Non-redundant roles for LXRα and LXRβ in atherosclerosis susceptibility in low density lipoprotein receptor knockout mice

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Abstract  The liver X receptors LXRα and LXRβ play critical roles in maintaining lipid homeostasis by functioning as transcription factors that regulate genetic networks controlling the transport, catabolism, and excretion of cholesterol. The studies described in this report examine the individual anti-atherogenic activity of LXRα and LXRβ and determine the ability of each subtype to mediate the biological response to LXR agonists. Utilizing individual knockouts of LXRα and LXRβ in the LDLr−/− background, we demonstrate that LXRα has a dominant role in limiting atherosclerosis in vivo. Functional studies in macrophages indicate that LXRα is required for a robust response to LXR ligands, whereas LXRβ functions more strongly as a repressor. Furthermore, selective knockout of LXRα in hematopoietic cells and rescue experiments indicate that the anti-atherogenic activity of this LXR subtype is not restricted to macrophages. These studies indicate that LXRα plays a selective role in limiting atherosclerosis in response to hyperlipidemia.—Bischoff, E. D., C. L. Daige, M. Petrowski, H. Dedman, J. Pattison, J. Juliano, A. C. Li, and I. G. Schulman. Non-redundant roles for LXRα and LXRβ in atherosclerosis susceptibility in low density lipoprotein receptor knockout mice. J. Lipid Res. 2010. 51: 900–906.

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The contribution of elevated cholesterol levels to the development of cardiovascular disease and atherosclerosis is well documented. Nevertheless the molecular signaling pathways that regulate cholesterol homeostasis at the blood vessel wall, particularly in response to elevated cholesterol levels, remain to be fully deciphered (1). A critical early event in the development of atherosclerosis is the recruitment of macrophages to the subendothelial space of vessel walls and the uncontrolled uptake of oxidized or aggregated low density lipoprotein particles. Continued accumulation of oxidized or aggregated LDL by macrophages and an associated inflammatory response leads to foam cell formation and the initiation of atherosclerosis (2).

The liver X receptors LXRα (NR1H3) and LXRβ (NR1H2), members of the nuclear hormone receptor superfamily of transcription factors, have been identified as important regulators of cholesterol homeostasis in multiple cell types, including macrophages (3). Treatment of cells with oxysterols, natural LXR ligands derived from cholesterol, or synthetic LXR agonists promotes the efflux of cholesterol by increasing expression of the ATP binding cassette transporters ABCA1 and ABCG1 and the apolipoprotein E (apoE) (4, 5). ABCA1, ABCG1, and apoE all participate in the transfer of intracellular and plasma membrane cholesterol to HDL, a process termed reverse cholesterol transport (6). Importantly, LXR agonists reduce atherosclerosis in animal models of cardiovascular disease, and upregulation of ABC transporters can be detected in the atherosclerotic lesions of treated animals (7, 8). The ability of LXRs to inhibit pro-inflammatory pathways may also contribute to the anti-atherogenic activities of these receptors (9). Previous work from our laboratory has demonstrated that LXR function in hema-

Abbreviations: ABCA1, ATP binding cassette transporter A1; ABCG1, ATP binding cassette transporter G1; apoE, apolipoprotein E; FPLC, fast-protein liquid chromatography; iNOS, inducible nitric oxide synthase; LXR, liver X receptor; MCP-1, monocyte chemotactic protein 1; NFκB, nuclear factor kappa B; SREBP, sterol-regulatory element binding protein; TNF-α, tumor necrosis factor-α.

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topoietic cells is necessary for the anti-atherogenic activity of LXR agonists (8), implicating macrophages as a critical site of action for the LXRs.

Each LXR subtype demonstrates a unique but overlapping expression pattern. LXXRa is highly expressed in cell types that modulate cholesterol and fatty acid synthesis, including the liver and intestine, whereas LXRβ is ubiquitously expressed (10–12). In the liver, LXXRa is the predominant subtype expressed, and studies indicate that LXXRa controls fatty acid synthesis via regulation of the gene encoding the sterol-regulatory element binding protein 1c (SREBP1c) (13, 14). Additionally LXXRa modulates cholesterol homeostasis by controlling expression of cholesterol 7α-hydroxylase (11), the rate-limiting enzyme in the conversion of cholesterol to bile acids, and the cholesterol transporters ABCG5 and ABCG8 (15). In other tissues, including the intestine and macrophages, both subtypes are coexpressed and appear competent to regulate LXR target genes (4, 16, 17).

The ability to induce reverse cholesterol transport spurred great interest in the LXRs as drug targets for cardiovascular disease (13, 14, 18). Increases in plasma triglycerides and cholesterol observed in animals treated with synthetic LXR agonists, however, has raised concerns regarding the therapeutic potential of LXR-based drugs (18). As described above, the effects on lipid levels in animals treated with LXR agonists appear to be selectively mediated by LXXRa in the liver (10, 15). In contrast, treatment with an LXR agonist reduced atherosclerosis in apoE −/−/LXXRa −/− mice (19), suggesting that LXRβ alone is sufficient to mediate the anti-atherogenic functions of LXR activation. Not surprisingly, several investigators have suggested that LXRβ-selective activators may have an optimized therapeutic potential (10, 12). To our knowledge, the anti-atherogenic activity of such selective modulators has not yet been described.

Although the case for LXRβ-selective agonists has been put forward, the individual anti-atherogenic potential of LXXRa and LXRβ has not yet been addressed. To examine the anti-atherogenic activity of each LXR subtype in vivo, genetic knockouts of LXXRa or LXRβ were crossed into the LDL receptor knockout mouse (Ldlr −/−) background. Analysis of atherosclerosis in these strains indicates that LXXRa plays a dominant role in limiting disease in response to elevated lipid levels and that this subtype is required for the full anti-atherogenic activity of LXR agonists. Experiments that selectively rescue LXXRa function in bone marrow–derived or nonbone marrow–derived cells suggest that LXXRa activity may be required in cell types other than macrophages to limit diet-induced cardiovascular disease.

METHODS

Animals and diets

All animal experiments were approved by the Exelixis Institutional Animal Care and Research Advisory Committee. Ldlr −/−/LXXRa −/− and Ldlr −/−/LXRβ −/− mice were created by mating Lxxa −/− and Lxb −/− mice (C57BL/6 genetic background) with LDLR +/− mice (male B6.129S7-Ldlr imHs/j) purchased from Jackson Laboratories (Bar Harbor, ME). Homozygous Lxxa −/−, Lxb −/−, and Lxxa −/−/Lxb −/− knockout mice on the C57BL/6 genetic background were from a colony established and maintained at Exelixis, Inc. (San Diego, CA). Mice were fed standard chow (Lab Diet 5001) ad libitum until put on the study.

To examine atherosclerosis in Ldlr −/−, Ldlr −/−/Lxxa −/−, and Ldlr −/−/Lxb −/− mice, 8–9 week old male animals were placed on a Western diet (21% fat/wt, 0.15% cholesterol wt/wt; Purina Test Diets #21551) as described in the figure legends. When described in the text, the LXR agonist T0901317 was administered by oral gavage.

Bone marrow transplantation

Bone marrow transplantations were carried as previously described (8, 20). After transplant, animals were allowed to recover on a standard diet (Lab Diet 5001) for 4 weeks before being fed the Western diet for an additional 8 weeks.

Quantitation of atherosclerosis

Atherosclerosis in root sections and en face preparations were quantitated as previously described (8, 20).

Lipid and lipoprotein analyses

Plasma total cholesterol and triglyceride levels were determined by colorimetric enzymatic assays that were adapted to 96-well plate as previously described (8). Terminal plasma samples from mice fed the Western diet for 20 weeks were pooled and fractionated by fast-protein liquid chromatography (FPLC) gel filtration on Superose 6 columns. Triglyceride and cholesterol concentrations of each fraction were determined as above.

Measurement of TNFα plasma levels

To measure tumor necrosis factor-α (TNFα) protein levels, mice of the appropriate genotypes were fed the Western diet for 2 weeks and then given a single injection of LPS (25μg). Plasma TNFα levels were determined by ELISA (R and D Systems) 1.5 h after the LPS treatment.

Macrophage isolation

Bone marrow–derived macrophages were isolated from 8–9 week old mice as previously described (21).

Quantitative real-time PCR

Quantitative analysis of gene expression was carried out as previously described (8).

Statistical analyses

Results were analyzed by one-way ANOVA (ANOVA) or Student’s unpaired t test using GraphPad Prism (GraphPad Software, Inc.).

RESULTS

Increased atherosclerosis in Lxxa −/− mice

To define the anti-atherogenic activity of the individual LXR subtypes, single LXXRa and LXRβ knockouts were crossed into the Ldlr −/− background to create Ldlr −/−/Lxxa −/− and Ldlr −/−/Lxb −/− double knockout strains. Animals from the three strains were placed on a Western diet (21% fat, 0.15% cholesterol), and plasma lipid levels were measured biweekly for 20 weeks (Fig. 1). Genetic deletion of LXXRa in the Ldlr −/− background resulted in a dramatic decrease in plasma triglyceride and total cholesterol concentrations (Fig. 1).
plasma triglyceride levels at all time points, confirming previous data identifying this receptor subtype as the principal regulator of fatty acid synthesis (10, 12) (Fig. 1A). Ldlr<sup>−/−</sup>/Lxr<sup>−/−</sup> mice also exhibited a significant decrease in total cholesterol levels beginning at week 4 (Fig. 1B). Plasma triglyceride and cholesterol levels were unchanged in Ldlr<sup>−/−</sup>/Lxrβ<sup>−/−</sup> animals for the first 12 weeks of the experiment (Fig. 1A, B). Beginning at week 12, however, there was a significant drop in lipid levels that coincided with an overall failure of these animals to thrive, including weight loss and a general decrease in activity (data not shown). FPLC analysis of pooled lipoprotein samples taken at completion of the study confirmed significant decreases in VLDL levels in both double knockout strains (see supplementary Fig. I).

Quantitation of atherosclerosis by serial section analysis of the aortic root (Fig. 2A) demonstrated that deletion of LXRα significantly increases atherosclerosis by 25%. There was also a trend toward an increase in Ldlr<sup>−/−</sup>/Lxrβ<sup>−/−</sup> animals that did not reach statistical significance (P = 0.09). Immunohistochemical staining did not detect significant differences in macrophage or collagen content when normalized to the lesion area (data not shown). To extend the analysis to additional areas beyond the aortic root, en face quantitation was carried out (Fig. 2B). Analysis of the arch and thoracic regions indicates there is an 80% increase in Ldlr<sup>−/−</sup>/Lxr<sup>−/−</sup> samples (Fig. 2B, C) with the most dramatic differences distal to the aortic arch (Fig. 2C). Deleting LXRβ, however, had no effect. A significant increase in atherosclerosis is also selectively detected in Ldlr<sup>−/−</sup>/Lxr<sup>−/−</sup> animals if the en face analysis is limited to aortic arch region (see supplementary Fig. II). Thus LXRα played a dominant role in limiting diet-induced atherosclerosis.

LXRα is required for the anti-atherogenic activity of LXR agonists

Synthetic LXR agonists have been shown to decrease atherosclerosis and promote the regression of lesions in Ldlr<sup>−/−</sup> mice (7, 8). To explore the contribution of each LXR subtype to the anti-atherogenic activity of LXR agonists, mice of the appropriate genotypes were exposed to the Western diet for 12 weeks and concurrently treated

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**Fig. 1.** Plasma lipid levels in double knockout mice. Ldlr<sup>−/−</sup> (n = 12), Ldlr<sup>−/−</sup>/Lxr<sup>−/−</sup> (n = 9), and Ldlr<sup>−/−</sup>/Lxr<sup>−/−</sup> (n = 12) mice were fed a Western diet for 20 weeks, and plasma triglyceride (A) and total cholesterol (B) levels were determined.

**Fig. 2.** Atherosclerosis in double knockout mice. Mice were fed a Western diet for 20 weeks, and atherosclerosis was quantitated by root section (A) and en face analysis of the aorta (B) as described in the “Methods.” (C) Representative Sudan IV stained aortas. *Significantly different from Ldlr<sup>−/−</sup> controls.
with the LXR agonist T0901317. As expected from previous studies, plasma triglycerides were significantly lower in \textit{Ldlr}^{-/-}/\textit{Lxr}^{-/-} mice, regardless of agonist treatment, while T0901317 elevated triglyceride levels in strains expressing LXR\textalpha{} (Fig. 3A). Importantly, \textit{Ldlr}^{-/-}/\textit{Lxr}\beta{}^{-/-} mice treated with 10 mg/kg T0901317 rapidly developed massive hypertriglyceridemia (data not shown) that could not be accurately measured, and 3 out of 10 mice died within 4 weeks of initiating treatment. At week 4, the dose for this group was dropped to 3 mg/kg, and the rest of the animals survived until study completion. Nevertheless, given the small number of animals that remained in this group, the conclusions regarding the effects of T0901317 on \textit{Ldlr}^{-/-}/\textit{Lxr}\beta{}^{-/-} mice should be interpreted with caution.

The LXR agonist-dependent hypertriglyceridemia observed in these animals suggests, at least in the \textit{Ldlr}^{-/-} background, that LXR\beta{} partially represses the ability of LXR\textalpha{} to regulate triglyceride levels. Little or no effect of T0901317 on total plasma cholesterol levels was detected (Fig. 3B).

Consistent with previous studies (7, 8, 22), treatment of \textit{Ldlr}^{-/-} mice with T0901317 significantly reduced atherosclerosis when quantitated by root section (Fig. 3C) or en face analysis (Fig. 3D). T0901317 also significantly reduced atherosclerosis at the aortic root in the absence of either of LXR\textalpha{} or LXR\beta{} (Fig. 3C), indicating that each LXR subtype alone can mediate an anti-atherogenic response to an LXR agonist. Once again, however, en face analysis uncovered a large increase in atherosclerosis in \textit{Ldlr}^{-/-}/\textit{Lxr}^{-/-} animals that was unaffected by treatment with T0901317 (Fig. 3D). Thus when atherosclerosis is measured throughout the aorta, LXR\textalpha{} is necessary for the anti-atherogenic activity of LXR agonists. In contrast, even at a lower dose, the LXR agonist remained effective in animals lacking LXR\beta{} (Fig. 3D).

**LXR\textalpha{} selective regulation of gene expression in macrophages**

We previously demonstrated that macrophages are an important site of action for the anti-atherogenic activity of LXR agonists (8, 20). Therefore to explore a mechanistic basis for the selective effect of LXR\textalpha{} deletion on atherosclerosis, the expression of LXR target genes was examined in bone marrow–derived macrophages isolated from C57BL/6, \textit{Lxr}^{-/-}, \textit{Lxr}\beta{}^{-/-}, and \textit{Lxr}^{-/-}/\textit{Lxr}\beta{}^{-/-} mice. As shown in Fig. 4A and 4B, the ability of T0901317 to induce expression of ABCA1 and ABCG1 was selectively impaired in \textit{Lxr}^{-/-}/\textit{Lxr}\beta{}^{-/-} cells (compare solid black lines to dotted lines). These results indicate that LXR\textalpha{} is required to maximally induce genes involved in reverse cholesterol transport. The subtype selective regulation of SREBP1c in response to agonist treatment is somewhat different.

**Fig. 3.** LXR agonist activity in double knockout mice. Mice were exposed to a Western diet for 12 weeks and dosed daily with vehicle or 10 mg/kg T090137. The \textit{Ldlr}^{-/-}/\textit{Lxr}\beta{}^{-/-} + T0901317 group was switched from 10 mg/kg to 3 mg/kg at week 4. After completion of the study plasma triglycerides (A) and total plasma cholesterol (B) were measured. *Significantly different from strain-matched vehicle controls. **Significantly different from vehicle-treated \textit{Ldlr}^{-/-} mice. Atherosclerosis was measured by quantitation of root sections (C) and by en face analysis of the entire aorta (D). *Significantly different from strain-matched vehicle controls. **Significantly different from vehicle-treated \textit{Ldlr}^{-/-} mice.
We did not, however, observe LXR subtype-specific differences in the ability of T0901317 to inhibit expression of monocyte chemotactic protein 1 (MCP-1), TNF-α, or inducible nitric oxide synthase (iNOS) in lipopolysaccharide-stimulated bone marrow derived macrophages (data not shown).

LXRα functions in bone marrow- and nonbone marrow–derived cells

Macrophages as well as other immune cells play a critical role in the development of atherosclerosis (2), and the ability of LXRs to regulate reverse cholesterol transport and inflammation in these cells is thought to underlie their anti-atherogenic activities (9). To determine if it is simply the absence of LXRα in the hematopoietic system that lead to the increased atherosclerosis observed in the Ldb1−/− background, bone marrow transplantation experiments were used to either selectively eliminate or selectively express LXRα in hematopoietic cells (Fig. 5). As expected, transplantation of Ldb1−/− mice with bone marrow from Ldb1−/−/Lxrα−/− animals (hematopoietic system knockout) increased atherosclerosis relative to controls (in Fig. 5, compare groups 1 and 2; P = 0.03). Nevertheless, atherosclerosis in Ldb1−/− mice transplanted with Ldb1−/−/Lxrα−/− marrow was not as great as that observed in the complete LXRα knockout (i.e., irradiated Ldb1−/−/Lxrα−/− mice that received Ldb1−/−/Lxrα−/− bone marrow; in Fig. 5, compare groups 2 and 4; P = 0.0001). Thus, eliminating LXRα activity in hematopoietic cells did not entirely recapitulate the phenotype of the complete knockout. Similarly, the introduction of LXRα positive bone marrow into Ldb1−/−/Lxrα−/− mice (hematopoietic system selective expression) decreased atherosclerosis (in Fig. 5, compare groups 4 and 5; P = 0.0001) but not to the levels measured in Ldb1−/− controls (in Fig. 5, compare groups 1 and 5; P = 0.01). Taken together, the results of the bone marrow transplantation experiments indicate that LXRα function in cell types other than macrophages may contribute to the antiatherogenic activity of this receptor.
DISCUSSION

The ability of LXRα to regulate reverse cholesterol transport in macrophages has stimulated great interest in understanding the anti-atherogenic activity of the two LXR subtypes. Analysis of single genetic deletions of each receptor in the Ldlr<sup>−/−</sup> background reveals a dominant effect for LXRα in the control of cardiovascular disease. Quantitation of atherosclerosis in animals exposed to a Western diet uncovered an 80% increase in Lxrα<sup>−/−</sup> animals, with dramatic increases in the lesion area in regions distal to the aortic arch. Little or no effect of deleting LXRβ was observed. Our results are consistent with reports by Bradley et al. (19) and Teupser et al. (24) that detected increased atherosclerosis in apoE<sup>−/−</sup>/Lxrα<sup>−/−</sup> mice and decreased atherosclerosis in Ldlr<sup>−/−</sup> mice over expressing LXRα, respectively. Neither of these studies, however, directly compared the two LXR subtypes.

Functional experiments using bone marrow–derived macrophages demonstrate that LXRα is quantitatively a stronger transcriptional activator of the ABCA1 and ABCG1 promoters in response to agonists than is LXRβ. The subtype selective response to agonist treatment measured in macrophages suggests an impaired ability of Lxrα<sup>−/−</sup> macrophages to induce gene expression and reverse cholesterol transport in response to elevated cholesterol levels. In support of the gene expression data, the anti-atherogenic activity of the agonist T0901317 is muted in the absence of LXRα. LXRβ, on the other hand, appears to be more responsible for setting the basal level of macrophage ABCA1 and ABCG1 expression by limiting transcription in the absence of activating signals.

The effect of the LXR agonist T0901317 on atherosclerosis in Ldlr<sup>−/−</sup>/Lxrα<sup>−/−</sup> mice differed depending on the method of analysis used. Serial section of the aortic root indicated that T0901317 reduced atherosclerosis in Ldlr<sup>−/−</sup>/Lxrα<sup>−/−</sup> mice (i.e., LXRβ alone can mediate an anti-atherogenic response). On the other hand, en face analysis of the aortic arch and thoracic region indicated that the agonist had no effect in the absence of LXRα. Additionally, the en face analysis uncovered a large increase in atherosclerosis distal to the aortic arch region in these animals. It is not unusual to see differences in atherosclerosis measured by these two methods. Serial sections provide cross-sectional information from a single area whereas en face analysis measures the surface area of the entire segment covered by lesions. In mouse models of atherosclerosis, lesions first develop in the aortic root and arch areas and then progress distally down the segment as animals age (25). The large increase in atherosclerosis observed in Lxrα<sup>−/−</sup> animals suggests that LXRα may selectively influence the kinetics of early lesion initiation and/or disease progression. After exposure to the Western diet for 20 weeks, such an acceleration of lesion progression may be easily visible at areas distal to the arch while less apparent at the older, more advanced areas measured at the aortic root. Zhu et al. (26) have also recently observed that LXRα is expressed at higher levels in the thoracic aorta compared with the arch region, raising the possibility that this subtype plays a unique or quantitatively dominant role in the thoracic region. LXRβ expression in these regions was not examined.

The function of LXRβ is more difficult to discern from these studies. After 12 weeks on the Western diet, there was a general failure of the Ldlr<sup>−/−</sup>/Lxrβ<sup>−/−</sup> mice to thrive. Although there was no obvious pathology at necropsy 8 weeks later, lymphoid hyperplasia has been observed in older Lxrβ<sup>−/−</sup> mice (27), which, when coupled with the Western diet, may have contributed to the poor health of these animals. Interestingly, in Lxrβ<sup>−/−</sup> macrophages, the basal levels of ABCA1 and ABCG1 were elevated, suggesting a role for this receptor in limiting reverse cholesterol transport in the absence of activating signals. Additionally, treatment of Ldlr<sup>−/−</sup>/Lxrβ<sup>−/−</sup> mice with T0901317 produced a massive increase in plasma triglycerides, indicating that LXRβ functions to limit the ability of LXRα to induce fatty acid synthesis (at least under hyperlipidemic conditions). A similar inhibitory effect of LXRβ on the agonist-dependent induction of SREBP1c was detected in macrophages. These results suggest opposing functions for the two subtypes, with LXRα functioning to drive transcription in the presence of agonists and LXRβ functioning to repress transcription when ligands are active.

CONCLUSION

The ability to regulate reverse cholesterol transport and inflammation at the site of atherosclerotic lesions has made macrophages the focus of LXR studies (8, 20). To address the sites of LXRα-dependent anti-atherogenic activity, we used bone marrow transplantations to selectively eliminate or selectively express LXRα in the hematopoietic system. The introduction of LXRα positive bone marrow into Ldlr<sup>−/−</sup>/Lxrα<sup>−/−</sup> mice did partially rescue the athero...
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REFERENCES


