Available online at www.sciencedirect.com

Metabolism

www.metabolismjournal.com

Oxysterol sulfation by cytosolic sulfotransferase suppresses liver X receptor/sterol regulatory element binding protein–1c signaling pathway and reduces serum and hepatic lipids in mouse models of nonalcoholic fatty liver disease

Qianming Bai[a,b], *Xin Zhang*[a,b], *Leyuan Xu*[a], *Genta Kakiyama*[a], *Douglas Heuman*[a], *Arun Sanyal*[a], *William M. Pandak*[a], *Lianhua Yin*[b], *Wen Xie*[c], *Shunlin Ren*[a,*]

[a]*Department of Medicine, Virginia Commonwealth University/Veterans Affairs McGuire Medical Center, Richmond, VA 23249, USA*
[b]*Department of Pathology and Pathophysiology, Fudan University Shanghai Medical College, Shanghai 200032, China*
[c]*Center for Pharmacogenetics, University of Pittsburgh, Pittsburgh, PA 15261, USA*

ARTICLE INFO

Article history:
Received 28 September 2011
Accepted 29 November 2011

ABSTRACT

Cytosolic sulfotransferase (SULT2B1b) catalyzes oxysterol sulfation. 5-Cholesten-3β-25-diol-3-sulfate (25HC3S), one product of this reaction, decreases intracellular lipids in vitro by suppressing liver X receptor/sterol regulatory element binding protein (SREBP)–1c signaling, with regulatory properties opposite to those of its precursor 25-hydroxycholesterol. Upregulation of SULT2B1b may be an effective strategy to treat hyperlipidemia and hepatic steatosis. The objective of the study was to explore the effect and mechanism of oxysterol sulfation by SULT2B1b on lipid metabolism in vivo. C57BL/6 and LDLR$^{-/-}$ mice were fed with high-cholesterol diet or high-fat diet for 10 weeks and infected with adenovirus encoding SULT2B1b. SULT2B1b expressions in different tissues were determined by immunohistochemistry and Western blot. Sulfated oxysterols in liver were analyzed by high-pressure liquid chromatography. Serum and hepatic lipid levels were determined by kit reagents and hematoxylin and eosin staining. Gene expressions were determined by real-time reverse transcriptase polymerase chain reaction and Western Blot. Following infection, SULT2B1b was successfully overexpressed in the liver, aorta, and lung tissues, but not in the heart or kidney. SULT2B1b overexpression, combined with administration of 25-hydroxycholesterol, significantly increased the formation of 25HC3S in liver tissue and significantly decreased serum and hepatic lipid levels, including triglycerides, total cholesterol, free cholesterol, and free fatty acids, as compared with controls in both C57BL/6 and LDLR$^{-/-}$ mice. Gene expression analysis showed that increases in SULT2B1b expression were accompanied by reduction in key regulators and enzymes involved in lipid

Author contributions: Qianming Bai: design and conduct of the study, data collection and analysis, data interpretation, and manuscript writing. Xin Zhang: conduct of the section of immunohistochemistry and animal treatments, and manuscript review. Leyuan Xu: conduct of the section of animal treatments. Genta Kakiyama: analysis of composition of sulfated oxysterols in liver tissue. Douglas Heuman: manuscript review. Arun Sanyal: manuscript review. William M. Pandak: data interpretation and manuscript review. Lianhua Yin: manuscript review. Wen Xie: manuscript review. Shunlin Ren: funding of the study, design of the study, data interpretation, and manuscript writing.

* *Corresponding author*. McGuire Veterans Affairs Medical Center/Virginia Commonwealth University, Research 151, Richmond, VA 23249, USA. Tel.: +1 804 675 5000x4973; fax: +1 804 675 5359.
E-mail address: sren@vcu.edu (S. Ren).

doi:10.1016/j.metabol.2011.11.014

metabolism, including liver X receptor α, SREBP-1, SREBP-2, acetyl-CoA carboxylase–1, and fatty acid synthase. These findings support the hypothesis that 25HC3S is an important endogenous regulator of lipid biosynthesis.

1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is emerging as the most common liver disease in the United States and worldwide [1,2]. The spectrum of NAFLD ranges from simple nonprogressive steatosis to progressive nonalcoholic steatohepatitis that may lead to cirrhosis and hepatocellular carcinoma [3-5]. The pathogenesis of steatosis and progression to nonalcoholic steatohepatitis and cirrhosis involve numerous metabolic and inflammatory pathways and remain incompletely understood [6]. However, the central role of lipid accumulation in the liver in the pathogenesis of NAFLD has been confirmed in clinical correlation studies and animal models [6,7]. Thus, decreasing serum and hepatic lipid levels are crucial to the prevention of NAFLD.

Recently, we found that overexpression of mitochondrial cholesterol delivery protein (steroidogenic acute regulatory protein) upregulated bile acid synthesis and secretion via the "acidic" pathway initiated by cholesterol 27-hydroxylase, and downregulated cholesterol and fatty acid biosynthesis. A search for the possible mechanism of these regulatory effects led to the finding of a novel sulfated oxysterol, 5-cholesten-3β-25-diol-3-sulfate (25HC3S) [8-10]. Addition of 25HC3S in human and rat hepatocytes and human acute monocytic leukemia cell line–derived macrophages decreased expression and processing of nuclear liver X receptor (LXR) and its target gene sterol regulatory element binding protein (SREBP)–1, and subsequently decreased intracellular levels of free fatty acids, cholesterol, and triglycerides. Furthermore, addition of exogenous 25HC3S increased cytosolic inhibitor of kappa B-alpha (IκBα) IκB expression, decreased nuclear factor–κB, and subsequently decreased inflammatory responses in hepatocytes [11,12]. The results suggest that 25HC3S could play an important role in preventing nonalcoholic steatohepatitis. However, the effects of endogenous 25HC3S in vivo on serum and hepatic lipid metabolism have not been investigated.

It is reported that 25HC3S is synthesized by cytosolic sulfotransferase (SULT2B1b) through sulfation of 25-hydroxycholesterol (25HC) [13,14]. Moreover, the regulatory properties of 25HC are opposite to those of 25HC3S, increasing nuclear LXR and SREBP-1 protein levels and increasing acetyl-CoA carboxylase–1 (ACC1) and fatty acid synthase (FAS) messenger RNA (mRNA) levels [11,12,14]. Thus, SULT2B1b, by determining the balance between 25HC and 25HC3S, could play an important role in lipid metabolism. SULT2B1b is expressed in hepatocytes and liver tissue, and highly regulated by duration of culture and insulin in primary rat hepatocytes [13,15,16]. Overexpression of SULT2B1b in primary human aortic endothelial cells decreases mRNA and protein levels of LXRα, adenosine triphosphate (ATP)–binding cassette transporter A1 (ABCA1), SREBP-1c, ACC, and FAS, and subsequently reduces cellular lipid levels [14]. The effects of SULT2B1b overexpression in the presence of 25HC are similar to those produced by direct addition of 25HC3S in cell culture [14].

In the present study, we further evaluated the effect of SULT2B1b on the lipid metabolism in serum and liver tissue in vivo in mouse NAFLD models. The results indicate that 25HC sulfation by SULT2B1b substantially decreases serum and hepatic lipid levels by inhibiting the LXR/SREBP-1c signaling pathway. This finding supports the hypothesis that 25HC3S is an important endogenous regulator of lipid biosynthesis.

2. Materials and methods

2.1. Animals and treatment

Eight-week-old female C57BL/6 mice were purchased from Charles River Laboratories (Cambridge, MA), and LDLR$^{-/-}$ mice were obtained from Jackson Laboratory (Sacramento, CA). Mice were hosted under a standard 12/12-hour light/dark cycle. Mice were fed with either a high-cholesterol diet (HCD, 3.1 kcal/g, 2% cholesterol and 5.7% fat) or a high-fat diet (HFD, 4.5 kcal/g, 0.2% cholesterol and 21.2% fat) for 10 weeks. Mice were then infected with recombinant adenovirus encoding cytomegalovirus (CMV)-driven SULT2B1b (1×10^8 pfu per mouse) through tail vein injection. The CMV-SULT2B1b was prepared as previously described [14]. Ad-CMV-β-Gal adenovirus was used as control. In addition, some mice were given an intraperitoneal injection of 25HC (0.01% wt/wt, about 0.2 mg per mouse)

Table 1 – Animal groups

	C57BL/6 mice_HCD		C57BL/6 mice_HCD + 25HC		LDLR$^{-/-}$ mice_HFD			
	Control	SULT2B1b	Control	SULT2B1b	Control		SULT2B1b	
Mouse type	C57BL/6	C57BL/6	C57BL/6	C57BL/6	LDLR$^{-/-}$		LDLR$^{-/-}$	
Mouse sex	F	F	F	F	F	M	F	M
Mouse number	5	5	5	5	3	3	4	3
Diet	HCD	HCD	HCD	HCD	HFD		HFD	
Virus infection	β-Gal	SULT2B1b	β-Gal	SULT2B1b	β-Gal		SULT2B1b	
IP inject 25HC	No	No	Yes	Yes	No		No	

F indicates female; M, male.

2 days after infection. Mice were killed following an overnight fast 6 days after adenovirus infection. The detailed information for the animal groups was shown in Table 1. All protocols were approved by the Institutional Animal Care and Use Committee of the McGuire VA medical center.

2.2. Immunohistochemistry

Formalin-fixed liver tissues were processed for histological analyses and stained with hematoxylin and eosin (H&E). Briefly, deparaffinized 4-μm sections were stained with rabbit anti-SULT2B1b antibody (Abcam, Cambridge, MA, USA). Immobilized antibodies were detected by the avidin-biotin-peroxidase technique (Vectastain ABC Kits, Vector Laboratories, Burlingame, CA, USA). 3-3'-Diaminobenzidine (DAB) was used as the chromogen, and hematoxylin was used as the nuclear counterstain.

2.3. Analysis of composition of sulfated oxysterols in liver tissue

Total lipids in liver tissue were extracted by the Folch method [17]. Briefly, 200 mg of mouse liver tissue was homogenized in 1 mL of phosphate-buffered saline. Twenty milliliters of chloroform:methanol (2:1, vol/vol) was added in the homogenates, sonicated for 1 to 2 hours, and filtered. Four milliliters of water and 100 μL of 1 mol/L K_2CO_3 were added, mixed, and allowed to stand for about 3 hours for the phase separation.

The water/methanol (upper) phase, which contains sulfated oxysterols, was evaporated under N_2 stream. The residue was resuspended in 0.5 mL of methanol, 3.5 mL of water, and 0.5 mL of NaOH (1 N) by sonication; and the suspension was passed through a preconditioned Sep-Pak C18 cartridge (Waters, Milford, MA) to remove nonsulfated oxysterols. After successively washing the cartridge with 8 mL of water, 3.5 mL of 15% acetone, and 8 mL of water again, the sulfated oxysterol fraction was eluted in 5 mL of 75% methanol, which was taken to dryness under N_2 stream below 40°C. The extracts were then hydrolyzed in 1 mL of sulfatase (2 mg/mL) at 37°C for 4 hours. Deconjugated oxysterols were extracted by Folch partition (chloroform:methanol, 2:1), and the chloroform phase was taken to dryness.

The oxysterol samples thus obtained from the methanol/water were oxidized with cholesterol oxidase as previously described [18]. To the oxysterol sample dissolved in 50 μL of 2-propanol were added 450 μL of water, 50 μL of 1 mol/L potassium phosphate buffer, and 1.5 μg of progesterone as an internal standard; and the resulting mixture was sonicated for 10 minutes. To the mixture, 0.4 U of cholesterol oxidase in 50 μL of potassium phosphate buffer was added and incubated at 37°C for 1 hour. Three hundred microliters of methanol was added to stop the reaction. The products, enones, were extracted 3 times with 2 mL of hexane; and the extracts were evaporated under N_2 stream. The residue was redissolved in 150 μL of 5% isopropanol in hexane, and 100 μL of the solution was subjected to high-pressure liquid chromatography (HPLC) as described below.

The HPLC analysis was conducted with Alliance 2695 separation module fitted with 2487 Dual λ absorbance detector (Waters). The separation was carried out on an Ultrasphere silica column (5 μm, 4.6 mm id × 250 mm; Beckman, Urbana, IL) and hexane:isopropanol:acetic acid (965:25:10, vol:vol:vol) as an eluent at a flow rate of 1.3 mL/min. The column temperature was kept constant at 30°C. The enones were monitored at 240-nm absorption.

2.4. Lipid levels in liver tissue and sera

Liver cholesterol and triglycerides were extracted and analyzed as previously described [14]. Briefly, mouse liver tissue, 100 mg, was homogenized in 1 mL of phosphate-buffered saline. The lipids in the homogenates were extracted overnight with 9 mL of chloroform:methanol (2:1, vol/vol), sonicated for 1 to 2 hours, and filtered. The extract, 100 μL, was evaporated to dryness and dissolved in 100 μL of isopropanol containing 10% Triton X-100 for cholesterol assay, dissolved in isopropanol for triglycerides assay, or in nonesterified fatty acid solution (0.5 g of EDTA-Na_2, 2 g of Triton X-100, 0.76 mL of 1 N NaOH, and 0.5 g of sodium azide per liter of H_2O, pH 6.5) for free fatty acids assay. Total and free cholesterol, triglycerides, and free fatty acids assays were performed according to the manufacturer's instructions.

For serum analysis, the lipid levels and the liver-specific cytosolic enzyme activities of alkaline phosphatase, alanine transaminase, and aspartate transaminase in serum of mice were determined by clinical biochemistry laboratory blood assays at the VA Medical Center. The lipoproteins of cholesterol (very low-density lipoprotein [VLDL], low-density lipoprotein [LDL], and high-density lipoprotein [HDL]) were measured by gel filtration using HPLC as previously described [19] with mild modifications. Briefly, serum was centrifuged at 2000 rpm for 2 minutes, and 100 μL of supernatant was subjected to HPLC with Pharmacia Superose 6HR 10/30 column (Oregon City, OR, USA) using mobile phase, 154 mmol/L NaCl and 0.1 mmol/L EDTA (pH 8.0) at flow rate of 0.2 mL/min. Each fraction was collected starting at 20 minutes, 1.2 minutes each (240 μL) for up to 100 minutes. A total of 180 μL of each fraction was transferred to a 96-well plate, and 20 μL Wako (Richmond, VA, USA) total cholesterol kit 10× reagent buffer was added. After incubating at 37°C for 3 hours in the dark, the optical density was read at 595 nm for cholesterol assay. The protein profile was monitored at an optical density of 280 nm as internal control.

2.5. Determination of gene expression involved in lipid metabolism

Nuclear and cytosolic proteins from mouse liver tissue were extracted according to the manufacturer's instructions. A total of 20 μg nuclear extracts or 50 μg cytosolic proteins was loaded on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis for detection of the specific proteins, including LXRα, SREBP-1, SREBP-2, ACC1, FAS, and SULT2B1b, using lamin B1 and β-actin as loading control for nuclear and cytosolic proteins, respectively. Western blot analysis was performed as previously described [20].

Total RNA in liver tissue was isolated by SV total RNA isolation kit (Promega, Wisconsin, WI) following the manufacturer's instructions. The relative mRNA levels were measured by real-time reverse transcriptase polymerase chain reaction as previously described [20]. Quantitative normalization of complementary DNA in each sample was performed using glyceraldehyde-3-phosphate dehydrogenase as

an internal control. The primer sets used in these assays are shown in Supplemental Table 1.

2.6. Statistical analysis

Data are expressed as mean ± SD. Significance of differences was determined using Student *t* test for unpaired samples. A value of $P < .05$ was considered statistically significant.

3. Results

3.1. Effect of adenovirus infection on liver toxicity

Following infection with different amount of recombinant adenovirus, serum activities of alkaline phosphatase, alanine transaminase, and aspartate transaminase and the

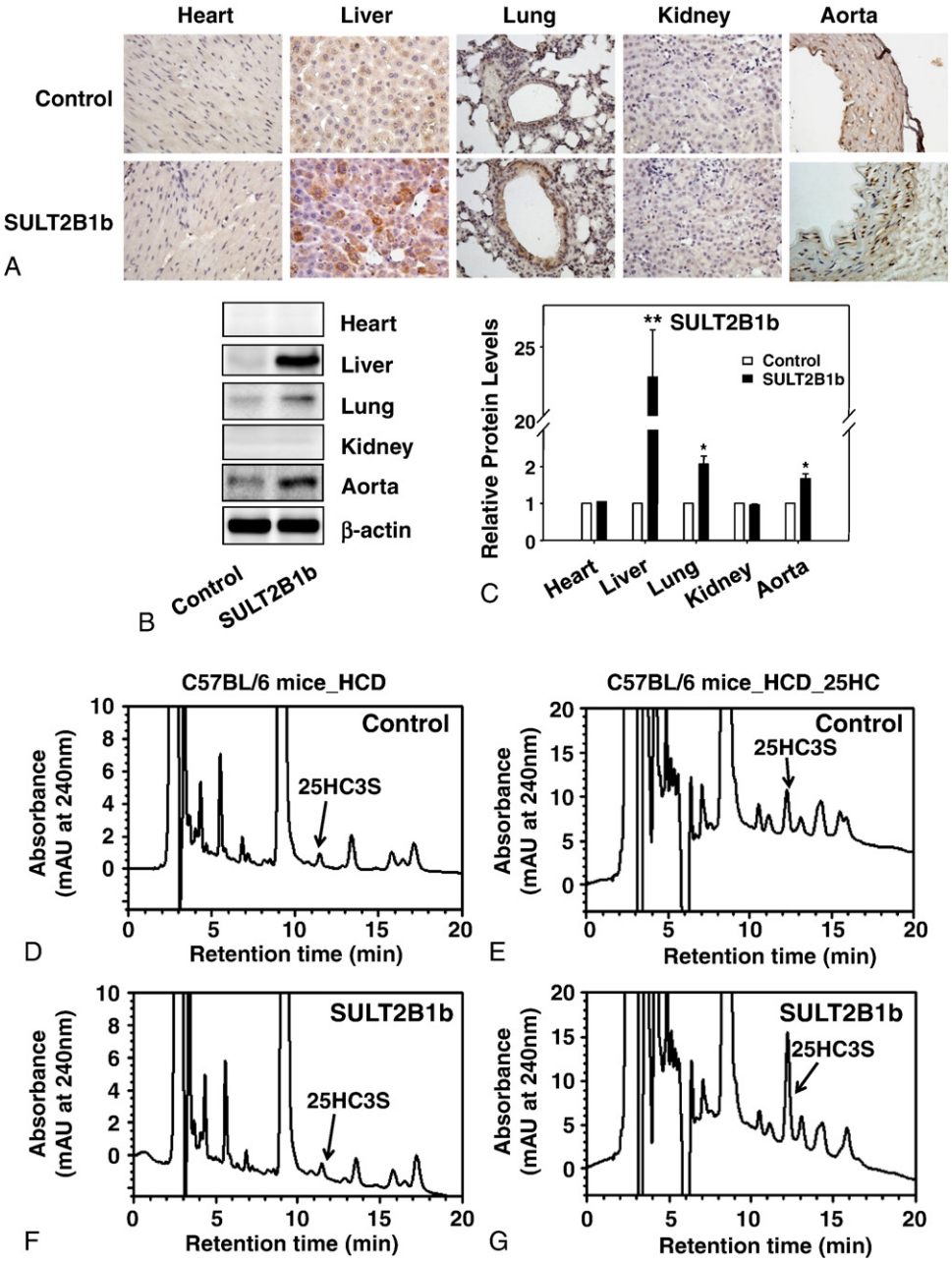


Fig. 1 – Determination of SULT2B1b expression in different tissues and SULT2B1b activities in liver tissue following infection with Ad-SULT2B1b. C57BL/6 mice, 8 weeks old, were fed with HCD for 10 weeks and infected with Ad-control or Ad-SULT2B1b (1×10^8 pfu) in the presence or absence of 25HC as indicated. SULT2B1b protein expressions in different tissues were analyzed by immunohistochemistry at day 6 following infection (A), and its protein levels were analyzed by Western blot (B and C). Total intracellular lipids were extracted with chloroform/methanol. Sulfated oxysterols in the liver infected with Ad-β-Gal (D and E) or Ad-SULT2B1b (F and G) were analyzed by HPLC. 24-Hydrocholesterol; 25HC; 27-hydrocholesterol; 24,25-epoxycholesterol; 7KC; 6βHC 7α-hydrocholesterol; and 7βHC were used as standard controls. *P < .05, **P < .01 vs control. n = 5 or 6.

Table 2 – Lipids in serum in C57BL/6 mice or LDLR$^{-/-}$ mice after infection with β-Gal control or SULT2B1b virus

	C57BL/6 mice_HCD		C57BL/6 mice_HCD + 25HC		LDLR$^{-/-}$ mice_HFD	
	Control	SULT2B1b	Control	SULT2B1b	Control	SULT2B1b
TG (mg/dL)	53 ± 7.3	49 ± 12.4	39 ± 3.4	32 ± 4.3*	328 ± 92.8	222 ± 47.2*
TC (mg/dL)	117 ± 12.7	120 ± 8.4	102 ± 8.2	107 ± 9.3	1338 ± 203.8	1233 ± 155.6

Values are mean ± SD. TG indicates triglycerides; TC, total cholesterol.
* $P < .05$ vs control.

ratios of liver to body weight were determined at day 6 as shown in Supplemental Fig. 1A and Supplemental Fig. 1B. The activities and the ratios did not significantly change as compared with Ad-β-Gal–infected mice, indicating that no liver injury occurred when less than 1 × 10^8 pfu of Ad-SULT2B1b per mouse was used. Thus, the dose of 1 × 10^8 pfu per mouse was selected to study the effect of SULT2B1b on lipid metabolism. Under this condition, no liver toxicity was detected in mice fed with HCD or HFD (Supplemental Fig. 1C).

3.2. *SULT2B1b expression in different tissues after infection with Ad-SULT2B1b*

Mice were infected with Ad-SULT2B1b or Ad-control through tail vein injection in the condition as described above. Immunohistochemistry analysis showed that SULT2B1b gene expression following infection was significantly increased in the liver, aorta, and lung tissues, but not in the heart or kidney (Fig. 1A). Consistently, Western blot analysis following Ad-SULT2B1b infection showed that SULT2B1b gene expression increased by 20-fold in the liver, with more modest increases of 1.5-fold in the aorta and 2-fold in the lung, as compared with control. No changes in expression of SULT2B1b were detected in the heart or kidney (Fig. 1B and 1C).

3.3. *Effect of SULT2B1b on oxysterols and sulfated oxysterols in liver tissues*

To determine the specific activities of SULT2B1b, oxysterols and sulfated oxysterols were extracted from liver tissues and analyzed by HPLC. The results showed that SULT2B1b overexpression in the presence of 25HC significantly increased sulfated oxysterols, especially 25HC3S (Fig. 1E and G); decreased nonsulfated oxysterols, including 7-ketocholesterol (7KC), 6β-hydroxycholesterol (6βHC), and 25HC (Supplemental Fig. 2B and Supplemental Fig. 2D). However, in the absence of 25HC, SULT2B1b overexpression alone did not significantly change the levels of oxysterols and sulfated oxysterols (Fig. 1D, F, Supplemental Fig. 2A and Supplemental Fig. 2C).

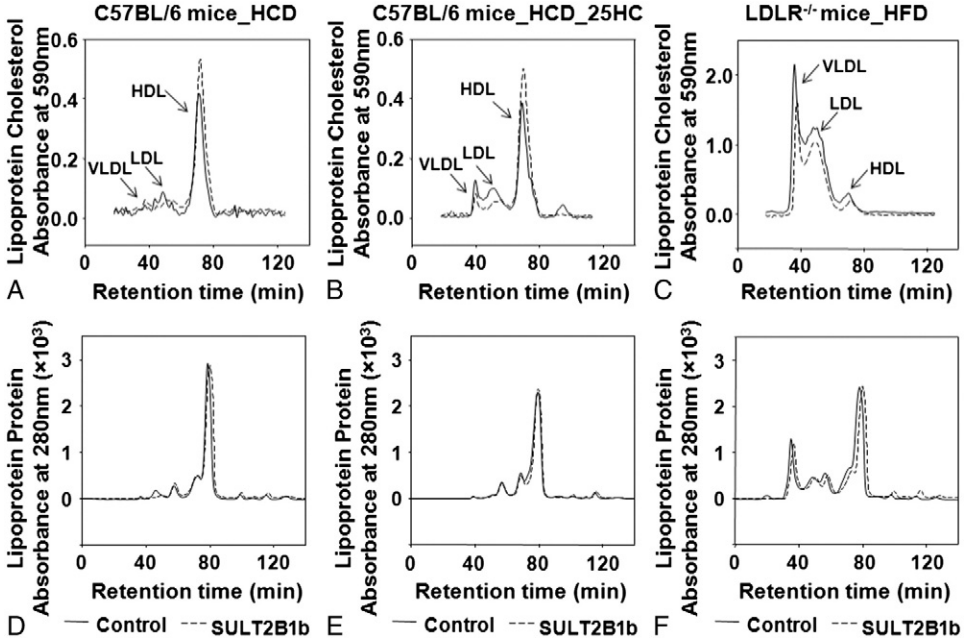


Fig. 2 – Effect of SULT2B1b overexpression on lipoprotein cholesterol in serum by HPLC. C57BL/6 mice and LDLR$^{-/-}$ mice, 8 weeks old, were fed with HCD or HFD for 10 weeks and then infected with Ad-control or Ad-SULT2B1b (1 × 10^8 pfu) in the presence or absence of 25HC as indicated. The lipoprotein (VLDL, LDL, and HDL) cholesterol levels in sera in both C57BL/6 mice and LDLR$^{-/-}$ mice were analyzed by HPLC (A, B, C). The lipoprotein protein levels were determined by absorbance at 280 nm (D, E, F). The data represent 1 of 3 separate experiments.

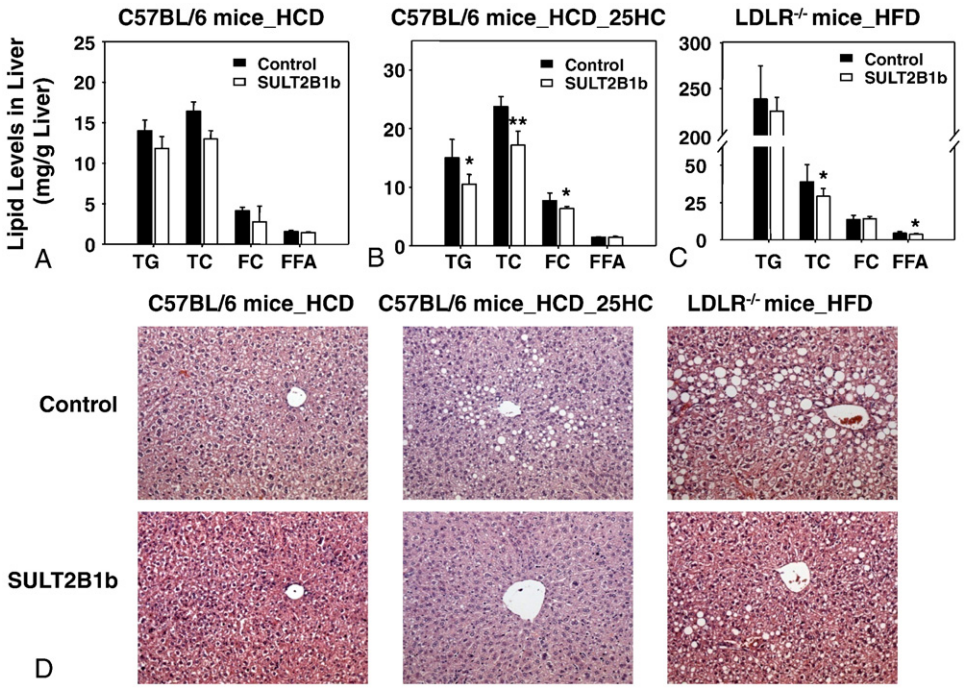


Fig. 3 – Effect of SULT2B1b overexpression on lipid levels in the liver tissues. The mice were fed and infected as stated in Fig. 2. Total intracellular lipids were extracted with chloroform/methanol. Triglycerides, free fatty acids, total cholesterol, and free cholesterol in liver in both C57BL/6 mice and LDLR$^{-/-}$ mice (A-C) were analyzed as described in "Materials and Methods." Liver morphology was examined by H&E staining (D). $^{*}P < .05$, $^{**}P < .01$ vs control. n = 5 or 6.

3.4. *Effect of SULT2B1b overexpression on lipid levels in sera and liver tissue*

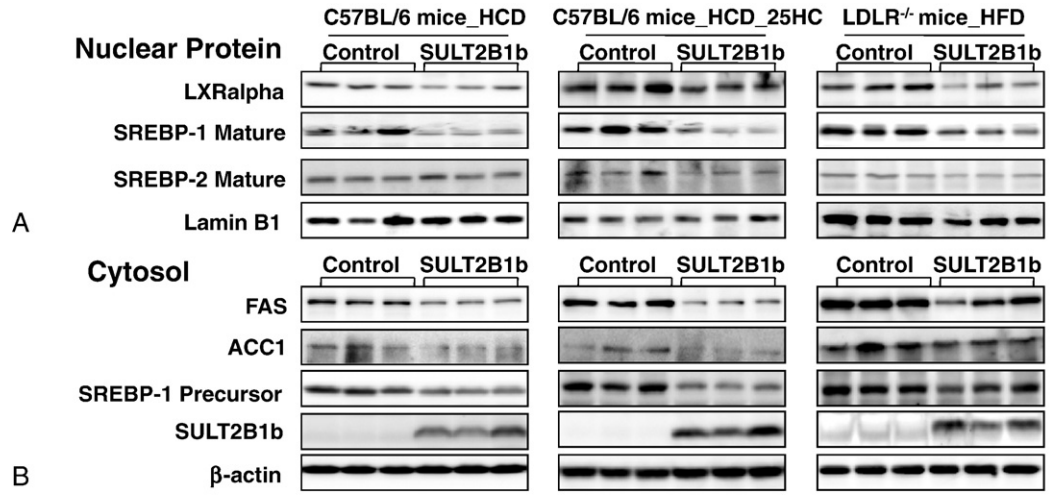
To study the effect of SULT2B1b on serum and hepatic lipid levels, C57BL/6 and LDLR$^{-/-}$ mice were infected by SULT2B1b adenovirus, with or without intraperitoneal injection of 25HC, as described above. Total cholesterol and triglycerides in sera were measured by clinical laboratory as shown in Table 2. Following the combination of SULT2B1b overexpression and 25HC administration, triglyceride levels in serum in C57BL/6 mice_HCD were significantly decreased by 18% as compared with control mice injected with β-Gal virus. No significant change was observed in the absence of 25HC. In LDLR$^{-/-}$ mice_HFD, triglyceride levels in serum were decreased by 32% following SULT2B1b overexpression as compared with the control injected with β-Gal virus. However, total serum cholesterol levels in both C57BL/6 and LDLR$^{-/-}$ mice were unchanged with SULT2B1b overexpression. Interestingly, SULT2B1b overexpression decreased cholesterol levels in VLDL and LDL fraction in C57BL/6 and LDLR$^{-/-}$ mice, whereas it increased cholesterol levels in HDL fraction in C57BL/6 mice (Fig. 2A, B, and C). The protein profile (internal control) was unchanged following SULT2B1b overexpression (Fig. 2D, E, and F). These effects were much stronger in the presence of 25HC (Fig. 2).

Quantitative analysis of hepatic lipid levels showed that overexpression of SULT2B1b significantly decreased hepatic triglyceride, total cholesterol, and free cholesterol levels in the presence of 25HC in C57BL/6 mice_HCD (Fig. 3B). No significant change was detected in the absence of 25HC as compared with control (Fig. 3A). In LDLR$^{-/-}$ mice_HFD, SULT2B1b overexpression significantly decreased total cholesterol and free fatty acids levels but did not change triglycerides and free cholesterol levels significantly (Fig. 3C). Consistently, H&E staining also showed that SULT2B1b overexpression substantially decreased total neutral lipids in liver tissue in both C57BL/6 mice and LDLR$^{-/-}$ mice (Fig. 3D).

3.5. *Effect of SULT2B1b on gene expressions involved in lipid metabolism*

To better understand the mechanism of effects of SULT2B1b on lipid metabolism, gene expressions involved in lipid metabolism were determined. As expected, overexpression of SULT2B1b significantly decreased LXRα and SREBP-1 in nuclear protein levels but not SREBP-2 in both C57BL/6 mice and LDLR$^{-/-}$ mice fed with HCD or HFD. Consistently, SULT2B1b overexpression also significantly decreased the cytosolic protein levels of FAS and ACC1 as shown in Fig. 4A and B.

Real-time reverse transcriptase polymerase chain reaction analysis of the gene expressions at mRNA level involved in lipid metabolism was shown in Table 3. Consistent with protein levels, SULT2B1b overexpression significantly decreased mRNA levels of LXRα, SREBP-1, SREBP-2, glycerol-3-phosphate acyltransferase (mitochondrial), acetyl-coenzyme A acetyltransferase 2, CYP27A, ABCA1, ATP-binding cassette transporter G1, and steroid sulfatase involved in lipid metabolism in the mice injected peritoneally with 25HC; in the absence of 25HC, SULT2B1b overexpression only decreased mRNA levels of LXRα, SREBP-1, and ABCA1.



Relative hepatic protein expression in C57BL/6 mice or LDLR$^{-/-}$ mice after infection with β-Gal control or SULT2B1b virus

	C57BL/6 mice_HCD		C57BL/6 mice_HCD + 25HC		LDLR$^{-/-}$ mice_HFD	
	Control	SULT2B1b	Control	SULT2B1b	Control	SULT2B1b
Nuclear protein						
LXRalpha	1.0 ± 0.32	0.54 ± 0.19	1.0 ± 0.20	**0.68 ± 0.06** *	1.0 ± 0.15	**0.51 ± 0.13** **
SREBP-1 Mature	1.0 ± 0.23	**0.35 ± 0.07** *	1.0 ± 0.38	**0.28 ± 0.11** *	1.0 ± 0.15	**0.55 ± 0.07** **
SREBP-2 Mature	1.0 ± 0.16	0.86 ± 0.03	1.0 ± 0.14	0.81 ± 0.17	1.0 ± 0.16	**0.65 ± 0.07** *
Cytosol						
ACC-1	1.0 ± 0.34	**0.54 ± 0.13** *	1.0 ± 0.20	**0.40 ± 0.03** *	1.0 ± 0.35	0.81 ± 0.03
FAS	1.0 ± 0.11	**0.49 ± 0.02** **	1.0 ± 0.18	**0.58 ± 0.14** *	1.0 ± 0.08	**0.54 ± 0.17** **
SREBP-1 Precursor	1.0 ± 0.15	**0.81 ± 0.01** *	1.0 ± 0.21	**0.50 ± 0.07** **	1.0 ± 0.09	**0.82 ± 0.10** *
SULT2B1b	1.0 ± 0.10	**3.04 ± 0.45** **	1.0 ± 0.07	**8.78 ± 2.43** **	1.0 ± 0.08	**2.53 ± 0.78** *

Values are mean ± SD; * $P < .05$, ** $P < .01$ vs Control

Fig. 4 – Effect of SULT2B1b overexpression on gene expressions involved in lipid metabolism at the protein level. The mice were fed and infected as stated in Fig. 2. Nuclear proteins (LXR*α*, SREBP-1 mature, SREBP-2 mature) and cytosolic proteins (FAS, ACC1, SREBP-1 precursor, SULT2B1b) in liver tissue were analyzed by Western blot with specific antibodies (A). Western blot data were quantitatively analyzed (B). Cytoplasmic proteins were normalized to *ß*-actin; nuclear proteins, to lamin B1. *P < .05, **P < .01 vs control. n = 5 or 6.

4. Discussion

The present study shows that in vivo SULT2B1b overexpression with 25HC supplementation in Western diet-fed mice increases 25HC sulfation and levels of its product 25HC3S, mainly in liver tissue. These effects are accompanied by decreases in serum and hepatic triglycerides, total cholesterol, free cholesterol, and free fatty acids, and by reduction in key regulators and enzymes in lipid metabolism, including SREBP-1, SREBP-2, ACC1, and FAS. The results are consistent with previous in vitro studies demonstrating that SULT2B1b overexpression in the presence of 25HC leads to 25HC3S formation and decreases intracellular lipid levels [11,12,14]. The present results in vivo confirm that oxysterol sulfation by SULT2B1b can play an important regulatory role in lipid metabolism and that oxysterol sulfation must be added to the list of signaling pathways involved in lipid homeostasis [14,21].

There is no significant difference in dietary intake between experimental and control mice (data not shown). Thus, serum and hepatic lipid levels depend on lipid synthesis and clearance. To confirm that the decreases in serum and hepatic lipid levels are mediated through suppression of lipid biosynthesis, low-density-lipoprotein receptor (LDLR) knockout mice have been used in the present study. Low-density-lipoprotein receptor is an important molecule in clearance of lipids from circulation [22-24]. Binding of lipoproteins to LDLR facilitates their endocytosis and delivery of lipids to target tissues [25]. Therefore, in this animal model, the serum lipid levels more directly represent the rates of lipid biosynthesis from liver tissue. Interestingly, overexpression of SULT2B1b in the knockout mice not only decreases neutral lipid levels in the liver but also changes the lipid profiles in the sera, indicating that SULT2B1b overexpression decreases VLDL and LDL synthesis and secretion from liver tissue and increases HDL synthesis (Fig. 2). High-density lipoprotein plays a key role in reverse cholesterol transport, which involves transport of cholesterol from peripheral tissues and cells to the liver, transforming it into bile acids and finally eliminating it from the body [26-28]. It has been shown that the patients with high VLDL and LDL and low HDL are at a higher risk of myocardial infarction and sudden death [29-32]. Whether these patients have lower expression of SULT2B1b or lower sulfated oxysterol levels is unknown and merits further investigation.

Three SULT isoenzymes in human and 4 in rat have been cloned and identified [33-36]. He et al [37] reported that SULT2B1b has been localized to the cytosol and nuclei of both human cells and tissues. Fuda et al [38] and Javitt et al

Table 3 – Relative hepatic mRNA expression in C57BL/6 mice or LDLR$^{-/-}$ mice after infection with β-Gal control or SULT2B1b virus

	C57BL/6 mice_HCD		C57BL/6 mice_HCD + 25HC		LDLR$^{-/-}$ mice_HFD	
	Control	SULT2B1b	Control	SULT2B1b	Control	SULT2B1b
Fatty acid metabolism						
SREBP-1c	1.0 ± 0.29	0.57 ± 0.19*	1.0 ± 0.16	0.53 ± 0.18†	1.0 ± 0.23	1.14 ± 0.27
ACC1	1.0 ± 0.37	0.93 ± 0.15	1.0 ± 0.27	0.79 ± 0.09	1.0 ± 0 22	0.84 ± 0.22
FAS	1.0 ± 0.41	0.76 ± 0.21	1.0 ± 0.50	0.57 ± 0.14	1.0 ± 0.27	1.01 ± 0.20
LXRa	1.0 ± 0.14	0.81 ± 0.09*	1.0 ± 0.30	0.63 ± 0.08*	1.0 ± 0.19	0.82 ± 0.11*
PPARa	1.0 ± 0.26	0.59 ± 0.17*	1.0 ± 0.29	0.58 ± 0.15*	1.0 ± 0.27	0.81 ± 0.19*
FABP4	1.0 ± 0.51	0.68 ± 0.19	1.0 ± 0.45	0.43 ± 0.16*	1.0 ± 0.27	0.87 ± 0.27
FATP	1.0 ± 0.39	0.59 ± 0.15*	1.0 ± 0.25	0.62 ± 0.17*	1.0 ± 0.33	0.51 ± 0.18†
Triglyceride metabolism						
GPAM	1.0 ± 0.62	0.94 ± 0.36	1.0 ± 0.33	0.69 ± 0.11*	1.0 ± 0.19	0.90 ± 0.17
MTTP	1.0 ± 0.55	0.93 ± 0.41	1.0 ± 0.28	0.81 ± 0.16	1.0 ± 0.17	0.89 ± 0.16
PLTP	1.0 ± 0.69	0.94 ± 0.51	1.0 ± 0.13	0.85 ± 0.20	1.0 ± 0.15	0.69 ± 0.11†
Cholesterol metabolism						
SREBP-2	1.0 ± 0.68	0.92 ± 0.50	1.0 ± 0.08	0.71 ± 0.07†	1.0 ± 0.14	0.94 ± 0.16
HMGR	1.0 ± 0.19	0.90 ± 0.08	1.0 ± 0.24	0.96 ± 0.24	1.0 ± 0.26	0.91 ± 0.29
LDLR	1.0 ± 0.20	1.13 ± 0.25	1.0 ± 0.39	0.53 ± 0.11*	1.0 ± 0.34	0.80 ± 0.22
ACAT1	1.0 ± 0.12	1.02 ± 0.35	1.0 ± 0.18	1.11 ± 0.29	1.0 ± 0 23	1.00 ± 0.13
ACAT2	1.0 ± 0.27	0.61 ± 0.10*	1.0 ± 0.21	0.73 ± 0.06*	1.0 ± 0.19	0.89 ± 0.10
Cholesterol efflux						
ABCA1	1.0 ± 0.22	0.65 ± 0.09*	1.0 ± 0.31	0.51 ± 0.16*	1.0 ± 0.26	0.89 ± 0.17
ABCG1	1.0 ± 0.23	1.15 ± 0.28	1.0 ± 0.29	0.64 ± 0.22*	1.0 ± 0.27	0.83 ± 0.19
Bile acid metabolism						
CYP7a	1.0 ± 0.33	0.78 ± 0.42	1.0 ± 0.35	0.57 ± 0.31	1.0 ± 0.20	0.90 ± 0.48
CYP27a	1.0 ± 0.27	0.80 ± 0.14	1.0 ± 0.31	0.50 ± 0.07*	1.0 ± 0.21	1.10 ± 0.21
Others						
SULT2B1b	1.0 ± 0.24	193.5 ± 46.1†	1.0 ± 0.17	754.1 ± 114.8†	1.0 ± 0.44	324.6 ± 36.7†
STS	1.0 ± 0.73	0.52 ± 0.23	1.0 ± 0.37	0.41 ± 0.06*	1.0 ± 0.57	1.28 ± 0.37

Values are mean ± SD; n = 5 to 6. PPARa indicates peroxisome proliferator activated receptor a; FABP4, fatty acid binding protein 4; FATP, fatty acid transport protein; GPAM, glycerol-3-phosphate acyltransferase (mitochondrial); MTTP, microsomal triglyceride transfer protein; PLTP, phospholipid transfer protein; HMGR, 5-hydroxy-3-methylglutaryl-coenzyme A reductase; ACAT, acetyl-coenzyme A acetyltransferase; ABCG1, ATP-binding cassette transporter G1; STS, steroid sulfatase.
*$P < .05$, †$P < .01$ vs control.

[33] reported that SULT2B1b could sulfonate a variety of oxysterols, including 7-KC. Cells with high expression of SULT2B1b are significantly more resistant to the cytotoxic effects of 7KC, and overexpression of SULT2B1b protects from 7KC-induced loss of cell viability [38]. Our previous studies also showed that SULT2B1b effectively sulfates 25HC in human aortic endothelial cells, with the main product of 25HC3S [14]. However, in the present study, overexpression of SULT2B1b in vivo only significantly increased 25HC3S , whereas other sulfated oxysterols such as 24HC-S, 27HC-S, 7KC-S, 6βHC-S, 7αHC-S, and 7βHC-S were almost unchanged or undetectable (Fig. 1D, E, and F and Fig. 2G). These results suggest that SULT2B1b may have a potential specificity for 25HC sulfation in mouse liver. Furthermore, hepatic SULT2B1b expression in mice with HFD decreased by 90% compared with those with normal-cholesterol diet or HCD. Thus, we hypothesized that lower level of SULT2B1b may be one of the major pathogenesis during occurrence and development of NAFLD.

Overexpression of SULT2B1b inactivates the response of liver oxysterol receptor (LXR) to multiple oxysterol ligands [21], suggesting that SULT2B1b is involved in LXR signaling pathway. It has been hypothesized that the oxysterol sulfation by SULT2B1b is an inactivation processing [21]. However, our laboratory studies showed that addition of exogenous 25HC3S to primary human hepatocytes and human acute monocytic leukemia cell line–derived macrophages, and overexpression of SULT2B1b in human aortic endothelial cells decrease SREBP-1/2 expression and block the activation of SREBP-1c; suppress the expression of key enzymes, including 3-hydroxy-3-methylglutaryl-CoA reductase, ACC1, and FAS involved in lipid metabolism; and subsequently decrease cholesterol and neutral lipid levels [11,12,14]. These results indicate that the sulfated oxysterol may act as an LXR antagonist rather than only an inactive form of LXR ligand and that SULT2B1b plays an important role in lipid homeostasis.

In conclusion, the present study adds to the convincing body of evidence implicating the oxysterol sulfate 25HC3S in regulation of serum and hepatic lipid metabolism in vivo. Oxysterol sulfation is catalyzed by SULT2B1b; and activity of this enzyme, by determining the balance between 25HC and 25HC3S, may play an important role in lipid homeostasis.

Appendix A. Supplementary data

Supplementary to this article can be found online at doi:10.1016/j.metabol.2011.11.014.

Funding

This work was supported by grants from the National Institutes of Health (R01 HL078898) and the Veterans Administration Department (VA Merit Review).

Acknowledgment

We acknowledge the excellent technical help from Patricia S. Cooper and Dalila Marques.

Conflict of Interest

The authors declare that they have no conflict of interest.

REFERENCES

[1] Clark JM, Brancati FL, Diehl AM. Nonalcoholic fatty liver disease. Gastroenterology 2002;122:1649-57.
[2] Lazo M, Clark JM. The epidemiology of nonalcoholic fatty liver disease: a global perspective. Semin Liver Dis 2008;28:339-50.
[3] Brunt EM. Nonalcoholic steatohepatitis. Semin Liver Dis 2004;24:3-20.
[4] Leite NC, Salles GF, Araujo AL, et al. Prevalence and associated factors of non-alcoholic fatty liver disease in patients with type-2 diabetes mellitus. Liver Int 2009;29:113-9.
[5] Miller MH, Ferguson MA, Dillon JF. Systematic review of performance of non-invasive biomarkers in the evaluation of non-alcoholic fatty liver disease. Liver Int 2011;31:461-73.
[6] Petta S, Muratore C, Craxi A. Non-alcoholic fatty liver disease pathogenesis. The present and the future Dig Liver Dis 2009;41:615-25.
[7] Day CP, James OF. Steatohepatitis: a tale of two "hits"? Gastroenterology 1998;114:842-5.
[8] Pandak WM, Ren S, Marques D, et al. Transport of cholesterol into mitochondria is rate-limiting for bile acid synthesis via the alternative pathway in primary rat hepatocytes. J Biol Chem 2002;277:48158-64.
[9] Ren S, Hylemon PB, Marques D, et al. Overexpression of cholesterol transporter StAR increases in vivo rates of bile acid synthesis in the rat and mouse. Hepatology 2004;40:910-7.
[10] Ren S, Hylemon P, Zhang ZP, et al. Identification of a novel sulfonated oxysterol, 5-cholesten-3beta,25-diol 3-sulfonate, in hepatocyte nuclei and mitochondria. J Lipid Res 2006;47:1081-90.
[11] Xu L, Bai Q, Rodriguez-Agudo D, et al. Regulation of hepatocyte lipid metabolism and inflammatory response by 25-hydroxycholesterol and 25-hydroxycholesterol-3-sulfate. Lipids 2010;45:821-32.
[12] Ma Y, Xu L, Rodriguez-Agudo D, et al. 25-Hydroxycholesterol-3-sulfate regulates macrophage lipid metabolism via the LXR/SREBP-1 signaling pathway. Am J Physiol Endocrinol Metab 2008;295:E1369-79.
[13] Li X, Pandak WM, Erickson SK, et al. Biosynthesis of the regulatory oxysterol, 5-cholesten-3beta,25-diol 3-sulfate, in hepatocytes. J Lipid Res 2007;48:2587-96.
[14] Bai Q, Xu L, Kakiyama G, et al. Sulfation of 25-hydroxycholesterol by SULT2B1b decreases cellular lipids via the LXR/SREBP-1c signaling pathway in human aortic endothelial cells. Atherosclerosis 2011;214:350-6.
[15] Shimizu C, Fuda H, Yanai H, et al. Conservation of the hydroxysteroid sulfotransferase SULT2B1 gene structure in the mouse: pre- and postnatal expression, kinetic analysis of isoforms, and comparison with prototypical SULT2A1. Endocrinology 2003;144:1186-93.
[16] Geese WJ, Raftogianis RB. Biochemical characterization and tissue distribution of human SULT2B1. Biochem Biophys Res Commun 2001;288:280-9.
[17] Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem 1957;226:497-509.
[18] Zhang Z, Li D, Blanchard DE, et al. Key regulatory oxysterols in liver: analysis as delta4-3-ketone derivatives by HPLC and response to physiological perturbations. J Lipid Res 2001;42:649-58.
[19] Ishibashi S, Brown MS, Goldstein JL, et al. Hypercholesterolemia in low density lipoprotein receptor knockout mice and its reversal by adenovirus-mediated gene delivery. J Clin Invest 1993;92:883-93.
[20] Ren S, Hylemon P, Marques D, et al. Effect of increasing the expression of cholesterol transporters (StAR, MLN64, and SCP-2) on bile acid synthesis. J Lipid Res 2004;45:2123-31.
[21] Chen W, Chen G, Head DL, et al. Enzymatic reduction of oxysterols impairs LXR signaling in cultured cells and the livers of mice. Cell Metab 2007;5:73-9.
[22] Hobbs HH, Russell DW, Brown MS, et al. The LDL receptor locus in familial hypercholesterolemia: mutational analysis of a membrane protein. Annu Rev Genet 1990;24:133-70.
[23] Kypreos KE, Zannis VI. LDL receptor deficiency or apoE mutations prevent remnant clearance and induce hypertriglyceridemia in mice. J Lipid Res 2006;47:521-9.
[24] Wouters K, Shiri-Sverdlov R, van Gorp PJ, et al. Understanding hyperlipidemia and atherosclerosis: lessons from genetically modified apoe and ldlr mice. Clin Chem Lab Med 2005;43:470-9.
[25] Brown MS, Goldstein JL. A receptor-mediated pathway for cholesterol homeostasis. Science 1986;232:34-47.
[26] Glomset JA. Physiological role of lecithin-cholesterol acyltransferase. Am J Clin Nutr 1970;23:1129-36.
[27] Lewis GF, Rader DJ. New insights into the regulation of HDL metabolism and reverse cholesterol transport. Circ Res 2005;96:1221-32.
[28] Cuchel M, Rader DJ. Macrophage reverse cholesterol transport: key to the regression of atherosclerosis? Circulation 2006;113:2548-55.
[29] Baigent C, Keech A, Kearney PM, et al. Efficacy and safety of cholesterol-lowering treatment: prospective meta-analysis of data from 90,056 participants in 14 randomised trials of statins. Lancet 2005;366:1267-78.
[30] Gordon DJ, Probstfield JL, Garrison RJ, et al. High-density lipoprotein cholesterol and cardiovascular disease. Four prospective American studies. Circulation 1989;79:8-15.
[31] Curb JD, Abbott RD, Rodriguez BL, et al. A prospective study of HDL-C and cholesteryl ester transfer protein gene mutations and the risk of coronary heart disease in the elderly. J Lipid Res 2004;45:948-53.
[32] Barter P, Gotto AM, LaRosa JC, et al. HDL cholesterol, very low levels of LDL cholesterol, and cardiovascular events. N Engl J Med 2007;357:1301-10.
[33] Javitt NB, Lee YC, Shimizu C, et al. Cholesterol and hydroxycholesterol sulfotransferases: identification, distinction from dehydroepiandrosterone sulfotransferase, and differential tissue expression. Endocrinology 2001;142:2978-84.
[34] Falany CN, He D, Dumas N, et al. Human cytosolic sulfotransferase 2B1: isoform expression, tissue specificity and subcellular localization. J Steroid Biochem Mol Biol 2006;102:214-21.

[35] Liu L, LeCluyse EL, Liu J, et al. Sulfotransferase gene expression in primary cultures of rat hepatocytes. Biochem Pharmacol 1996;52:1621-30.
[36] Watabe T, Ogura K, Satsukawa M, et al. Molecular cloning and functions of rat liver hydroxysteroid sulfotransferases catalysing covalent binding of carcinogenic polycyclic arylmethanols to DNA. Chem Biol Interact 1994;92:87-105.
[37] He D, Frost AR, Falany CN. Identification and immunohistochemical localization of Sulfotransferase 2B1b (SULT2B1b) in human lung. Biochim Biophys Acta 2005;1724:119-26.
[38] Fuda H, Javitt NB, Mitamura K, et al. Oxysterols are substrates for cholesterol sulfotransferase. J Lipid Res 2007;48:1343-52.