in accordance with the animal care guidelines set by the University of California at Los Angeles.

Lipid and Lipoprotein Analyses

Plasma triglyceride, cholesterol, high-density lipoprotein cholesterol (HDL-C), and free fatty acids were measured as described.21 In addition, plasma from multiple mice (n=8) was combined and 50 or 400 μL of plasma was analyzed using fast-performance liquid chromatography (fast protein liquid chromatography [FPLC]) and cholesterol concentration determined in individual fractions.22

Quantitative Reverse-Transcription Polymerase Chain Reaction

RNA was extracted using Trizol reagent (Invitrogen, Calif) and mRNA levels then determined by quantitative reverse-transcription polymerase chain reaction (qRT-PCR) using iQ SYBR Green Supermix (Bio-Rad, Calif). The primer sequences for qRT-PCR are provided in supplemental Table I (available online at http://atvb.ahajournals.org).

Peritoneal Macrophages

Mice were injected with 1 mL of 3% thioglycollate and peritoneal exudates collected by lavage after 4 days. Peritoneal macrophages were isolated by centrifugation, washed in phosphate-buffered saline (PBS), and resuspended in Dulbecco’s modified Eagle’s medium (DMEM)/10% fetal bovine serum (FBS). Similar numbers of peritoneal macrophages were recovered from Ldlr−/− and DKO mice (data not shown) and allowed to adhere to either cover slips or 10 cm culture dishes. After 4 hours, the culture dishes and cover slips were washed three times with PBS to remove nonadhered cells. RNA was extracted from the macrophages on culture dishes, whereas oil red O staining was performed to stain macrophages on cover slips.

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.23 The surface lesion area was quantified with commercially available software (Image-Pro Plus, Media Cybernetics) as previously described.23

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 Display a Proatherogenic Plasma Lipid Profile

Fxr−/− mice, originally generated by Sinal et al, were on a mixed genetic background.14 These mice were backcrossed to C57BL/6 mice for seven additional generations before being used in the current study. Nonetheless, consistent with the initial report by Sinal et al,14 compared with their wild-type littermates, Fxr−/− 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−/− 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−/− mice was consistent with decreased hepatic expression of scavenger receptor class B, type I (SR-BI) (Figure 1E), the scavenger receptor that facilitates HDL-C administration of a Western diet for 3 months, no significant levels of atherosclerosis were detected in the aortic roots or aortic arches.

FXR Null Mice Do Not Develop Atherosclerosis

The proatherogenic lipid profile of Fxr−/− 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<sup>−/−</sup>/Ldlr<sup>−/−</sup> Double Knockout Mice**

FXR<sup>−/−</sup>/Ldlr<sup>−/−</sup> (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<sup>−/−</sup> 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<sup>−/−</sup> 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<sup>−/−</sup> mice fed a chow diet (data now shown).

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

To investigate the potential role of FXR in atherosclerosis, both Ldlr<sup>−/−</sup> 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<sup>−/−</sup> mice (Figure 2A). The mechanism underlying the differences in body weight between the two 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<sup>−/−</sup> mice (Figure 2B and 2C; P<0.001). The data of Figure 2B show that lesions of Ldlr<sup>−/−</sup> female mice fed the western diet were reduced as compared with male Ldlr<sup>−/−</sup> mice (24% versus 42%; P<0.05). However, lesions of female Ldlr<sup>−/−</sup> and DKO mice fed the Western diet were not significantly different (Figure 2B). Thus, loss of FXR reduced lesion size in Ldlr<sup>−/−</sup> 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<sup>−/−</sup>/Ldlr<sup>−/−</sup> 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 and FFA levels were elevated in both male and female DKO mice as compared with Ldlr<sup>−/−</sup> mice. Although plasma total cholesterol levels were not significantly different between male DKO and Ldlr<sup>−/−</sup> mice (Figure 3B), plasma HDL-C levels were reduced by 40% in the male DKO mice (Figure 3C). In contrast, female DKO
mice responded differently than males to the Western diet feeding; plasma cholesterol levels increased (Figure 3B), whereas HDL-C levels were similar between the two groups (Figure 3C).

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<sup>−/−</sup> 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 increased VLDL-C, and unchanged LDL-C and HDL-C levels (Figure 4B), with the result that total plasma cholesterol levels increased ~37% (Figure 3B). Figure 4C shows that plasma LDL-C levels decreased ~50% in male DKO mice to levels that are seen in both female Ldlr<sup>−/−</sup> 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<sup>−/−</sup> and DKO mice have both similar plasma concentrations of LDL-C (Figure 4C) and similar levels of atherosclerotic lesions (Figure 2B).

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

Atherosclerosis is an inflammatory disease. 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. 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.

Macrophage internalization of modified lipoproteins via scavenger receptors versus cellular lipid efflux via SR-B1 has been thought to play an important role in the generation of foam cells and the initiation of atherosclerosis. Consequently, we isolated peritoneal macrophages from male DKO and Ldlr<sup>−/−</sup> 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<sup>−/−</sup> 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-1β, 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<sup>−/−</sup> and DKO mice.
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-type or Ldlr\(^{-/-}\) mice. In the latter study, Hanniman et al demonstrated that administration of FXR agonists may prove to be useful. Thus the finding that loss of FXR function, at least in male DKO mice, results in decreased atherosclerosis is surprising. 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\(^{-/-}\) Apo\(^{-/-}\) mice exhibited increased aortic sinus lesions, even though the DKO peritoneal macrophages exhibited reduced lipid accumulation in vitro.

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. However, the role of ADRP pathway in atherogenesis in the Fxr\(^{-/-}\) Ldlr\(^{-/-}\) mice remains to be determined. Because FXR is not expressed in macrophages (supplemental Table II), 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\(^{-/-}\) Apo\(^{-/-}\) mice also gained less body weight than Apo\(^{-/-}\) mice. Together, these data suggest that a nul 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. Because hypertriglyceridemia, hypercholesterolemia, and insulin resistance are all risk factors for atherosclerosis, 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. Agonists have been shown to promote dissociation of the corepressors and recruitment of coactivators; the result is increased transcription of target genes. 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. 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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Disclosures
None.

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## Supplementary Table I

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

<table>
<thead>
<tr>
<th>Gene name</th>
<th>qPCR primer sequence</th>
<th>Gene name</th>
<th>qPCR primer sequence</th>
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</thead>
<tbody>
<tr>
<td>SR-BI</td>
<td>5'-CCTTCAATGACACAAGCAGACACCG-3' (F)</td>
<td>IL-6</td>
<td>5'-CTGCAAGAGACACTCCATCCAGTT-3' (F)</td>
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<td></td>
<td>5'-CCATGCACCTGTCAGTGTGACTGCT-3' (R)</td>
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<td>5'-GAAGTGAGGGAGGCCGCGTGG-3' (R)</td>
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<td>CD36</td>
<td>5'-GCACAATTGCAACATGACATG-3' (F)</td>
<td>Cox2</td>
<td>5'-TGCTGGAAGGTTCTTCTACGG-3' (F)</td>
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<td></td>
<td>5'-TCTCAATGTCCAGACTTTTCAAC-3' (R)</td>
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<td>5'-GAACCCAGTGTCCTGCCTATG-3' (R)</td>
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<td>SHP</td>
<td>5'-GTCCCAAAGGATAGTGCAGCT-3' (F)</td>
<td>SR-A</td>
<td>5'-GCAAAATTTGCTCCCTGGAGA-3' (F)</td>
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<td>5'-GCTATGTCGAGGAGGGC-3' (R)</td>
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<td>ICAM-1</td>
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<td>LXRα</td>
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<td></td>
<td>5'-CGAAAGTCCGGAGGCTCC-3' (R)</td>
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<td>P-selectin</td>
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<td>IL-1β</td>
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<td>tPA</td>
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<td>SAA-2</td>
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<td>5'-CAGCTTGCTTAGCAGCCAGAG-3' (F)</td>
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<td>5'-GCTGCACTGTGAGATTTTC-3' (R)</td>
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<td>TNFα</td>
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<td>5'-GAGCGGTGAGGCCCCCT-3' (R)</td>
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Supplementary Table II

Supplementary Table II. Gene Ct values in *Ldlr*⁻⁻ macrophages.

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<tr>
<td>IL-1β</td>
<td>26.3±2.8</td>
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<tr>
<td>IL-6</td>
<td>28.1±4.5</td>
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<tr>
<td>TNFα</td>
<td>24.5±2.5</td>
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<tr>
<td>Cox2</td>
<td>25.9±2.8</td>
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<tr>
<td>CD36</td>
<td>21.3±1.4</td>
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<tr>
<td>SR-A</td>
<td>24.6±2.4</td>
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<tr>
<td>SR-BI</td>
<td>27.6±2.1</td>
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<tr>
<td>ApoE</td>
<td>22.1±2.1</td>
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<tr>
<td>LXRα</td>
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<tr>
<td>LXRβ</td>
<td>25.8±2.1</td>
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<tr>
<td>ADRP</td>
<td>21.6±3.4</td>
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<tr>
<td>FXR</td>
<td>35.2±1.7</td>
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Peritoneal macrophages from *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.