LXRα is uniquely required for maximal reverse cholesterol transport and atheroprotection in ApoE-deficient mice

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Abstract The liver X receptor (LXR) signaling pathway is an important modulator of atherosclerosis, but the relative importance of the two LXRs in atheroprotection is incompletely understood. We show here that LXRα, the dominant LXR isotype expressed in liver, plays a particularly important role in whole-body sterol homeostasis. In the context of the ApoE−/− background, deletion of LXRα, but not LXRβ, led to prominent increases in atherosclerosis and peripheral cholesterol accumulation. However, combined loss of LXRα and LXRβ on the ApoE−/− background led to an even more severe cholesterol accumulation phenotype compared to LXRα−/−ApoE−/− mice, indicating that LXRβ does contribute to reverse cholesterol transport (RCT) but that this contribution is quantitatively less important than that of LXRα. Unexpectedly, macrophages did not appear to underlie the differential phenotype of LXRα−/−ApoE−/− and LXRβ−/−ApoE−/− mice, as in vitro assays revealed no difference in the efficiency of cholesterol efflux from isolated macrophages. By contrast, in vivo assays of RCT using exogenously labeled macrophages revealed a marked defect in fecal sterol efflux in LXRα−/−ApoE−/− mice. Mechanistically, this defect was linked to a specific requirement for LXRα−/− in the expression of hepatic LXR target genes involved in sterol transport and metabolism. These studies reveal a previously unrecognized requirement for hepatic LXRα for optimal reverse cholesterol transport in mice.—Hong, C., M. N. Bradley, X. Rong, X. Wang, A. Wagner, V. Grijalva, L. W. Castellani, J. Salazar, S. Realegeno, R. Boyadjian, A. M. Fogelman, B. J. Van Lenten, S. T. Reddy, A. J. Lusis, R. K. Tangirala, and P. Tontonoz. LXRα is uniquely required for maximal reverse cholesterol transport and atheroprotection in ApoE-deficient mice. J. Lipid Res. 2012. 53: 1126–1133.

Supplementary key words atherosclerosis • nuclear receptor • cholesterol metabolism • apolipoprotein

High levels of plasma LDL cholesterol have long been recognized to be a primary driver of atherosclerotic lesion formation (1). More recently, the ability of an animal to remove excess cholesterol from tissues and the body through the process of reverse cholesterol transport (RCT) has begun to be appreciated as an important determinant of susceptibility (2, 3). During atherogenesis, macrophages infiltrate the artery wall and accumulate lipoprotein-derived cholesterol via scavenger receptors. Macrophages attempt to restore their cellular cholesterol homeostasis by effluxing cholesterol to lipid-poor acceptors such as apoA-1 and HDL, which in turn carry the excess cholesterol back to the liver and possibly intestine for excretion (2, 4). Mounting evidence indicates that disruption of the pathways for macropage cholesterol efflux or RCT by HDL promotes atherogenesis in animal models.

The primary transcriptional regulators of the RCT pathway are liver X receptor (LXRα) and LXRβ, closely related members of the nuclear hormone receptor superfamily. These receptors function as “cholesterol sensors,” responding to elevated cellular concentrations of their oxysterol ligands by activating the expression of numerous genes linked to sterol transport and metabolism. Other studies have defined a role for the LXRs in repressing inflammatory genes and autoimmunity (5, 6). Although the two LXRs appear to regulate a similar set of target genes, their expression patterns differ. LXRβ is ubiquitously expressed in a wide variety of tissues and cell types, whereas LXRα is more restricted in expression and is particularly abundant in the liver (7). Although both LXRα and LXRβ are required for atheroprotection in vivo, the relative importance of the two receptors in this process is incompletely understood. Here, we show that LXRα plays a particularly important role in whole-body sterol homeostasis. In the context of the ApoE−/− background, deletion of LXRα, but not LXRβ, led to prominent increases in atherosclerosis and peripheral cholesterol accumulation. However, combined loss of LXRα and LXRβ on the ApoE−/− background led to an even more severe cholesterol accumulation phenotype compared to LXRα−/−ApoE−/− mice, indicating that LXRβ does contribute to reverse cholesterol transport (RCT) but that this contribution is quantitatively less important than that of LXRα. Unexpectedly, macrophages did not appear to underlie the differential phenotype of LXRα−/−ApoE−/− and LXRβ−/−ApoE−/− mice, as in vitro assays revealed no difference in the efficiency of cholesterol efflux from isolated macrophages. By contrast, in vivo assays of RCT using exogenously labeled macrophages revealed a marked defect in fecal sterol efflux in LXRα−/−ApoE−/− mice. Mechanistically, this defect was linked to a specific requirement for LXRα−/− in the expression of hepatic LXR target genes involved in sterol transport and metabolism. These studies reveal a previously unrecognized requirement for hepatic LXRα for optimal reverse cholesterol transport in mice.

Abbreviations: E/α KO (knockout) mice, ApoE−/− LXRα−/− mice; E/β KO (knockout) mice, ApoE−/− LXRβ−/− mice; LXR, liver X receptor; RCT, reverse cholesterol transport; RXR, retinoid X receptor; TKO (total knockout) mice, ApoE−/− LXRα−/− LXRβ−/− mice.

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expressed, whereas LXRα is highly expressed liver, intestine, adipose, and macrophages. Interestingly, the liver is one of the few tissues that express predominantly LXRα rather than LXRβ (7–10). Prior studies have suggested that LXR isoforms may have differential impacts on atherosclerosis, but the physiological basis for these effects have remained unclear (7, 11, 12).

Considerable evidence indicates that macrophages contribute to the antiatherogenic effects of the LXR pathway (6). It is clear that complete loss of LXR activity in the macrophage predisposes the cell to cholesterol overload and foam-cell formation. Transplantation of LXRα−/−LXRβ−/− bone marrow into LDLR−/− mice leads to greatly increased atherosclerotic lesion formation (11). In addition, the ability of LXR ligands to reduce atherosclerotic lesion formation is reduced when both LXRα and LXRβ are deleted from the bone marrow compartment (13, 14). With the notable exception of the LXRα-specific target gene AIM/api6, prior gene expression analyses have suggested that most LXR target genes are comparably regulated by both LXR isotypes and that combined deletion of the two isotypes is required to prevent target gene induction by LXR agonists (15–17).

We show here that ApoE−/− mice lacking LXRα but not LXRβ show enhanced atherosclerosis and peripheral cholesterol accumulation, indicating that LXRα plays a role in atherosclerosis susceptibility that is not redundant with LXRβ. Unexpectedly, this phenotype cannot be explained by differential function of the two LXR isotypes in macrophages. Rather, we show that LXRα is uniquely required for maximal postmacrophage RTC. Moreover, we show that the defect in LXRα-deficient mice is linked with a specific requirement for LXRα in the expression of hepatic LXR target genes involved in sterol transport.

METHODS

Animals and diets

LXRα−/−, LXRβ−/−, and LXRαβ−/− mice (C57Bl/6, greater than 10 generations backcrossed) were provided by David Mangelsdorf and bred with C57Bl/6 ApoE−/− mice from the Jackson Laboratory (18). Male mice were fed either standard chow, Western diet (21% fat, 0.21% cholesterol: D12079B; Research Diets, Inc.). For ligand treatment studies, mice were gavaged with vehicle or 40 mg/kg GW3965 once a day for 3 days. Tissues were harvested 4 h after the last gavage. Atherosclerotic lesion analysis was done as described (12). Animal experiments were approved by the UCLA Institutional Animal Care and Research Advisory Committee.

RNA analysis, cell culture, and reagents

Total RNA was isolated from tissues using TRIzol (Invitrogen) and analyzed by real-time PCR using an Applied Biosystems 7900HT sequence detector. Results show averages of duplicate experiments normalized to 36B4. The primer sequences are 7900HT sequence detector. Results show averages of duplicate experiments normalized to 36B4. The primer sequences are

RESULTS

We previously reported that mice lacking both ApoE and LXRα exhibit a dramatic increase in susceptibility to atherosclerosis (12). However, the relative importance of LXRα and LXRβ expression in sterol homeostasis and atherogenesis in the context of the ApoE−/− background has not been addressed. To explore this, we generated ApoE−/− mice lacking LXRα, LXRβ, or both (C57Bl6 background; more than 10 generations backcrossed). ApoE−/−LXRα−/− (hereafter denoted E/α KO) mice and ApoE−/−LXRβ−/− (E/β KO) mice were obtained at the

Tissue and plasmid lipid analysis

Lipids were extracted from tissues using the Folch method. Briefly, chloroform extracts were dried under nitrogen and re-solubilized in water. Cholesterol content was determined using a commercially available enzymatic kit (Sigma-Aldrich). Data are expressed as milligrams of cholesterol per gram of tissue weight. For plasma lipid analysis, mice were fasted overnight and euthanized. Blood was collected from the abdominal vena cava. Aliquots of plasma were analyzed for cholesterol content and plasma lipoproteins were fractionated using an FPLC system.

Histological and lesion analysis

Immunohistochemistry of skin sections and preparation and staining of frozen and paraffin-embedded sections from tissues were performed as described previously. Atherosclerosis in the aortic roots and the descending aortas (en face) were quantified by computer-assisted image analysis. Atherosclerotic lesions at the aortic valve were analyzed as described. P<0.05 was considered significant.

Cholesterol efflux

Peritoneal macrophages cells were labeled with [3H]cholesterol (1.0 μCi/ml) in the presence of acyl-CoA:cholesterol O:acyltransferase inhibitor (2 μg/ml) either with DMSO or with ligand for LXR and RXR (1 μM GW3965; 50 nM LG268). After equilibrating the cholesterol pools, cells were washed with PBS and incubated in DMEM containing 0.2% BSA in the absence or presence of ApoA-I (15 μg/ml) or HDL (50 μg/ml) for 6 h. Radioactivity in the medium and total cell-associated radioactivity was determined by scintillation counting (12).

RTC

[1]74 murine macrophages were cultured for 2 days in the presence of 5 μg/ml [3H]cholesterol and 100 μg/ml Ac-LDL in 10% FBS, 1% Pen Strep, and DMEM for 48 h. The cells were collected and washed twice with cold 1× PBS and delivered to the mice by intraperitoneal injection. Two days after injection, fecal pellets were collected, lipid was extracted using the Folch METHOD, and radioactive counts were determined (3).
expected Mendelian ratios; however, LXRβ and E/β mice exhibited dramatically reduced fertility. At eight months of age, E/α mice developed an obvious external phenotype characterized by thickening of the skin and alopecia (Fig. 1A), consistent with prior results on a mixed C57BL6/SV129 background (12). Unexpectedly, however, E/β KO mice did not exhibit this phenotype, suggesting that loss of LXRα and LXRβ differentially affect whole body sterol homeostasis on the ApoE KO background.

To explore this further, cohorts of each genotype were placed on Western diet for 15 weeks. FPLC analysis of plasma lipids revealed reduced plasma triglycerides and VLDL in E/α KO mice compared to E/β KO mice or ApoE KO controls (Fig. 1B). These findings suggest that LXRα expression is a particularly important determinant of plasma triglyceride levels in ApoE KO mice, and are consistent with earlier studies of mixed background LXR single KO mice (9, 11). Plasma cholesterol profiles of both E/α mice and E/β mice revealed elevated plasma cholesterol content compared to ApoE KO controls (Fig. 1C). Despite the similar plasma cholesterol levels of E/α mice and E/β mice, however, analysis of tissue lipid content revealed marked accumulation of cholesterol in the skin of only the E/α KO mice (Fig. 1D). Gene expression studies revealed increased CD68 and CD11b expression in the skin of E/α mice, indicative of macrophage infiltration into the skin, corresponding with the cholesterol deposition (Fig. 1E).

Next we analyzed atherosclerotic lesion formation after 15 weeks of Western diet. En face lesion analysis demonstrated a marked increase in lesion area in the aortas of E/α KO mice compared to E/β KO or ApoE KO controls (Fig. 2A, B). A similar degree of increase lesion area was also observed in of E/α KO mice compared to E/β KO or ApoE KO controls in analysis of aortic root sections (Fig. 2C, D).

We also analyzed a limited number of ApoE−/−LXRα−/−LXRβ−/− (TKO) mice. Due to the infertility of LXRβ−/− mice on a C57Bl6 background, TKO mice were exceedingly difficult to generate and most did not survive past 10 weeks of age. Although we were unable to obtain sufficient numbers to perform atherosclerotic lesion analysis, histological analysis of tissues from young TKO mice revealed a dramatic macrophage foam-cell accumulation phenotype. Lipid-laden cells with the histological appearance of macrophages were readily identified in the spleen, dermis, and liver of 8-week-old TKO mice on a chow diet (Figs. 3A and 4A). Staining for the macrophage marker F4/80 confirmed the identity of these cells as macrophages (Fig. 4B). Few macrophage foam cells were identified in either E/α KO or E/β KO mice at this age. Furthermore, it was clear that peripheral cholesterol overload phenotype of the TKO mice was much more severe than that of the E/α KO mice. These data strongly support the conclusion that combined loss of LXRα and LXRβ has synergistic effect on lipid accumulation. Collectively, phenotypic analysis of compound ApoE/LXR-deficient mice suggests that both LXRα and LXRβ contribute to reverse cholesterol on the ApoE−/− background, but that LXRα plays a quantitatively more important role that becomes increasingly apparent as the mice age.
Role of LXRα in atheroprotection of ApoE-deficient mice

We initially suspected that the increased susceptibility of E/α KO mice to atherosclerosis compared with E/β KO mice might reflect a specific role for LXRα in the macrophage. However, we observed no difference in acetylated LDL loading after 24 h between isolated E/α KO and E/β KO macrophages (Fig. 5A). TKO macrophages loaded more readily than either E/α KO or E/β KO cells, again confirming that both LXR isotypes contribute to macrophage cholesterol efflux. Accordingly, we found that sterol transport-related genes such as ABCA1, ABCG1, and Idol were

Fig. 2. LXRα-selective effect on atherogenesis in ApoE-null mice. (A, B) En face analysis quantified lesion coverage of the aorta. (C, D) Aortic root sections were stained with CD68 (red) and quantified. (n = 12–19 per group). Statistical significance was determined using one-way ANOVA.

Fig. 3. Synergistic effect of LXRα and LXRβ deletion on macrophage foam-cell formation in tissues. H and E stains of spleen (A) and skin (B) from 8-week-old female E KO, E/α KO, E/β KO and TKO mice.
Comparably expressed and responsive to LXR ligand (GW3965) in peritoneal macrophages derived from E/α KO and E/β KO mice (Fig. 5B). Similar results were obtained using naturally occurring oxysterol LXR agonists (supplementary Fig. I). In vitro analysis of LXR agonist-dependent cholesterol efflux revealed no difference in the efflux capacity between primary E/α KO and E/β KO peritoneal macrophages to either ApoA-I or HDL acceptors (Fig. 5C, D). Thus, the difference in lesion formation between E/α KO and E/β KO mice did not correlate with a differential ability of the isolated macrophages to efflux cholesterol.

An alternative explanation was that loss of a single LXR isotype might have preferential effect on macrophage inflammatory responses. For example, it has been suggested based on transfection assays that LXRβ may be a stronger repressor of inflammatory gene expression than LXRα (11). We therefore considered the possibility that loss of ApoE expression in RTC and suggest that this may be due to its control of hepatic ABCG5 and ABCG8 expression.

**DISCUSSION**

Dysregulation of cholesterol homeostasis has important consequences for cardiovascular disease risk. Numerous studies have established an integral role for the LXRαs in regulating cholesterol metabolism. Activation of LXRα induces the transcription of genes involved in cholesterol uptake, excretion, and transport in a tissue-selective manner. Many LXR-dependent pathways are potentially relevant to atherosclerosis, but the relative importance of
Role of LXR<sub>α</sub> in atheroprotection of ApoE-deficient mice

We hypothesized that this phenotype was most likely due to the fraction loss of LXR activity from the macrophage, given the well-documented importance of macrophage cholesterol efflux in atherogenesis. The observation that various processes, for example macrophage cholesterol efflux or hepatic sterol excretion, is unknown. In addition, the tissue-specific functions of the two LXR isotypes remain incompletely understood. Previous work by our laboratory revealed accelerated atherosclerosis and peripheral cholesterol overload in E/α KO mice (12). We hypothesized that this phenotype was most likely due to the fraction loss of LXR activity from the macrophage, given the well-documented importance of macrophage cholesterol efflux in atherogenesis.

**Fig. 5.** LXR<sub>α</sub> and LXR<sub>β</sub> exhibit comparable ability to control lipid metabolic gene expression and cholesterol efflux in macrophages. (A) Bone marrow-derived macrophages were loaded for 24 h with AcLDL and stained with DAPI (blue) and Bodipy (green). (B) Thioglycollate-elicited peritoneal macrophages from E KO, E/α KO, and E/β KO mice were treated for 24 h with 1 μM GW3965. Gene expression for known LXR targets, ABCA1, ABCG1, and IDOL was measured by real-time PCR. Thioglycollate-elicited peritoneal macrophages from E KO, E/α KO, and E/β KO mice were loaded with [3H]cholesterol (1.0 μCi/ml) in the presence of acyl-CoA:cholesterol O-acyltransferase inhibitor (2 μg/ml) either with DMSO or with ligand for LXR and RXR (1 μM GW3965, 50 nM LG268). Efflux was measured in the presence of (A) ApoA-I or (B) HDL. Experiments were conducted in triplicate. Data are expressed as mean ± SEM. DMSO versus GW+LG *P< 0.05, **P< 0.01; NS, non-significant.

**Fig. 6.** LXR<sub>α</sub> and LXR<sub>β</sub> exhibit comparable ability to control inflammatory gene expression in macrophages. Thioglycollate-elicited peritoneal macrophages from E KO, E/α KO and E/β KO mice were treated for 24 h with 1 μmol/L GW3965 then stimulate with LPS (100 nM) for 4 h. Gene expression of inflammatory genes was examined by real-time PCR.
the ability of an LXR agonist to inhibit lesion development was preserved in the absence of LXRα provided further support for the idea that both LXRα and LXRβ contribute to maximal macrophage cholesterol efflux (13). Here, we report the unexpected observation that loss of either LXRα or LXRβ alone has differential consequences for peripheral cholesterol overload on the ApoE-null background, pointing to a previously unappreciated quantitative importance of the LXRα-dependent gene expression in atherosclerosis susceptibility in mice.

Our characterization of E/α and E/β mice provides strong support for the idea that LXRα and LXRβ play largely redundant roles in macrophage cholesterol homeostasis and inflammatory gene expression. Both LXRs are fully competent to stimulate cholesterol efflux and to suppress inflammatory mediator production in response to natural and synthetic LXR ligands. Furthermore, loss of both LXRα and LXRβ on the ApoE-null background leads to profound and synergistic effects on peripheral cholesterol accumulation. TKO mice die at a young age and show massive foam-cell accumulation in numerous tissues. At the same time, the fact that E/α KO but not E/β KO mice show a less dramatic but significant cholesterol overload phenotype is inconsistent with our original hypothesis that the macrophage was solely responsible for this phenotype. Loss of either LXRα or LXRβ alone leads to minimal functional deficit in isolated macrophages, suggesting that the phenotype of E/α KO mice must involve LXRα-dependent pathways in another tissue or cell type.

The fact that macrophage cholesterol efflux was not different between E/α KO and E/β KO mice suggested that the process of RCT downstream of macrophage efflux to ApoA-I was compromised in E/α KO mice. Indeed, when wild-type cholesterol-loaded macrophages were used as the source of labeled cholesterol in in vivo RCT assays, we observed a defect in label appearance in the feces in E/α KO but not in E/β KO mice. Collectively, our data suggest that LXRα and LXRβ play redundant roles in macrophage cholesterol efflux, but that LXRα is uniquely important for maximal postmacrophage RCT.

Analysis of LXR-dependent gene expression in a variety of cell types suggested that the liver was the principal tissue in which LXRα has a dominant effect on LXR target gene expression.
responses. Whereas loss of either LXRα or LXRβ alone has minimal effect on target gene expression in macrophages and intestine, prominent deficits were observed in liver of LXRα-deficient mice. These observations are consistent with the early characterization of LXRα-deficient mice by Mangelsdorff and colleagues (8, 9). Although LXRαs are expressed in several cell types that contribute to peripheral cholesterol efflux and subsequent transport out of the body, including macrophages, hepatocytes, and intestinal epithelium, hepatocytes are the only cell type in which the two LXRαs are expressed at dramatically different levels.

Mechanistically, the phenotype of E/α correlates with a specific requirement for LXRα for optimal sterol-dependent ABCG5 and ABCG8 transporter expression in the liver. The liver and intestinal compartments are key areas where these transporters return cholesterol from the enterocytes to luminal cavity for excretion, and ABCG5/8 have previously been shown to be critical for LXR-dependent sterol transport in these tissues (20–24). Perturbations in ABCG5/8 have been linked to excess cholesterol accumulation in mice and humans (22, 25, 26). Interestingly, Lazar and colleagues have identified macrophage liver X receptors as inhibitors of atherosclerosis (Proc. Natl. Acad. Sci. USA. 99: 11880–11901).

Together these findings underscore the critical importance of LXR signaling for whole-body cholesterol homeostasis, as loss of both LXRαs in the ApoE background leads to early lethality. Our results further expand our understanding of the contributions of the different LXR isotypes to atherosclerosis susceptibility and their tissue-selective functions in sterol homeostasis.

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