REV-ERB α Regulates CYP7A1 Through Repression of Liver Receptor Homolog-1[§]

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ABSTRACT

Nuclear heme receptor reverse erythroblastosis virus (REV-ERB) α (a transcriptional repressor) is known to regulate cholesterol 7 α -hydroxylase (CYP7A1) and bile acid synthesis. However, the mechanism for REV-ERB α regulation of CYP7A1 remains elusive. Here, we investigate the role of Lrh-1 in REV-ERB α regulation of CYP7A1 and cholesterol metabolism. We first characterized the tertiary amine *N*-(4-chloro-2-methylbenzyl)-*N*-(4-chlorobenzyl)-1-(5-nitrothiophen-2-yl)methanamine (GSK2945) as a highly specific Rev-erb α /REV-ERB α antagonist using cell-based assays and confirmed expression of Rev-erb α in mouse liver. GSK2945 treatment increased hepatic mouse cholesterol 7 α -hydroxylase (Cyp7a1) level and lowered plasma cholesterol in wild-type mice. Likewise, the compound increased the expression and microsomal activity of Cyp7a1 in hypercholesterolemic mice. This coincided with

reduced plasma and liver cholesterol and enhanced production of bile acids. Increased levels of Cyp7a1/CYP7A1 were also found in mouse and human primary hepatocytes after GSK2945 treatment. In these experiments, we observed parallel increases in Lrh-1/LRH-1 (a known hepatic activator of Cyp7a1/CYP7A1) mRNA and protein. Luciferase reporter, mobility shift, and chromatin immunoprecipitation assays revealed that Lrh-1/LRH-1 was a direct Rev-erb α /REV-ERB α target gene. Furthermore, conditional deletion of Lrh-1 in the liver abrogated the regulatory effects of Rev-erb α on Cyp7a1 and cholesterol metabolism in mice. In conclusion, Rev-erb α regulates Cyp7a1 and cholesterol metabolism through its repression of the Lrh-1 receptor. Targeting the REV-ERB α /LRH-1 axis may represent a novel approach for management of cholesterol-related diseases.

Introduction

Cholesterol is a sterol molecule biosynthesized in animal cells. In addition to its importance for cell integrity, cholesterol also serves as a precursor for biosynthesis of hormones and bile acids. However, excessive cholesterol is a major risk factor for developing cardiovascular diseases (e.g., angina, heart attack, and stroke), a main cause of poor health and death (D'Agostino et al., 2008). Bile acid synthesis is the primary pathway for cholesterol catabolism. Cholesterol is converted into bile acids mainly through the multistep classic (or neutral) pathway, wherein human cholesterol 7 α -hydroxylase (CYP7A1) is the first and rate-limiting enzyme (Ishibashi et al., 1996). A deficiency in CYP7A1 is associated with hypercholesterolemia, leading to cardiovascular and gallstone diseases (Pullinger et al., 2002). To maintain bile acid and cholesterol homeostasis, CYP7A1 is under the control of the farnesoid X receptor (Chiang et al., 2000). In excess, bile acids (e.g., cholic acid and chenodeoxycholic acid) activate the farnesoid X receptor to decrease

CYP7A1 expression via small heterodimer partner (SHP)-dependent and SHP-independent mechanisms, thereby downregulating their own biosynthesis (Inagaki et al., 2005). In addition to the farnesoid X receptor, many other transcription factors [e.g., liver X receptor, LXR-1, E4BP4, HNF4 α , vitamin D receptor, PPAR α , DBP, DEC2, and reverse erythroblastosis virus (REV-ERB) α] also participate in the regulation of CYP7A1 (Noshiro et al., 2007; Chow et al., 2014). Of note, DBP, DEC2, and REV-ERB α may contribute to the circadian rhythm of CYP7A1 (Noshiro et al., 2007).

REV-ERB α (NR1D1) and its paralog REV-ERB β (NR1D2), the two members of the nuclear receptor (NR) 1D subfamily, are important components of the mammalian clock machinery (Preitner et al., 2002; Cho et al., 2012). REV-ERBs were initially regarded as orphan NRs. They are no longer orphan receptors after heme was identified as their endogenous ligand (Raghuram et al., 2007; Yin et al., 2007). REV-ERBs serve as clock repressors that negatively regulate the expression of circadian and metabolic genes, thereby integrating circadian rhythms with cell metabolism (Everett and Lazar, 2014; Ercolani et al., 2015). The receptors bind to their specific reverse erythroblastosis virus response element (RevRE) (consisting of a NR half-site AGGTCA and an A/T-rich 5' extension) and repress gene transcription by recruiting the corepressor complex containing the core proteins NR

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ABBREVIATIONS: ChIP, chromatin immunoprecipitation; CYP7A1, human cholesterol 7 α -hydroxylase; Cyp7a1, mouse cholesterol 7 α -hydroxylase; EMSA, electrophoretic mobility shift assay; GSK2945, *N*-(4-chloro-2-methylbenzyl)-*N*-(4-chlorobenzyl)-1-(5-nitrothiophen-2-yl)-methanamine; LBD, ligand-binding domain; NR, nuclear receptor; REV-ERB, human reverse erythroblastosis virus; Rev-erb, mouse reverse erythroblastosis virus; RevRE, reverse erythroblastosis virus response element; SHP, small heterodimer partner; ZT, Zeitgeber time.

corepressor 1 and histone deacetylase 3 (Harding and Lazar, 1995; Duez and Staels, 2009; Yin et al., 2010; Ercolani et al., 2015). REV-ERBs have been implicated in regulation of various physiologic processes (e.g., cell differentiation, adipogenesis, inflammation, and lipid and glucose metabolism), and thus are regarded as potential therapeutic targets for cancers and metabolic disorders such as dyslipidemia, obesity, and diabetes (Duez and Staels, 2009; Yin et al., 2010; Ercolani et al., 2015).

Although both REV-ERB paralogs are necessary in the generation of circadian rhythms and maintenance of metabolic homeostasis, REV-ERB α appears to be of greater importance (Bugge et al., 2012; Cho et al., 2012). Rev-erb α knockout in mice causes moderate disruptions to circadian rhythms and metabolic homeostasis, whereas loss of Rev-erb β shows no significant effects (Preitner et al., 2002; Bugge et al., 2012; Cho et al., 2012). The regulatory role of REV-ERB α in hepatic lipid homeostasis has received considerable attention (Duez and Staels, 2008). REV-ERB α is shown to control the expression of the genes involved in lipid metabolism such as apoC-III and ELOVL3 (Raspé et al., 2002; Anzulovich et al., 2006). REV-ERB α also participates in regulation of bile acid synthesis since its absence leads to decreased bile acid accumulation in mice (Duez et al., 2008; Le Martelot et al., 2009). This regulation is attained through a modulatory effect of REV-ERB α on CYP7A1 expression. However, the exact mechanisms for REV-ERB α regulation of CYP7A1 remain elusive (Duez et al., 2008; Le Martelot et al., 2009).

Although REV-ERB α is known to be involved in bile acid synthesis, little is known about its effects on cholesterol homeostasis. In addition, the ability of REV-ERB α to be targeted by a drug is of great interest due to a critical role in circadian biology and cell metabolism (Solt et al., 2012; Trump et al., 2013). However, drug evaluation of synthetic ligands of REV-ERB α is limited by poor pharmacokinetics or the lack of NR selectivity (Trump et al., 2013; Ercolani et al., 2015). Therefore, the objectives of this study were to investigate the role of Lrh-1 in REV-ERB α regulation of cholesterol 7 α -hydroxylase (Cyp7a1) and to clarify the impact of REV-ERB α on cholesterol metabolism using a small-molecule probe. We identified the tertiary amine *N*-(4-chloro-2-methylbenzyl)-*N*-(4-chlorobenzyl)-1-(5-nitrothiophen-2-yl)methanamine (GSK2945) as an *in vivo* functional probe (antagonist) of Rev-erb α , and demonstrated that administration of GSK2945 to mice led to induction of Cyp7a1 and reduction of cholesterol via upregulation of Lrh-1. Luciferase reporter, electrophoretic mobility shift assay (EMSA), and chromatin immunoprecipitation (ChIP) assay supported a direct role for Rev-erb α /REV-ERB α in repression of Lrh-1/LRH-1, thereby identifying the Rev-erb α /Lrh-1 axis as a novel regulatory pathway for CYP7A1 expression and cholesterol homeostasis.

Materials and Methods

The materials, primers, antibodies, plasmids, synthesis method for GSK2945, and procedures for the pharmacokinetic studies are provided in the Supplemental Material.

Animal Studies. Male C57BL/6 mice were obtained from Beijing HFK Bioscience Co., Ltd. (Beijing, China). Mice were housed in a temperature-controlled room (22°C $\pm$ 2°C) on a standard 12/12 hour light/dark cycle (7:00 AM to 7:00 PM), with access to food and water *ad libitum*. In each set of studies, GSK2945 was administered intraperitoneally to mice at doses of 0 or 10 mg/kg twice every day [at Zeitgeber time (ZT) 0 and ZT12] for 7 days. First, normal diet-fed mice (8–10 weeks of age, *n* = 5 per group) were treated with GSK2945, and blood and livers were harvested on day 8 at ZT0, ZT4, ZT8, ZT12, ZT16, and ZT20. In the second study, hypercholesterolemic mice fed a Western diet (1.25% cholesterol; Trophic Animal Feed High-tech Company, Jiangsu, China) for

4 weeks were developed. GSK2945 was given to both normal and hypercholesterolemic mice (10–12 weeks of age, *n* = 6 per group). On day 8 (ZT12), the blood and tissues (small intestine, gallbladder, and liver) were harvested. The feces were collected over a 48-hour period (from day 6 to day 8). All animal procedures were performed with isofluran anesthesia and analgesia with buprenorphine prior to blood and tissue harvest. All of the animal procedures were approved by the Institutional Animal Care and Use Committee of Jinan University and conform to the National Institutes of Health guidelines (Guide for the Care and Use of Laboratory Animals; <https://grants.nih.gov/grants/olaw/olaw.htm>).

Determination of Bile Acid Pool Size. Bile acid pool was extracted following previously published procedures (Alnouti et al., 2008). Dehydrocholic acid was used as the internal standard. Samples were analyzed using the Waters Acquity UPLC/Synapt G2 QTOF with an electrospray ionization source in the negative ion mode (Waters (Milford, MA). Chromatographic separation was performed on a Luna Omega C18 column (1.6 μ m, 100 mm $\times$ 2.1 mm; Phenomenex, Torrance, CA.) at a flow rate of 0.3 ml/min. The mobile phases consisted of 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The gradient program was 25% B at 0–3 minutes, 25%–35% B at 3–7 minutes, 35%–40% B at 7–18 minutes, 40%–50% B at 18–23 minutes, 50%–90% B at 23–25 minutes, 90% B at 25–28 minutes, and 90%–25% B at 28–30 minutes. Calibration curves of reference standards were prepared to quantify the bile acids as described previously (Lee et al., 2008).

Immunohistochemical Staining. Mouse livers were perfused with phosphate-buffered saline and fixed overnight in 4% paraformaldehyde at 4°C.

Fig. 1. Rev-erb α (Nr1d1) protein tissue distribution. (A) Protein expression of Rev-erb α in mouse liver (L), kidney (K), brain (Br), and ileum (I). HEK293 cells transfected with pcDNA3.1 (–) and pcDNA3.1-Nr1d1 (+) are used as negative and positive controls, respectively. Data are presented as mean $\pm$ S.D. (*n* = 3). **P* < 0.05 (*t* test). N.D., not detected. (B) Liver immunostaining showing Rev-erb α protein (arrows) within nuclei of mouse hepatocytes.

Fig. 2. Identification of GSK2945 as an antagonist of Rev-erb α (NR1d1)/REV-ERB α (NR1D1). (A) Chemical structure of GSK2945. (B) Gal4 cotransfection assays with HEK293 cells demonstrating the antagonistic activity of GSK2945 vs. agonistic activity of GSK4112 (also known as SR6452). (C) NR specificity assay illustrating a highly specific action of GSK2945 on REV-ERB α . The compound was tested at a concentration of 20 μM . The format of the assay was a cotransfection assay with a Gal4 DNA-binding domain/NR LBD fusion in HEK293 cells. (D) Cotransfection assays with full-length Rev-erb α and a Bmal1 luciferase reporter in HEK293 cells demonstrating the antagonistic activity of GSK2945. (E) Modulation of the expression of Rev-erb α target genes by GSK2945 in HepG2 cells. Data are presented as mean $\pm$ S.D. ($n = 3$). * $P < 0.05$ (t test) drug vs. vehicle treatment.

Then, 4- μm -thick paraffin-embedded sections were heated at 65°C for 1 hour, dewaxed in xylene, and rehydrated in descending concentrations of ethanol. Antigen retrieval was achieved by boiling samples at 100°C in a citrate buffer solution (pH = 6.0) for 10 minutes. The sections were preblocked with 5% goat serum and incubated overnight with the primary anti-Rev-erb α antibody (1:50; Sigma-Aldrich, St. Louis, MO). After washing with phosphate-buffered saline, the sections were incubated with the secondary goat anti-mouse horseradish peroxidase antibody at room temperature for 1 hour, followed by staining with diaminobenzidine tetrahydrochloride and counterstaining with hematoxylin. The sections were imaged with a Nikon Eclipse Ti-SR microscope (Nikon Inc., Tokyo, Japan).

Mouse/Human Primary Hepatocytes. Mouse (male, CD1) and human (male, Caucasian) primary hepatocytes were obtained from XenoTech, LLC

(Lenexa, KS), and plated in collagen I 24-well plates (Biocoat, Corning, NY). After 8-hour incubation at 37°C (for cell attachment), cells were overlaid with 0.25 mg/ml Matrigel in OptiCulture Media. On the next day, cells were treated with vehicle (0.5% dimethylsulfoxide) or 20 μM GSK2945, and harvested at 3, 6, 12, or 24 hours.

Real-Time Polymerase Chain Reaction. All primer sequences are summarized in Supplemental Tables 1 and 2. Total RNA extraction and quantitative reverse transcriptase polymerase chain reaction were performed as previously described (Zhang et al., 2015). mRNA levels were first normalized to cyclophilin b or glyceraldehyde-3-phosphate dehydrogenase, and then expressed as relative mRNA expression of the control.

Western Blotting. Western blotting was performed as previously described (Song et al., 2008). Total proteins were separated on 10% sodium dodecyl sulfate-

Fig. 3. Rev-erb α regulates the mRNA expression of cholesterol metabolism-related genes in the liver. C57BL/6 mice were administered repeated doses of GSK2945 (10 mg/kg, i.p.) twice daily at ZT0 and ZT12 for 7 days and groups of mice ($n = 5$) were sacrificed and gene expression was assessed by quantitative polymerase chain reaction. (A) Expression of *Cyp7a1* in the livers of vehicle-treated vs. GSK2945-treated mice. (B) Changes of blood and liver cholesterol in response to GSK2945 treatment. (C) Expression of *Cyp7a1*-regulatory transcriptional factors in the livers of vehicle-treated vs. GSK2945-treated mice. (D) Expression of Rev-erb α target genes in the livers of vehicle-treated vs. GSK2945-treated mice. (E) Expression of cholesterolgenic regulatory genes in the livers of vehicle-treated vs. GSK2945-treated mice. Data are presented as mean $\pm$ S.D. ($n = 5$). Statistical analysis on the circadian expression data were performed using a Student's t test comparing levels of gene expression of vehicle treatment vs. drug treatment at individual circadian times. * $P < 0.05$.

polyacrylamide gels, and transferred to polyvinylidene difluoride membranes. After probing with primary and secondary antibodies, protein bands were visualized by enhanced chemiluminescence and analyzed by the Quantity One software (Hercules, CA). Protein levels were normalized to glyceraldehyde-3-phosphate dehydrogenase or β -actin.

Liver Microsomal Cyp7a1 Activity. Mouse liver microsomes were prepared by sequential ultracentrifugation, first at 9000g for 10 minutes and then at 100,000g for 60 minutes. The microsomal Cyp7a1 activity toward cholesterol was determined using the published procedures involving the oxidation of 7 α -hydroxycholesterol to 7 α -hydroxy-4-cholesten-3-one by cholesterol oxidase (Chow et al., 2009).

Plasma and Tissue Cholesterol. Total plasma cholesterol was measured using the LabAssay Cholesterol Kit (Wako Chemical, Osaka, Japan). Total cholesterol was extracted from mouse livers as previously described (Patel et al., 2011), and quantified using the Total Cholesterol Assay Kit (Jiancheng Bioengineering Institute, Nanjing, China).

Nuclear Receptor Specificity Assay. The potential of GSK2945 to modulate the activities of all 48 human NRs was assessed using a Gal4 cotransfection assay system as described previously (Kumar et al., 2010). In the Gal4 cotransfection assay, only specific ligand of test NR can bind to the ligand-binding domain (LBD) to activate gene transcription. In brief, HEK293 cells were cotransfected with a GAL4-NR LBD construct, pGL4.35[luc2P/9XGAL4UAS/Hygro] vector, and pRL-TK vector using the HET Transfection Kit (Biowit Technologies, Shenzhen, China). On the next day, GSK2945 (20 μ M) or dimethylsulfoxide was added and incubated with the cells for 24 hours. The luciferase activities were measured with the Dual Luciferase Reporter Assay Kit (Promega, Madison, WI) and Glomax 20/20 Luminometer (Promega).

Luciferase Reporter Cotransfection Assays. Cotransfection assays were performed in HEK293 or HepG2 cells using the HET Transfection Kit (Biowit Technologies) as described previously (Zhao et al., 2016). Ligands were added at 16–20 hours post-transfection. Cells harvested 6 hours later were assayed for luciferase activities. The relative luciferase activity was initially derived as the ratio of firefly over Renilla luciferase activity. The relative luciferase activity values of treated cells were normalized to that of control cells.

Electrophoretic Mobility Shift Assay. After transfection of HEK293 cells with Rev-erb α /REV-ERB α , nuclear extracts were prepared using a cytoplasmic/nuclear protein extraction kit (Beyotime, Shanghai, China). EMSA assays were performed using a chemiluminescent EMSA kit (Beyotime). 6 μ g of nuclear extract was mixed in EMSA binding buffer. After 10-minute preincubation on ice, 200-fmol of biotin-labeled probe was added and incubated for 20 minutes at room temperature. Reaction products were subjected to 5% polyacrylamide gel electrophoresis. After transferring to Hybond-N⁺ membrane (Amersham, Buckinghamshire, United Kingdom), the products were visualized by enhanced chemiluminescence reagent. All Oligonucleotide sequences for EMSA assays can be found in Supplemental Table 3.

Chromatin Immunoprecipitation Assay. ChIP assays were performed using a SimpleChIP Enzymatic Chromatin IP Kit (Cell Signaling Technology, Beverly, MA) according to the manufacturer's instructions. Mouse liver was fixed in 1% formaldehyde for 20 minutes at room temperature, followed by digestion with micrococcal nuclease and shearing with sonication. An aliquot of sheared chromatin was immunoprecipitated with anti-Rev-erb α or normal rabbit IgG (control) by overnight incubation at 4°C. Immunoprecipitated chromatin was decrosslinked at 65°C for 4 hours and DNAs were purified

Fig. 4. Rev-erb α regulates the protein expression of cholesterol metabolism-related genes in the liver. C57BL/6 mice were administered repeated doses of GSK2945 (10 mg/kg, i.p.) twice daily at ZT0 and ZT12 for 7 days and groups of mice ($n = 5$) were sacrificed and protein expression was assessed by Western blotting. (A) Expression of Cyp7a1 in the livers of vehicle-treated vs. GSK2945-treated mice. (B) Expression of Lrh-1 in the livers of vehicle-treated vs. GSK2945-treated mice. (C) Expression of Cyp7a1-regulatory transcriptional factors in the livers of vehicle-treated vs. GSK2945-treated mice. Data are presented as mean $\pm$ S.D. ($n = 5$). Statistical analysis on the circadian expression data were performed using a Student's t test comparing levels of protein expression of vehicle treatment vs. drug treatment at individual circadian times. $*P < 0.05$.

using spin columns. The purified DNAs were used as a template for quantitative real-time polymerase chain reaction with specific primers (Supplemental Table 4).

Statistical Analysis. All data are presented as mean $\pm$ S.D. Statistical analysis on the circadian expression data were performed using a Student's t test comparing levels of gene/protein expression of vehicle treatment versus drug treatment at individual circadian times. The Student's t test was also used to test for statistical differences between treatment and control groups. The level of significance was set at $P < 0.05$.

Results

Rev-erb α Protein Tissue Distribution. Nuclear Rev-erb α protein was found at a similar level in mouse liver and ileum (Fig. 1A). The protein level of Rev-erb α was 2.1-fold higher in the brain compared with the liver or ileum (Fig. 1A). By contrast, Rev-erb α protein was not detected in the kidney (Fig. 1A). The presence of Rev-erb α protein within nuclei of mouse hepatocytes was further confirmed by liver immunostaining (Fig. 1B).

Identification of GSK2945 as a Specific Rev-erb α /REV-ERB α Antagonist. GSK2945 (Fig. 2A) was synthesized by reductive amination (Supplemental Material), and its chemical structure was verified through ^1H NMR, ^{13}C NMR, and mass spectrometric analyses (Supplemental Fig. 1). The compound displayed a superior pharmacokinetic profile with much higher systemic and liver exposures compared

with SR8278, the first synthetic antagonist of REV-ERBs (Supplemental Fig. 2) (Kojetin et al., 2011). The activity of GSK2945 was first assessed in HEK293 cells coexpressing a chimeric receptor (i.e., the DNA-binding domain of Gal4 is fused to the LBD of Rev-erb/REV-ERB) and a Gal4-responsive luciferase reporter. In line with previous studies (Alnouti et al., 2008; Solt et al., 2012), the agonist GSK4112 enhanced the transcriptional repression activities of Rev-erb α /REV-ERB α in the Gal4 chimeric assay (Fig. 2B). By contrast, GSK2945 dose dependently inhibited the repressor activities of Rev-erb α /REV-ERB α , but showed no effects on Rev-erb β /REV-ERB β activities (Fig. 2B) (Rev-erb α EC_{50} = 21.5 μM ; REV-ERB α EC_{50} = 20.8 μM). The compound did not exhibit activities toward other types of NRs, demonstrating its exclusive action on the REV-ERB α receptor (Fig. 2C). In addition, GSK2945 dose dependently enhanced the transcriptional activity in a cotransfection assay using full-length Rev-erb α and a Bmal1 (a target gene of REV-ERBs) luciferase reporter (Fig. 2D) (EC_{50} = 2.05 μM). Also, the compound blocked the agonistic activity of GSK4112, resulting in induction of transcription at doses of ≥ 5 μM (Fig. 2D) (EC_{50} = 2.47 μM). Furthermore, GSK2945 increased the mRNA expression of BMAL1 and PEPCK (i.e., known target genes of REV-ERBs) in HepG2 cells in a dose-dependent fashion (Fig. 2E), a similar effect as observed when intracellular heme was reduced by succinylacetone (Supplemental Fig. 3). Taken together, GSK2945 acted as an antagonist of Rev-erb α /REV-ERB α probably by blocking the action of endogenous heme.

Fig. 5. GSK2945 changes Cyp7a1/CYP7A1 and Lrh-1/LRH-1 expression in mouse and human primary hepatocytes. (A) Mouse primary hepatocytes showed increased Cyp7a1 and Lrh-1 expression 24 hours after GSK2945 (20 μ M) treatment. (B) Human primary hepatocytes showed increased Cyp7a1 and Lrh-1 expression 24 hours after GSK2945 (20 μ M) treatment. * P < 0.05 (t test) drug vs. vehicle treatment at different time points.

REV-ERB α Regulates Hepatic Cyp7a1 and Cholesterol Homeostasis in Normal Diet-Fed Mice. The improved pharmacokinetic properties of GSK2945 allowed us to explore the functional effects of REV-ERB α in vivo. No overt toxicity was observed in mice treated with GSK2945 based on hematology and liver function tests (Supplemental Fig. 4; Supplemental Table 5). GSK2945 treatment of mice resulted in significant upregulation of hepatic *Cyp7a1*, the key gene involved in

cholesterol catabolism (Fig. 3A). The protein level of Cyp7a1 was also elevated in response to GSK2945 administration (Fig. 4A). This was accompanied by significant decreases in plasma and liver cholesterol (Fig. 3B). Of Cyp7a1-regulatory transcriptional factors, Lrh-1 was unregulated at both mRNA and protein levels (Figs. 3C and 4B). Although *E4bp4* and *Insig2* mRNAs were also increased, no significant differences were noted in their protein levels (Figs. 3D and 4C).

Fig. 6. GSK2945 reduces cholesterol and enhances bile acid production by upregulating hepatic Lrh-1 and Cyp7a1 in hypercholesterolemic mice. (A) Plasma and liver total cholesterol were reduced after GSK2945 treatment. (B) Hepatic Cyp7a1 expression and activity were increased after GSK2945 treatment. (C) Hepatic Lrh-1 mRNA and protein levels were increased after GSK2945 treatment. (D) GSK2945 treatment increased bile acid pool size, plasma bile acid level, and fecal bile acid excretion. [#] $P < 0.05$ (t test) Western diet vs. normal diet; ^{*} $P < 0.05$ (t test) vehicle-treated vs. GSK2945-treated hypercholesterolemic mice.

GSK2945 treatment caused significant increases in mRNA and protein expression of Bmal1 and Pepck (two direct target genes of Rev-erb α) consistent with Rev-erb α antagonism (Fig. 3D; Supplemental Fig. 5). Expression of the cholesterologenic regulatory genes (Srebp2 and Hmgcr) were not altered by GSK2945 (Fig. 3E). Of other cholesterol-related genes, expression levels of Bsep and Abcg5/g8 (the target genes of Lrh-1) were also increased (Supplemental Fig. 6).

REV-ERB α Regulates Cyp7a1/CYP7A1 Expression in Mouse and Human Primary Hepatocytes. Impact of Rev-erb α on Cyp7a1 and Lrh-1 was confirmed in isolated mouse primary hepatocytes. GSK2945 (20 μ M, a concentration close to the EC₅₀ value) treatment of cells led to significant increases in mRNA and protein expression (at 24 hours) of Cyp7a1 (Fig. 5A). Likewise, mRNA and protein levels

(at 24 hours) of CYP7A1 were increased in human primary hepatocyte after exposure to 20 μ M GSK2945 (Fig. 5B). Of Cyp7a1/CYP7A1-regulatory transcriptional factors, only Lrh-1/LRH-1 (a hepatic activator of Cyp7a1/CYP7A1) was upregulated in response to GSK2945 treatment (Fig. 5). These data support a critical role for Lrh-1/LRH-1 in REV-ERB α upregulation of Cyp7a1/CYP7A1.

REV-ERB α Regulates Cholesterol Catabolism through Upregulation of Cyp7a1 and Lrh-1 in Hypercholesterolemic Mice. The effects of GSK2945 on Cyp7a1 and cholesterol metabolism were further evaluated using hypercholesterolemic mice, which showed markedly increased liver and plasma cholesterol (Fig. 6A). GSK2945 treatment of hypercholesterolemic mice led to significant reductions in plasma cholesterol (22.6%) and liver cholesterol (29.6%) (Fig. 6A). This coincided with

Fig. 7. Rev-erb α /REV-ERB α is a transcriptional repressor of Lrh-1/LRH-1. (A) Luciferase reporter assays driven by Bmal1 or Cyp7a1 promoter in HEK293 and HepG2 cells, showing that Rev-erb α failed to directly regulate Cyp7a1. $*P < 0.05$ (t test) Rev-erb α plasmid treatment vs. control. (B) Luciferase reporter assays driven by Lrh-1 promoter in HEK293 and HepG2 cells, showing Rev-erb α directly repressed transcription of Lrh-1. $*P < 0.05$ (t test). (C) Luciferase reporter assays with truncated and/or mutated versions of mouse Lrh-1 promoter. The open boxes in the lines denote potential RevRE sites, and the peaks in the lines represent the construct with RevRE site mutated (sequences shown). $*P < 0.05$ (t test) Rev-erb α plasmid treatment vs. control. (D) EMSA assay with biotin-labeled probes [Bmal1-RevRE probe (AAAGTAGGTAA) as control], showing Rev-erb α bound to the mRevRE2 site of Lrh-1. (E) Luciferase reporter assays with truncated and/or mutated versions of human LRH-1 promoter. The open boxes in the lines denote potential RevRE sites, and the peaks in the lines represent the construct with the hRevRE site mutated (sequences shown). $*P < 0.05$ (t test) Rev-erb α plasmid treatment vs. control. (F) EMSA assay with biotin-labeled probes [Bmal1-RevRE probe as control], showing REV-ERB α bound to the hRevRE site of LRH-1. (G) ChIP assay with anti-Rev-erb α antibody (IgG as control) and mouse liver, showing Rev-erb α bound to the mRevRE2 site of Lrh-1 in vivo. Data are repressed as mean $\pm$ S.D. ($n = 3$). $*P < 0.05$ (t test). RLU, relative luciferase unit.

significantly increased Cyp7a1 mRNA levels (1.91-fold) and protein levels (1.52-fold) as well as microsomal activity (1.58-fold) in the liver (Fig. 6B). Increased expression of Cyp7a1 may be accounted for by upregulation of Lrh-1 (Fig. 6C). Owing to upregulated Lrh-1, expression of Cyp8b1 was also increased (Supplemental Fig. 7). In addition, both bile acid pool size and fecal excretion of bile acids were significantly increased after GSK2945 treatment (Fig. 6D; Supplemental Figs. 8 and 9). Likewise, there was a higher amount of bile acids in the plasma (Fig. 6D). These data suggest increased bile acid production because of enhanced Cyp7a1 activity.

Rev-erb α /REV-ERB α Is a Transcriptional Repressor of Lrh-1/LRH-1. Luciferase reporter assays were performed to determine whether Cyp7a1 is directly regulated by Rev-erb α . In line with the literature, Rev-erb α and Rev-erb β directly repressed Bmal1 transcription (Fig. 7A). However, neither showed regulatory effects on Cyp7a1

transcription (Fig. 7A). Consistently, transcription of Cyp7a1 was unaffected in the presence of GSK2945 (Fig. 7A). Therefore, an indirect mechanism is necessary for regulation of Cyp7a1 by the Rev-erb α receptor as indicated previously (Duez et al., 2008; Le Martelot et al., 2009). We further performed luciferase reporter assays using proximal Lrh-1 promoter (-700/+3 base pairs). Rev-erb α significantly decreased Lrh-1 promoter activity (Fig. 7B). Addition of GSK2945 blocked the action of Rev-erb α and eliminated its repressor activity (Fig. 7B). These data revealed Rev-erb α as a transcriptional repressor of Lrh-1.

Sequence analysis of mouse Lrh-1 promoter suggested two potential Rev-erb response elements (mRevRE1 and mRevRE2) (at positions -647 and -167, respectively). Accordingly, multiple truncated and/or mutated versions of Lrh-1 luciferase reporters were generated and tested for repression activities (Fig. 7C). The repressor ability of Rev-erb α was

Fig. 8. Lrh-1 participates in Rev-erb α regulation of Cyp7a1 and cholesterol homeostasis. (A) mRNA expression of Cyp7a1 and Lrh-1 in the livers of Alb-Cre;Lrh-1^{fl/fl} mice after vehicle or GSK2945 treatment. (B) Effects of hepatic Lrh-1 deletion on plasma cholesterol in response to GSK2945 treatment. Data are presented as mean $\pm$ S.D. ($n = 5$). * $P < 0.05$ (t test) drug vs. vehicle treatment. (C) A schematic representation showing that Lrh-1 links Rev-erb α to Cyp7a1 regulation and cholesterol metabolism. Rev-erb α targeting by GSK2945 derepresses Lrh-1 to increase Cyp7a1 and enhance cholesterol catabolism, resulting in a reduced level of cholesterol in plasma and liver.

unaffected when the mRevRE1 site was deleted or mutated, but completely lost when the mRevRE2 site was mutated (Fig. 7C). This indicated that mRevRE2 rather than mRevRE1 was responsible for the repressor activity of Rev-erb α . EMSA experiments with biotinylated oligonucleotides showed that Rev-erb α bound directly to the mRevRE2 site, forming a distinct DNA-protein complex (Fig. 7D). Formation of this complex was markedly diminished in the presence of unlabeled competitor (Fig. 7D). In a similar manner, a REV-ERB α response element (at position -79) within the human LRH-1 promoter was identified (Fig. 7, E and F). To confirm the interaction of Rev-erb α with Lrh-1 promoter in vivo, ChIP assays were performed using mouse liver samples at ZT8 (corresponding to peak Rev-erb α expression). We observed significant recruitment of Rev-erb α to the mRevRE2 site (Fig. 7G). Overall, these data indicate that Rev-erb α represses transcription of Lrh-1 through its specific binding to the mRevRE2 site (i.e., the -178- to -167-base pair region).

Lrh-1 Is Required for Regulation of Cyp7a1 by Rev-erb α In Vivo. To determine whether Lrh-1 is an actual mediator for Rev-erb α regulation of Cyp7a1, we performed in vivo studies using genetic mice. Conditional deletion of Lrh-1 in liver (Alb-Cre;Lrh^{fl/fl}) abrogated the changes in Cyp7a1 expression (Fig. 8A) and plasma cholesterol (Fig. 8B) as noted in wild-type mice, supporting a critical role for Lrh-1 in control of Cyp7a1 and cholesterol metabolism by Rev-erb α .

Discussion

In this study, we established the tertiary amine GSK2945 as an in vivo probe (antagonist) of Rev-erb α and observed a cholesterol-lowering effect in mice after GSK2945 treatment. The cholesterol-lowering effect was associated with an elevation in expression of Cyp7a1 (the rate-limiting enzyme in the classic pathway of bile acid synthesis), which led to increased production of bile acids (Fig. 6D). The enhanced bile acid synthesis was primarily responsible for reduction in body cholesterol since

no significant changes were found in cholesterologenic genes Srebp2 and Hmgcr (Fig. 3E). We further observed parallel expression changes in Lrh-1 (a positive regulator of Cyp7a1), thus predicted a critical role for Lrh-1 in upregulation of Cyp7a1. Through luciferase reporter, EMSA, and ChIP assays, we revealed that Rev-erb α directly repressed transcription of Lrh-1 via binding to its specific response element (Fig. 7). Therefore, upregulation of Cyp7a1 by Rev-erb α works through derepression of Lrh-1 (Fig. 8C). This is the first demonstration that Rev-erb α can be targeted to alter Cyp7a1 expression and cholesterol homeostasis.

Previous studies have consistently shown that Cyp7a1 and bile acid synthesis are under the control of Rev-erb α using engineered mice with Rev-erb α deletion or overexpression (Duez et al., 2008; Le Martelot et al., 2009). However, the exact mechanisms for this regulation remained unresolved. Duez et al. (2008) believe that Rev-erb α represses SHP and E4bp4 (two potential repressors of Cyp7a1) to increase Cyp7a1 in mice. On the contrary, Le Martelot et al. (2009) proposed that upregulation of Cyp7a1 by Rev-erb α is through downregulation of Insig2, although the authors acknowledged the lack of direct evidence. Repressive action of Rev-erb α on SHP in vivo is questioned by our study and others (Le Martelot et al., 2009). This was because no change in SHP expression was observed in GSK2945-treated wild-type mice (Fig. 3C) or Rev-erb α knockout mice, and increased expression of SHP was found in transgenic mice overexpressing hepatic Rev-erb α (Le Martelot et al., 2009). We and others also argue against a major role for E4bp4 in Cyp7a1 regulation (Le Martelot et al., 2009). First, E4bp4^{-/-} mice did not show increased Cyp7a1 expression compared with wild-type mice (Le Martelot et al., 2009). Second, expression of E4bp4 was increased in wild-type mice in response to Rev-erb α antagonism (Fig. 3C). Furthermore, we believed that Cyp7a1 upregulation was independent of Insig2 because expression of Insig2 was increased in GSK2945-treated mice (Fig. 3C).

We propose that the Rev-erb α /Lrh-1 axis is involved in regulation of Cyp7a1, highlighting Lrh-1 as a key mediator for indirect regulation of Cyp7a1 by Rev-erb α . Small-molecule targeting of Rev-erb α

derepresses Lrh-1, a hepatic activator of Cyp7a1 (Nitta et al., 1999; Noshiro et al., 2007; Out et al., 2011), to increase Cyp7a1 expression and lower cholesterol (Fig. 8C). This novel mechanism of Cyp7a1 upregulation has greater potential utility in treating hypercholesterolemia compared with the vitamin D receptor activation by 1,25(OH) $_2$ D $_3$ (Chow et al., 2014). The latter is concerned with the hypercalcemic side effect of 1,25(OH) $_2$ D $_3$. The proposed mechanism also helps to explain why Rev-erb α activation by synthetic agonists (SR9009 and SR9011) results in a decrease in Cyp7a1 and why Rev-erb α knockout leads to a reduction in liver cholesterol, which were unexpected previously due to proposed positive control of Cyp7a1 by Rev-erb α (Cho et al., 2012; Ercolani et al., 2015). We observed parallel increases in expression of other Lrh-1 target genes such as Cyp8b1, Cyp27a1, Bsep, and Abcg5/g8, supporting the upregulation of Lrh-1 in response to Rev-erb α antagonism (Supplemental Figs. 6 and 7) (Freeman et al., 2004; Lee et al., 2008; Song et al., 2008). Although Cyp8b1 participates in classic bile acid synthesis, a contributing role of Cyp8b1 upregulation to reduced cholesterol can be ruled out because Cyp8b1-mediated reaction is not a limiting step to the overall bile acid synthesis (Li and Chiang, 2014). On the other hand, the increase of Cyp27a1 might contribute to reduced cholesterol because the enzyme initiates the alternative (acidic) pathway of bile acid synthesis (Li and Chiang, 2014). However, the contribution, if any, would be rather limited because the acidic pathway accounts for a minor portion (<10%) of bile acid synthesis (Li and Chiang, 2014).

We provided strong evidence that GSK2945 functions as an antagonist of Rev-erb α . First, GSK2945 dose dependently antagonizes the repressor action of Rev-erb α in the chimeric Gal4-LBD assay (Fig. 2B). Second, GSK2945 derepresses the transcription of Bmal1 reporter and blocks the action of GSK4112 in a cotransfection assay with full-length Rev-erb α (Fig. 2D). Third, GSK2945 increases the expression of Rev-erb α target genes in HepG2 and hepatocytes as well as in mice by antagonizing the action of endogenous heme (Figs. 2–5). All these cell-based and in vivo data were consistent with the antagonism of Rev-erb α . It was noted that GSK2945 behaved like a REV-ERB α agonist in a previous study (Trump et al., 2013). The authors observed agonistic effects in nonstandard activity assays (i.e., THP-1 interleukin 6 repression and U2OS reporter assays) (Trump et al., 2013). Although the exact reasons for this contradiction remained unknown, there was a possibility that the agonistic versus antagonistic action of GSK2945 was cell-type (tissue) dependent. This is because the activity of the REV-ERB receptor is strongly affected by the cellular microenvironments such as the redox state, small-molecule gasses (e.g., NO and CO), and the types of cofactors (Marvin et al., 2009; Pardee et al., 2009; Trump et al., 2013; Matta-Camacho et al., 2014). Modifications of ligand-bound REV-ERB by redox conditions and gasses are likely the key determinants to ligand switching and functional effects (Kojetin et al., 2011). The high sensitivity of REV-ERB activity to the conformational changes in the ligand-bound receptor complex is also evidenced by the fact that structurally related compounds [e.g., GSK2945 vs. GSK4112; cobalt protoporphyrin IX vs. heme] demonstrate different types of actions on REV-ERB (i.e., antagonist vs. agonist) (Kojetin et al., 2011).

In summary, we characterized GSK2945 as a Rev-erb α antagonist with sufficient pharmacokinetic parameters for in vivo uses. GSK2945 increased the expression of Cyp7a1 and reduced total cholesterol in mice. This regulatory effect was ascribed to Rev-erb α -mediated derepression of Lrh-1, a hepatic activator of Cyp7a1. Therefore, targeting the REV-ERB α /LRH-1 axis may represent a novel approach for management of cholesterol-related diseases.

Authorship Contributions

Participated in research design: Zhang, Zhao, Wu.

Conducted experiments: Zhang, Zhao, Lu, Wang, Yu, Guo.

Contributed new reagents or analytic tools: Wen.

Performed data analysis: Zhang, Zhao, Wu.

Wrote or contributed to the writing of the manuscript: Zhang, Zhao, Wu.

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