Genome-wide analysis of LXRα activation reveals new transcriphional networks in human atherosclerotic foam cells

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ABSTRACT

Increased physiological levels of oxysterols are major risk factors for developing atherosclerosis and cardiovascular disease. Lipid-loaded macrophages, termed foam cells, are important during the early development of atherosclerotic plaques. To pursue the hypothesis that ligand-based modulation of the nuclear receptor LXRα is crucial for cell homeostasis during atherosclerotic processes, we analysed genome-wide the action of LXRα in foam cells and macrophages. By integrating chromatin immunoprecipitation-sequencing (ChIP-seq) and gene expression profile analyses, we generated a highly stringent set of 186 LXRα target genes. Treatment with the nanomolar-binding ligand T0901317 and subsequent auto-regulatory LXRα activation resulted in sequence-dependent sharpening of the genome-binding patterns of LXRα. LXRα-binding loci that correlated with differential gene expression revealed 32 novel target genes with potential beneficial effects, which in part explained the implications of disease-associated genetic variation data. These observations identified highly integrated LXRα ligand-dependent transcriptional networks, including the APOE/C1/C4/C2-gene cluster, which contribute to the reversal of cholesterol efflux and the dampening of inflammation processes in foam cells to prevent atherogenesis.

INTRODUCTION

Cardiovascular and metabolic disorders have become an enormous burden for human health. Homeostasis of cholesterol at the blood vessel wall is important to cope with elevated cholesterol levels that contribute to increased rates of atherosclerosis and cardiovascular disease (1). A key event in the development of atherosclerosis consists of the uncontrolled uptake of oxidized low-density lipoproteins (oxLDL) by macrophages recruited at the subendothelial space of vessel walls (2). In concert with local inflammatory reactions, this process leads to the formation of lipid-loaded macrophages termed foam cells (3). The liver x (nuclear) receptors, LXRα and LXRβ, are ligand-dependent factors that regulate cholesterol homeostasis and reverse cholesterol transport; hence, they are interesting drug targets for treating cardiovascular diseases (4). Oxysterols are natural ligands and activators of LXRα (5), which is the predominant LXR subtype required for the full anti-atherogenic action of LXR agonists in inhibiting atherosclerosis (6). Evolutionary analysis of the sequences of both LXR subtypes suggested that a single LXR gene duplicated during mammalian evolution (7) and resulted in one general and one more specialized factor. Consistent with this hypothesis, LXRβ was found to be ubiquitously expressed, whereas LXRα is more restricted to cell types that modulate cholesterol and fatty acid metabolism, most importantly in liver and macrophages (8). Hypothesis-driven research revealed important insights, including LXRα regulation at selected genomic loci of target genes, such as the ATP-binding cassette transporters ABCA1 and ABCG1, or apolipoprotein E, to understand mechanistic aspects of foam cell development and atherosclerosis (9).

Here, we applied genome-wide analysis to shed light on the contribution of ligand-based LXRα regulation to these processes. We performed integrative genomic analyses with the aim to decipher LXRα-dependent functional features and transcriptional regulation pathways, including the effects of anti-atherosclerotic drug treatment. Key networks determined by LXRα activation led to biological function such as counteracting lipid-overload...
in macrophages, which could in principle not be fully derived from single gene events and analysis thereof. To analyze the role of LXR modulation in atherosclerosis and related diseases we used a well-validated human macrophage and foam cell model and applied the efficient synthetic LXR agonist T0901317.

MATERIALS AND METHODS

Cell models

Human monocytic leukaemia THP1 cells were maintained in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS, Biochrom) and differentiated for 48 h using 10-8 M phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich). Human primary macrophages were isolated from at least four individual buffy coats donated by healthy volunteers (kindly provided by DRK-Blutspendedienst Ost Gemeinnützige GmbH Institut Berlin). Peripheral blood monocytes (PBMs) were isolated from buffy coats with Ficoll Paque and MACS Monocyte Isolation Kit II with MACS LS columns (Miltenyi Biotec). Monocytes were differentiated for 7 days. Primary and THP1 macrophages were treated similarly either with 0.01% dimethylsulfoxide (DMSO) or 1 mM M T0901317 or 0.1% DMSO for 24 h. Cell formation was induced by 100 μg/ml oxLDL (Autogen Bioclear UK Ltd) for 48 h and subsequently treated with 0.01% DMSO or 1 μM T0901317 (Sigma-Aldrich) for 24 h. Cholesterol loading and treatment was controlled by Oil Red O staining; detailed cholesterol composition was assessed with the fluorometric method of Amplex Red O staining; detailed cholesterol composition was assessed with two individual biological replicates for macrophages and T0901317 treated macrophages. Sequence reads of 36 bp were obtained using the second-generation Genome Analyzer and the Solexa Analysis Pipeline (Illumina). Reads were mapped to human genome assembly (February 2009, GRCh37/hg19) using Bowtie (13). Sequencing data were submitted to EBI and can be accessed via www.ubi.ac.uk/ena/data/view/ERP001502.

Peak calling and LXR binding profiles were generated genome-wide using the model-based analysis of ChIP-seq algorithm [MACS, (14)]. Validation of sequencing results was performed with ChIP-quantitative polymerase chain reaction (qPCR) (SYBR Green PCR Master Mix, Applied Biosystems). The relative occupancy of the immunoprecipitated factor at a locus was estimated using the following equation: 2 x (Ct input – Ct ChIP).

LXR knockdown

For siRNA-mediated LXRβ knockdown, differentiated THP1 cells were transfected with TransIT-TKO transfection reagent (Mirus) and 15 nM LXRβ Silencer Validated siRNA (ID 5458) and 15 nM LXRβ Silencer Select Validated siRNA (ID s14684) or 30 nM Silencer Select Negative Control #1 (all from Ambion). Transfection was carried out for 48 h followed by treatment of macrophages and foam cells with 10 μM T0901317 or 0.1% DMSO for 24 h.

Western blotting

Western blotting was performed with commercial antibodies against LXRα [Abcam, ab 41902, (10,11)], LXRβ [Abcam, ab56237, (12)], RXRα (Santa Cruz Biotechnology, sc-774 X) and β-actin (Santa Cruz Biotechnology, sc-47778, C4). Secondary antibodies were horseradish peroxidase (HRP)-labelled anti-mouse and anti-rabbit (Santa Cruz Biotechnology). Densitometry was performed using ImageQuant TL (GE Healthcare).

Chromatin immunoprecipitation and sequencing

Chromatin immunoprecipitation (ChIP) was performed with Diagenode’s Transcription Factor ChIP Kit (Diagenode). For immunoprecipitation, we used the well-validated antibodies against LXRα (Abcam, ab 41902), LXRβ (Abcam, ab56237), H3K4me3 (pAB-003-050, Diagenode), anti-H4K20me1 (ab9051, Abcam) and negative control IgG (kch-819-015, Diagenode). For one LXRα ChIP-seq reaction, we pooled at least four individual ChIP reactions. Sequencing of LXRα was performed twice (individual biological replicates) for macrophages and T0901317 treated macrophages. Sequence reads of 36 bp were obtained using the second-generation Genome Analyzer and the Solexa Analysis Pipeline (Illumina). Reads were mapped to human genome assembly (February 2009, GRCh37/hg19) using Bowtie (13). Sequencing data were submitted to EBI and can be accessed via www.ubi.ac.uk/ena/data/view/ERP001502.

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LXRα ChIP qPCR was normalized against IgG ChIP qPCR of the same locus. Detailed description of ChIP procedure, peak calling and filtering can be found in the Supplementary Methods and Supplementary Datasets S1.

Peak analysis

To compare different ChIP-seq lanes and to detect differential LXRα binding across investigated cell models, we performed LXRα peak-enrichment normalization (15) for all cell models. Differential binding events were based on a change of 1.5-fold. For visualization, raw tag data was loaded to seqMINER (16). Genomic distributions were determined using the Cis-regulatory Element Annotation System (17).

Annotation of genes controlled by nearby peaks

As we assumed that one LXRα-binding site can regulate multiple surrounding target genes (18,19), we decided to annotate all potential LXRα target genes with a maximal distance of 200 kb from peak centre (Supplementary Datasets S2). Therefore, we used the Peak Center Annotation script (peak2gene) from the Cistrome Analysis Pipeline (cistrome.org). Gene definitions were taken from the UCSC Genome Browser’s RefGene table (20).

Formaldehyde-assisted isolation of regulatory elements followed by sequencing

Formaldehyde-assisted isolation of regulatory elements (FAIRE) was performed as described previously (21). For relative openness analysis, enrichment intensities were extracted, quantile normalized and averaged for each genomic region. Detailed descriptions can be found in Supplementary Methods.

LXRα motif analyses

Motif search was performed de novo with top 100 bound sequences for each defined peak set individually using the
MEME-ChIP tool from MEME suite [http://meme.sdsc.edu, (22)] with default settings. Derived motifs with P-value of <10e-7 were scanned using available position weight matrices from the Transfac database (23). Motif distribution in all LXRα peak set sequences was determined by the find motif tool FIMO (MEME suite tool) with threshold P-value of 10e-4.

Report gene assays

Report gene assays were performed with natural and mutated LXR response elements (LXREs). Therefore, five copies of the LXREs were cloned into pGL4.31 vector (Promega) with the In-Fusion HD EcoDry cloning system (Clontech Takara Bio Europe). Full-length LXRα and RXRα were cloned from cDNA fragments (Source BioScience clone IRATp970C0271, Gene ID: 7376 and clone IOH39435, Gene ID: 6256) into pBIND vector (Promega). Detailed descriptions can be found in supplementary experimental procedures. For reporter gene assay, HEK293T cells were co-transfected with the pGL4.31–LXRE–Luc, pBIND–LXRα and pBIND–RXRα in 0.25% Lipofectamine (Invitrogen) for 4 h and subsequently treated with 10 μM T0901317 or 0.1% DMSO for 24 h. Reporter activity was determined with Dual Luciferase Reporter System (Promega). Activity was measured in relative luciferase units and finally visualized as fold-change of T0901317 treatment from vehicle control.

Gene expression

RNA extraction, microarray and quantitative PCR analyses were performed as described elsewhere (24). Differential expression analysis was performed on background subtracted data with cubic spline normalization and Benjamini Hochberg false discovery rate (FDR) correction. Significant data were considered to have a detection P-value of <0.01 and differential P-value of <0.05. Gene expression data were submitted in MIAME-compliant form to the ArrayExpress database under accession number E-MTAB-1106 (www.ebi.ac.uk/arrayexpress). Detailed descriptions and gene lists can be found in Supplementary Methods and Supplementary Datasets S3.

Correlation analyses

For correlation, we compared differentially expressed genes (versus vehicle treated macrophages) with binding site associated genes. For visualization, we chose to plot the normalized LXRα peak enrichment and differential gene expression sorted in 4 up- and 4 down- quantiles according to the expression fold-change. For gene activity prediction, we determined promoter-specific changes of H3K4me3, H4K20me1 and chromatin accessibility at genes with a nearby binding (25). Further, we generated a relative histone and FAIRE-seq signal of our cell models versus untreated macrophage and built a mean signal ±1.5 kb of the target gene transcription start site (TSS) and plotted the mean signal and differentially expressed genes in quintiles for each cell model (26). For prediction, we chose the mean signal fold-change of expression quantile 4. To determine a significant impact of LXRα binding on gene expression, we correlated LXRα peak locations and differential expression of nearby genes and compared this with a randomly shuffled peak set. Initially, we investigated this relationship as described by Boeva et al. (27). The significance of this spatial relationship was tested by three statistical hypothesis tests, hypergeometric test, Kolmogorov-Smirnov test (K-S test) and empirical cumulative distribution function (ECDF)-based test (Anderson-Darling test) (28).

Gene ontology analysis

Gene ontology (GO)-enrichment analysis was performed using the Database for Annotation, Visualization and Integrated Discovery [DAVID, http://david.abcc.ncifcrf.gov/(29)] for the model-specific and knockdown-validated LXRα target genes with a binding site. We performed functional annotation clustering for GO term Bioprocess_FAT with highest classification stringency.

Pathway analysis

Pathway analysis was also performed with DAVID analysing the Kyoto Encyclopedia of Genes and Genomes (KEGG) and BIOCARDATA pathways with the functional annotation chart option. Information on regulation of lipid metabolism by peroxisome proliferators-activated receptor alpha (PPARα) was extracted from Reactome pathway database [www.reactome.org, (30)]. Pathway analysis for T0901317-specific LXRα target genes in foam cells was performed with Ingenuity Pathways Analysis [IPA, www.ingenuity.com, (31)]. We performed core analysis using the Ingenuity Knowledge Base as reference set and considered direct and indirect relationships with high or experimentally observed confidence.

Association of LXRα-binding sites with genome-wide association studies

Correlation of LXRα-binding data with genome-wide association studies (GWAS) was processed by overlapping the NHGRI GWAS catalogue (32) single-nucleotide polymorphism (SNPs) positions with our defined LXRα-binding sites. We chose a P-value threshold of <10e-5. Additionally, we controlled that the LXRα-binding sites of interest were within the linkage disequilibrium (LD) block [DistilLD Database, (33)] of the SNP. Further, we considered just LXRα peak-associated genes that were also reported in the GWAS. Detailed list can be found in Supplementary Datasets S4.

Functional network analysis

Interaction networks were derived from the FANTOM4-EdgeExpress Database (34) and STRING (35). Networks were visualized using Cytoscape (36). Differentiation between already known and new LXRα target genes and interactors were done with BIOGRID, NEXTBIO, Nuclear Receptor Resource [(37,38); http://nrresource.org] databases and most recent publications on LXRα (19,39).
Statistical analysis

The Student’s t-test was used to calculate the statistical significance. When multiple samples were analyzed the ANOVA test was applied followed by Dunnett’s multiple comparison test with $P < 0.05$ considered as statistically significant. All results represent the mean ± standard deviation.

RESULTS

LXRα binding is highly ligand dependent in THP1 and human primary macrophage models

Knowledge about the binding of LXRα to the human genome is required to lay the foundation for deciphering specific sets of regulatory regions of this ligand-dependent nuclear receptor. LXRα is by nature a flexible sensor for diverse metabolites in the human body, which can respond quickly to changing ligand concentrations. To generate stringent data sets for further analyses, we considered only LXRα-binding sites, which resulted in differential gene expression and were LXR knockdown sensitive (Supplementary Figure S1A).

First, we examined the amount of LXRα and LXRβ proteins and their heterodimerization partner RXRα. Because of ligand-based activation in human macrophages, LXRα is increasingly expressed in an auto-regulatory fashion, whereas comparably low proteins amounts of the LXRβ subtype and RXRα showed no significant change (Figure 1A, Figure 1B and Supplementary Figure S1B). Notably, mouse macrophages do not show a feed-forward loop of LXRα expression (40), indicating that activation of LXRα in human cells differs strikingly from mouse foam cells. Cholesterol loading and beneficial effects of T0901317 were further confirmed by Oil red O staining (Supplementary Figure S1C) and cholesterol composition analysis (Figure 1C). To determine LXRα binding in THP1-derived macrophages and foam cells in presence and absence of synthetic LXRα ligand T0901317, we applied ChIP using a well-validated and previously applied antibody (10,41) followed by massively parallel deep sequencing. Macrophages and T0901317-treated macrophages were sequenced in biological duplicates and reached correlation values of $r = 0.98$ and $r = 0.92$, respectively (Supplementary Figure S1B). In ligand-free macrophages with low amounts of LXRα (Figure 1B), its genome-wide enrichment at potential binding sites was mostly below the defined threshold for detection of significantly enriched LXRα-binding sites (Supplementary Figure S1E). To exclude any bias during ChIP-seq data processing, we selected LXRα-binding sites and validated successfully 21 ChIP-seq peaks by ChIP–
qPCR analysis, including confirmation of the extremely low abundance of LXR\(\alpha\) in the absence of ligand (Figure 1D and Supplementary Figure S1F and G). Characteristically, LXR\(\alpha\) binding was highly induced by its synthetic ligand T0901317 in contrast to ligand-independent, constitutive binding of the LXR\(\beta\) subtype. As LXR\(\alpha\) was 38 times more abundant in T0901317-treated foam cells than its \(\beta\)-subtype, we consistently observed up to 80-fold enrichment of LXR\(\alpha\), whereas we found only 2.5-fold enrichment for LXR\(\beta\), as detected at the \(ABCA1\) LXR response element (LXRE) locus (Figure 1D and Supplementary Table S1).

To validate the THP1 model, we used human PBM-derived macrophages and produced foam cells thereof, and obtained similar LXR\(\alpha\)-binding profiles (Figure 1D). Interestingly, we detected spurious ligand-free LXR\(\alpha\)-binding background only in PBM-derived macrophages, indicating experimentally interfering lipids observed in the donor blood. THP1 cells were required to gain sufficient and standardized material for further analyses and were, therefore, the experimental resource of choice.

Three sets of LXR\(\alpha\) genomic loci define gene regulation in macrophages and foam cells

To understand oxLDL- and/or T0901317-induced cell-specific and shared binding events of LXR\(\alpha\), we applied comparative ChIP-seq analyses (15) and could categorize three different sets of LXR\(\alpha\) genome binding (Figure 2A). Fifty-five per cent of LXR\(\alpha\) peaks (1459 peaks) were different among cell models. Thereof, 29% (769 peaks) showed prominent enrichment in T0901317 treated macrophages and foam cells (T0901317 specific set), and we observed an enrichment of 26% (690 peaks) of LXR\(\alpha\) sites in foam cells over T0901317-treated cells, which we subsumed as foam cell-specific set. Forty-five per cent of LXR\(\alpha\) peaks (1193 peaks) were shared among all cell models, which we termed shared peak set. Open chromatin is associated with active transcriptional regulators and pre-determines transcription factor binding (42,43). Using FAIRE-seq analysis, we observed pronounced chromatin openness of LXR\(\alpha\) loci of shared binding sites and T0901317-specific binding sites compared with foam cell-specific–binding sites, similarly in all cell models.

Figure 2. Differential LXR\(\alpha\)-binding sites in human macrophage and foam cell models. (A) Comparative LXR\(\alpha\) ChIP-seq enrichment heatmap and mean tag density ±1.5 kb from peak center. Peaks were sorted according to their degree of variability, calculated as log2 fold-change between models. The following sets were defined: i) T0901317-specific set (29%); ii) all models that share similar LXR\(\alpha\) enrichment are referred to as shared peaks (45%); iii) foam cell-specific peak set (26%). Additionally to the LXR\(\alpha\) ChIP-seq, we also plotted the average chromatin openness of the three peak sets derived from FAIRE-seq analyses. Macrophages are represented in grey, T0901317-treated macrophages in brown, foam cells in green, T0901317-treated foam cells in blue and average FAIRE in black. (B) Representative tag alignment tracks of LXR\(\alpha\) ChIP-seq, which were normalized against IgG control lane tags of same loci. Set-specific examples of genomic regions with differential or shared LXR\(\alpha\) binding in our models. \(APOC1\) locus (T0901317 specific set), \(ABCA1\) locus (shared set) and \(PARP1\) locus (foam cell specific set). Arrows indicate transcription start sites and orientation of transcription. Black arrows under tracks show peaks.
(Figure 2A and Supplementary Figure S2A). Further, we observed that open LXR\(\alpha\)-binding sites were enriched with other co-localized transcription factor-binding sites (TFBS, data extracted from Encode) compared with oxysterol-induced foam cell-specific-binding sites (Supplementary Figure S2A, TFBS). Although T0901317 treatment apparently induced LXR\(\alpha\) and co-activator assembly at highly accessible transcriptional hotspots, oxoLDL treatment seemed to trigger less pronounced LXR\(\alpha\) binding to DNA. As shown in Figure 2B and Supplementary Figure S2B and C, characteristic loci for each set of the LXR\(\alpha\)-binding site included the well-known LXR\(\alpha\) target genes \(ABCA1\), LXR\(\alpha\) itself and the \(APOE/C2/C4\)-gene cluster, or unexpected new target genes as poly(ADP-ribose)polymerase-1, \(PARP1\) or pre-B-cell leukemia homeobox 4, \(PBX4\).

**LXR\(\alpha\) peak enrichment at transcriptional start sites of target genes is sharpened by T0901317 treatment**

To further characterize shared and differential LXR\(\alpha\)-binding sites, we performed \textit{de novo} motif search for each of the three sets of specific LXR\(\alpha\) genomic sites. Only in the shared and T0901317-specific LXR\(\alpha\) peak sets we found significant enrichment of the direct repeat spacer 4 (DR4) motif, which is known from targeted gene analyses [Figure 3A. (44)]. In contrast, the foam cell-specific set of LXR\(\alpha\)-binding site sequences did not significantly enrich any motif. Similar was true for the targeted scanning of \textit{de novo} extracted LXR:RXR motif from the shared peak set. With a fairly stringent \(P\)-value cut-off of 10e-4, we found that 15\% (398 peaks) of all LXR\(\alpha\)-binding sites occupied this DR4 motif, but only 2\% thereof were derived from foam cell-specific set (Supplementary Figure S3A). To biochemically characterize the derived \textit{de novo} consensus LXR:RXR motif, we performed transient reporter gene assays (Supplementary Figure S3B). Surprisingly, single-nucleotide mutations of LXR response elements did hardly change reporter gene activity. Major mutations in LXR\(\alpha\) halfsites were required to significantly decrease reporter activity. These observations were consistent with other nuclear receptor studies, suggesting that LXR\(\alpha\) could bind to fairly degenerated DR elements (19,39).

We also tested the observed higher degree of conservation in the spacer region at positions 8 and 9. Mutations in the spacer region resulted in a strong decrease of reporter gene expression, indicating potential stereochemical disturbance of LXR\(\alpha\) binding.

To further analyse the spatial characteristics of LXR\(\alpha\) binding, we determined the genomic positions of LXR\(\alpha\)-binding sites. This analysis revealed that T0901317-induced LXR\(\alpha\) binding increased enrichments around transcription start sites and promoters, whereas oxysterol induced foam cell specific LXR\(\alpha\) binding sites were enriched downstream of genes (Figure 3B). This analysis suggested a more pronounced shaping of transcriptional initiation by the nanomolar binding ligand T0901317 in contrast to only micromolar binding by natural sterols.

To study the effects of genome-wide LXR\(\alpha\) binding on transcriptional networks, we performed gene expression and LXR knockdown analyses of the investigated cellular models. By correlating the differentially expressed target genes \((P \leq 0.05\) versus macrophage\) with the genome-wide LXR\(\alpha\) binding map, 19\% of the LXR\(\alpha\)-binding sites overlapped with differentially expressed genes, thereof 8\% were associated with 186 genes that were significantly sensitive to LXR knockdown (Figure 3C, stringent set). The observed enrichment of differentially expressed genes close to LXR\(\alpha\)-binding sites was statistically significant \((P < 0.005\) for K–S test and hypergeometric test and \(P < 0.01\) for ECDF test) and was absent in a randomly shuffled set of binding sites (Supplementary Figure S3C).

In addition to gene expression data, we generated for all cell models genome-wide maps from histone modifications H3K4me3 and H4K20me1, which are important transcriptional initiation or elongation marks, respectively (45). Combined with FAIRE-seq data of open chromatin, we determined promoter-specific changes of potential LXR\(\alpha\) target genes that harbour enriched LXR\(\alpha\)-binding sites. These changes correlated well with gene expression profiles (Supplementary Figure S3D and E, (25)) and could be used to identify 492 additional LXR\(\alpha\) target genes. This approach further increased the total correlation of LXR\(\alpha\)-binding sites with target genes to 30\% (Figure 3C). Correlation analysis between gene expression and LXR\(\alpha\) peak enrichment at differentially regulated LXR\(\alpha\) target genes revealed a bimodal regulation of gene expression in all cell models (Figure 3D). Expression levels separated in quantiles showed increased LXR\(\alpha\)-binding enrichment in quantiles \(-2 \rightarrow -4\) and \(2 \rightarrow 3\). Remarkably, expression quantile 4 in all cell models was correlated with decreased LXR\(\alpha\) peak enrichment, suggesting minor effects of LXR\(\alpha\) on this gene set.

Despite similar expression profiles, we observed less enriched LXR\(\alpha\) binding at differentially regulated target genes in untreated foam cells compared with T0901317-treated foam cells. This was also consistent with mentioned observations of less efficient positioning of LXR\(\alpha\) binding to promoter sites and DR4 sequence motifs. Taken together, we observed a bimodal expression profile in all described cell models with less pronounced binding properties in foam cells, which could be substantially sharpened by T0901317 treatment.

**Main functions of LXR\(\alpha\) in cholesterol metabolism, apoptosis and interaction with the PPAR\(\alpha\) signalling pathway**

For the functional annotation, we first analysed the stringently validated set of 186 knockdown-sensitive LXR\(\alpha\) target genes and deciphered specific pathways enriched in foam cells, T0901317-foam cells and T0901317-treated macrophages (Supplementary Table S2). In vehicle-treated foam cells, we discovered “organic acid biosynthetic process” and “regulation of cholesterol storage”. Further, we found 14 genes associated with the regulation of apoptosis, indicating increasing cell death that could contribute to the formation of atherosclerotic plaques in vivo. Foam cells treated with the synthetic LXR\(\alpha\) ligand T0901317 did not significantly enrich this disease-
associated pathway. Instead they showed enrichment of the BIOCARTA pathway “nuclear receptors in lipid metabolism and toxicity”, and the bioprocess “negative regulation of cholesterol storage”, which is one of the main functions of this anti-atherogenic compound. Interestingly, the PPARα signalling pathway was enriched in T0901317-treated and -untreated foam cells, indicating tight interactions of LXRα- and PPARα-regulated pathways, as reported recently for macrophages (39). Further investigation of the involvement of LXRα in PPARα signalling pathway in T0901317-treated foam cells revealed a major proportion of shared target genes (Supplementary Figure S4). Strikingly, the LXRα complex also regulated the expression of two major co-activators of PPARα, Cbp/p300 interacting transactivator with Glu/Asp-rich carboxy terminal domain 2 (CITED2) and peroxisomal proliferator-activated receptor A interacting complex 285 (PRIC 285). For set-specific functional annotation, we found similar results as for the stringent set of LXRα target genes (Supplementary Table S3), with the addition of defence response enrichment in shared peak set and the involvement of Ras protein signal transduction in foam cell-specific peaks set.

LXRα binding and genetic variants associated with complex diseases reveal key target genes in transcriptional networks

Numerous genome-wide association studies (GWAS) were successful in associating genetic variants and genomic loci to common diseases (46). But in many cases, genetic variation associated to disease phenotypes could not functionally explain the underlying mechanisms. To discover the potential impact of genetically influenced...
LXRz-induced transcriptional networks on complex diseases, we searched for significant, disease relevant SNPs. We considered SNPs in linkage disequilibrium of cell model-specific LXRz-dependent–binding sites that showed differentially expressed target genes. As listed in Table 1, we found a number of LXRz-dependent genes with central impact on metabolism and inflammation. The new LXRz target gene homeobox protein PBX4 (Supplementary Figure S2C) showed striking association with LDL cholesterol metabolism, whereas another LXRz target gene, the LDL-associated phospholipase A2 (PLA2G7) was associated with “lipoprotein-associated lipase activity and mass”. The most striking connection between LXRz binding and disease related loci was found with SNP rs4420638, which was associated to “C-reactive protein” with a P-value of 9e-139 and “LDL cholesterol” with a P-value of 6e-60 in meta-analyses (Table 1). The SNP rs4420638 is located close to the transcription termination site of APOC1 and is encircled by two LXRz-binding sites that are in 5.7- and 6.5-kb distance (Figure 4A). LXRz-binding peaks detected at this locus were T0901317 specific (Figures 2B and 4A), whereas the natural ligand oxLDL was not efficient in recruiting LXRz to this locus. Consistently, T0901317 treatment led to an improved expression profile of the whole-gene cluster (Supplementary Figure S5). In this context, it is interesting to note that APOC1 gene is not only located within a cluster of genes that are implicated in plasma lipid metabolism [APOE, APOC4 and APOC2, (47)] but also surrounded by genes (BCL3, RELB, PVRL2) that are involved in biological processes, such as inflammation, immunity, differentiation, cell growth, tumorigenesis and apoptosis (first two via NF-κB signalling). To provide a frame on the global impact of APOC1 and surrounding genes on lipid metabolism and inflammatory response, we performed transcriptional network analysis of this LXRz locus. To embed the gene cluster around APOC1 into a broader metabolic context, we further considered well-known LXRz target genes and generated a network with differential expression data of the foam cell using the STRING database (Figure 4B). This network illustrated the tight relationship between direct and indirect LXRz target genes in foam cells and their involvement in major processes, such as lipid metabolism, inflammation and apoptosis. Thus, we can conclude that LXRz-dependent activation of the APOE/C1/C4/C2 cluster supported lowering cholesterol levels (48) by triggering a defined network of genes.

**Atheroprotective potential of 32 novel LXRz target genes in T0901317-treated foam cells**

The atheroprotective potential of the synthetic LXRz ligand T0901317 has been previously shown, but the underlying gene networks were only partly understood.

### Table 1. Correlation of LXRz-binding data with GWAS

<table>
<thead>
<tr>
<th>Function</th>
<th>Disease/term</th>
<th>SNP</th>
<th>P-value</th>
<th>Genes</th>
<th>Distance to SNP (kb)</th>
</tr>
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<tbody>
<tr>
<td>Metabolism</td>
<td>LDL cholesterol</td>
<td>rs10401969</td>
<td>2.0E-08</td>
<td>PBX4</td>
<td>311.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rs4420638</td>
<td>1.0E-60</td>
<td>APOC1</td>
<td>5.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rs2142672</td>
<td>2.0E-08</td>
<td>MYLIP</td>
<td>52.1</td>
</tr>
<tr>
<td>Triglycerides</td>
<td></td>
<td>rs17216525</td>
<td>4.0E-11</td>
<td>PBX4</td>
<td>57.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rs4420638</td>
<td>3.0E-13</td>
<td>APOC1</td>
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</tr>
<tr>
<td>HDL cholesterol</td>
<td></td>
<td>rs7679</td>
<td>7.0E-11</td>
<td>PLTP</td>
<td>33.3</td>
</tr>
<tr>
<td>Lipoprotein-associated phospholipase A2 activity and mass</td>
<td></td>
<td>rs7679</td>
<td>4.0E-09</td>
<td>PLTP</td>
<td>33.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rs1805017</td>
<td>6.0E-14</td>
<td>PLA2G7</td>
<td>40.4</td>
</tr>
<tr>
<td>Inflammation</td>
<td>Mean platelet volume</td>
<td>rs12485738</td>
<td>6.0E-31</td>
<td>ARHGEF3</td>
<td>16.0</td>
</tr>
<tr>
<td></td>
<td>Rheumatoid arthritis</td>
<td>rs2062583</td>
<td>2.0E-06</td>
<td></td>
<td>84.4</td>
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<tr>
<td></td>
<td>C-reactive protein</td>
<td>rs4420638</td>
<td>9.0E-13</td>
<td>APOC1</td>
<td>5.7</td>
</tr>
<tr>
<td>Heart</td>
<td>Myocardial infarction (early onset)</td>
<td>rs9992601</td>
<td>6.0E-11</td>
<td>MRP6</td>
<td>17.2</td>
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<tr>
<td></td>
<td>Coronary heart disease</td>
<td>rs9982601</td>
<td>4.0E-10</td>
<td>SLC12A9</td>
<td>53.6</td>
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<tr>
<td></td>
<td>D-dimer levels</td>
<td>rs7801190</td>
<td>3.0E-08</td>
<td>CD300LF</td>
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</tr>
<tr>
<td></td>
<td>Resting heart rate</td>
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<td>ABCA4</td>
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<td></td>
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<td></td>
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<td></td>
<td></td>
<td>rs1530440</td>
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</tr>
<tr>
<td>Brain</td>
<td>Alzheimer’s disease</td>
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<td>2.0E-44</td>
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</tr>
<tr>
<td></td>
<td>Information processing speed</td>
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<td>2.0E-07</td>
<td>TRIB3</td>
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</tr>
<tr>
<td></td>
<td>Multiple sclerosis</td>
<td>rs17174870</td>
<td>1.0E-08</td>
<td>MERTK</td>
<td>93.7</td>
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<tr>
<td></td>
<td>Amyloid A levels</td>
<td>rs2896526</td>
<td>4.0E-22</td>
<td>LDHA</td>
<td>92.5</td>
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<tr>
<td>Skin</td>
<td>Melanoma</td>
<td>rs3219090</td>
<td>9.0E-08</td>
<td>PARP1</td>
<td>72.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rs2230926</td>
<td>1.0E-17</td>
<td></td>
<td>36.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rs610604</td>
<td>9.0E-12</td>
<td>TNAIP3</td>
<td>33.2</td>
</tr>
</tbody>
</table>

For screening potential overlaps between LXRz-binding sites and validated SNPs that were genetically associated with common diseases, we chose the NHGRI GWAS catalogue. We considered only those LXRz target sites that were within LD to the reported SNPs. Columns indicate the general category, associated diseases or terms and the SNP ID. The P-value indicates the degree of certainty of disease association of the SNP. Next column depicts the reported and potentially disease relevant genes in proximity to the associated SNP. The last column shows the distance between LXRz binding sites and SNPs within the LD block, presented in kilobases (kb).
obtained 32 novel LXR target genes. We selected LXR knockdown-sensitive genes with LXR knockdown in T0901317-treated foam cells compared with untreated, next step, we performed differential expression analysis using IPA (Figure 5C). We found two genes of the classical LXR/RXR activation pathway (IPA top canonical pathway) and their further subsequent impact on foam cell development. Therefore, we selected differentially expressed and LXR knockdown-validated target genes in foam cells and generated a network with interaction data from the FANTOM and STRING database (Figure 5A). The introduced oxLDL stimulus led to activation of 160 genes and repression of 93 genes. The involvement of various transcription factors such as CCAAT-enhancer-binding proteins CEBPB and CEBPG or NF-kB1 illustrated once more the broad impact of ligand-induced LXR activation on multiple metabolic processes regulated by LXR itself, interacting transcription factors and other genes. For functional classification, we applied Ingenuity Pathway Analysis (IPA) and could annotate molecular functions, such as lipid metabolism, inflammation and cell death. The apoptotic function of these cells was already observed in the stringent set of LXR target genes (Supplementary Table S2). In the next step, we performed differential expression analysis of T0901317-treated foam cells compared with untreated, diseased foam cells. We selected LXR knockdown-sensitive genes with LXR binding site in this model and obtained 32 novel LXR target genes and six previously described genes. We subjected all 38 genes to IPA and revealed all 38 genes to be direct LXR target genes. Bold circles represent genes with an enriched LXR binding site close by.

(49). First, we analysed the direct effects of LXR binding and their further subsequent impact on foam cell development. Therefore, we selected differentially expressed and LXR knockdown-validated target genes in foam cells and generated a network with interaction data from the FANTOM and STRING database (Figure 5A). The introduced oxLDL stimulus led to activation of 160 genes and repression of 93 genes. The involvement of various transcription factors such as CCAAT-enhancer-binding proteins CEBPB and CEBPG or NF-kB1 illustrated once more the broad impact of ligand-induced LXR activation on multiple metabolic processes regulated by LXR itself, interacting transcription factors and other genes. For functional classification, we applied Ingenuity Pathway Analysis (IPA) and could annotate molecular functions, such as lipid metabolism, inflammation and cell death. The apoptotic function of these cells was already observed in the stringent set of LXR target genes (Supplementary Table S2). In the next step, we performed differential expression analysis of T0901317-treated foam cells compared with untreated, diseased foam cells. We selected LXR knockdown-sensitive genes with LXR binding site in this model and obtained 32 novel LXR target genes and six previously described genes. We subjected all 38 genes to IPA and obtained the most enriched network functions in carbohydrate metabolism, molecular transport and lipid metabolism (Figure 5B and Supplementary Figure S6A). To gain more insight in the involvement of the underlying genes in classical LXR/RXR activation pathway (IPA top canonical pathway P-value 1.65e-3), we performed an overlap analysis using IPA (Figure 5C). We found two genes directly connected with LXR (SCD, MYLIP, Supplementary Figure S6B) and 10 indirect interactions in this pathway, the latter are tightly bound on interactions with the nuclear factor of kappa light polypeptide gene enhancer in B-cells (NF-kB) complex and tumour necrosis factor-α (TNFA) signalling. Our previous analysis revealed all 38 genes to be direct LXR target genes, indicating a strong bond between LXR, NF-kB and TNFA signalling. Interestingly, arresten [COL4A1, collagen chain of basement membranes, (50)] and chemokine orphan receptor 1 [CXCR7, member of the G-protein coupled receptor family, (51)] were associated with cardiovascular disease (IPA top disorder, P-value 1.07e-3). Both genes were significantly upregulated after T0901317 treatment in foam cells. The highest upregulation (~6-fold) showed the interferon-induced guanylate-binding protein 2 (GBP2, Supplementary Figure S6B). This GTPase has not been previously related to LXR but was found to be associated with diseases such as psoriasis, rheumatoid arthritis and experimentally induced diabetes. One of the highest downregulated (~2-fold) genes was protein inhibitor of activated STAT 4 (PIAS4, Supplementary Figure S6B), which interacts with the NF-kB complex (52).

In summary, this analysis revealed a number of so far unknown factors of the artheroprotective network that were found to be enriched in functions, such as carbohydrate metabolism, molecular transport and lipid metabolism. These new genes can be of important value for thorough understanding and future investigations of transcriptional regulation in cellular atherosclerosis processes and ligand-specific activation of LXRα.

Figure 4. Functional connection between LXRα binding to APOE/C1/C2 locus and disease-relevant SNPs. (A) UCSC genome browser picture of the rs4420638 SNP and in linkage disequilibrium (DistiLD Database, Palleja et al., 2011) surrounding area (chr19: 45247846-45544890). SNP position is indicated with a black arrow on top of normalized tag alignment tracks of LXRα ChIP-seq. LXRα-binding sites (black arrows under track) around apolipoprotein C1 gene (APOC1). LXRα enrichment in macrophages (grey), in T0901317 treated macrophages (brown), in foam cells (green) and in T0901317-treated foam cells (blue) is depicted. (B) STRING interaction network of genes within the extended APOE/C1/C4/C2-gene cluster with addition of well-known LXRα target genes. Differential expression of foam cells compared to macrophages is presented. Red circles indicate upregulated genes, and blue circles show downregulated genes. Grey lines represent the STRING network confidence view. Asterisk indicates LXR knockdown-sensitive genes. Bold circles represent genes with an enriched LXRα-binding site close by.
DISCUSSION

Comprehensive analyses are important to decode the complex molecular networks that underlie cell physiology and common diseases (53). In contrast to conventional molecular biological studies that usually focus on individual target genes of a nuclear receptor, we aimed to identify gene networks of LXRα that coherently respond to natural ligands or to pharmacological intervention in foam cells and macrophages. To make the analysis most stringent, and to largely exclude any potential off-target effects of LXR ligands, we considered in our network analyses only LXRα loci that showed significant effects on gene expression and were additionally sensitive to LXR knockdown.

In this study, we analysed human macrophages, which in stark contrast to mouse macrophages show a characteristic auto-regulatory loop of LXRα activation. In general, studies on atherosclerotic plaque development in mouse...

Figure 5. Beneficial effects of LXRα modulation with synthetic ligand T0901317 in foam cells. (A) LXRα target gene network in foam cells. The differential expression of LXR knockdown-sensitive genes is displayed in foam cells compared with macrophages. Well-known and physiologically relevant target genes in lipid metabolism as well as in inflammation and apoptosis are emphasized. Red circles indicate upregulated genes, and blue circles show downregulated genes. Grey lines represent the FANTOM and STRING network. Bold circles represent genes with an enriched LXRα-binding site close by. (B) T0901317-specific LXRα target genes in foam cells. The differential expression of LXR knockdown-sensitive genes in T0901317 foam cells is shown compared to foam cells. Red circles display upregulated genes, and blue circles indicate downregulated genes. Bold circles represent genes with an enriched LXRα-binding site close by. Grey lines represent LXR knockdown-validated interaction with LXRα. The top associated network functions assigned by IPA are carbohydrate metabolism, molecular transport and lipid metabolism. (C) LXR/RXR activation pathway with direct involvement of T0901317-specific LXRα target genes on T0901317 treatment in foam cells compared to untreated foam cells are visualized in red for significantly upregulated genes and in blue for significantly downregulated genes. Unregulated gene expression of central gene products is displayed in white. Bold circles represent an enriched LXRα-binding site near the gene.
In contrast to the shared and T0901317-specific peaks at known lipid metabolism relevant target genes LXRz and APOC1, we discovered for the foam cell-specific peaks unexpected gene loci: for example the novel LXRz target gene PARP1, which represents an enzyme that is known to be involved in DNA damage repair. Recently, it has been shown that inhibition of PARP1 leads in particular in brown adipose tissue and skeletal muscle to enhanced mitochondrial metabolism via activation of the histone deacetylase SIRT1, which culminates in protection against metabolic disease (61). Interestingly, in diseased foam cells, we detected increased oxLDL triggered LXRz binding at the PARP1 locus but striking absence of LXRz binding at this locus under T0901317 therapy. Thus, LXRz modulation by an efficient synthetic LXR ligand may play a so far unexplored role in counteracting the effects of (basal) PARP1 gene binding effects of oxLDL-activated LXRz.

Recent changes in human life style and nutrition may overstrain the natural balance in foam cells to reverse cholesterol transport. The synthetic LXRz ligand was more efficient in triggering sequence-specific binding and thereby controlling more stringently LXRz transcriptional regulation. This genome-wide effect form increased atheroprotective effects. In summary, we identified disease-causing and pharmacologically treatable gene networks that can be modulated by specific intervention. Altogether, we found 32 novel LXRz target genes with atheroprotective potential. In contrast to natural oxysterols, T0901317 treatment resulted in striking binding of LXRz at the APOE/C1/C4/C2-gene cluster and its surrounding genes. This genomic region has been found to be highly significantly associated with the disease terms “LDL-cholesterol” and “C-reactive protein”, which are found to be highly significantly associated with the disease terms “LDL-cholesterol” and “C-reactive protein”, which suggests that specific ligand-induced LXRz binding—in conjunction with other transcriptional regulators—at this locus is important for establishing a key network of cholesterol transport and anti-inflammatory genes.

Quantitative LXRz-binding studies using clinical samples of diseased patients and control subjects may reveal sequence-dependent variation in LXRz binding efficiency, and thus explain the functional impact of genetic variation in the APOE/C1/C4/C2-gene locus for cardiovascular or other diseases.

We have generated LXRz genomic loci and related expression data in human foam cells, which may also provide a resource for developing further mechanistic studies, including the use of alternative small molecule activators of LXR, such as GW3965 (62) to analyse ligand-dependent fine-tuning of LXRz binding—in conjunction with other transcriptional regulators—at this locus important for establishing a key network of cholesterol transport and anti-inflammatory genes.

In summary, our data revealed an LXRz ligand-dependent network of transcriptional regulation, which is vulnerable to molecules that efficiently activate LXRz and shape gene expression patterns. Single gene analysis approaches may hit the limits of reductionism when biological function emerges on the interaction network level of genes or gene products (63). Although genome-wide DNA binding of LXRz may be deduced from binding or reporter gene assay efficiencies, expression can vary because of ligand-dependent differential interaction with...
further transcriptional co-factors leading to varying response on the gene network level (24).

Integration of gene, protein or metabolic networks (64,65), will potentially generate an additional layer of complementary information to provide an even more comprehensive understanding of complex interconnected molecular pathways in cell metabolism during atherogenesis and to optimize treatment of cardiovascular disease.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Tables 1–3, Supplementary Figures 1–6, Supplementary Methods, Supplementary Datasets 1–4 and Supplementary References [66–70].

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