25-Hydroxycholesterol activates the expression of cholesterol 25-hydroxylase in an LXR-dependent mechanism

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**Running title:** Activation of LXR induces CH25H expression
**Abbreviations used:** ACC1, acetyl-CoA carboxylase 1; CH25H, cholesterol 25-hydroxylase; FASN, fatty acid synthase; HMGCR, HMG-CoA reductase; 25-HC, 25-hydroxycholesterol; ISG, IFN-stimulated gene; LXR, liver X receptor; LXRE: LXR response element; SCAP, SREBP cleavage-activating protein; SREBP2, sterol regulatory element binding protein 2; T317, T0901317
Abstract

Cholesterol 25-hydroxylase (CH25H) catalyzes the production of 25-hydroxycholesterol (25-HC), an oxysterol which can play an important role in different biological processes. However, the mechanisms regulating CH25H expression have not been fully elucidated. In this study, we determined that CH25H is highly expressed in mouse liver and peritoneal macrophages. We identified several liver X receptor (LXR) response elements (LXREs) in the human CH25H promoter. In HepG2 cells, activation of LXR by 25-HC or other oxysterols and synthetic ligands [T0901317 (T317) and GW3965] induced CH25H protein expression which was associated with increased CH25H mRNA expression. 25-HC or T317 activated CH25H transcription in an LXRE-dependent manner. Thus, high expressing LXRα or LXRβ activated CH25H expression, and the activation was further enhanced by LXR ligands. In contrast, inhibition of LXRα/β expression attenuated 25-HC or T317-induced CH25H expression. Deficiency of interferon γ expression reduced, but did not block, LXR ligand-induced hepatic CH25H expression. Activation of LXR also substantially induced macrophage CH25H expression. In vivo, administration of GW3965 to mice increased CH25H expression in both liver and peritoneal macrophages. Taken together, our study demonstrates that 25-HC can activate CH25H expression in an LXR-dependent manner, which may be an important mechanism to exert the biological actions of 25-HC.

Key words: cholesterol 25-hydroxylase; hepatocytes; 25-hydroxycholesterol; interferon-γ; liver X receptor
Introduction

25-Hydroxycholesterol (25-HC) is produced from cholesterol. Although several cytochrome P450 enzymes may complete the conversion of cholesterol into 25-HC in vitro, cholesterol 25-hydroxylase (CH25H) has been demonstrated to play a critical role in the production of 25-HC, both in vitro and in vivo(1). 25-HC can play an important role in different biological processes, particularly in the cholesterol metabolism. For instance, at the cellular level, 25-HC inhibits the activity of HMG-CoA reductase (HMGCR) to reduce cholesterol biosynthesis through inactivation of the sterol regulatory element binding protein 2 (SREBP2)(2). However, mice with deficiency of CH25H expression still have intact cholesterol metabolism(3), that challenges if 25-HC physiologically functions as a negative regulator of SREBP2 activity and cholesterol biosynthesis. 25-HC also substantially enhances cholesterol esterification by activating ACAT(4).

Liver X receptor (LXR) α and β are ligand-activated transcription factors. LXR can play several important roles in the regulation of macrophage cholesterol metabolism and hepatic lipogenesis(5). For instance, LXR activation by synthetic ligands increases ATP-binding cassette transporter A1 (ABCA1) or ABCG1 to enhance excess cellular free cholesterol efflux to the extracellular cholesterol acceptor, apolipoprotein AI (apoA-I) or high-density lipoprotein (HDL), thereby inhibiting formation of lipid-laden macrophage/foam cells and the development of atherosclerosis(6). In addition to synthetic ligands, such as T0901317 (T317) and GW3965, several oxysterols including 25-HC, can function as endogenous LXR ligands. Thus, 25-HC can also induce macrophage ABCA1/ABCG1 expression in an LXR-dependent manner(7). However, the effect of 25-HC on foam cell formation appears controversial.

In
vitro, treatment of macrophages with 25-HC increases cellular cholesterol accumulation and foam cell formation. In vivo, a high 25-HC level is found in the atherosclerotic lesion areas of ApoE deficient (ApoE−/−) mice (8). Meanwhile, activation of LXR induces expression of SREBP-1 and its target genes in hepatocytes, such as fatty acid synthase (FASN) and acetyl-CoA carboxylase 1 (ACC1), which can result in severe lipid accumulation in the liver(9).

The effects of 25-HC on inflammation may need more investigation since the controversial results have also been reported. Activation of macrophages lacking CH25H expression leads to overproduction of inflammatory interleukin-1 (IL-1) family cytokines(3), which indicates the anti-inflammatory effects of 25-HC. 25-HC also reduces LPS-induced macrophage TNF-α expression and secretion(10). In contrast, 25-HC enhances poly I:C-induced macrophage IL-6 production(11). In endothelial cells, 25-HC induces expression of adhesion molecules including intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1) and E-selectin, in an LXR-independent manner(12). 25-HC is also involved in other immunological processes. For instance, 25-HC secreted from activated macrophages can suppress TLR-induced immunoglobulin A production(13).

Expression of CH25H protein in bone marrow-derived dendritic cells and macrophages can be up-regulated by interferons (IFNs)(14). This finding has led to identification of CH25H as one of the IFN-stimulated genes, and 25-HC as a potent inhibitor of viral infection(15,16).

Cellular 25-HC production is mainly determined by CH25H activity indicating that CH25H expression can greatly influence 25-HC actions. For example, suppression of the IL-1
family cytokines and immunoglobulin A production by 25-HC is substantially impaired in mice lacking of CH25H expression (3,13). Macrophage CH25H expression can be activated by TLR4 ligands (e.g., LPS) and IFNs via an LXR independent manner (16). However, it remains unknown if the CH25H expression can be regulated by other mechanisms. Interestingly, injection of mice with TLR ligand induces CH25H expression in multiple tissues, with the greatest effect in the liver. LPS treatment also elevates serum 25-HC levels which solely depends on CH25H expression (13). Collectively, these findings indicate that both macrophages and hepatocytes may be an important source for 25-HC production. Indeed, we determined high CH25H expression in mouse liver and peritoneal macrophages in this study. By completing a sequence alignment analysis, we identified several LXR response elements (LXREs) in the human CH25H promoter. Combining the facts that 25-HC is an endogenous LXR ligand, both LXR expression and oxysterol production can be detected in the cells with high activity of cholesterol metabolism, such as hepatocytes and macrophages, we hypothesized that 25-HC can activate CH25H expression in an LXR-dependent manner, which may be an important mechanism to exert the biological actions of 25-HC.
Materials and methods

Reagents

25-HC, 22(R)-hydroxycholesterol and 22(S)-hydroxycholesterol were purchased from Sigma-Aldrich (St. Louis, MO). Synthetic LXR ligands, GW3965 and T317, were purchased from Cayman Chemical (Ann Arbor, MI). Rabbit anti-CH25H, anti-SREBP2, anti-FASN, anti-HMGCR, anti-β-actin and anti-GAPDH polyclonal antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX). Rabbit anti-ABCA1 polyclonal antibody was purchased from Novus Biologicals (Littleton, CO). Rabbit anti-LXRα, anti-LXRβ and anti-ABCG1 polyclonal antibodies were purchased from Proteintech (Chicago, IL). Dual-Luciferase assay kit was purchased from Promega (Madison, WI).

Cell lines

RAW264.7 and HepG2 cell lines were purchased from ATCC (Manassas, VA), and cultured in complete RPMI 1640 and DMEM medium containing 10% FBS, 50 μg/ml streptomycin, 100 units/ml penicillin and 2 mM glutamine, respectively. Peritoneal macrophages and primary hepatocytes were isolated from male C57BL/6 mice and IFNγ deficient (IFNγ−/−) mice (C57BL/6 background), respectively, as described(17,18). The protocols for experiments with mice were approved by the Ethics Committee of Nankai University and conformed to the Guide for the Care and Use of Laboratory Animals published by NIH. Cells received treatment in serum-free medium.

cDNA synthesis and quantitative real time PCR (qRT-PCR)
Total RNA was extracted from cells followed by cDNA synthesis as described (19). Real-time PCR was conducted using SYBR Green PCR Master Mix (Bio-Rad) and the following primers: homo-CH25H forward, 5’-ATGTTGACCACGTGGAAGGT-3’, and homo-CH25H backward, 5’-TGAAAACTGTTTTGTTGAG-3’; mus-CH25H forward, 5’-CCAGCTCTTAAGTCACGTC-3’, and mus-CH25H backward, 5’-TGGGAACTGTTTTCTTTGGG-3’; homo-GAPDH forward, 5’-ACAACCTTTGCTACTTTGTGAA-3’, and homo-GAPDH backward, 5’-GATGCAGGGATGATGTTCTG-3’, mus-β-actin forward, 5’-GATGCAGGGATGATGTTCTG-3’, and mus-β-actin backward, 5’-TTCTTTGAGCTCCTTCGTTGTTG-3’. CH25H mRNA expression was normalized by GAPDH or β-actin mRNA in the corresponding samples.

**Western blot analysis**

Expression of CH25H, LXRα, LXRβ, SREBP1, SREBP2, FASN, HMGCR, ABCA1, ABCG1 and GAPDH protein was determined by Western blot with cellular proteins extracted from HepG2 cells, primary hepatocytes, RAW264.7 cells, peritoneal macrophages or mouse liver as described (20). Briefly, after treatment, cells were washed with PBS and then lysed in an ice-cold lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 1 mM PMSF, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 50 μg/ml aprotinin and leupeptin). A piece of mouse liver was removed and homogenized in the same lysis buffer above. Cellular lysate or liver homogenate was centrifuged for 10 min at 4°C at 13,000 rpm with a Microfuge. The supernatant was collected as the whole cellular extract or
whole tissue protein. After determination of concentration, an equal amount of protein from each sample was loaded on a SDS-PAGE. After electrophoresis, the proteins were transferred onto a nylon-enhanced nitrocellulose membrane. The membrane was blocked with a solution of 0.1% Tween 20/PBS (PBS-T) containing 5% dry fat-free milk for 1 h followed by incubation with primary antibody overnight at 4°C. After re-blocking with PBS-T containing 5% milk, the blot was incubated with goat horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody or rabbit HRP-conjugated anti-mouse secondary antibody for 1 h at room temperature (RT). After washing 3 times for 10 min of each time with PBS-T, the membrane was incubated for 1 min in a mixture of equal volume of Western blot chemiluminescence reagent 1 and 2. The membrane was then exposed to X-ray film or subjected to C-DiGit Blot Scanner (Li-cor, Lincoln, NE).

**Immunofluorescent staining**

After treatment, expression of CH25H, HSPA5 or ATP1A1 protein in HepG2 cells was determined by immunofluorescent staining as described(21). Briefly, cells were fixed with 4% paraformaldehyde for 30 min, washed with PBS for 10 min, and blocked with 2% BSA for 2 h at RT. After incubation with primary antibody overnight at 4°C, cells were incubated with either Rhodamine- or FITC-conjugated secondary antibody for 2 h at RT. Cells were also stained with DAPI solution for the nucleus. Cells were observed under a fluorescence microscope (Leica, Wetzlar, Germany), and the images were photographed.

**Construction of LXRα or LXRβ expression vector, normal or mutated CH25H promoter(s),**
and determination of promoter activity

The cDNAs encoding LXRα and LXRβ cloned into pEGFP-C2 (C2) vector were constructed, and expression of exogenous LXRα and LXRβ protein was confirmed by Western blot as described(20).

The DNA for the human CH25H promoter (from -962 to +64) was generated by PCR with genomic DNA isolated from HepG2 cells and the following primers: forward, 5’-GGTACCTTGACGAACAACGCAGGTGG-3’; backward, 5’-GATATCGAGCAGTTGTGGCAGCTCAT-3’. The DNA was then ligated into pGL4.10 luciferase reporter vector, and the constructed normal CH25H promoter was named as pCH25H. The promoters with LXRE mutation(s) as indicated in the inserted box in Figure 3E was constructed with pCH25H and the primers containing mutated sequences using the Phusion Site-Directed Mutagenesis kit (New England Biolabs), respectively.

To determine normal or mutated pCH25H promoter activity, HepG2 cells were transfected with DNA for normal or mutated pCH25H promoter plus DNA for Renilla (for normalization of transfection efficiency) using Lipofectamine™ 2000 (Invitrogen). After 20 h of transfection plus treatment, cells were lysed with the lyse buffer supplied in the Dual-Luciferase Reporter Assay System (Promega, Madison, WI). The cellular lysate was then used to determine firefly and Renilla luciferase activity, respectively, as described(22). The promoter activity in each sample was initially obtained by dividing firefly luciferase activity with Renilla luciferase activity in the same sample. It was further normalized to the promoter activity of the control group (normal pCH25H promoter or normal pCH25H plus the C2 empty vector), and the promoter activity in the control group was defined as 1.
CRISPR-Cas9-mediated deletion of LXRα or LXRβ expression

HepG2 cells lacking LXRα or LXRβ expression were established using the clustered regulatory interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein 9 (Cas9) technology, respectively(23). The guide RNA was designed by the online CRISPR Design Tool (http://tools.genome-engineering.org). The sequences of the guide oligos for LXRα and LXRβ are 5’-TCGGCTTCGCAAATGCCGTC-3’ and 5’-ACCCCGGCAGGCATAGCGCC-3’, respectively. After ligation, HepG2 cells were transfected with plasmid of pSpCas9 (BB)-2A-Puro vector or vector ligated with guide oligos, respectively. The selection of mutated clonal cell lines was completed using the standard protocol(23), and the deletion of target gene expression was confirmed by Western blot. The cells lacking LXRα and LXRβ expression were defined as CRISPR-LXRα and CRISPR-LXRβ cells, respectively, while the corresponding control cells were defined as CRISPR-Ctrl cells. To further determine the role of LXRs in the CH25H-induction, CRISPR-LXRα cells were transfected with LXRβ siRNA followed by T317 treatment. The siRNA sequence against LXRβ was purchased from Santa Cruz Biotechnology (catalog #: sc-45316).

Chromatin immunoprecipitation (ChIP)-qPCR analysis

The binding of LXREs in the human CH25H promoter with LXRα or LXRβ protein was determined by ChIP-qPCR methods. Briefly, 5x10⁷ HepG2 cells were treated with 25-HC or T317 overnight followed by isolation of chromatin(22). The input PCR was conducted with DNA extracted from the sonicated chromatin after reversal of the cross-linking.
Immunoprecipitation was conducted with the same amount of chromatin from each sample based on the input and anti-LXRα, anti-LXRβ or anti-STAT1 (as a positive control antibody) polyclonal antibody, or normal IgG (as a negative control antibody). The qPCR was conducted with the detailed protocol as described (24) and the corresponding primers were listed in the legend of Figure 3.

_Determination of cellular total cholesterol and triglyceride levels_

HepG2 cells in serum-free medium were treated with T317 or 25-HC for 16 h. After treatment, total cellular proteins and total lipids were extracted, respectively, followed by determination of total cholesterol and triglyceride levels using assay kits, and normalized by cellular protein levels (25).

_Isolation of primary hepatocytes and in vivo studies_

Both C57BL/6 wild type mice and IFN-γ deficient (IFN-γ−/−, C57BL/6 background) mice were purchased from the Animal Center of Nanjing University, and the protocol for the animal study was approved by the Animal Ethics Committee of Nankai University.

Mouse primary hepatocytes were isolated from C57BL/6 or IFN-γ−/− mice by a collagenase perfusion method. Briefly, after the animals were anesthetized, the midline laparotomy was performed, and the inferior vena cava was cannulated with an angiocatheter. The liver was then perfused with 1 ml heparin (320 U/ml), 40 ml solution I (Kreb’s solution containing 0.1 mM EGTA) and 30 ml solution II (Kreb’s solution containing 2.74 mM CaCl2 and 0.05% collagenase I) at 37°C, sequentially. The perfused liver was then passed through a
400 μm screening size filter by flushing with the cold DMEM medium. The isolated hepatocytes were collected after centrifuge for 5 min at 50 g, re-suspended with DMEM medium and plated in 6-well plates (the cell density is ~1x10⁶ cells/well). The viability of the isolated hepatocytes was ~90% which was determined by the trypan blue exclusion method.

To determine if LXR activation can induce CH25H expression in mouse liver and peritoneal macrophages, C57BL/6 mice (males, ~8-week old) were randomly divided into two groups (5/group), and they then received the following treatment for one week: Control group, mice were fed normal chow; GW3965 group, mice were fed normal chow containing GW3965, a synthetic LXR ligand, at a dose of 20 mg/day/kg bodyweight. At the end of treatment, mice were anesthetized and sacrificed. Mouse blood, peritoneal macrophage and liver samples were collected. Blood was used to prepare serum. A piece of liver was used to prepare frozen sections or extract total lipids. The hepatic lipid content was determined by Oil-Red O staining of liver frozen sections and triglyceride (TG) quantitative assay with the liver total lipid extract as described(26). Total cellular protein and RNA were extracted from a piece of liver or peritoneal macrophages followed by determination of CH25H protein and mRNA expression by Western blot and qRT-PCR, respectively.

Data analysis

All experiments were repeated at least three times, and the representative results are presented. Data were presented as mean ± standard errors, and analyzed by a Student’s t-test using Prism 5 (GraphPad Software, Inc.). The significant difference was considered if P<0.05 (n≥3).
Results

Activation of LXR induces CH25H protein expression in HepG2 cells

Cellular 25-HC is mainly produced from cholesterol in the reaction catalyzed by CH25H. Therefore, CH25H protein levels in tissue/cell types may decide the rate of 25-HC production. We initially extracted total cellular proteins from mouse tissues and peritoneal macrophages, and then determined CH25H protein expression by Western blot. As shown in Figure 1A, CH25H protein is expressed in multiple tissues, particularly in the liver. Meanwhile, a high expression of CH25H protein was found in mouse peritoneal macrophages. In addition, we determined that CH25H mRNA levels in mouse brain, intestine, heart, adipose tissue and muscle are much lower than that found in peritoneal macrophages, by using a qRT-PCR method (Figure 1B). Therefore, in mice, liver or macrophages could be one of the main sources for CH25H expression as well as 25-HC production in vivo.

By completing a sequence alignment analysis, we identified several LXREs in the promoter of the human CH25H gene (Figure 3D). Thus, we speculated that CH25H expression can be induced by LXR activation. If this is in the case, 25-HC may also activate CH25H expression in an LXR-dependent manner, based on the fact that 25-HC is an endogenous LXR ligand. To test this hypothesis, we treated HepG2 cells, a human hepatic cell line, with synthetic LXR ligands (T317 and GW3965) and 25-HC at different concentrations for 16 h. The results in Figure 1C demonstrate that hepatic CH25H protein expression was induced by T317, GW3965 and 25-HC, in a concentration-dependent manner. The time course study (Figure 1D) shows that LXR ligands induced hepatic CH25H expression after a few hours of treatment, with quickest by 25-HC. In addition, the induction
of CH25H expression by these LXR ligands can last for 24 hours after treatment (Figure 1D).

In addition to 25-HC, other oxysterols can also function as endogenous LXR ligands to activate expression of LXR target genes. For instance, 22(R)-hydroxycholesterol is a potent LXR ligand inducing macrophage ABCA1 expression, while its stereo isoform, 22(S)-hydroxycholesterol, can also induce ABCA1 expression but to a lesser extent(7). In this study, we determined that both 22(R)-hydroxycholesterol and 22(S)-hydroxycholesterol induced CH25H expression, with a greater effect by 22(R)-hydroxycholesterol (Figure 1E). Thus, induction of CH25H expression by oxysterols may depend on its ability to activate LXR.

To further confirm the induction of hepatic CH25H protein expression by LXR activation, we conducted an immunofluorescent staining study with intact HepG2 cells following treatment with 25-HC and T317. Similar to the results found from our Western blots, Figure 1F demonstrates that 25-HC or T317 substantially increased CH25H protein expression in HepG2 cells.

In addition, by completing a co-immunofluorescent staining study with anti-CH25H and anti-HSPA5 (heat shock protein 5, a marker of ER) or anti-ATP1A1 (Na⁺/K⁺-transporting ATPase subunit α-1, a marker of plasma membrane) antibodies, we determined that CH25H co-localized with HSPA5 protein (up panel, Figure 1G), but not the ATP1A1 protein (bottom panel, Figure 1G), indicating that CH25H likely is an ER protein.

*Activation of LXR induces CH25H expression at the transcriptional level*

The results above demonstrate that activation of LXR by T317 and 25-HC can induce
CH25H protein expression. To determine if the induction of CH25H protein occurs at a transcriptional or/and post-transcriptional level, we treated HepG2 cells with cycloheximide to arrest protein synthesis in the absence or presence of T317 or 25-HC. We then determined the rate of CH25H protein degradation by Western blot analysis. As shown in Figure 2A, cycloheximide alone caused cellular CH25H protein to decline with time of treatment. Meanwhile, addition of T317 to cycloheximide did not change the rate of CH25H protein degradation (Figure 2A, upper panel). In contrast, the co-treatment of cells with cycloheximide and 25-HC caused a decrease in the rate of CH25H protein degradation (Figure 2A, lower panel). Therefore, both transcriptional and post-transcriptional regulation of CH25H expression by LXR activation occur.

To further define LXR activates CH25H transcription, we initially investigated the effect of LXR activation on CH25H mRNA expression. As shown in Figures 2B and 2C, both 25-HC and T317 increased CH25H mRNA levels in both concentration- and time-dependent manners. To directly determine the effect of LXR activation on CH25H transcription, we constructed a CH25H promoter, and found that activity of the normal CH25H promoter was increased by T317 or 25-HC treatment (Figure 3A); and that high expressing LXRα or LXRβ also induced CH25H promoter activity (Figure 3B, 3C).

By completing a sequence alignment analysis, we found 5 putative LXREs in the proximal region (~1,000 bp) of the human CH25H promoter (Figure 3D). LXRE is also named as a direct repeat 4 (DR4) since the 6 nucleotides on each side, which are separated by any four nucleotides, are repeated. We found that 5 of 6 nucleotides on each side are repeated in the LXRE1, which is more than any other LXREs, indicating that LXRE1 could be most
active. To determine the role of these LXREs in CH25H transcription, we constructed several CH25H promoters with mutation of each LXRE, and a promoter with mutations of all the LXREs (the mutated sequences are shown in the inserted box in Figure 3E). We determined that the normal CH25H promoter was activated by 25-HC and the high expressing LXRα/β. Meanwhile, we found that the mutation in each LXRE reduced CH25H promoter activity, with most occurring in the LXRE1 mutation, at the basal level. In addition, we determined that the promoter with the mutation in a single LXRE was still activated by LXR activation. Consequently, the mutations of all the 5 LXREs totally abolished the promoter basal activity, and no induction occurred to this mutated CH25H promoter by LXR activation (Figure 3E). Compared with other LXRE, we determined that the mutation of LXRE1 reduced promoter basal activity most, and the LXRE1 mutated CH25H promoter is barely activated by LXR activation (Figure 3E). These results further confirm that LXRE1 is important for CH25H transcription induced by LXR activation.

Next, we conducted a ChIP-qPCR to analyze the binding of LXRE1 with LXRα/β protein. In addition to anti-LXRα or anti-LXRβ antibody, we used normal IgG as a negative control antibody and anti-STAT1 as a positive control antibody because of an IFNγ-activated site (GAS) element in the CH25H promoter. At the basal level, compared with the substantial binding of the STAT1 protein with GAS, the binding of LXRα or LXRβ protein with LXRE1 was relatively moderate in control sample, (Figure 3F). However, treatment of cells with 25-HC or T317 clearly increased the binding of LXRα or LXRβ protein to the LXRE1 (left and middle panels, Figure 3F).
LXR expression is critical for induction of CH25H expression by 25-HC and T317

To determine if the induction of CH25H expression by T317 or 25-HC is directly mediated by LXR, we initially transfected HepG2 cells with an LXRα or LXRβ expression vector, and then treated the transfected cells with 25-HC. Figure 4A shows that high expressing LXRα or LXRβ induced CH25H expression, and the induction was further enhanced by 25-HC treatment.

Next, we established an LXRα or LXRβ knockout HepG2 cell line by the CRISPR-Cas9 method(23), and defined them as CRISPR-LXRα or CRISPR-LXRβ cells. The deletion of LXRα or LXRβ protein expression in these cells was confirmed by Western blot (Figure 4B). As shown in Figure 4C-4F, both CH25H protein and mRNA expression were activated by T317 and 25-HC in the control (CRISPR-Ctrl) cells. However, the activation of CH25H protein expression was partially abolished in cells lacking either LXRα or LXRβ expression (Figure 4C, 4D). Similarly, activation of CH25H mRNA expression by T317 or 25-HC was reduced in CRISPR-LXRα or CRISPR-LXRβ cells (Figure 4F, 4G). These results suggest that either LXRα or LXRβ can induce CH25H expression.

To strengthen our conclusions, we conducted another experiment to determine if LXR ligands can regulation CH25H expression in cells with inhibition of both LXRα and LXRβ expression. CRISPR-LXRα cells were transfected with LXRβ siRNA followed by T317 treatment. Compared to the T317-induced CH25H expression which was associated with increased LXRβ expression in CRISPR-Ctrl cells (Figure 4G, left panel), T317 barely activated CH25H expression in CRISPR-LXRα cells transfected with LXRβ siRNA (Figure 4G, right panel). Taken together, the data in Figures 3 and 4 strongly suggests that CH25H
expression can be activated by 25-HC or T317 in an LXR-dependent manner.

Activation of LXR has been demonstrated to play an important role in lipid metabolism. For instance, LXRα induces lipogenesis by activating the regulatory element binding protein 1 (SREBP1) which consequently enhances FASN expression(9). In contrast to activation of LXR and SREBP1 expression, 25-HC inactivates SREBP2 to inhibit 3-hydroxy-3-methylglutaryl CoA reductase (HMGCR) expression and cholesterol biosynthesis(2). In this study, we determined the effect of 25-HC or T317 on cholesterol and triglyceride (TG) biosynthesis in HepG2 cells. After treatment with T317 or 25-HC overnight, we found that both T317 and 25-HC inactivated SREBP2 by reducing the precursor of SREBP2 and mature SREBP2. Consequently, HMGCR expression was reduced by both T317 and 25-HC (Figure 5A). Associated with inhibition of HMGCR expression, we found that cellular total cholesterol levels were reduced (Figure 5C). Meanwhile, we observed that T317 and 25-HC influenced expression of SREBP-1 and FASN differently. T317 substantially induced SREBP-1 and FASN expression, while 25-HC had a slight effect on both (Figure 5B). As a result, cellular TG levels were increased by T317, but not by 25-HC (Figure 5D).

Activation of LXR can induce ABCA1 and ABCG1 expression to enhance cellular cholesterol efflux. We also determined that both T317 and 25-HC increased ABCA1 and ABCG1 levels in HepG2 cells (Figure 5E), which may be another mechanism contributing to the reduction of cellular cholesterol levels in response to T317 or 25-HC treatment (Figure 5C).

Deficiency of IFN-γ expression reduces, but does not block, LXR-induced hepatic CH25H expression
Expression of CH25H has been reported to be induced in LPS- or IFN-activated macrophages. We previously reported that IFN-γ is an LXR target gene(22). Thus, we speculated that IFN-γ might be involved in LXR-induced CH25H expression. To address this hypothesis, we isolated primary hepatocytes from wild type and IFN-γ knockout (IFN-γ−/−) mice, and treated these cells with 25-HC or T317. We found that deficiency of IFN-γ expression reduced CH25H expression at basal levels in control cells indicating the importance of IFN-γ in controlling CH25H expression. However, expression of CH25H was still activated by 25-HC or T317 in IFN-γ−/− cells (Figure 6A). In addition, we determined that 25-HC or T317 induced CH25H expression in a time-dependent manner, in hepatocytes isolated from wild type or IFN-γ−/− mice (Figure 6B). Taken together, the results in Figure 6 suggest that IFN-γ is important for CH25H expression; but the induction of CH25H expression by T317 or 25-HC occurs in an IFN-γ independent manner.

Activation of LXR also induces macrophage CH25H expression

Previous reports have demonstrated that macrophage CH25H is an IFN-induced gene(15,16). Macrophage is a major cell type producing IFNs, such as IFN-γ. Our results in this study demonstrate that the macrophage is another cell type which highly expresses CH25H (Figure 1A). Therefore, we completed the following experiment to determine if activation of LXR can also induce macrophage CH25H protein expression. Similar to hepatocytes, we determined that 25-HC, T317 or GW3965 induced CH25H protein expression in a dose-dependent manner (Figure 7A), and the induction occurred quickly (Figure 7B), in peritoneal macrophages collected from wild type mice.
In RAW264.7 macrophage cell line, we also observed that CH25H protein expression was activated by T317, GW3965 or 25-HC in both concentration- and time-dependent manners (Figure 7C, 7D). Combining the results we obtained from the studies with mouse primary hepatocytes (Figure 6) and HepG2 cells (Figures 1-4), we can make a conclusion that induction of CH25H expression by LXR activation can be independent of cell type or species.

**Activation of LXR induces CH25H expression in vivo**

To confirm if activation of LXR can induce CH25H expression in vivo, particularly in hepatocytes and macrophages, we fed wild-type mice normal chow or normal chow containing GW3965 at a dose of 20 mg/day/kg bodyweight for 1 week. After treatment, we collected mouse blood, liver and peritoneal macrophage samples individually. Similar to our in vitro studies, we found that expression of CH25H mRNA in liver and macrophages was increased by GW3965 (Figure 8A). Associated with increased CH25H mRNA expression, CH25H protein expression in liver or macrophages was also elevated by GW3965 treatment (Figure 8B).

Activation of LXR can induce hepatic lipogenesis and hypertriglyceridemia. Similar to previous reports(6), in this study we also determined that after one week of GW3965 treatment, TG levels in both serum and liver were increased (Figure 8C). Meanwhile, hepatic lipid accumulation induced by GW3965 was confirmed by Oil-Red O staining of liver frozen sections (Figure 8D).
Discussion

25-HC has emerged as an important molecule in different biological processes by functioning as a negative regulator of SREBP-2 activity and cholesterol biosynthesis, a mediator of inflammation and a potent inhibitor of viral infection. 25-HC can be generated from cholesterol by enzymatic or/and non-enzymatic reactions. Although a very small amount of 25-HC can be found in cholesterol crystals after a long time of autoxidation, it is difficult to find 25-HC in lipoproteins, such as LDL, even under strong oxidizing conditions in vitro(27). While it is possible to produce 25-HC as a side-product in a few enzymatic reactions. For instance, 25-HC can be produced in the reactions catalyzed by cytochrome P450 27A1 (CYP27A1, sterol 27-hydroxylase), CYP46 (cholesterol 24-hydroxylase) and CYP3A4, the enzymes responsible for production of 27-hydroxycholesterol, 24(S)-hydroxylcholesterol and 4-hydroxycholesterol, respectively(28-30). However, it still remains unclear if these enzymes can generate 25-HC in vivo.

CH25H does not belong to the cytochrome P450 enzyme family. It mainly localizes in the ER. The following evidence supports the role of CH25H in the production and actions of 25-HC in vivo. Macrophage CH25H can be up-regulated by LPS in a TLR4/TRIF-dependent manner. Compared with controls, much higher concentrations of 25-HC in serum can be determined in healthy volunteers receiving LPS injection(31). The overproduction of IL-1 family cytokines in activated macrophages isolated from CH25H deficient mice can be reduced to normal by 25-HC treatment(3). TLR activation induces immunoglobulin A production in cells lacking CH25H expression. However, the induction is blocked by extracellular 25-HC indicating that CH25H expression is critical for the production and
actions of 25-HC(13).

In this study, we demonstrated that the activated CH25H protein is localized in the ER (up panel, Figure 1F), which may be linked to the physiological relevance of its product, 25-HC, in the regulation of cholesterol biosynthesis. In the ER, 25-HC enhances the binding of SCAP (SREBP cleavage-activating protein) to Insigs (insulin-induced genes), thereby abrogating the movement of the SCAP-SREBP-2 complex to the Golgi apparatus, inactivating SREBP-2 and inhibiting HMGCR expression(2).

Regulation of CH25H expression in the immune system, particularly in macrophages, has been well defined. LPS can increase macrophage CH25H mRNA expression and 25-HC production in a TLR4-dependent manner(31). Activation of TLR4 by Kdo2-Lipid A (KDO) and activation of other TLRs by the corresponding ligands (TLR2 by peptidoglycan from Staphylococcus aureus and TLR2/6 by lipoteichoic acid) can also activate macrophage CH25H expression(11,13). Treatment of macrophages or dendritic cells with IFN-α, β or γ induced CH25H expression in a STAT1-dependent manner(14,16). Taken together, these studies indicate that CH25H expression in the immune system can be regulated by multiple pathways. Interestingly, administration of KDO can activate CH25H expression in mouse tissues with the greatest effect on the liver(13), suggesting that CH25H can be expressed by different cell types, and CH25H expression can be regulated by different mechanisms. Consistently, in this study, we observed that activation of LXR by 25-HC and synthetic LXR ligands increased CH25H expression in both hepatocytes and macrophages (Figure 1, 6, 7). And in vivo, we determined that administration of GW3965 to mice increased CH25H expression in both liver and peritoneal macrophages (Figure 8).
Expression of macrophage CH25H can be induced by IFNs. Previously, we identified that IFN-\(\gamma\) is an LXR target gene(22). Activation of LXR by ligands induces IFN-\(\gamma\) expression and secretion(22,32). Therefore, the LXR-induced CH25H expression may be involved by activation of IFN-\(\gamma\). Indeed, we found that lack of IFN-\(\gamma\) expression reduced the basal levels of CH25H protein indicating the importance of IFN-\(\gamma\) in the activation of CH25H expression. However, LXR activation still induced CH25H expression in cells lacking IFN-\(\gamma\) expression (Figure 6). Thus, we conclude that IFN-\(\gamma\) can enhance, but not determine, LXR-induced CH25H expression. Our results also indicate the interaction between 25-HC and IFN-\(\gamma\) which may suggest that LXR has different pathways to regulate CH25H expression in a cell type-dependent manner. For instance, in cells high expressing IFN-\(\gamma\), such as macrophages, LXR activation can induce CH25H expression in two ways: by direct induction via activation of LXRE in CH25H, and by indirect induction via activation of IFN-\(\gamma\). However, in cells with high cholesterol metabolic activity that have little IFN-\(\gamma\) expression, the direct induction may dominate the effects of LXR on CH25H expression.

In summary, based on the fact that 25-HC is an endogenous LXR ligand, we identified LXREs in the CH25H promoter, and determined that activation of LXR by ligands including 25-HC can induce CH25H expression at the transcriptional level. In addition, we determined that induction of CH25H expression by LXR activation is independent of cell type, species and IFN-\(\gamma\) expression. Induction of CH25H expression can occur in vivo in response to LXR ligand administration. We believe that the results in this study advance our understanding of the regulation of CH25H expression as well as 25-HC biological actions.
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Figure 1. Activation of LXR induces hepatic CH25H protein expression

A: Total cellular proteins were extracted from C57BL/6 mouse lung, kidney, liver, spleen, lymph node and peritoneal macrophages; B: Total RNA was extracted from mouse peritoneal macrophages (PM), brain (B), intestine (I), heart (H), adipose tissue (F) and muscle (M); C: 

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HepG2 cells in serum-free medium were treated with T317, GW3965 or 25-HC for 16 h; D: HepG2 cells were treated with T317 (100 nM), GW3965 (100 nM) or 25-HC (4 μM) for the indicated times; E: HepG2 cells were treated with 25-HC (2 μM), 22(R)-hydroxycholesterol (22R, 2 μM), 22(S)-hydroxycholesterol (22S, 2 μM) or T317 (200 nM) for 16 h. Expression of CH25H protein (A, C-E) was determined by Western blot. The bands from three repeated experiments were scanned and the density was quantified with statistical analysis. \(^{a}P<0.05;\) \(^{b}P<0.01\) vs. control (n=3); Expression of CH25H mRNA (B) was determined by qRT-PCR. \(^{**}P<0.01\) vs. peritoneal macrophages (n=4); F, G: HepG2 cells were treated with 25-HC (2 μM) or T317 (200 nM) for 16 h. Expression of CH25H protein (F, G), or HSPA5 and ATP1A1 protein (G) was determined by immunofluorescent staining, respectively. The white arrows indicate the co-localization of CH25H and HSPA5 proteins, while the triangles indicate the different localization of CH25H protein from the plasma membrane.
Figure 2. Activation of LXR regulates CH25H protein stability and induces CH25H mRNA expression

A: HepG2 cells were treated with cycloheximide (CHX, 5 μM), or CHX (5 μM) plus T317 (100 nM) or 25-HC (2 μM) for the indicated times. Total cellular proteins were extracted, and CH25H protein expression was determined by Western blot. *P<0.05 vs. control;
ns: not significantly different (n=3); B, C: HepG2 cells were treated with T317 or 25-HC at the indicated concentrations for 16 h (B); or with T317 (100 nM) or 25-HC (4 μM) for the indicated times (C), respectively. Total cellular RNA was extracted, and CH25H mRNA expression was determined by qRT-PCR. *P<0.05 vs. control in the corresponding group (n=3).
Figure 3. LXR activation induces CH25H transcription in the LXRE-dependent manner

A: HepG2 cells were transfected with the normal human CH25H promoter (pCH25H) for 4 h followed by treatment with T317 or 25-HC at the indicated concentrations overnight; B, C: HepG2 cells in 48-well plates were co-transfected with pCH25H plus LXRα or LXRβ expression vector for 20 h; D: The sequence of human CH25H promoter (from -962 to +64)
with indication of positions for LXRE1, 2, 3, 4 and 5, and the 6 nucleotides on each side of these LXREs were underlined; E: Cells were transfected with DNA for the normal pCH25H promoter or mutated pCH25H promoter(s) (the mutated nucleotides in each LXRE were underlined in the sequences in the inserted box) plus LXRα/β expression vectors for 4 h followed by the indicated treatment for 16 h. Cells were also transfected with Renilla luciferase for internal control. After treatment, cellular proteins were extracted and used to determine activity of firefly and Renilla luciferase with the Dual-Luciferase Reporter Assay System. The promoter activity of each sample was normalized to the promoter activity of the control sample (normal pCH25H alone, A, E; or normal pCH25H plus C2 empty vector, B, C). The activity of control sample was defined as 1. *P<0.05; **P<0.01; ***P<0.001 vs. normal pCH25H alone (A, E) or normal pCH25H plus C2 empty vector (B, C) in the corresponding group (n=4); F: after treatment with T317 (100 nM) or 25-HC (2 μM) for 12 h, chromatin was isolated from HepG2 cells followed by immunoprecipitation with normal IgG (negative control), anti-LXRα, anti-LXRβ or anti-STAT1 antibody (positive control). The qPCR was conducted with the primers for LXRE1 and GAS, respectively. The primers for LXRE1 (from -886 to -871) are, forward (from -988 to -967), 5’-AGGCTTCAGGAACTCACATCTC-3’, and reverse (from -884 to -867), 5’-CTCCCAGTCTGCACATCTC-3’; for GAS (from -513 to -505, the conserved sequence for GAS is TTCNNNGAA and central complementary) are, forward (from -623 to -606), 5’-CTTTCTAGTCTGACGACATT-3’, and reverse (from -358 to -341), 5’-CCGTTTACATTGCACCA-3’. *P<0.05; **P<0.01 vs. control in the corresponding group (n=3).
Figure 4. Activation of CH25H expression by 25-hydroxycholesterol and T317 depends on LXR expression

A: HepG2 cells in 6-well plates were transfected with LXRα or LXRβ expression vector for 4 h followed by 25-HC treatment for 16 h; B: deficiency of LXRα or LXRβ expression in CRISPR-LXRα or CRISPR-LXRβ cells was confirmed; C-F: CRISPR-Ctrl, CRISPR-LXRα and CRISPR-LXRβ cells received the indicated treatment for 16 h; G: CRISPR-LXRα cells
were transfected with LXRβ siRNA followed by the indicated treatment for 16 h. Expression of LXRα, LXRβ and CH25H protein (A-D, G) was determined by Western blot. aP<0.05, bP<0.01 vs. control or as indicated; ns: not significantly different (n=3); Expression of CH25H mRNA (E, F) was determined by qRT-PCR. *P<0.05 vs. control in the corresponding group (n=6).
Figure 5. T317 and 25-HC regulate expression of the genes for biosynthesis of cholesterol or triglyceride and cholesterol efflux in HepG2 cells

A, B, E: HepG2 cells were treated with T317 and 25-HC for 16 h. Expression of cholesterol biosynthesis related genes [precursor of SREBP2 (P) and mature SREBP2 (M), HMGCR] (A), triglyceride biosynthesis related genes (SREBP1 and FASN) (B), and cholesterol efflux related genes (ABCA1 and ABCG1) (E) were determined by Western blot, respectively. *P<0.05, **P<0.01 vs. control (n=3); C, D: HepG2 cells were treated with T317 (200 nM) or 25-HC (2 μM) for 16 h followed by determination of cellular total cholesterol levels (C) and triglyceride (TG) levels (D) using assay kits. *P<0.05; **P<0.01 vs. control in the corresponding groups (n=3).
Figure 6. Lack of IFN-γ expression reduces, but does not abolish, LXR-activated hepatic CH25H protein expression

Primary hepatocytes were isolated from wild type and IFN-γ−/− mice, respectively. A: comparison of CH25H protein basal levels or induction of CH25H protein expression by 25-HC or T317 treatment between wild type and IFN-γ−/− hepatocytes; B: wild type and IFN-γ−/− hepatocytes were treated with 25-HC (2 μM) and T317 (200 nM) for the indicated times, respectively. Expression of CH25H protein was determined by Western blot. aP<0.05, bP<0.01 vs. control or as indicated (n=3).
Figure 7. LXR activation induces macrophage CH25H protein expression

Peritoneal macrophages isolated from C57BL/6 wild type mice (A, B) or RAW264.7 macrophages (C, D) were treated with T317, GW3965 and 25-HC at the indicated concentrations for 16 h (A, C), or with T317 (200 nM), GW3965 (200 nM) and 25-HC (2 μM) for the indicated times (B, D), respectively. Expression of CH25H protein was determined by Western blot. *P<0.05, **P<0.01 vs. control (n=3).
Figure 8. LXR activation induces CH25H expression in mouse liver and peritoneal macrophages in vivo

C57BL/6 mice were randomly divided into two groups. Control group was fed normal chow while GW3965 group was fed normal chow containing GW3965 (20 mg/day/kg bodyweight) for 7 days. At the end of the experiment, mice were anesthetized and sacrificed followed by collection of blood, liver and peritoneal macrophage samples. Expression of CH25H mRNA or protein in peritoneal macrophages and liver was determined by qRT-PCR (A) or Western blot (B). Triglyceride levels in serum and liver were determined by quantitative TG analysis with serum samples and total lipid extracted from liver, respectively (C). The lipid accumulation in the liver was also determined by Oil-Red O staining of liver frozen sections (D). *P<0.05 vs. control (n=3); *P<0.05, **P<0.01 vs. control (n=5).