Accumulation of Foam Cells in Liver X Receptor–Deficient Mice

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Background—The nature of some of the target genes for liver X receptors (LXRs)-α and -β, such as sterol regulatory element binding protein-1 and ATP-binding cassette transporter proteins, suggests a pivotal role of these nuclear receptors in the regulation of fatty acid and cholesterol homeostasis. The present study aimed to elucidate the physiological relevance of both LXRs with regard to lipid metabolism and macrophage cholesterol efflux.

Methods and Results—Mice depleted for LXRα, LXRβ, or both were fed low-fat rodent chow for 18 months before investigations. The combined deficiency of LXRα and LXRβ was linked to impaired triglyceride metabolism, increased LDL and reduced HDL cholesterol levels, and cholesterol accumulation in macrophages (foam cells) of the spleen, lung, and arterial wall.

Conclusions—Our data demonstrate the physiological importance of both LXRs in lipid metabolism and strongly indicate that both LXRs have a protective role against the development of atherosclerosis. (Circulation. 2002;106:1147-1153.)

Key Words: receptors, liver X α atherosclerosis β lipoproteins α fatty acids β cholesterol

During the past few years, the liver X receptors (LXRα and LXRβ), for which different oxysterols serve as ligands, have been recognized as important regulators of lipid and lipoprotein metabolism by identification of various target genes.

In vitro, both LXRα and LXRβ regulate the transcription of cholesterol 7α-hydroxylase (Cyp7a), the rate-limiting enzyme in bile acid biosynthesis. In response to dietary cholesterol, however, only LXRα−/− but not LXRβ−/− mice fail to upregulate the hepatic conversion of cholesterol into bile acids. This was associated with increased total cholesterol, LDL cholesterol, and 24- and 27-hydroxycholesterol levels in the serum.

By regulating the expression of the sterol regulatory element binding protein-1 (SREBP-1) and acetyl CoA decarboxylase, fatty acid synthase, and lipoprotein lipase, LXRs influence metabolism of fatty acids, triglycerides, and triglyceride-rich lipoproteins. In addition, LXRs promote the transcription of the cholesterol ester transfer protein and apolipoprotein E (apoE). Furthermore, in vitro studies have shown that LXRα and LXRβ regulate several ATP-binding cassette transporter proteins (ABCs). In the enterocytes, LXRs activate the transcription of ABCA1, ABCG5, and ABCG8 and thus regulate intestinal cholesterol absorption. In macrophages, LXRs affect the cholesterol efflux into HDLs, by regulating the expression of ABCA1 and ABCG1. As the formation of foam cells by cholesterol accumulation in arterial wall macrophages is a crucial first step in atherogenesis, an antiatherogenic effect of LXRs may be postulated.

The majority of data on the role of LXRs in cholesterol and triglyceride metabolism, however, has been generated either in vitro or under nonphysiological conditions, such as cholesterol challenge in mice. Neither LXRα−/− nor LXRβ−/− mice showed any morphological alterations or any disturbances in cholesterol metabolism when fed standard rodent chow. Thus, the physiological relevance of LXRs for lipid homeostasis and atherogenesis remains to be convincingly demonstrated in vivo. To establish whether LXRs play such a physiological role, mice depleted for LXRα, LXRβ, or both were fed a low-fat rodent chow and followed for 18 months. In particular, our investigation focused on cholesterol efflux from macrophages and on hepatic lipid and serum lipoprotein metabolism. For the first time, we were able to show that combined deficiency of LXRα and LXRβ leads to increased cholesterol accumulation in macrophages (foam cells) of spleen, lung, and arterial wall in aging animals fed a normal diet. This was associated with increased LDL and reduced HDL cholesterol, despite normal total cholesterol levels in the serum. Our data substantiate the view that LXRs have a protective role in atherogenesis.

Methods

Animals
LXRα−/−, LXRβ−/−, and wild-type mice were generated in our laboratory as described previously. LXRα−/−β−/− mice were ob-
tained by several mating steps, beginning with \( \text{LXR} \alpha^{\text{lop}} \) and cre-transgenic \( \text{LXR} \beta^{\text{lop}} \) founder animals. All mice used in our study (\( \text{LXR} \alpha^{+/-}, \text{LXR} \beta^{+/-}, \text{LXR} \alpha^{+-}\beta^{-/-} \) mice and wild-type controls) had a similar mixed genetic background based on 129/Sv and C57Bl/6 strains. Mice with pure Svl29 background were backcrossed in C57BL/6 mice for 2 generations, unless otherwise stated. Animals were housed with a regular 12-hour/12-hour light/dark cycle and fed a low-fat standard rodent chow diet (R36 Lactamin AB) ad libitum. Experiments were approved by the local ethics committee for animal experiments.

Chemical Analysis of Serum and Tissue

Blood was drawn by cardiac puncture under light methoxyflurane anesthesia before tissues were collected. Total cholesterol and triglycerides were determined individually using a Monarch automated analyser (ILS Laboratories Scandinavia AB). The kit for the determination of triglycerides detects free glycerol; therefore, the serum triglyceride value for each animal was corrected with regard to the respective glycerol content. Size-fractionation of lipoproteins was performed on 10 μL of pooled sera from each group, using a micro fast protein liquid chromatography column (FPLC; 30×0.32 cm Suprose 6B, Amersham Pharmacia Biotech AB) at a flow rate of 0.4 μL/min, as previously described. FPLC separation of sera from individual animals was performed to quantitate cholesterol and triglyceride content in the different lipoprotein fractions, calculated as described (EZ Chrom, Scientific Software Inc). Values were used to calculate the LDL/HDL ratio; individual data were expressed as a percentage of the mean of wild-type mice before statistical analysis.

Histology and Immunohistochemistry

Lungs, spleens, and livers were fixed in 4% formaldehyde solution, (Sigma) embedded in paraffin, and stained with hematoxylin and eosin. For cryosections, spleen, lung, liver, heart, and proximal aorta were snap-frozen in Tissue-Tek (Sakura Finetek Europe BV). Lipid deposits were visualized by staining with hematoxylin and oil red O on 10-μm cryosections as previously described. Macrophages were detected using the monoclonal antibody against CD11b (Mac-1; PharMingen), followed by biotinylated anti-mouse IgG, horseradish peroxidase–labeled avidin, and diaminobenzidine/H\(_2\)O\(_2\) (Vector Laboratory). Aortic lipid infiltration was quantified by measuring the oil red O–stained surface area inside the external elastic lamina (ie, intima and media) in cross-sections at 0-, 200-, and 400-μm distances from the aortic valves. Measurements were made with a computer program written in Quips language, by use of a Leica DMRB microscope operating at 200× magnification linked with a Leica Q500IW image analysis system.

Northern Blot Analysis

Northern blot analysis was performed on 2 to 5 μg hepatic poly (A+) RNA using \( ^3\text{P} \)-labeled cDNA probes as described. Signals on Northern blot filters were quantitated by use of a PhosphorImage analyzer (Fuji Sverige AB) and normalized according to GAPDH mRNA expression; data represent results from 4 to 9 independent determinations. cDNA probes were generated by reverse transcription–polymerase chain reaction from mouse liver as previously described or by following oligonucleotides: ABCA1, 5’-CTGATGAGGTTGGAGATAGC-3’ and 5’-AGCAGAGATGGAGGCAGATGG-3’.

Statistics

Data are presented as mean±SEM. The significance of differences between groups was tested by 1-way ANOVA, followed by post-hoc comparisons according to Dunnett’s tests, by planned comparisons or by Tukey’s honestly significant difference test (Statistica software, Stat Soft).

Results

Serum Lipids and Lipoprotein Pattern

Analysis of total cholesterol in serum showed no differences between wild-type controls, \( \text{LXR} \alpha^{+-}, \text{LXR} \beta^{+-}, \) or \( \text{LXR} \alpha^{+-}\beta^{-/-} \) mice (Figure 1A), in line with previously published data on 12- to 16-week-old LXR single-mutant mice fed standard rodent chow. In this study, however, pronounced differences in cholesterol content of the different lipoprotein classes were observed between mouse lines after separation by FPLC (Figure 1B). \( \text{LXR} \alpha^{+-} \) mice tended to have an increase in LDL (\( +46\%\pm25\%\); \( P=\text{NS} \)) and a decrease in HDL cholesterol (\( -17\%\pm7.4\%\); \( P=\text{NS} \)) compared with that of wild-type controls. No distinct changes were observed in \( \text{LXR} \beta^{-/-} \) mice. Young LXR single-mutant mice had no differences in lipoprotein distribution. In double-mutant mice, cholesterol content was increased by \( 83\%\pm16\%\); \( P<0.05 \) in LDL particles and tended to be decreased (\( 26\%\pm2.6\%\); \( P=\text{NS} \)) in HDL particles. These
changes in lipoprotein cholesterol distribution resulted in 86±45% (P<0.05) and 150±26% (P<0.001) increases of the LDL/HDL cholesterol ratio in LXRα−/− mice and double-mutant mice, respectively.

Compared with wild-type controls, serum triglycerides were reduced by 70% in double-mutant mice, whereas no significant differences were observed in LXRα−/− or LXRβ−/− mice (Figure 1C; P<0.001). VLDL triglycerides were reduced by 79±4.3% (P<0.01) and LDL triglycerides by 63±6.9% (P<0.01) in LXRα−/−β−/− mice (Figure 1D). No distinct changes were observed in LXRα−/− mice, whereas there was a trend toward a reduction of VLDL and LDL triglycerides in LXRβ−/− mice (−38±12%, P=NS; and −20±12%, P=NS; respectively).

The changes in lipid composition of lipoproteins prompted an analysis of the electrophoretic mobility of lipoproteins. No differences in mobility were observed among the groups (data not shown).

**Lipid Content in Tissues**

In the spleen, analysis of cholesterol content revealed no changes in LXRα−/− or LXRβ−/− mice. However, there was a 2-fold increase (P<0.001) in total cholesterol in the spleen from LXRα−/−β−/− mice (Figure 2A). Triglyceride content tended to decrease in spleens from LXRβ−/− and LXRα−/−β−/− mice (Figure 2A). No changes in triglyceride levels were observed in LXRα−/− spleens. In lungs, a 3-fold increase (P<0.001) in total cholesterol was observed in double-mutant mice (Figure 2B). No changes in LXRα−/− or LXRβ−/− were found with regard to cholesterol or triglyceride levels, but a 70% reduction (P<0.05) of triglycerides was observed in lungs from LXRα−/−β−/− mice (Figure 2B). In livers, an increase in cholesterol (3-fold; P<0.001) was found in LXRα−/−β−/− mice (Figure 2C) but not in 16-week-old LXRα−/− or LXRβ−/− mice. Furthermore, hepatic triglycerides dramatically decreased (by 90%) in double-mutant mice (Figure 2C; P<0.001). In LXRβ−/− mice, triglycerides were reduced by 80% (P<0.05), whereas only a tendency to a decrease was observed in LXRα−/− mice.

**Tissue Morphology**

Histological analysis revealed accumulation of foam cells in the spleens of LXRα−/−β−/− mice (Figure 3A). In contrast, foam cells were not seen in LXRα−/− or LXRβ−/− mice (Figure 3C and 3D). To characterize these cells further, cryosections were prepared and stained for lipids with oil red O (Figure 3E and 3F). Cells from LXRα−/−β−/− mice (Figure 3E) were filled with lipid droplets, and staining of parallel sections indicated that they expressed CD11b and were thus of macrophage origin (Figure 3G). Large foam cells and coalescent lipid accumulations were found in the center of the white pulp, whereas smaller foam cells were scattered throughout the red pulp.

Substantial foam cell accumulations were also observed in the lungs of LXRα−/−β−/− mice, particularly in the alveolar saccs, surrounded by normal epithelial cells (Figure 4A). Oil red O staining confirmed that foam cells were filled with coalescent lipid droplets (Figure 4B). The lungs from LXRα−/− and LXRβ−/− mice were normal (Figure 4C and 4D). None of the mice showed accumulation of foam cells in the livers, irrespective of their genotype (Figure 5). Oil red O staining of hepatic cryosections revealed a less pronounced intracellular lipid accumulation in LXRα−/−β−/− mice compared with wild-type controls (Figure 5E and 5F). Macrophages could not be detected in the livers from LXRα−/−β−/− mice as judged by CD11b staining of parallel sections (Figure 5G and 5H).

Cryosections of the aortic root of LXR knockout mice showed lipid accumulation after staining with oil red O (Figure 6). Occasional lipid deposits were also observed in the aortic root from mice deficient in either LXRα or LXRβ (Figure 6C and 6D), but the larger infiltrates were confined to mice deficient in both LXR (Figure 6A and 6E). Wild-type mice did not exhibit any aortic lipid accumulations (Figure 6B). In the aortas of LXRα−/−β−/− mice, lipids were present both extracellularly in the subendothelial region and in lipid-laden foam cells (Figure 6E). The latter cells were identified as CD11b+ by immunohistochemical staining of parallel sections (data not shown).

To quantitate lipid infiltration, the oil red O–stained area was measured in aortic cross-sections of LXRα−/−, LXRβ−/−, LXRα−/−β−/−, and wild-type mice (Table). The aortas in LXRα−/− and LXRβ−/− mice exhibited a 3.3-fold and a 5-fold increase, respectively, in lipid infiltrated area compared with those of wild-type mice (P=NS). In the absence of both LXR, the lipid content of the intima-media cross-sectional...
area was increased 27-fold (P<0.05; both versus wild-type and single-mutant mice).

**Hepatic mRNA Levels and Lipoprotein Receptor Expression**

We then examined the relative mRNA levels of several genes regulating cholesterol and lipid metabolism in the livers from LXR<i>α</i><sup>−/−</sup>, LXR<i>β</i><sup>−/−</sup>, LXR<i>α</i><sup>−/−</sup><i>β</i><sup>−/−</sup>, and wild-type mice (Figure 7). The mRNA levels of ABCA1 were significantly reduced in both LXR<i>β</i><sup>−/−</sup> and LXR<i>α</i><sup>−/−</sup><i>β</i><sup>−/−</sup> mice (to 42% and 26% of controls), whereas no significant reduction was observed for ABCG1. Furthermore, LDL receptor mRNA expression was increased 2-fold in LXR<i>α</i><sup>−/−</sup><i>β</i><sup>−/−</sup> mice, whereas SREBP-2 and apoE expression was unchanged. Expression of SREBP-1 increased 2-fold in LXR<i>α</i><sup>−/−</sup><i>β</i><sup>−/−</sup> mice but decreased to 35% in LXR<i>α</i><sup>−/−</sup><i>β</i><sup>−/−</sup> mice (Figure 7).

**Discussion**

Several new and important conclusions can be drawn from the present study. For the first time, we show that combined deficiency of both LXRs results in foam cell accumulation in several organs, including the aorta. Interestingly, these changes were observed in animals on a normal diet, displaying distinct abnormalities of circulating lipoproteins. In general, mice are resistant to atherosclerosis, unless they are challenged with a specific high-fat diet<sup>9,10</sup> or carry deletions of key genes in cholesterol metabolism, such as apoE or the LDL receptor, leading to drastic elevations of cholesterol-rich plasma lipoproteins.<sup>10</sup> Despite the fact that the animals were followed for 18 months, the lesions in the aorta of LXR<i>α</i><sup>−/−</sup><i>β</i><sup>−/−</sup> mice only consisted of foam cell accumulation and did not progress into raised lesions or advanced atheromas. Therefore, LXRs are important for macrophage cholesterol efflux, but defects in the macrophages or elsewhere based on the deletion of both LXRs do not cause advanced atherosclerosis.
genes for the LXRs are ABCA1, ABCG1, and apoE. The ABC proteins have recently been shown to be important for apoAI-mediated phospholipid and cholesterol efflux from different cell types, particularly macrophages. LXR s regulate the transcriptional activity of the ABCA1 and ABCG1 genes and thus promote the oxysterol-induced cholesterol efflux to HDL. Similar to what is observed for the ABCs, LXRs increase the basal gene expression of apoE in macrophages in response to oxysterols. These observations lead to the hypothesis that formation of cholesterol-loaded macrophages owing to LXR deficiency results from the inability to upregulate the ABC- and apoE-mediated cholesterol efflux from macrophages, the initial step of reverse cholesterol transport. An increased 27-hydroxysterol/cholesterol ratio is also present in LXRαβ mice (G.U. Schuster, unpublished observation, 2002), probably in line with a higher availability of substrate for sterol 27 hydroxylase, secondary to the impaired cholesterol efflux from macrophages. In LXRαβ mice, foam cells accumulate in the spleen, lungs, and aorta. Except for the aorta, a similar phenotype is also seen in ABCA1 mice, an animal model resembling the human disorder Tangier disease, which is characterized by low HDL cholesterol levels and cholesterol loading of reticuloendothelial cells. Although data on the degree of foam cell accumulation in ABCAI mice are somewhat conflicting, LXRαβ mice seem to display more foam cells in the peripheral organs compared with those in ABCAI mice. This would be in concert with the view that LXR has a more general and thus more important role than ABCA1 in determining the degree of cholesterol accumulation in macrophages.

Foam cells were not observed in livers from LXRαβ mice, but a significant increase in cholesterol accumulation occurred within the hepatocytes. It is of interest to note that the expression of ABCA1 was reduced significantly in the livers of these animals. Recently, it has been shown that ABCAI mice have an enhanced cholesterol synthesis in peripheral organs, like spleen and adrenals, but not in the liver. Thus, disruption of both LXRs does not result in an identical phenotype to that observed in ABCAI mice. Surprisingly, LXRαβ mice, in contrast to ABCAI mice, had only minor reduction in HDL cholesterol. Hence, it
seems that in $\text{LXR}^{-/-}\beta^{-/-}$ mice, HDL synthesis is not dependent on ABCA1 gene expression in the liver.

In addition to the expected finding of increased cholesterol content in the spleen, liver, and lungs of LXR double-mutant mice, triglycerides were drastically reduced in these tissues, combined with low serum triglycerides. These findings could be explained by the regulation of expression of SREBP-1, acetyl CoA carboxylase, and fatty acid synthase by LXR in the liver. Indeed, an LXR-dependent increase in triglyceride levels in vivo has recently been described. The observed 2-fold induction of hepatic SREBP-1 expression, together with reduced liver triglyceride levels, in $\text{LXR}^{-/-}$ mice suggests that in vivo, LXR$\beta$ might regulate triglyceride metabolism differently from LXR$\alpha$ and independently of SREBP-1. In association with the subtle decrease in HDL cholesterol, analysis of lipoproteins revealed a significant increase in LDL cholesterol in $\text{LXR}^{-/-}\beta^{-/-}$ mice. Such a pattern, although of a greater magnitude, has also been described in combined LDL-receptor$^{-/-}$ and APOBEC-1$^{-/-}$ mice. However, in that mouse model, serum triglyceride levels were increased 2-fold, whereas a 70% reduction in serum triglycerides was observed in $\text{LXR}^{-/-}\beta^{-/-}$ mice, presumably owing to an impaired fatty acid and triglyceride metabolism. Triglycerides were decreased in the fractions corresponding to VLDL and LDL particles in $\text{LXR}^{-/-}\beta^{-/-}$ mice. Low triglyceride levels in lipoproteins of VLDL/LDL size are also seen in apoE-deficient mice. In such animals, however, these lipoproteins mainly consist of chylomicron remnants, whereas in the LXR double-mutant mice there was no accumulation of remnants. Another clear difference between apoE$^{-/-}$ mice and LXR double-mutant mice is in hepatic triglyceride levels. ApoE$^{-/-}$ mice accumulate triglycerides in the liver, whereas LXR double-mutant mice had lower hepatic triglycerides. Disruption of the apoE gene results in a clear reduction of VLDL secretion. Similar to what is observed in macrophages from LXR double-mutant mice, the basic expression of apoE in the liver was not altered. Considering triglyceride availability as a rate-limiting

**Morphometric Analysis of Aortic Lipid Infiltration in LXR-Deficient Mice**

<table>
<thead>
<tr>
<th>Group</th>
<th>Oil Red O-Stained Area, % of Lesion Cross-Section Area</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>0.72 ± 0.22</td>
</tr>
<tr>
<td>$\text{LXR}^{-/-}$</td>
<td>2.25 ± 0.52*</td>
</tr>
<tr>
<td>$\text{LXRB}^{-/-}$</td>
<td>3.57 ± 0.63*</td>
</tr>
<tr>
<td>$\text{LXR}^{-/-}\beta^{-/-}$</td>
<td>17.46 ± 1.88†</td>
</tr>
</tbody>
</table>

Data show oil red O-stained area (μm$^2$) ×100/cross-section area of aorta (μm$^2$); mean ± SEM (n = 4). Mice used for these determinations were backcrossed for 3 generations into C57BL/6. No differences in serum lipids were observed in mice, backcrossed for 2 or 3 generations into C57BL/6.

*P<0.001 vs $\text{LXR}^{-/-}\beta^{-/-}$ mice.
†P<0.001 vs wild-type mice.
factor for hepatic VLDL production, we hypothesize that VLDL secretion from the liver is reduced in LXR double-mutant mice, independently of apoE.22

In conclusion, we have shown that disruption of LXRα and LXRβ leads to foam cell formation in ageing mice, even on a normal diet, and to impaired triglyceride metabolism. We hypothesize that the cholesterol accumulation in macrophages is owing to the combination of an enhanced cholesterol deposition—secondary to increased LDL cholesterol—and a failure to adequately increase the ABC- and apoE-mediated cholesterol efflux from these cells. The LXR double-knockout mouse should provide a useful animal model to study the early interaction between lipoproteins and the arterial wall.

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