Identification of human low-density lipoprotein receptor as a novel target gene regulated by liver X receptor alpha

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Abstract Liver X receptor alpha (LXRα) is a member of the nuclear receptor superfamily that is activated by oxysterols, and plays a pivotal role in regulating the metabolism, transport and uptake of cholesterol. Here, we demonstrate that LXRα also regulates the low-density lipoprotein receptor (LDLR) gene, which mediates the endocytic uptake of LDL cholesterol in the liver. An LXR agonist induced the expression of LDLR in cultured hepatoblastoma cells. Moreover, the LDLR promoter contained an LXR response element that was recognized by LXRα/RXRα heterodimers in hepatoblastoma cells. These results suggest a novel pathway whereby LXRα might modulate cholesterol metabolism.

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1. Introduction

The hepatic low-density lipoprotein receptor (LDLR) mediates the endocytic uptake of LDL cholesterol, thereby controlling cholesterol levels in cells and plasma [1]. Cholesterol regulates LDLR transcription through a negative-feedback mechanism [2]. When cellular cholesterol levels rise, LDLR transcription is reduced. When cellular cholesterol storage is depleted, LDLR transcription is activated. This regulation is controlled through specific interactions of the sterol-regulatory element (SRE) of the LDLR promoter and the family of SRE-binding proteins (SREBP-1 and SREBP-2) [3]. SREBP-1 preferentially activates genes involved in lipogenesis, whereas SREBP-2 preferentially activates genes involved in the cholesterol biosynthetic pathway [4].

Liver X receptor alpha (LXRα) is a member of the nuclear receptor superfamily that forms a functional heterodimer with retinoid X receptors (RXRs) [5]. LXRα/RXR heterodimers bind to DR4-type sequence elements, known as LXR response elements (LXREs), in their target genes. LXRα is highly expressed in the liver, intestine, macrophages and adipose tissue. It is reported to be activated by a specific class of oxidized derivatives of cholesterol [6,7].

Studies in the liver, activation of LXRα induces the transcription of multiple genes involved in bile-acid synthesis (CYP7A1) and sterol efflux (ABCG5 and ABCG8) [6,7], whereas LXRα regulates modulates de novo fatty-acid biosynthesis by directly facilitating SREBP-1c gene expression [6,7]. These effects have led to the suggestion that LXRα is a sensor of the balance between cholesterol and fatty-acid metabolism.

Both LXRα and LDLR play a pivotal role in the control of the cholesterol levels in the liver. Thus, we aimed to evaluate the relationship between LXRα and LDLR. In the current study, we show that a synthetic LXR ligand regulates expression of the LDLR gene in hepatoblastoma cells. Consequently, we demonstrate that the LDLR is a direct target for gene expression by LXRα/RXRα heterodimers. These results suggest a novel pathway whereby LXRα might become as a factor of cholesterol homeostasis.

2. Materials and methods

2.1. Materials

We obtained cholesterol, 25-hydroxycholesterol (25-HC), sodium mevalonate and lipoprotein-deficient serum (LPDS) from Sigma. T0901317 was obtained from Cayman Chemical. The HMG-CoA reductase inhibitor, pitavastatin, was obtained from Kowa Co. LTD., Japan.

2.2. Plasmid constructs

The expression plasmid for human LXRα (pcDNA3-hLXRα) was constructed as described previously [8]. To generate human LDLR promoter reporter plasmids, LDLR promoter containing ~4000 to ~57 bp (LDLR4000) was obtained by PCR with human genomic DNA from HepG2 cells. The promoter was cloned into the pGL4.10[luc2] vector (Promega). A deletion construct and site-directed
mutations were introduced into the LDLR promoter by PCR methods. The nucleotide sequences of these plasmids were confirmed by an ABI PRISM® 310 Genetic Analyzer (Applied Biosystems).

2.3. Cell culture
Huh-7 cells were cultured in DMEM medium containing 7.5% foetal bovine serum (FBS). For ligand treatments, cells were cultured in DMEM, supplemented with 5% charcoal/dextran treated FBS (HyClone) or 5% LPDS and LXR ligands. In some experiments, cells were supplemented either with 10 µg/ml cholesterol plus 1 µg/ml 25-HC or 50 µM pitavastatin plus 50 µM mevalonate.

2.4. Quantitative real-time PCR
Real-time PCR was performed as described previously [9], using 25 ng template cDNA for each reaction. 18S rRNA was used for normalizing the expression data.

2.5. Immunoblot analysis
Each cell extract (25 µg) was resolved by 7.5% SDS-PAGE, and electroblotted to nitrocellulose membranes. Western blot analyses were carried out using anti-human LDLR antibody (R&D System) or anti-GAPDH antibody (Chemicon). The signals were visualized using SuperSignal West Dura Extended Duration chemiluminescence substrate (Pierce).

2.6. Transfections and luciferase assay
The luciferase assay was performed as described previously [9]. Briefly, HepG2 cells were seeded at a density of 1.6 × 10^5 cells/well on 96-well plates in the presence of 10 µg/ml cholesterol and 1 µg/ml 25-HC 14-18 h before transfection. The cells were transfected with 50 ng LDLR reporter plasmid, 40 ng pgluc-luc2Z and 20 ng expression vector (pcDNA3 or pcDNA3-hLXRα). Twenty-four hours after transfection, the cells were incubated with DMSO or 5 µM T0901317 in a medium containing 10% LPDS, 10 µg/ml cholesterol and 1 µg/ml 25-HC. After 24 h, assays for both luciferase and β-galactosidase were performed. We used pGL4.10(luc2) as a negative control vector and corrected each value of the reporter gene expression as compared to the value of the control vector. These values are expressed as fold activation compared with the control (CMV/DMSO: control vector and DMSO treatment) in each LDLR-reporter construct.

2.7. Electrophoretic mobility shift assay (EMSA)
Nuclear extracts were prepared from cultured HepG2-tet-off-hLXRα cells in the absence of doxycycline for 4 days. EMSAs were performed as described previously [8,9]. In the competition studies, a 10-fold or 50-fold molar excess of unlabeled DNAs were added to the reaction mixture. Supershift assays were performed by using anti-human LDLR antibody (R&D System) or RXRα (K5808, Perseus Proteomics) antibodies. Double-stranded DNAs composed of the following sequences were used for the binding and competition assays: human LDLR LXRE wild type, 5'-GGTCCA-GACCTGCCCCTGACCTCTAG-3'; LDLR LXRE mutant, 5'-GGTCCA-GACCTGCCCCTGACCTCTAG-3'; and rat CYP7A1 LXRE wild type, 5'-GGTCCA-GACCTGCCCCTGACCTCTAG-3' (mutated bases are denoted by lowercase letters).

2.8. Chromatin immunoprecipitation (ChIP) assay
ChIP assays were performed as described previously [9]. HepG2-tet-off-hLXRα cells were cultured in the absence of doxycycline for 4 days. The cells were treated with 5 µM T0901317 for 8 h. Antibodies for LXRα (PPZ0412, Perseus Proteomics) or RXRα (K5808, Perseus Proteomics) antibodies. Double-stranded DNAs composed of the following sequences were used for the binding and competition assays: human LDLR LXRE wild type, 5'-GGTCCA-GACCTGCCCCTGACCTCTAG-3'; LDLR LXRE mutant, 5'-GGTCCA-GACCTGCCCCTGACCTCTAG-3'; and rat CYP7A1 LXRE wild type, 5'-GGTCCA-GACCTGCCCCTGACCTCTAG-3' (mutated bases are denoted by lowercase letters).

2.9. Statistical analysis
All data are presented as the mean ± standard error (S.E.M.). Statistical analyses were performed by unpaired Student’s t-test or Dunn-Bonferroni’s test using Stata/SE version 2.0 (OMS, Japan).

3. Results and discussion
3.1. LXR agonist induces LDLR expression in human hepatoblastoma cells
LDLR expression is regulated by intracellular sterol concentrations [1,2]. Therefore, various cellular sterol conditions were used to analyze the regulation of LDLR gene expression in human hepatoblastoma cells. The mRNA expression of LDLR gene was increased by the synthetic LXR agonist T0901317 in sterol-depleted cells (pitavastatin or LPDS treated cells), in which levels of nuclear SREBP-1 were expected to be high (Fig. 1A). A significant induction of LDLR expression was also observed in LPDS medium supplemented with sterols (cholesterol), under which conditions SREBP cleavage was suppressed. This induction effect by T0901317 was detected in LDLR protein levels, as well as mRNA levels (Fig. 1B). Thus, the LXR agonist induced the expression of LDLR in hepatocytes under various cellular sterol conditions.

3.2. LXR transactivates the human LDLR promoter
To investigate the effect of T0901317 on the transcription of LDLR, we cloned this gene from human genomic DNA and constructed a luciferase reporter plasmid containing its 5'-flanking region (−4000 to +57 bp) (Fig. 2A). Human hepatoblas-
toma cells were transiently transfected with the LXRα expression vector along with the luciferase reporter constructs. As shown in Fig. 2 B, the LDLR promoter (LDLR4000) was strongly enhanced by LXRα in a ligand-dependent manner. However, LDLR expression was also regulated by SREBPs through the SRE [3,10]. Because LXRs activate SREBP-1 expression by binding to LXRE in the SREBP-1 promoter [6,7], it is possible that LXRα-mediated regulation of the LDLR gene is secondary to the induction of SREBP-1. In fact, we also detected the induction of SREBP-1 by the LXR agonist in hepatoblastoma cells (data not shown). To investigate whether LXRα can directly regulate LDLR expression in a SREBP-1-independent manner, we generated a construct containing a point mutation in the SRE (C065 to C056 bp) sequence. The 4.8-fold induction of luciferase activity mediated by LXRα on the wild-type promoter construct (LDLR4000) was reduced to 1.8-fold induction when the SRE was mutated (LDLR-Sm). This result demonstrated that LXRα can up-regulate the LDLR promoter, even within the mutations of the SRE sequence.

This finding raises the possibility of the existence of an LXRE in the promoter of the human LDLR gene. LXRα/RXR heterodimers are known to bind to DR4. We analyzed the sequence of the human LDLR (−4000 to +57 bp) for LXRE, as predicted by NUBIScan [11], and found a potential binding site (−3788 to −3773 bp) (Fig. 2A). In order to certify that the effects of LXRα on the human LDLR promoter are mediated by this potential LXRE, we analyzed the reporter gene assays using LDLR promoter constructs carrying mutations and deletions (Fig. 2B). Mutation of the potential LXRE site partly reduced the magnitude of LXRα activation (LDLR-Lm), and simultaneous mutations of both the potential LXRE and the SRE sites virtually abolished the effect of LXR agonist (LDLR-LSm). Furthermore, deletion of the sequence between −4000 and −3773 bp (LDLR3772) partly reduced reporter activity as well as LDLR-Lm. When the SRE of the LDLR3772 construct was mutated, LXRα-mediated transactivation was fully abolished.

We further tested whether the potential LXRE could function as a cis-acting element. We placed three copies of this sequence upstream of a minimal thymidine kinase promoter and luciferase reporter gene (TK-LXREx3). When co-transfected with the LXRα expression vector, the TK-LXREx3 reporter was activated (1.7-fold induction) by the LXR agonist, whereas the empty vector TK was not responsive (Fig. 2C). Collectively, these data are consistent with regulation of the LDLR promoter by LXRα.

3.3. LXRα/RXRα heterodimers bind directly to the LXRE in the LDLR promoter

Fig. 3A shows the consensus sequence for canonical LXRE [7]. The sequence of the LDLR LXRE was very similar to

![Diagram of LXRE](image)

Fig. 2. LXRα modulates human LDLR promoter activity via an LXRE located between −3788 and −3773 bp. (A) Scheme representing the luciferase reporter gene containing the LDLR promoter sequence. Wild-type and mutated LXRE or SRE sequences (underlined) are shown. (B, C) Reporter genes were co-transfected into HepG2 cells with empty vector or LXRα expression vector. Transfected cells were treated with DMSO or 5 µM T0901317. These assays were performed in a medium containing 10 µg/mL cholesterol plus 1 µg/mL 25-HC. Data are presented as luciferase activity normalized to β-galactosidase activity. All values are expressed as the mean ± S.E.M. (n = 3–5). The statistical significance is indicated by *P < 0.05 or **P < 0.01.

Fig. 3. LXRα/RXRα heterodimers bind to the LXRE in the −3788/−3773 region of the human LDLR gene. (A) Sequence of LXRE consensus, LDLR LXRE, and mutant LXRE. (B) A radiolabeled DNA probe corresponding to nucleotides −3788 to −3773 of the LDLR promoter was incubated with nuclear extracts, with or without an excess of unlabeled probe corresponding to LDLR-LXRE (LDLR), the mutated LXRE motif (LDLRmut) or the identified LXRE from the rat CYP7A1 gene (CYP7A). (C) Supershift experiments were performed with anti-LXRα (LXR), anti-RXRα (RXR) and anti-SR-A (SRA) monoclonal antibodies. Closed and open arrowheads indicate the specific shift bands and the supershift bands, respectively.
consensus sequence. To determine whether LXRα/RXR heterodimers bind to this LXRE site in the LDLR promoter, an EMSA was performed using this response element as a radio-labeled probe (Fig. 3B). A labeled probe was shifted by the incubation of nuclear extracts from human LXRα stable transfectants of HepG2 cells (lane 2). This labeled complex formation was disrupted by increasing amounts (10-fold and 50-fold excess) of unlabeled self-competitor (LDLR) and rat CYP7A1 LXRE fragments (CYP7A), but not by the mutated LXRE (LDLRmut) probes (lanes 3–8). Furthermore, no protein–DNA complexes were observed when using the mutated LXRE (LDLRmut) probe (lane 9). Monoclonal antibodies directed against LXRα (LXR) and RXRα (RXR) gave super-shifted bands (Fig. 3C, open arrowheads in lanes 11 and 12). As expected, a control antibody (scavenger receptor A; SRA) had no significant effect (lane 13). These results demonstrate that LXRα/RXRα heterodimers bind to the LXRE site at –3788 to –3773 bp.

To investigate whether LXRα/RXRα heterodimers bind to the LXRE in cells, we performed ChIP assays in human LXRα stable transfectants of HepG2 cells. Fragmented chromatin from formaldehyde cross-linked cells was incubated with an anti-LXRα (LXR) or anti-RXRα (RXR) antibody. The precipitated LXRE was detected by PCR amplification of the LDLR promoter fragment (–3865 to –3678 bp) including the LXRE (–3788 to –3773 bp). Specific amplification was detected when antibody for LXRα or RXRα was used, but not in the case of the control IgG (Fig. 4). These results indicate that LXRα/RXRα heterodimers bind to the LXRE in the LDLR promoter in hepatoblastoma cells. LXRα is known to regulate a number of genes involved in cholesterol homeostasis [6,7]. The current study suggests that LXRα directly regulates the human LDLR gene. The mRNA expression of LDLR gene was induced by the LXR agonist T0901317 in HepG2 cells under various cellular sterol conditions (Fig. 1A). We also examined the up-regulation of LDLR mRNA by adding T0901317 to human functional liver 4 cells (FLC4) (data not shown). When we examined the effect of T0901317, this LXR agonist increased the expression of LDLR protein (Fig. 1B). In particular, in comparison with vehicle, the LXR agonist strongly induced the LDLR expression under a high cholesterol condition which suppresses endogenous SREBPs activity. These observations support that LXRα directly regulates the LDLR expression. Here, a functional LXRE has been identified in the human LDLR promoter (Fig. 2). Simultaneously, an LXR agonist influenced the SRE in the LDLR promoter. It is noteworthy that similar data have recently been reported for fatty acid synthase (FAS) [12] and ileal bile acid-binding protein (I-BABP) [13], suggesting that LXR and SRE independently but additively confer the LXR responsiveness. In fact, we also investigated that T0901317 and pitavastatin can additively activate the LDLR promoter (data not shown). Thus, the LDLR gene can be directly as well as indirectly up-regulated by LXRα. Finally, we elucidated that an LXRE in the human LDLR promoter was bound by LXRα/RXRα heterodimers in hepatoblastoma cells (Figs. 3 and 4).

The rodent LDLR LXRE has not been identified yet. There might be species differences between human and rodents. However, Rowe et al. [14] reported that T0901317 increased the mRNA expression of LDLR gene in murine macrophage J774A.1 cells. Masson et al. [15] reported the similar induction in the liver from C57BL6 mice by T0901317. It has been suggested that the effects of T0901317 might regulate sensitivity to alternations in the cholesterol content. We have shown here that LXRα regulates human LDLR expression through the direct interaction with the LDLR promoter. Further analyses are needed to determine whether an LXRE is presented or not in the LDLR promoter in rodents.

In conclusion, we have demonstrated for the first time that the SREBP-independent activation of LDLR expression by an LXR agonist occurs via a LXRE. Therefore, we suggest that LXRα affects plasma cholesterol levels and that LXRα agonists might be useful for the treatment of hyperlipidemia.

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