Liver X Receptor Activation Promotes Polyunsaturated Fatty Acid Synthesis in Macrophages
Relevance in the Context of Atherosclerosis

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Objective—Liver X receptors (LXRs) modulate cholesterol and fatty acid homeostasis as well as inflammation. This study aims to decipher the role of LXRs in the regulation of polyunsaturated fatty acid (PUFA) synthesis in macrophages in the context of atherosclerosis.

Approach and Results—Transcriptomic analysis in human monocytes and macrophages was used to identify putative LXR target genes among enzymes involved in PUFA biosynthesis. In parallel, the consequences of LXR activation or LXR invalidation on PUFA synthesis and distribution were determined. Finally, we investigated the impact of LXR activation on PUFA metabolism in vivo in apolipoprotein E–deficient mice. mRNA levels of acyl-CoA synthase long-chain family member 3, fatty acid desaturases 1 and 2, and fatty acid elongase 5 were significantly increased in human macrophages after LXR agonist treatment, involving both direct and sterol responsive element binding protein-1–dependent mechanisms. Subsequently, pharmacological LXR agonist increased long chain PUFA synthesis and enhanced arachidonic acid content in the phospholipids of human macrophages. Increased fatty acid desaturases 1 and 2 and acyl-CoA synthase long-chain family member 3 mRNA levels as well as increased arachidonic acid to linoleic acid and docosahexaenoic acid to eicosapentaenoic acid ratios were also found in atheroma plaque and peritoneal foam cells from LXR agonist–treated mice. By contrast, murine LXR-deficient macrophages displayed reduced expression of fatty acid elongase 5, acyl-CoA synthase long-chain family member 3 and fatty acid desaturases 1, as well as decreased cellular levels of docosahexaenoic acid and arachidonic acid.

Conclusions—Our results indicate that LXR activation triggers PUFA synthesis in macrophages, which results in significant alterations in the macrophage lipid composition. Moreover, we demonstrate here that LXR agonist treatment modulates PUFA metabolism in atherosclerotic arteries. (Arterioscler Thromb Vasc Biol. 2015;35:1357-1365. DOI: 10.1161/ATVBAHA.115.305539.)

Key Words: arachidonic acid ■ liver X receptor ■ macrophages ■ n-3 polyunsaturated fatty acid

Liver X receptors (LXRs) α and β (NR1H3 and NR1H2, respectively) are nuclear receptors activated by oxidized derivatives of cholesterol and by intermediates of the cholesterol synthesis pathway.1,2 LXRs are involved in the regulation of cholesterol homeostasis, in the control of inflammation and the innate immune response.3,4 Notably, LXR activation stimulates the reverse cholesterol transport pathway through the coordinated activation of cellular cholesterol efflux, plasma cholesterol transport, and finally hepatic and intestinal cholesterol excretion. Besides regulating cholesterol homeostasis, LXRs also play an important role in fatty acid metabolism because several genes of the fatty acid biosynthesis pathway such as acetyl-CoA carboxylase, fatty acid synthase (FASN), and steroyl-CoA desaturase (SCD1) have been shown as direct LXR targets.5-8 Key lipogenic transcription factors including the sterol responsive element binding protein 1c (SREBP1), carbohydrate-response element-binding protein, and peroxisome proliferator activated receptor γ are also directly regulated by LXR.9-11 Accordingly, pharmacological activation of LXRs results in a marked stimulation of lipogenesis in vivo, particularly in the liver. As a consequence, the administration of a synthetic LXR agonist such as T0901317 in mice induces...
Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AA</td>
<td>arachidonic acid</td>
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<tr>
<td>ApoE−/− mice</td>
<td>apolipoprotein E−deficient mice</td>
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<tr>
<td>ACSL</td>
<td>acyl-CoA synthase long-chain family member 3</td>
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<tr>
<td>DHA</td>
<td>docosahexaenoic acid</td>
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<td>ELOVL5</td>
<td>fatty acid elongase 5</td>
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<tr>
<td>EPA</td>
<td>eicosapentaenoic acid</td>
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<td>FADS</td>
<td>fatty acid desaturase</td>
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<td>FASN</td>
<td>fatty acid synthase</td>
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<td>LA</td>
<td>linoleic acid</td>
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<tr>
<td>LPCAT3</td>
<td>lysophosphatidylcholine acyl transferase 3</td>
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<tr>
<td>LXR</td>
<td>liver X receptor</td>
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<tr>
<td>MUFA</td>
<td>monounsaturated fatty acid</td>
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<tr>
<td>NCoR</td>
<td>nuclear receptor corepressor</td>
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<tr>
<td>PUFAs</td>
<td>polyunsaturated fatty acids</td>
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<tr>
<td>SCD</td>
<td>sterol-CoA desaturase</td>
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<td>SREBP1</td>
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a massive increase in the synthesis of fatty acids and triglycerides in the liver, resulting in hepatomegaly and steatosis as well as increased plasma triglyceride levels.5,12 This hepatic steatosis, driven mainly by the LXRα isoform, is a major undesirable side effect which has to date limited the therapeutic development of synthetic LXR agonists.13 Although the impact on the liver is the most dramatic side effect, LXR activation also induces lipogenesis and triglyceride accumulation in other cells/tissues such as the pancreas and skeletal muscle cells.14,15 In adipocytes, the role of LXRs in controlling lipogenesis seems to be more complex with recent studies suggesting that LXRs regulate fatty acid synthesis in subcutaneous and visceral adipose tissues in different ways.16 At this stage, the impact of LXRs on lipogenesis in macrophages has been less documented. Nevertheless, LXR activation in macrophages is known to increase the expression of lipogenic genes such as SREBP1 and SCDS.17,18

From a qualitative standpoint, LXR activation has been reported to induce the preferential formation of C16 and C18 monounsaturated fatty acids (MUFAs) belonging to the n-7 or n-9 families, resulting in an increased MUFA/saturated fatty acid ratio in the liver. This LXR-driven synthesis of MUFAs may be important to prevent the lipotoxicity induced by the intracellular accumulation of fatty acids.19 Interestingly, some of the fatty acids whose synthesis is stimulated by LXRs such as 9Z-palmitoleic acid possess specific insulin-sensitizing and anti-inflammatory properties.2,20 In contrast to MUFAs, much less is known about the role of LXRs on long chain polyunsaturated fatty acid (PUFA) metabolism. Although it has been demonstrated that some PUFAs such as arachidonic acid (AA; C20:4 n-6) or eicosapentaenoic acid (EPA; C20:5 n-3) act as LXR antagonists,21,22 it is unknown whether LXRs are reciprocally able to interfere with PUFA metabolism and to modulate the synthesis of these molecules from their C18 precursors. Nevertheless, there is some evidence in support of such roles for LXRs. Indeed, it has been shown that LXR agonists increased AA levels in vitro in hepatoma cells.23 A study from our group showed that LXR could increase the total amount of AA in polar lipids in human macrophages.24 Finally, it has been demonstrated that nuclear receptor corepressor (NCoR) deficiency in mice results in the derepression of long chain o-3 fatty acid synthesis probably through a LXR-dependent mechanism.25 The aim of this present work was therefore to investigate whether LXR activation could directly affect PUFA synthesis in macrophages in vitro and in vivo. Primary macrophages were treated with LXR agonists and mRNA levels of genes involved in PUFA synthesis were evaluated in parallel to fatty acid levels and their distribution.

Materials and Methods

Materials and Methods are available in the online-only Data Supplement.

Results

Activation of LXRs Results in the Induction of Genes Involved in PUFA Synthesis in Human Macrophages

Transcriptomic analysis was performed in human primary monocytes from 2 healthy donors treated or not with the synthetic LXR agonist T0901317 at 10 µmol/L for 48 hours. As expected, LXR activation resulted in the marked induction of well-established LXR target genes involved in saturated fatty acid and MUFA synthesis such as acetyl-CoA carboxylase, FASN, and SCD1. LXR activation also induced the master transcriptional regulator of lipogenesis, SREBP1 (Figure 1A). Interestingly, PUFA synthesis was also affected as depicted by the significant induction of Δ5 and Δ6 desaturases (fatty acid desaturase 1 [FADS1] and FADS2, respectively) after LXR agonist treatment as well as the induction of acyl-CoA synthase long-chain family member 3 (ACSL3) and the fatty acid elongase 5 (ELOVL5). The acyl-CoA synthase long-chain family member 4 (ACSL4) was significantly induced in 1 of the 2 donors.

Microarray data were further supported by real-time polymerase chain reaction analysis in primary human macrophages. As shown in Figure 1B, treatment with T0901317 at 1 µmol/L for 24 hours resulted in a marked induction of FADS1 and FADS2 mRNA levels (+30-fold increases; P<0.05) as compared with dimethyl sulfoxide controls. ELOVL5 and ACSL3 were also significantly induced by LXR agonist treatment (10- and 5-fold increases, respectively; P<0.05). In contrast, no significant induction of ACSL4 was observed (data not shown). Similar results were obtained with macrophages from distinct healthy donors. Treatment with different concentrations of T0901317 induced a dose-dependent increase of mRNA levels for all the genes with a maximal induction at 10 µmol/L (Figure 1 in the online-only Data Supplement). In order to determine whether the T0901317-mediated induction of these genes was mediated by a specific LXR isoform, differentiated human macrophages
LXRs Regulate Genes Involved in PUFA Synthesis Through Both Direct and SREBP1-Dependent Manner

LXRs are known to regulate genes involved in fatty acid synthesis through both direct and indirect mechanisms via the activation of the lipogenic transcription factor SREBP1. In order to evaluate the possible contribution of SREBP1, differentiated human macrophages were transfected with negative or SREBP1 small interfering RNAs and, 48 hours later, were treated with T0901317 at 1 μmol/L or dimethyl sulfoxide for an additional 24-hour period. SREBP1 knockdown significantly reduced mRNA levels of all the target genes under T0901317 conditions indicating that SREBP1 contributed in part to the LXR-mediated induction of FADS1, FADS2, ACSL3, and ELOVL5 (Figure 2A).

In a further step, we took advantage of the unique properties of desmosterol, which is a physiological LXR agonist as well as an inhibitor of SREBP1 proteolytic cleavage and its subsequent nuclear processing.26 As shown in Figure 2B, desmosterol treatment strongly inhibited FADS1 and FADS2 expression and tended to increase ELOVL5 and ACSL3 mRNA levels. As expected, we observed that desmosterol treatment significantly increased mRNA levels of ABCA1 or lysophosphatidylcholine acyl transferase 3 (LPCAT3), which are known to be direct LXR target genes (Figure 2B and Figure III in the online-only Data Supplement). Interestingly, desmosterol treatment also increased SCD1 but not FASN mRNA levels.

To further delineate the underlying mechanism with regards to the desmosterol-dependent inhibition of FADS1/FADS2, we treated vehicle-treated or LXR agonist–treated human primary macrophages along with the translation inhibitor cycloheximide. Cycloheximide treatment totally abolished the LXR response of FADS1 and FADS2 (Figure IV in the online-only Data Supplement). The picture was different with ACSL3 and ELOVL5 as induction of their expression was maintained in T0901317-treated macrophages in the presence of cycloheximide. These data therefore suggest that FADS1 and FADS2 are mainly regulated through the LXR-SREBP1 cascade, whereas ACSL3 and ELOVL5 could also be regulated directly by LXRs. A functional LXRE has been previously reported in the ACSL3 promoter.27 Interestingly, a recent Chip-sequencing survey performed in human macrophages showed a potential LXRE binding site in ELOVL5 gene ≈10000 bp upstream from the transcription start site.28 To address the functionality of this potential LXRE in the ELOVL5 gene, the corresponding element (DR4 element: GGGTAAattccCGGGCA) was cloned upstream of a thymidine kinase promoter-luciferase vector or added to the proximal ELOVL5 promoter (−500 bp) cloned into a pGL3 luciferase reporter vector. In both cases the activity of the promoters was increased when cotransfected with LXRXα and RXRXα expression vectors and treated with T0901317 (Figure 2C). These inductions were abolished by mutation of the LXRx element (Figure 2C).

LXR Activation Increases Arachidonate Levels in Polar Lipids in Primary Human Macrophages

To determine whether the changes observed at the transcriptional level translated into modifications of the fatty acid profile, total fatty acid composition of human macrophages were transfected with negative, LXRXα or LXRXβ small interfering RNAs for 24 hours prior being treated with T0901317 at 1 μmol/L or dimethyl sulfoxide for an additional 24-hour period. LXRXα knockdown significantly reduced ELOVL5 and ACSL3 mRNA levels under the T0901317 treatment conditions, whereas a nonstatistically significant reduction was observed for FADS1 and FADS2. LXRXβ silencing did not affect the expression of target genes whether macrophages were treated or not with T0901317. Conversely, LXRXα/β silencing significantly reduced ELOVL5, ACSL3, and FADS2 mRNA levels in the T0901317 treated cells only (Figure II in the online-only Data Supplement).
treated or not with LXR agonist was assessed by gas chromatography-mass spectrometry. As shown in Figure 3A and in accordance with the induction of FASN, acetyl-CoA carboxylase, and SCD1, LXR activation induced significant increases in the net amount and relative proportion of C16 and C18 MUFAs. Interestingly, an increase in the total amount of AA was also observed. Further analysis of the fatty acid composition of neutral lipids (triglycerides and cholesteryl esters) and polar lipids (glycerophospholipids and sphingolipids) revealed that the increased AA content primarily affected the polar lipid fraction (Figure 3B and 3C).

LXR Deficiency Alters PUFA Metabolism in Mouse Macrophages

To assess the intrinsic function of LXRs in the regulation of PUFA metabolism in the absence of pharmacological activation, bone marrow–derived macrophages were prepared from WT or Lxrαβ-deficient (Lxrαβ−/−) mice. As shown in Figure 4, Lxrαβ−/− macrophages displayed a moderate but significant phenotype including decrease mRNA levels of...
enzymes (Elovl5, Acsl3, and Fads1) involved PUFA metabolism (Figure 4A), significant decrease of AA and docosahexaenoic acid (DHA) content (Figure 4B) along with a significant reduction in the proportion of PUFA in phosphatidylcholines (Figure 4C). As previously reported, we observed here that LXR deficiency could also derepressed some target genes such as Abca1 or Abcg1 (Figure VI in the online-only Data Supplement). Interestingly a similar derepression was also retrieved for genes involved in fatty acid metabolism such as Scd2 (Figure VI in the online-only Data Supplement).

**LXR Activation Promotes the Synthesis of n-6 and n-3 PUFAs From C18 Substrates**

To determine whether the increased AA levels were because of increased uptake, decreased catabolism, or activation of synthesis, we directly measured the synthesis of AA in human macrophages at the basal state or after stimulation by LXR agonists (T0901317 or desmosterol). Primary macrophages were pretreated for 24 hours with T0901317 at 1 μmol/L or desmosterol at 10 μmol/L and then incubated for 24 hours in the presence of a tracer dose of deuterated linoleic acid (LA; C18:2 n-6) to measure its conversion into deuterated AA. The conversion rate was low in nonstimulated macrophages as deuterated AA represented <5% of the total deuterated molecules (Figure 5A). In contrast, the conversion was dramatically higher in LXR agonist–treated macrophages, with deuterated AA levels corresponding to ~25% of the total deuterated molecules. In accordance with the previous transcriptomic data, treatment with desmosterol induced no stimulation and even tended to decrease the biosynthesis of AA.

Because the enzymatic steps for the synthesis of long-chain n-3 and n-6 PUFAs are identical, we repeated the experiment using deuterated C18-3 n-3 as the substrate. As shown in Figure 5B, LXR agonist treatment induced the formation of n-3 long chain PUFAs, notably C20:4 n-3 and docosapentaenoic acid (C22:5 n-3). We could not detect the formation of DHA which requires a specific pathway for its synthesis.

**LXR Activates PUFA Synthesis In Vivo in Atherosclerotic Arteries and Foam Cells**

To address the pathophysiological relevance of our in vitro observations, we studied the impact of LXR agonists on the synthesis of PUFAs in atheroma plaque in vivo. To this end,
ApoE−/− mice, treated or not with an LXR agonist (T0901317), were fed a Western type diet for 8 weeks. This well-described approach leads to the development of atheroma in the aorta and aortic valves along with the development of massive lesions in the brachiocephalic and in the left common carotid arteries. Moreover, LXR agonists are known to exert atheroprotective effects in this model. Left common carotid and brachiocephalic arteries were harvested and cleaned under a dissecting microscope and used for transcriptomic and fatty acid analysis, respectively. Analysis of the total fatty acid profile revealed an increase in the proportion of MUFAs including 9Z-palmtoleic acid (C16:1 n-7). A similar, although not statistically significant, trend was also observed for several PUFAs such as 22:6 n-3, C22:4 n-6, and C20:4 n-6 (Figure 6A). The AA:LA ratio, which is considered a reliable marker of desaturase activities and AA biosynthesis, was significantly increased in LXR-treated mice. About n-3 fatty acids, the C18 precursor α-linolenic acid was barely detectable in the arteries but the DHA/EPA ratio was significantly higher in LXR agonist–treated mice suggesting an increased conversion of EPA to DHA.

About the enzymes involved in PUFA biosynthesis, significant inductions of Fads1, Fads2, and Acscl3 mRNA levels were observed in the arteries from T0901317-treated mice (Figure 6B). However, in contrast to our in vitro observations in human macrophages, we did not find any significant increase in Elov15 mRNA levels in LXR agonist–treated mice. Genes involved in MUFAs synthesis such as Srebpl, Fasn, Scd1, and Scd2 were also significantly induced (Figure VII in the online-only Data Supplement). Interestingly, mRNA levels of several proinflammatory genes such as Il1β and Cox2 were decreased in the arteries of T091317–treated mice (Figure VII in the online-only Data Supplement). Because atherosclerotic plaque composition is complex, dynamic and involves various cell types, we aimed to confirm our results in a more homogeneous population of macrophage–foam cells. The protocol initially described by Li et al was used to generate peritoneal foam cells in vivo from ApoE−/− mice. Similar results to atheroma plaque were obtained, including significant induction of Fads1, Fads2, Acscl3 but not Elov15 mRNA levels after LXR stimulation (Figure VIII in the online-only Data Supplement). Increased AA/LA and DHA/EPA ratios and increased DHA content were also retrieved (Figure VIII in the online-only Data Supplement). In contrast to our in vitro experiments, we did not retrieve an enrichment of phosphatidylincholines and phosphatidylethanolamines in AA. This discrepancy might be related to the activated state of the cells because of thioglycolate stimulation. Nevertheless, an enrichment of phosphatidylethanolamines as well as cholesteryl esters with DHAs was observed after LXR agonist treatment (Figure IX in the online-only Data Supplement). No differences in the hydroperoxy-eicosatetraenoic acids were observed (Figure IX in the online-only Data Supplement). Liver samples from LXR agonist–treated ApoE−/− mice were also collected to have an overall picture on the impact of LXR agonist treatment on body lipid metabolism. As expected, LXR treatment resulted in liver steatosis and in the induction of LXR target genes such as Scd1 and Srebpl (Figure X in the online-only Data Supplement). Surprisingly, the genes involved in PUFA metabolism were not induced or even slightly inhibited in the liver of LXR-agonist treated mice (Figure X in the online-only Data Supplement). Although there was an increase in the total amounts of AA and DHA in the liver of LXR–treated mice, AA/LA and DHA/EPA ratios were unchanged.

**Discussion**

In the present study, we demonstrated that activation of LXRs in macrophages induced the expression of all key enzymes of the PUFA synthesis in a coordinated manner. Consistent with these transcriptomic data, we found an increase in the rate of PUFA synthesis from either n-6 or n-3 precursors as well as the enrichment of phospholipids with AA. Reciprocally, LXR deficiency in mouse macrophages resulted in decrease expression of main enzymes involved in PUFA metabolism, as well as a reduced AA and DHA content. Noteworthy, in vitro data were further confirmed in vivo in mouse in atherosclerotic arteries and foam cells as we showed that LXR activation...
modulates in situ the PUFA composition and increases the expression of genes that control PUFA synthesis. Synthesis of PUFA is a multistep process requiring the initial activation of the substrate fatty acid into an acyl-CoA by an acyl-CoA synthase and the successive actions of elongases and desaturases (Figure 7). Interestingly, LXR activation in human macrophages resulted in the coordinated induction of these 3 classes of enzymes. Although there are at least 5 long chain fatty acyl CoA synthases, they display differential tissue distribution and specificity toward fatty acids. Interestingly, ACSL3 exhibits a preference for C18-C20 PUFAs over saturated and monounsaturated fatty acids. The regulation of ACSL3 by LXR has been described in placental trophoblast cells but to our knowledge no data on ACSL3 regulation by LXR in macrophages has been reported to date. As for ACSLs, several fatty acid elongases are present in mammals with different specificities. Data obtained in Elovl5−/− mice indicate that ELOVL5 is required for the synthesis of long chain n-3 and n-6 PUFA since Elovl5−/− mice accumulate n-6 and n-3 C18 precursors in their tissues at the expense of the downstream products AA and DHA. Indirect regulation of ELOVL5 by LXRα through a SREBP1-dependent pathway has been reported in mouse liver. We showed here that ELOVL5 is also a direct LXR target gene in human macrophages and that a potential LXR-MRE identified previously by Chip-sequencing is fully functional. In contrast to ACSLs and elongases, no redundancy exists for the Δ5 and Δ6 desaturases (FADS1 and FADS2), which are unique in the human genome and are absolutely required for PUFA synthesis. Moreover, Δ6 desaturation has been described as the limiting step for long chain PUFA synthesis. It has been demonstrated that FADS1 and FADS2 are regulated by peroxisome proliferator activated receptors and by SREBP1. In accordance with these data, the results of the present study strongly suggest that the T0901317-mediated induction of FADS1 and FADS2 at least in human macrophages is fully dependent on SREBP1 because desmosterol, an LXR agonist, but also an inhibitor of the SREBP1 cleavage, strongly repressed FADS1 and FADS2 expression in vitro.

Although the role of LXRs in MUFAs synthesis is well established, there are only few data on the impact of LXRs in PUFA metabolism. Nevertheless, some interactions between PUFA- and LXR-regulated pathways have been suggested. Indeed, several long chain PUFAs such as AA, EPA, and DHA are known to be potent LXR antagonists and inhibitors of SREBP1 transcription. This mechanism probably accounts for the ability of unsaturated fatty acid to inhibit fatty acid and triglyceride synthesis. Reciprocally, it has been recently demonstrated that LXRs modulate PUFA distribution in macrophages and liver through the induction of LPCAT3. In human primary macrophages, LPCAT3 preferentially promoted the incorporation of AA at the sn-2 position of lysophosphatidylcholines resulting in an enrichment of phosphatidylcholines with AA after LXR agonist treatment. Interestingly, when LPCAT3 was inhibited, LXR agonists triggered an increase in AA levels in the neutral lipid fraction, suggesting an increased availability of AA in the cells that was independent of LPCAT3. Recently, Li et al. reported that specific deletion of NCoR in mouse macrophages resulted in the derepression of several enzymes involved in PUFA synthesis such as ELOVL5 and FADS2 in a LXR-dependent manner. This was associated with increased levels of n-3 long chain PUFAs such as EPA and DHA. Although most of our data are in accordance with this study, it is worth noting some important differences. First, Li et al did not activate LXR directly with specific agonists, but they observed a derepression of some LXR target genes after NCoR deletion in macrophages. Second, the levels of long chain n-6 PUFA such as AA were not assessed in this study; however, because the same enzymes are required for long chain n-3 and n-6 PUFA synthesis, it would be of high interest to investigate whether NCoR deficiency in mouse macrophage also increases the level of AA in parallel to DHA and EPA.

Although it is reported here that the activation of PUFA synthesis by LXR agonists involved the direct regulation of some target genes, it was also highly dependent on the activation of SREBP1, an indirect pathway that is particularly relevant for FADS1 and FADS2. Thus, desmosterol, an inhibitor of SREBP1-cleavage, repressed the expression of both desaturases and does not stimulate the synthesis of AA. Importantly, this did not occur with the synthetic LXR agonist T0901317, thus highlighting a major difference in the biological activity of nonsteroidal versus steroidal LXR agonists. In contrast, desmosterol
ApoE and in the development of atherosclerosis.40,41 Because LXRs to be involved in the regulation of the inflammatory response lar wall.42,43

Finally, LXR regulation of PUFA metabolism in macrophages is likely to have significant biological consequences because these molecules and their oxygenated derivatives are potent biological mediators with both pro- and anti-inflammatory properties. We previously observed that the LXR-mediated induction of LPCAT3 primed human macrophages for subsequent eicosanoid secretion by increasing the pool of AA in the phospholipid fraction. In contrast, derepression of n-3 PUFA synthesis by NCoR deficiency in macrophages resulted in an anti-inflammatory, insulin-sensitive phenotype in mice.29 We observed in the present study that LXR agonist treatment of ApoE−/− mice led to significant changes of the PUFA profile in atherosclerotic arteries with increases in both the AA/LA and the DHA/EPA ratio and was associated with the decreased expression of proinflammatory genes such as Cox2 and Il1β. These data therefore suggest that local production of PUFA and derived lipid mediators triggered by LXR within the atheroma plaque could affect inflammation and atherosclerosis development, a hypothesis that deserves further investigation. Nevertheless, the activation of specific targets in macrophages within the atheroma plaque is essential for the atheroprotective effect of LXR agonists.38,39 Furthermore, PUFA and their oxidized derivatives are known to be involved in the regulation of the inflammatory response and in the development of atherosclerosis.40,41 Because LXRs are able to promote the synthesis of long chain PUFA from both n-3 and n-6 substrates, their overall impact is likely to be dependent on the quality and the relative abundance of the initial n-6 or n-3 substrates. Interestingly, although some interspecies differences were previously reported on LXR target genes, PUFA metabolism seems to be regulated in a similar way by LXR in humans and mice. Nevertheless, it is worth to note that DHA synthesis, as compared with AA, seems to be preferentially regulated by LXR in mice, an effect we did not retrieve in vitro in human macrophages. Finally, our data bring some support to the hypothesis that the antithromogenic activity of LXR agonists could be directly related to their lipogenic activity in the vascular wall.42,43

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Disclosures
None.

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LXRs Promote PUFA Synthesis in Macrophages

Significance

Liver X receptors are oxysterol-activated nuclear receptors that control different pathways such as cholesterol and fatty acid metabolism or inflammation. We demonstrate that in macrophages, liver X receptors coordinately regulate the enzymes responsible for the synthesis of long chain polyunsaturated fatty acids, including activation into acylCoA, elongation, and formation of additional double bonds. As a consequence, liver X receptor activation increases the amounts of n-3 and n-6 long chain fatty acid such as docosahexaenoic acid and arachidonic acid in macrophages. Importantly, we observed that this activation of polyunsaturated fatty acid synthesis occurred in situ in the atheroma plaque in mouse. Because PUFA and their oxidized derivatives are known to be involved in the regulation of the inflammatory response and in the development of atherosclerosis, our data bring some support to the hypothesis that the antiatherogenic activity of liver X receptor agonists could be directly related to their lipogenic activity in the vascular wall.
Liver X Receptor Activation Promotes Polyunsaturated Fatty Acid Synthesis in Macrophages: Relevance in the Context of Atherosclerosis
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Materials and Methods

Cell culture: Human Peripheral-blood Monocytes were obtained from two healthy donors who had provided informed consent, in accordance with the Declaration of Helsinki. Samples were collected by the Etablissement Français du Sang (Besançon, France). Mononuclear cells were isolated by Ficoll gradient centrifugation and monocyte negative selection was performed via magnetic activated cell sorting using the Monocyte Isolation Kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Monocytes were differentiated into macrophages for 6 days with 100 ng/mL of M-CSF in RPMI medium supplemented with 10% Fetal Bovine Serum (FBS) in 5% CO$_2$ at 37 °C. For transfection experiments, on day 7, macrophages were transfected with negative siRNAs (Ambion, Invitrogen) or siRNAs targeting LXR$\alpha$, LXR$\beta$ or SREBP1c (Ambion, Invitrogen, Illkirch, France) using Interferin (Polyplus, Illkirch, France) according to the manufacturer's instructions. The medium was changed 24 hours after transfection and the cells were treated with or without 1µM of synthetic LXR agonist T0901317 for 24 or 48 hours. Levels of silencing were 75% with LXR$\alpha$ SiRNAs and 80% with LXR$\beta$ SiRNAs either in the single or double knock down conditions. For cycloheximide experiments, macrophages were treated with T0901317 (5µM) or DMSO for 8hours in the presence of cycloheximide (2.5 µM) or control treatment.

Wild Type or LXR$\alpha/\beta^{-}\$ mouse$^1$ bone marrow cells were isolated from femurs and cultured for 6 days on plastic plates in RPMI 1640 medium with glutamax-I (Invitrogen, Illkirch, France) supplemented with 10% (vol/vol) fetal bovine serum (FBS; Invitrogen) in the presence of 50 ng/mL of M-CSF, in an atmosphere of 95% air and 5% CO2 at 37°C. Subsequently, floating cells were removed and macrophage differentiation was observed by fibroblast-like shape changes visualized with an Olympus IX70 microscope.

Microarray analysis: Total RNA was isolated using Trizol Reagent (Ambion, France). RNA quality was checked using the Agilent RNA 6000 Nano Kit on the Agilent 2100 Bioanalyzer (Agilent Technologies). RNA was quantified by measuring A260nm on the ND-1000 Spectrophotometer (NanoDrop Technologies). RNA samples (1 µg) were used for the amplification and labeling step using the Agilent Low RNA Input Linear Amp Kit (Agilent Technologies) in the presence of cyanine-3-CTP (Perkin Elmer, USA). Yields of cRNA and the dye incorporation rate were measured using the ND-1000 Spectrophotometer (NanoDrop Technologies). 1.65 µg of cy3-labeled fragmented cRNA in hybridization buffer was hybridized overnight at 65 °C to Agilent Whole Human Genome Oligo Microarrays. Following hybridization, the microarrays were washed twice with SSPE Buffer and then with acetonitrile. Fluorescence signals of the microarrays were detected using the Agilent Microarray Scanner System (Agilent Technologies). The Agilent Feature Extraction Software (FES) was used to read out and process data. Gene expression was determined using the Rosetta Resolver Gene Expression Data Analysis System (Rosetta Biosoftware). The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE13407 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE13407).

PUFA biosynthesis: Tetradeuterated linoleic acid (390150) and tetradecadeuterated $\alpha$-linolenic acid (9000433) and T0901317 were obtained from Cayman chemical (Ann Arbor, Michigan, USA). Fatty acid free bovine serum albumin (BSA) (A7030) and desmosterol.
were obtained from Sigma-Aldrich. RPMI 1640 medium with GlutaMAX™ (61870) and fetal bovine serum (FBS) (10270) were obtained from Gibco. Linoleic acid d4 or α-linolenic acid d14, final concentration 4µM were incubated with 0.8 mg/mL fatty acid free BSA in RPMI medium for 2 hours at 37°C, and then sterile filtered using 0.22 µm filter units. Human primary macrophages, pre-treated for 24 hours with or without 1 µM T0901317 or 10 µM desmosterol in RPMI medium supplemented with 10% FBS at 37°C and 5% CO2, were then incubated with the deuterated fatty acid/BSA mix and treated again with or without 1 µM T0901317 or 10 µM desmosterol for 24 hours at 37°C and 5% CO2. Total lipids were extracted using the Folch method and the quantity of corresponding synthetized omega 6 deuterated fatty acids (C20:4 n-6 d4) or omega 3 deuterated fatty acids (C18:4 n-3 d12, C20:3 n-3 d14, C20:4 n-3 d12, C20:5 n-3 d10, C22:5 n-3 d10, C22:6 n-3 d10, C24:5 n-3 d10 and C24:6 n-3 d10) was determined by GC-MS.

Real time PCR Analysis: Total RNA was extracted using the RNeasy Mini Kit (Qiagen, USA) according to the manufacturer’s instructions. 100 ng of total RNA was reverse transcribed using M-MLV Reverse Transcriptase, random primers and RNaseOUT inhibitor (Invitrogen, USA). cDNA obtained were quantified by real time PCR using the SYBR Green RT-PCR Kit (Invitrogen, USA) and using a LightCycler 2.0 (Roche Diagnostics, Meylan, France). The ΔΔCt method was used to determine the relative mRNA levels of each gene and Ct were normalized using Cyclophilin A mRNA levels for human and GAPDH mRNA levels for mouse. Sequences of the different primers used are available upon request.

Fatty acid profile: Aminopropyl bonded phase columns were used to isolate and purify three fractions corresponding to neutral lipids, free fatty acids and polar lipids, respectively, following the procedure described by Kaluzny et al. The fatty acid composition of each fraction was then determined by gas-chromatography mass spectrometry following the protocol detailed previously.

Phospholipid analysis: Phospholipid analysis: Dimyristoylphosphatidylcholine, lauroylsphingomyelin and dimyristoylphosphatidylethanolamine (Avanti Polar Lipids, USA) were used as internal standards, and 50 mg/L of butylated hydroxytoluene was added to the solvent. Total Lipids were extracted according to the method of Folch. An aliquot the organic phase was evaporated under vacuum and dried lipids were then dissolved in 100 µL of Chloroform/Methanol (4/1 v/v). Chromatography was performed on a 1200 HPLC system (Agilent technologies) equipped with a Zorbax RX-SIL 2.1x100mm 1.8µ column (Agilent Technologies). Elution was performed at a flow-rate of 0.3 mL/min with a binary gradient of solvent A (5 mM Ammonium Acetate in Chloroform/Methanol 4/1 v/v) and solvent B (5 mM Ammonium Acetate in Chloroform/Methanol/Water 6/3.4/0.6 v/v/v). Positive Electrospray Ionization Mass Spectrometry was performed on a quadrupole mass spectrometer (QqQ 6460; Agilent Technologies). The precursor ion ([M+H]+ → 184 Da) mode was used for phosphatidylcholine and sphingomyelin molecular species and the neutral loss ([M+H]+ → [M+H-141]+) mode for phosphatidylethanolamine species.

CEs and HETEs analysis : Cholesteryl esters (16:0, 17:0, 18:0, 18:1, 18:2, 24:4) and were purchased from Avanti Polar Lipids (Coger, Paris, France). 15-HETE-d8 was purchased from Cayman Chemical (Bertin Pharma, Montigny le Bretonneux, France). Chemicals of the highest grade available were purchased from Sigma Aldrich (Saint-Quentin Fallavier, France). LCMSMS quality grade
solvents were purchased from Fischer Scientific (Illkirch, France). Cells pellets (3 million cells) were suspended in 180 µL saline and spiked with 10 µg of 17:0-CE and 30 ng of 15-HETE-d8 used as internal standard. Lipids were extracted with 900 µL chloroform/methanol (2/1) for 2 h. After addition of 120 µL of saline, extracts were centrifuged for 5 min 10000g. The organic phase was then collected and divided in two equal parts for the analysis of cholesteryl esters and HETE respectively as follows.

**Quantitation of cholesteryl-esters by LC-MS²**

One half of the total lipid extract (ca 250 µL) was evaporated under vacuum and dried lipids were solubilized with 100µL of chloroform/methanol/water (60/30/4.5). Six microliters were then injected on a 1200-LC 6460-QqQ system equipped with an ESI-Jet stream source (Agilent technologies). Cholesteryl-esters were separated on a Zorbax Eclipse Plus C8 2.1x100 mm, 1.8 µm column (Agilent technologies) at a flow rate of 0.25 ml/min, 55°C, with a linear gradient of Methanol/ammonium acetate 10mM (90/10 v/v) containing acetic acid 1mM (solvent A) and Isopropanol/Methanol (90/10 v/v) containing ammonium acetate 10mM and acetic acid 1mM (solvent B) as follows : 5% B for 1 min, up to 80% B in 16 min, up to 100% in 1 min and maintained at 100% for 1 min. Acquisition was performed in positive Multiple Reaction Monitoring (MRM) mode (source temperature : 200°C, nebulizer gas flow rate was 10 L/min, sheath gas flow 11 L/min, temperature 200°C, capillary 3500 V, nozzle 1000 V, fragmentor 106-126 V, collision energy 20 V). Transitions [M+NH4]+ → 369.3 were used for the detection of cholesteryl esters. Calibration curves using the closest cholesteryl-ester external standard were used for cholesteryl-ester concentration determination. Quadratic regression was used for calculations.

**Quantitation of hydroxy-eicosatetraenoic acids (HETEs) by LC-MS-MS**

The second half of the lipid extract was evaporated under vacuum. HETE were further extracted according to the method of Yoshida et al. as follow. Dried lipids were mixed with 500 µL of BHT/ethanol (50 mg/L). The reduction of hydroperoxides was achieved with 500 µL of a solution of sodium borohydride (7.4 g/L in saline) at room temperature for 5 min. Reduced samples were then saponified under argon with 500 µL of 1M KOH in methanol for 45 min in the dark at 40°C. Samples were extracted with 2 mL of acetic acid/water (4/1 v/v) and 5 mL chloroform/ethyl-acetate (4/1 v/v). Lower organic phase was collected and evaporated under vacuum. Extracts were then solubilized with 100 µL of methanol 70% and 10 µL was injected on a 1200-LC 6460-QqQ system equipped with an ESI-Jet stream source (Agilent technologies). HETEs were separated on a Zorbax Eclipse Plus C18 2.1x100 mm, 1.8 µm column (Agilent technologies) at a flow rate of 0.2 ml/min, 30°C, with a linear gradient of ammonium acetate 5mM (solvent A) and acetonitrile/methanol (95/5 v/v) (solvent B) as follows : 23% B for 10 min, up to 75% B in 3 min, and maintained at 75 % for 7 min. Acquisition was performed in negative Multiple Reaction Monitoring (MRM) mode (source temperature : 325°C, nebulizer gas flow rate was 10 L/min, sheath gas flow 11 L/min, temperature 400°C, capillary 3500 V, nozzle 1000 V, fragmentor 115 V, collision energy 4 V). Transitions 319.3→219.2, 319.3→163.2, 319.3→115.2, 327.3→226.2 were used for quantitation of 15HETE, 12HETE, 5HETE and 15HETE-d8 respectively. Quantitation of HETEs was performed by calculating their relative response ratios to 15HETE-d8 used as internal standard.

**Luciferase transactivation assays:** Human Embryonic Kidney 293T (HEK293T) cells were transiently transfected with expression vector pSG5-LXR and pCMV-β-Galactosidase and
luciferase reporter vectors TkpGL3 or basic pGL3 containing different constructs of the human ELOVL5 gene promoter, using Lipofectamine 2000 reagent (Invitrogen, USA) and according to the manufacturer's instructions. The medium was changed 24 hours after transfection and cells were treated with or without 1 µM T0901317 for 24 hours. Luciferase activity was measured using the ONE-Glo Luciferase Assay System (Promega, USA) according to the manufacturer's instructions. Values obtained on a luminometer (Wallac 1420 Multilabel Counter; Perkin Elmer, USA) were normalized with β-Galactosidase activity that was determined by measuring hydrolysation of Chlorophenolred-β-D-Galactopyranoside (CPRG; Roche Diagnostics GmBH, Germany) at 560 nm using a spectrophotometer (Wallac 1420 Multilabel Counter; Perkin Elmer, USA). Primer sequences are available upon request.

**Animal studies:** ApoE−/− mice were fed control or T0901317-supplemented western type diets (21% fat and 0.2% cholesterol, Safe, Augy, France), corresponding approximately to 10 mg of T0901317/kg of body weight/day for 8 weeks and then sacrificed to collect the brachiocephalic and the left common carotid arteries. The arteries were immediately snap frozen in liquid nitrogen and used for fatty acid analysis and mRNA analysis, respectively. To collect peritoneal foam cells, ApoE−/− mice were placed on a western type diet for 4 weeks and then treated with T901317 at 20 mg/kg/d for 4 days by gavage. After two days of T0901317 treatment, mice were injected intraperitoneally with thioglycollate. Mice were sacrificed 48h later and liver as well as thioglycollate-elicited peritoneal macrophages were collected. All of the experimental procedures were conducted in accordance with the local guidelines for animal experimentation. Protocol no. 7105 was approved by the Animal Care and Use Committee of the University of Burgundy.

**Statistical analysis:** Significance of the data was determined using the Kruskall-Wallis test and Mann-Whitney U test. Values are usually the mean +/- S.D. of three independent determinations and are representative of at least two independent experiments with individual donors for the lipidomic data in human primary macrophages. Real time PCR data are representative of at least 6 independent experiments with individual donors

**Références**

Supplemental Figure I: Dose-dependent effect of T0901317 on genes involved in PUFA synthesis. Primary human macrophages were treated with T0901317 at the indicated doses or with DMSO (Ctrl) for 24 hours and mRNA levels of target genes were evaluated by real time PCR. Each value represents mean ± S.D. of at least six independent determinations. * P<0.05 Vs DMSO; **P<0.05 Vs DMSO and 0.1 µM; ***P<0.05 Vs DMSO, 0.1 µM and 1 µM.
**Supplemental figure II: Impact of LXR alpha and LXR beta silencing on the expression of target genes.**

Macrophages were transfected with Negative, LXRα and LXRβ siRNA and treated with T0901317 (1μM) or DMSO for 24 hours and mRNA levels of target genes were evaluated by real time PCR. Each value represents mean ± S.D. of at least three independent determinations. * P<0.05 vs DMSO same siRNA, § P<0.05 Vs negative siRNA same treatment.
Supplemental figure III: Impact of desmosterol treatment on the expression of LXR target genes in human primary macrophages. Human primary macrophages were treated with desmosterol at 10 µM for 48 hours. mRNA levels of target genes were evaluated by real time PCR * p<0.05 vs. cells treated with vehicle. Each value represents mean ± S.D. of at least three independent determinations.
Supplemental figure IV: Impact of cycloheximide on the response of genes involved in PUFA metabolism to an LXR agonist stimulation. Macrophages were treated with T0901317 (5µM) or DMSO for 8 hours in the presence of cycloheximide (2.5 µM) or control treatment (DMSO) and mRNA levels of target genes were evaluated by real time PCR. Each value represents mean ± S.D. of 6 independent determinations. * P<0.05 vs DMSO same conditions, § P<0.05 Vs no cycloheximide.
Supplemental figure V: Impact of LXR agonist treatments on phosphatidylcholines (PCs) and phosphatidylethanolamine (PEs) composition in human macrophages. Human macrophages were treated with vehicle or T0901317 (1 µM) for 48 hours before lipid analysis (A) Relative amounts of diacylglycerophosphatidylcholine; alk: alkyl; ac: acyl; AA-containing PCs are 38:4, 38:5 and 36:4 (B) Relative amounts of diacylglycerophosphatidylethanolamine molecules in human macrophages; AA-containing PEs are 38:4 and 38:5 Values are mean ±S.D. of three independent experiments *: significantly different from DMSO conditions. P<0.05, Mann-Whitney test.
**Supplemental figure VI: LXR deficiency alters PUFA metabolism in mouse macrophages.** Bone marrow derived macrophages were obtained from WT or Lxr deficient mice (A) Relative mRNA levels of selected LXR target genes involved in fatty acid and cholesterol metabolism. (B) AA/LA and DHA/EPA ratio in WT and LXR deficient BMD macrophages (C) Relative proportions of individual PEs in WT and LXR deficient macrophages *: significantly different from WT mice. P<0.05, Mann-Whitney test (n=5 individual mice for each group)
Supplemental figure VII: Impact of LXR agonist treatment on genes involved in FA synthesis (A) and inflammation (B). *Apoe<sup>-/-</sup> mice were fed control (white bars) or T0901317-enriched (black bars) western-type diets (10 mg/kg/day for T0901317) for eight weeks, and left common carotid arteries were collected to evaluate the relative mRNA levels of genes of interest by real time PCR. Each bar represents the mean ± S.D. of six mice. *: p<0.05 vs mice fed the control diet, Mann-Whitney test.
Supplemental figure VIII: Impact of LXR agonist treatment on gene involved in fatty acid metabolism and PUFA composition in peritoneal macrophages-foam cells. ApoE−/− mice were fed a western type diet for four weeks and then were treated with T0901317 (20 mg/kg/day) or vehicle for 4 days. Two days before sacrifice, mice received an intraperitoneal injection of thioglycolate. (A) Total amounts of individual fatty acids were determined by GC-MS. (B) mRNA levels of target genes were determined by real time PCR. Each bar represents the mean ± S.D. of six mice. *: P<0.05 vs mice fed the control diet, Mann Whitney test.
Supplemental figure IX: Impact of LXR agonist treatment on the composition of phosphatidylcholines (PCs) (A), phosphatidylethanolamine (PEs) (B), cholesteryl esters (CEs) (C) and Hydroperoxy-eicosatetraenoic acids (HETEs) (D) composition in peritoneal macrophages-foam cells. *: P<0.05 vs mice fed the control diet, Mann Whitney test.
Supplemental figure X: Impact of LXR agonist treatment on gene involved in fatty acid metabolism and PUFA composition in the liver. ApoE−/− mice were fed a western type diet for four weeks and then were treated with T0901317 (20 mg/kg/day) or vehicle for 4 days. Liver were collected to evaluate fatty acid composition and relative mRNA levels of genes of interest, respectively. (A) Total amounts of individual fatty acids were determined by GC-MS. (B) mRNA levels of target genes were determined by real time PCR. Each bar represents the mean ± S.D. of six mice. *: P<0.05 vs mice fed the control diet, Mann Whitney test.