Biosynthesis of the regulatory oxysterol, 5-cholesten-3β,25-diol 3-sulfate, in hepatocytes

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Abstract **Cellular cholesterol homeostasis is maintained through coordinated regulation of cholesterol synthesis, degradation, and secretion. Nuclear receptors for oxygenated cholesterol derivatives (oxysterols) are known to play key roles in the regulation of cholesterol homeostasis. We recently identified a sulfated oxysterol, 5-cholesten-3β,25-diol 3-sulfate (25HC3S), that is localized to liver nuclei. The present study reports a biosynthetic pathway for 25HC3S in hepatocytes. Assays using mitochondria isolated from rats and sterol 27-hydroxylase (Cyp27A1) gene knockout mice indicated that 25-hydroxycholesterol (25HC) is synthesized by CYP27A1. Incubation of cholesterol or 25HC with mitochondrial and cytosolic fractions in the presence of 3′-phosphoadenosyl 5′-phosphosulfate resulted in the synthesis of 25HC3S. Real-time RT-PCR and Western blot analysis showed the presence of insulin-regulated hydroxycholesterol sulfotransferase 2B1b (SULT2B1b) in hepatocytes. 25HC3S, but not 25HC, decreased SULT2B1b mRNA and protein levels. Specific small interfering RNA decreased SULT2B1b mRNA, protein, and activity levels. These findings demonstrate that mitochondria synthesize 25HC, which is subsequently 3β-sulfated to form 25HC3S.—** Li, X., W. M. Pandak, S. K. Erickson, Y. Ma, L. Yin, P. Hylemon, and S. Ren. **Biosynthesis of the regulatory oxysterol, 5-cholesten-3β,25-diol 3-sulfate, in hepatocytes.** ***J. Lipid Res.*** **2007.** 48: **2587–2596.**

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The metabolism of cholesterol to primary bile acids occurs via two main pathways in hepatocytes (1). The "neutral" pathway is considered to be the major pathway, at least in humans and rats (2–4). The "acidic" pathway is initiated by the mitochondrial enzyme sterol 27-hydroxylase (CYP27A1). Recently, reports showed that transport of cholesterol to mitochondria is the rate-limiting step for bile acid synthesis via the "acidic" pathway (5, 6). Oxysterol intermediates generated by this pathway, including 27-hydroxycholesterol (27HC) and 25-hydroxycholesterol (25HC), have been implicated in the regulation of cholesterol homeostasis (7, 8), in part by regulating the expression of genes involved in cholesterol biosynthesis and transport (9–11). Increased CYP27A1 activity in peripheral tissues downregulates cholesterol synthesis through the sterol-regulatory element binding protein pathway and enhances the efflux of cholesterol and its elimination (12). The acidic pathway is the only bile acid biosynthetic pathway expressed in fetal and neonatal life, a time at which no bile acid/bile is needed for digestion. In addition to the synthesis of bile acids, it is believed that the acidic pathway is related to cell differentiation and cell growth (13, 14). However, the physiological significance of the acidic pathway is not clearly defined (13, 14).

Recently, a novel oxysterol, 5-cholesten-3β,25-diol 3-sulfate (25HC3S), was found in human liver nuclei, and its levels were increased dramatically in nuclei after overexpression of a mitochondrial cholesterol delivery protein, StarD1, in primary rat hepatocytes (15). This sulfated oxysterol was believed to be initially synthesized by CYP27A1 in mitochondria (15). It has been reported that the addition of 25HC3S to primary human hepatocytes decreases cholesterol and fatty acid biosynthesis by blocking the activation of sterol-regulatory element binding protein-1c and inhibiting its expression (16). These results suggest that 25HC3S plays an important role in the maintenance of intracellular cholesterol and lipid homeostasis. However, its biosynthetic pathway in the hepatocytes is unknown.

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Biosynthesis of this molecule involves at least two reactions: 25-hydroxylation of cholesterol and 3β-sulfation. A sterol 25-hydroxylase and a hydroxysteroid sulfotransferase (SULT) likely are involved in its generation. Because no sulfated cholesterol was detected in mitochondria after the overexpression of StarD1 in primary rat hepatocytes (15), it is reasonable to hypothesize that the first step is the 25-hydroxylation of cholesterol followed by 3β-sulfation. The presence of 25HC3S in nuclei and mitochondria after the overexpression of StarD1 (15), a transport protein that facilitates cholesterol transport to mitochondria where CYP27A1 is located in hepatocytes (5, 6), indicated that 25HC3S is initially synthesized in mitochondria and translocated to nuclei. Lund et al. (17) reported that a purified CYP27A1 from pig liver was able to synthesize 25HC. Thus, CYP27A1 in mitochondria is likely responsible for the biosynthesis of 25HC. Several laboratories reported that steroid sulfotransferases are able to sulfate cholesterol and oxysterols (18–20). Furthermore, Fuda et al. (21) and Javitt et al. (22) reported that hydroxycholesterol sulfotransferase 2B1b (SULT2B1b) effectively sulfates a variety of oxysterols. Three SULT isoenzymes in human and four in rat were recently cloned and identified (19, 22–24). He, Frost, and Falany (25) reported that SULT2B1b has been localized to the cytosol and nuclei of both human cells and tissues. Falany et al. (19) reported that SULT2b1b is located in the cytosol of human prostate epithelial cells, prostate benign prostatic hyperplasia, prostate adenocarcinoma, and LNCaP prostate adenocarcinoma cells but is almost totally localized in the nuclei of synchiotrophoblast cells in human term placenta. Thus, the location of SULT2B1b varies depending on tissue specificity and cell differentiation stages. Recently, Chen et al. (26) reported that overexpression of sulfotransferase SULT2B1b inactivates the response of liver oxysterol receptor to multiple oxysterol ligands, suggesting that SULT2B1b is involved in the liver oxysterol receptor signaling pathway. However, to date, little is known about the role of SULTs in cholesterol metabolism in the liver. It is likely that SULT2B1b is involved in oxysterol sulfation, which plays an important role in lipid metabolism in liver (16).

In the present study, we provide evidence that CYP27A1 is responsible for the synthesis of 25HC in hepatocyte mitochondria and that the insulin-regulated cytosolic SULT2B1b is responsible for its sulfation. We propose a novel biosynthetic pathway for the synthesis of 25HC3S in hepatocytes.

MATERIALS AND METHODS

Materials

25HC and 27HC were purchased from Research Plus, Inc. (Bayonne, NJ); [^{3}H]25HC and [^{14}C]cholesterol were from Perkin-Elmer (Waltham, MA). *Streptomyces sp.* cholesterol oxydase was from Calbiochem (La Jolla, CA). β-Cyclodextrin was from Cyclodextrin Technologies Development, Inc. (High Springs, FL). Secondary antibody Alexa Fluor 488 goat anti-rabbit IgG was purchased from Molecular Probes (Eugene, OR). Human liver tissues and primary human hepatocytes from human liver were provided by the Liver Tissue Procurement and Distribution System (N01 DK-9-2310). All other reagents were from Sigma Chemical Co. (St. Louis, MO), unless indicated otherwise. Antibodies against human SULT2B1b and SULT2A1 proteins were kindly provided by Dr. Charles N. Falany (Department of Pharmacology, University of Alabama at Birmingham). Recombinant SULT2B1b proteins were kindly provided by Dr. Charles N. Falany and Dr. Hirotoshi Fuda (National Institutes of Health, National Institute of Child Health and Human Development, Bethesda, MD).

Culture of primary rat hepatocytes

Primary rat hepatocytes were prepared as described previously (27). Cells were plated on 60 or 150 mm tissue culture dishes in Williams' E medium containing dexamethasone (0.1 μM). Three hours after plating, culture medium was removed and fresh medium was added. The cells were harvested at the times as indicated. HepG2 hepatocellular carcinoma cells were purchased from the American Type Culture Collection and maintained according to the supplier's protocols.

Cyp27A1 gene knockout mice

Cyp27A1 gene knockout heterozygote breeding pairs from the Veterans Affairs Medical Center, San Francisco, colony (7) were used to found the colony at the Richmond Veterans Affairs Medical Center. At 3 months of age, male mice were euthanized in the morning, and livers were immediately collected for preparation of mitochondria as described below.

Preparation of subcellular fractions from rat/mouse livers

Mitochondria were isolated essentially as described previously (28). The excised livers were homogenized in buffer

TABLE 1. Primer sets used for real-time RT-PCR

Genes	GenBank Numbers	Sense Primers	Antisense Primers
Human SULT2A1	NM_003167	ACAGGACACAGGAAGAACCATAG	CCACTACATAATCAACACTCAGGAG
Human SULT2B1a	NM_004605	GTGTCACCACTTTACAGAAGAGGGACTGAG	AGATGATCTCGATCATCCAGGTCGTGCCGT
Human SULT2B1b	NM_177973	CTCGGAAATCAGCCAGAGTTG	GTCGTCGTCCCGCACATC
Human GAPDH	NM_002046	CAATGACCCCTTCATTGACC	TTGATTTTGGAGGGATCTCG
Rat SULT2A1	XM_574371	CCCATCCATCTCTTCTCCAA	CCTTGGAGGAACCATTCAAA
Rat SULT2A2	NM_001025131	ACCTTTTCCTGCCTTTTGGT	GGTCATGAGTCGTGGTCCTT
Rat SULT2B1a	AY827147	AGGATCATCGTGGAATGGAG	GGTCCCCATCTTTCAGGATT
Rat SULT2B1b	AY827148	CTATGGGGCTCATTGGAGAA	GGGGTAGGTGACGATGAAGA
Rat GAPDH	NM_017008	TGCCACTCAGAAGACTGTGG	TTCAGCTCTGGGATGACCTT

SULT, hydroxycholesterol sulfotransferase.

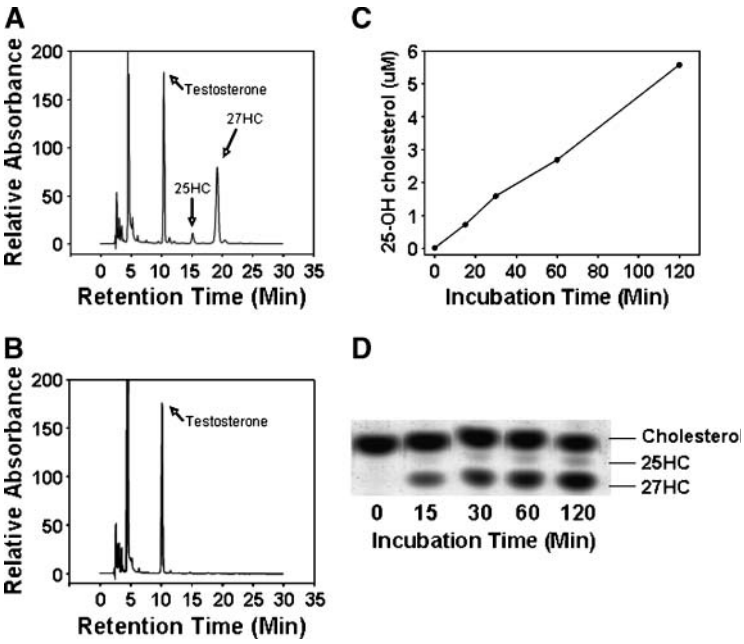

Fig. 1. HPLC and TLC analyses of 25-hydroxycholesterol (25HC) in mitochondria after incubation with cholesterol. A: HPLC analysis of oxysterol products after incubation of the isolated mitochondria with cholesterol; testosterone was used as an internal HPLC standard. B: Sodium cholate-treated mitochondria as a negative control. C: Time course of 25HC synthesis. D: TLC analysis of the [^{14}C]cholesterol derivatives synthesized by mitochondria. 27HC, 27-hydroxycholesterol.

(0.25 M sucrose, 0.5 mM EDTA, and 10 mM potassium phosphate, pH 7.4). The homogenates were centrifuged at 600 *g* at 4°C for 15 min. The supernatant was then centrifuged at 6,700 *g* at 4°C for 20 min, and the pellets (mitochondria-enriched fraction) were twice washed with the homogenization buffer. The supernatant was centrifuged at 100,000 *g* at 4°C for 2 h. The pellets (microsomal fraction) and supernatants (cytosolic fraction) were collected for further analysis. Protein concentration was determined by the Bradford dye reagent method (Bio-Rad, Hercules, CA).

Determination of the synthesis of 25HC and 27HC in hepatocyte mitochondria

The synthesis reactions were incubated in a total volume of 500 μl containing 40 nmol of cholesterol or 0.18 μCi of

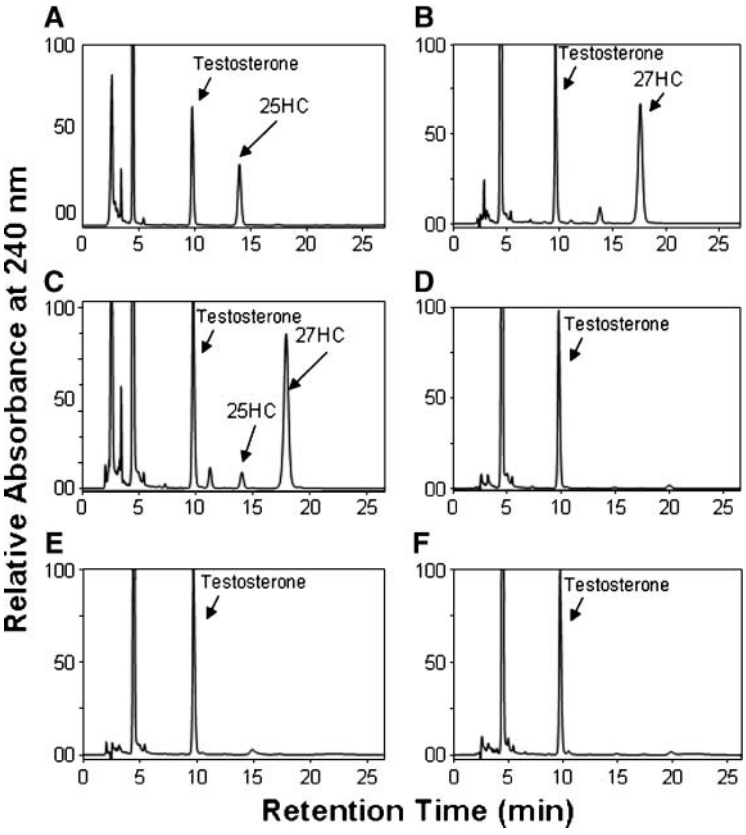

Fig. 2. Sterol 27-hydroxylase (CYP27A1) is responsible for the synthesis of 25HC in hepatocyte mitochondria. HPLC analysis of cholesterol derivative products generated by mitochondria isolated from wild-type or Cyp27A1 knockout mouse liver tissues. A, B: 25HC and 27HC standards. C, E: Wild-type mitochondria. D, F: Cyp27A1 knockout mitochondria. C and D represent the incubation of mitochondria with cholesterol, and in E and F, mitochondria were treated with sodium cholate. Testosterone was used as an internal standard during the lipid extraction.

[^{14}C]cholesterol dissolved in 10 μl of β-cyclodextrin (45% in water), 500 μg of mitochondrial protein, 100 mM sodium phosphate, pH 7.5, 0.2 mM EDTA, 1 mM DTT, 5.0 mM trisodium isocitrate, and 0.2 units of isocitrate dehydrogenase. Reactions were initiated by adding 60 μl of 10 mM β-NADPH and incubated with shaking at 37°C for 60 min. The reactions were stopped by adding 40 μl of 40% sodium cholate. Blanks were prepared by adding sodium cholate before adding mitochondria. After stopping the reaction, 1.5 μg of testosterone was added to the reaction mixture as an internal standard. The sterol products were incubated with 2 units of cholesterol oxidase at 37°C for 20 min. The oxidation reaction was terminated by adding 1.5 ml of methanol followed by 0.5 ml of saturated KCl. The derived ketosterols were extracted twice using 3 ml of hexane. The hexane phase was collected and evaporated under a stream of nitrogen. The residues were dissolved in mobile phase solvents for HPLC and TLC analysis as described previously (8, 15).

HPLC and TLC analyses of 25HC and 27HC

The sterol products synthesized as described above were analyzed by HPLC on an Ultrasphere Silica column (5 μm × 4.6 mm × 25 cm; Beckman) using an HP Series 1100 solvent delivery system (Hewlett-Packard) at a flow rate of 1.3 ml/min. The chromatograph was run in a solvent system of hexane-isopropanol-glacial acetic acid (965:25:10, v/v/v) as the mobile phase. The elution profiles were monitored at 240 nm. The column was calibrated with cholesterol, 25HC, testosterone, and 27HC as described previously (8, 15). The individual [^{14}C]oxysterols were identified by TLC in a solvent system of toluene-ethyl acetate (2:3, v/v) and visualized with a PhosphorImager.

Determination of the expression of SULTs in hepatocytes

RT-PCR analysis of SULT mRNA expression. Total RNAs were purified from human livers, primary human hepatocytes, HepG2 cells, rat livers, and primary rat hepatocytes using the SV Total RNA Isolation Kit (Promega, Madison, WI). Two micrograms of total RNA was used for first-strand cDNA synthesis as recommended by the manufacturer (Invitrogen). Chosen genes were amplified by PCR. The PCR fragments were visualized on a 1.5% agarose gel containing 5 μg/ml ethidium bromide. The fragments were further sequenced at the DNA Core Facility at Virginia Commonwealth University.

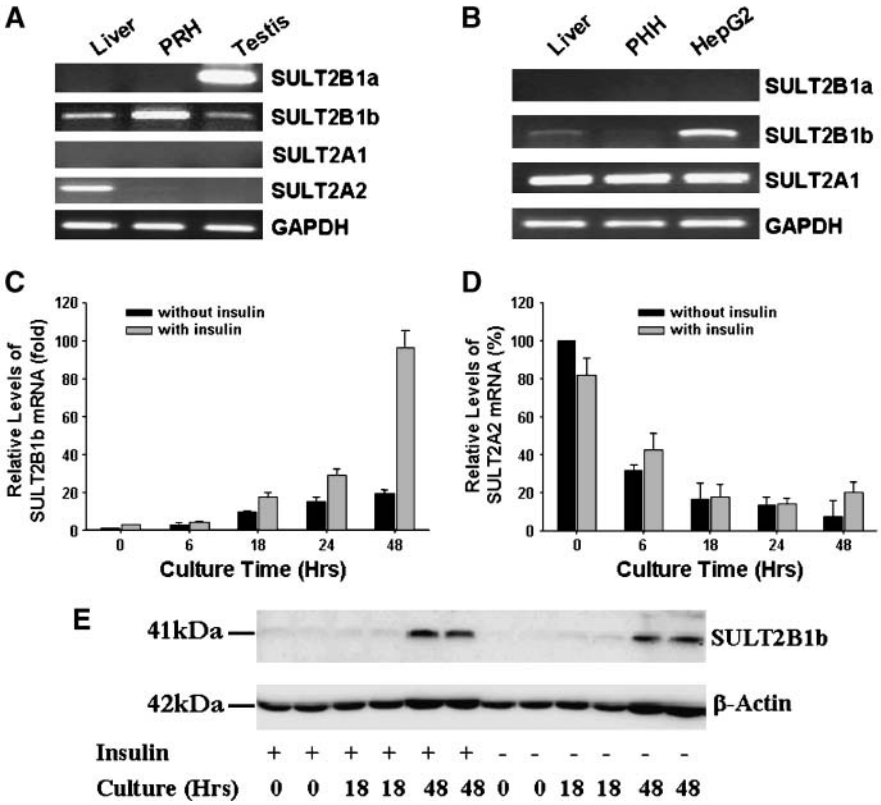

Fig. 3. The presence of hydroxysteroid sulfotransferase (SULT) mRNAs in rat and human liver. Total RNAs were purified from rat and human liver, primary human and rat hepatocytes, and HepG2 cells and analyzed by RT-PCR. A: Expression of SULTs in rat liver and primary rat hepatocytes (PRH). B: Human liver and hepatocytes. An equivalent of total RNA for each sample was loaded, and 30 cycles for each sample were used for PCR analysis. C, D: Real-time RT-PCR analysis of SULT2B1b and SULT2A2 mRNA levels in primary rat hepatocytes during culture. Primary rat hepatocytes were cultured in the absence or presence of insulin. The cells were harvested at different times, as indicated. The mRNA levels in cultured cells were compared with those in the freshly prepared cells. Two micrograms of total RNA was used for cDNA preparation (RT), and 10 ng of cDNA was used for real-time RT-PCR. The mRNA levels were normalized to β-actin. C shows SULT2B1b mRNA levels, and D shows SULT2A2. The data points represent means of triplicate determinations ± SD. E: Western blot analysis of SULT2B1b protein levels during culture as described above. Fifty micrograms of cell lysate protein was loaded in each lane. The data represent one of three separate experiments.

Real-time RT-PCR analysis of SULT mRNA levels. Real-time RT-PCR was performed using SYBR Green on the ABI 7500 Fast Real-Time PCR System (Applied Biosystems). The final reaction mixture contained 10 ng of cDNA, 100 nM of each primer, 10 μl of 2×SYBR Green PCR Master Mix (Applied Biosystems), and RNase-free water to complete the reaction mixture volume to 20 μl. All reactions were performed in triplicate. The PCR was performed with a hot-start denaturation step at 95°C for 10 min and then was carried out for 40 cycles at 95°C for 15 s and 60°C for 1 min. The fluorescence was read during the reaction, allowing a continuous monitoring of the amount of PCR product. The data were normalized to an internal control, GAPDH. The sequences of primers used in PCR and real-time PCR are shown in **Table 1**.

Transfection of small interfering RNAs. Transfection experiments were performed using a nonviral transfection method, which combines electrical parameters and cell-type solutions to drive small interfering RNAs directly into the cell nucleus, as described by the manufacturer (Amaxa, Cologne, Germany). Fresh isolated primary rat hepatocytes (1×10^6 cells) were resuspended in 100 μl of Amaxa Rat Hepatocyte Nucleofector Solution. Small interfering RNAs or scrambled RNAs (Dharmacon, Lafayette, CO), 60 nM and 100 nM, respectively (final concentration), were added and cells were immediately transfected using the Amaxa Nucleofector II device (program Q-025) to interrupt rat SULT2B1b (GenBank accession number NM_001039665) expression. Cells were subsequently resuspended in normal medium culture on six-well plates and processed after 48 h for relevant experiments.

Western blot analysis of SULTs. Samples were separated on 7.5% SDS-PAGE gels and transferred onto a polyvinylidene difluoride membrane using a Bio-Rad Mini-Blot transfer apparatus as described previously (28). Recombinant SULT2B1b proteins were used as a positive control (19). Membranes were blocked in TBS containing 5% nonfat dried milk for 1 h. The immunoblotting was performed at 4°C overnight with shaking using the rabbit anti-SULT2B1b antiserum. After washing, the membrane was incubated in a 1:4,000 dilution of a secondary antibody (goat anti-rabbit IgG-HRP conjugate; Bio-Rad) at room temperature for 1 h in TBS washing buffer (containing 0.5% Tween 20). After washing, protein bands were visualized using Western Lightning Chemiluminescence Reagent (Perkin-Elmer) and developed on Biomax Light Film (Kodak).

Immunofluorescence microscopic detection of SULT2B1b in primary rat hepatocytes. Primary rat hepatocytes were cultured on coverslips on six-well plates. Cells on coverslips were washed with PBS and fixed with PBS/3.7% formaldehyde for 30 min at 4°C and then rinsed three times with PBS alone at room temperature. They were then permeabilized in PBS containing 0.2% Triton X-100 and washed with PBS before blocking by incubation with 5% normal goat serum in PBS for 2 h at room temperature. For interaction with primary antibodies, cells were incubated with 2.5% normal goat serum in PBS containing SULT2B1b antibody overnight at 4°C in an incubator. Cells were washed in PBS/0.05% Tween 20 (3 × 20 min), and the bound primary antibodies were visualized with Alexa Fluor 488 goat anti-rabbit IgG. After washing, coverslips containing primary rat hepatocytes were mounted on slides and viewed with a Zeiss LSM 510 Meta confocal microscope. Normal rabbit IgG and preincubated antibody with recombinant protein served as negative controls.

Detection of the synthesis of 25HC3S in hepatocytes

Mitochondrial and cytosol fractions were prepared from rat liver as described above. To test whether 25HC3S can be synthesized from 25HC as substrate, cytosol (250 μg of protein) was

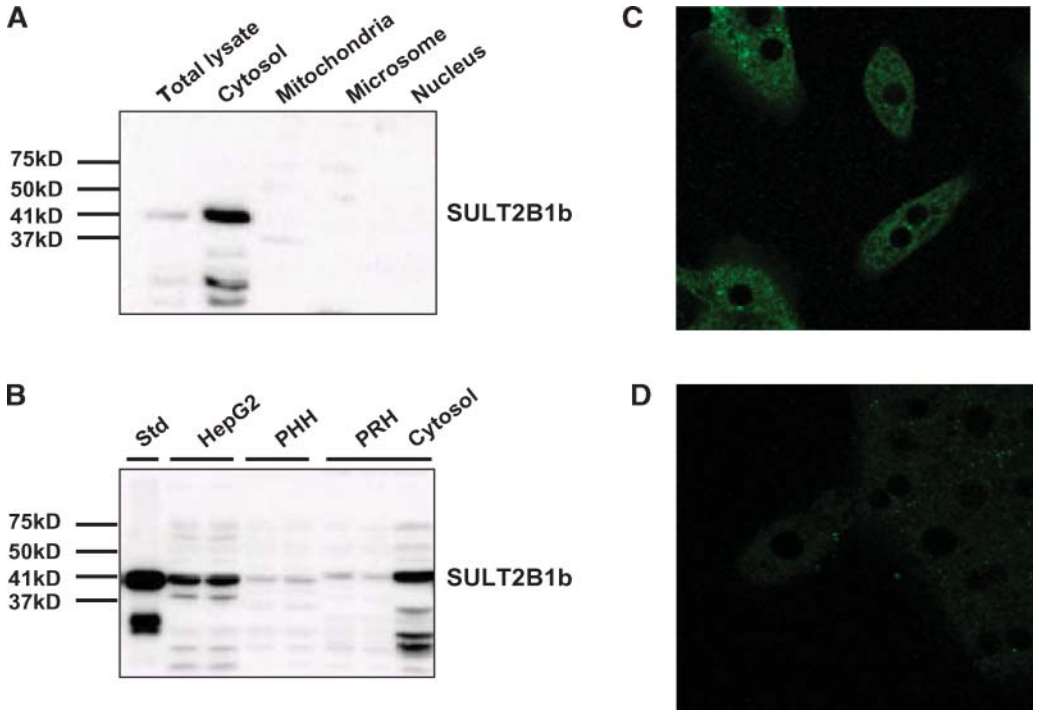

Fig. 4. Expression and distribution of SULT2B1b protein in liver tissues and hepatocytes. A: Subcellular fractions of rat liver tissues. B: Total cell lysates of HepG2, primary human hepatocytes (PHH), and primary rat hepatocytes (PRH); recombinant SULT2B1b protein served as a positive standard control (Std). C: Immunocytochemical staining of SULT2B1b in cultured primary rat hepatocytes. D: Negative control using preincubated serum with recombinant SULT2B1b protein.

incubated with [^{3}H]25HC (0.75 μCi) dissolved in 7.5 μl of cyclodextrin, 5 mM $MgCl_2$, 4% (v/v) ethanol, and 100 μM 3′-phosphoadenosyl 5′-phosphosulfate (PAPS) in 250 μl of 100 μM Tris buffer at 37°C for 1 h. To determine whether 25HC3S can be synthesized with cholesterol as substrate, mitochondrial (750 μg of protein) and cytosolic (750 μg of protein) fractions were incubated in 0.5 ml of substrate solution containing [^{14}C]cholesterol (1.5 μCi in cyclodextrin), 100 mM phosphate, pH 7.5, 1 mM DTT, 1.2 mM β-NADPH, 5.0 mM trisodium isocitrate, 0.2 units of isocitrate dehydrogenase, 5 mM $MgCl_2$, and 100 μM PAPS at 37°C for 4 h. Lipids were extracted with 10 volumes of chloroform-methanol (2:1, v/v). The extracts were dried under a stream of nitrogen gas, and the pellets were dissolved in the mobile phase (water-acetonitrile-methanol, 45:5:50, v/v/v) and analyzed by HPLC using a silica gel column with a gradient elution system. A binary system of solvent A (5% acetonitrile in water, v/v) and solvent B (5% acetonitrile in methanol, v/v) was used beginning at 45% A and 55% B with an initial flow rate of 0.5 ml/min, increasing to 100% B over a 25 min period, increasing the flow rate linearly to 1.5 ml/min, and followed by an additional isocratic period of 15 min, for a total duration of 40 min, as described previously (22). Each fraction per minute was collected otherwise as indicated. The radioactivity counts in eluted fractions were determined by liquid scintillation counting.

Sulfatase treatment of the synthesized 25HC3S

The purified [^{3}H]25HC3S or [^{14}C]25HC3S derivatives were digested with 2 mg/ml sulfatase (type H-1) in 0.5 ml of 50 mM acetic buffer, pH 5.0, by incubation at 37°C for 4 h. The lipids were extracted into hexane by adding 1.5 ml of methanol, 0.5 ml of saturated KCl, and 3 ml of hexane into the reaction solution. The products were analyzed by HPLC using an Ultrasphere Silica column as described previously.

Statistics

Data are reported as means ± SD. Where indicated, data were subjected to *t*-test analysis and determined to be significantly different at $P < 0.05$.

RESULTS

CYP27A1, mitochondrial cholesterol 27-hydroxylase, is responsible for the synthesis of 25HC in hepatocyte mitochondria

The synthesis of 25HC was assessed in mitochondria isolated from rat liver, human liver, and HepG2 cells. HPLC analysis of cholesterol derivatives after incubation of the mitochondrial fraction with cholesterol showed that 28% was converted to 27HC and 3% was converted to 25HC (**Fig. 1A**). Pretreated mitochondrial fraction with sodium cholate (detergent to solubilize mitochondria and denature proteins) failed to synthesize 27HC and 25HC (Fig. 1B), indicating that the synthesis of 27HC and 25HC

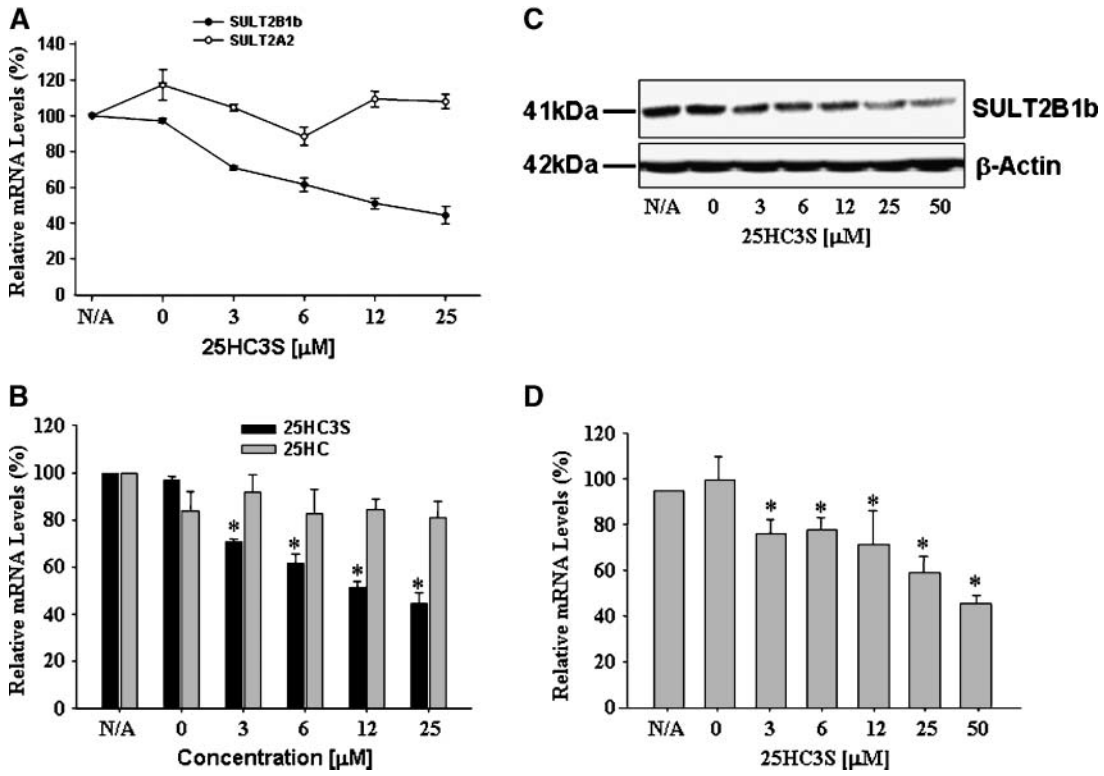

Fig. 5. Effects of 5-cholesten-3β,25-diol 3-sulfate (25HC3S) on the expression of SULT2B1b in primary rat hepatocytes. After preparation of primary rat hepatocytes, different concentrations of 25HC3S and/or 25HC were added as indicated. The cells were harvested at 6 h after the addition. A: Real-time RT-PCR analysis of the effects of 25HC3S on SULT2A2/SULT2B1b mRNA levels. B: Effects of 25HC3S and 25HC on SULT2B1b mRNA expression. C: Western blot analysis of the effects of 25HC3S on SULT2B1b protein expression. D: Summary of SULT2B1b protein levels normalized to β-actin. The data represent one of three experiments. * Significant difference compared with no addition ($P < 0.01$). The mRNA data represent one of two experiments performed in triplicate; each data point represents the mean ± SD.

is based upon intact mitochondria. The levels increased with time (Fig. 1C, D).

To confirm that mitochondrial CYP27A1 is responsible for the synthesis of 25HC, mitochondria were isolated from livers of wild-type and Cyp27A1 gene knockout mice. As shown in **Fig. 2D**, the mitochondria isolated from the gene knockout mice failed to synthesize 27HC and 25HC, suggesting that Cyp27A1 is responsible for their synthesis. When mitochondria from either wild-type or Cyp27A1 gene knockout livers were pretreated with sodium cholate, they failed to synthesize these oxysterols (Fig. 2E, F). These results support the notion that 25HC was specifically synthesized by Cyp27A1 and was not the result of auto-oxidation.

SULT2B1b is responsible for the synthesis of 25HC3S

To examine the expression of SULT(s) in rat and human hepatocytes, each SULT isoform mRNA was determined by RT-PCR using specific primer sets as shown in Table 1. The results show the presence of SULT2B1b and SULT2A2 mRNAs but the absence of expression of SULT2B1a and SULT2A1 in rat liver and primary rat hepatocytes (**Fig. 3A**). SULT2A1 and SULT2B1b mRNAs also were detected in human liver, primary human hepatocytes, and HepG2 cells. However, SULT2B1a mRNA was not detectable when a published primer set was used (Fig. 3B) (22). The expression of SULT2B1b and SULT2A2 is highly regulated. During culture of primary rat hepatocytes, the levels of SULT2B1b gradually increased (Fig. 3C) while those of SULT2A2 decreased (Fig. 3D). After the cells were cultured for 48 h, the levels of SULT2B1b increased by ~20-fold and those of SULT2A2 decreased by ~16-fold (Fig. 3C, D). In the presence of insulin in the culture medium, the levels of SULT2B1b mRNA increased by ~100-fold, significantly greater than those in the absence of insulin at 18, 24, and 48 h ($P < 0.01$) (Fig. 3C). However, insulin had no effect on SULT2A2 mRNA levels ($P > 0.05$) (Fig. 3D). Insulin also led to a significant increase in the protein levels of SULT2B1b (~20-fold; $P < 0.001$) (Fig. 3E). The increased protein levels qualitatively correlated with the increase in mRNA levels but did not quantitatively fit the magnitude of the documented increase in mRNAs. The reason for this is not entirely clear.

SULT2B1b protein expression and distribution in liver were detected by Western blot and immunocytochemical analyses. The results showed that only one major band with a molecular mass of 41 kDa, which is the same size as that of the standard recombinant SULT2B1b protein (19), was expressed in rat liver and located in the cytosol. This band was not detected in mitochondrial, microsomal, and nuclear fractions (**Fig. 4A, B**). Primary human hepatocytes and HepG2 cells also expressed SULT2B1b (Fig. 4B). Immunocytochemical localization of SULT2B1b in the cultured primary rat hepatocytes, using anti-SULT2B1b antibody, revealed a widespread cytoplasmic distribution (Fig. 4C). Preincubated antibody with recombinant SULT2B1b exhibited no staining (Fig. 4D).

The expression of SULT2B1b mRNA, but not of SULT2A2, was downregulated by the addition of 25HC3S to primary rat hepatocytes (**Fig. 5A**). Its expression was not significantly affected by 25HC (Fig. 5B). Western blot analysis showed that 25HC3S also decreased the protein levels of SULT2B1b (Fig. 5C, D) but not SULT2A2 (data not shown). 25HC did not affect SULT2B1b protein levels (data not shown). The addition of 25HC3S and 25HC for 6 h did not cause cytotoxicity based on [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium], viability assay (Promega) (data not shown).

At 48 h after transfection of its specific small interfering RNAs, SULT2B1b mRNA, protein, and activity levels in primary rat hepatocytes were decreased significantly by 70, 55, and 40%, respectively, but the levels of scrambled RNAs were not (**Fig. 6A–C**). These results indicate that SULT2B1b is responsible for the oxysterol sulfation.

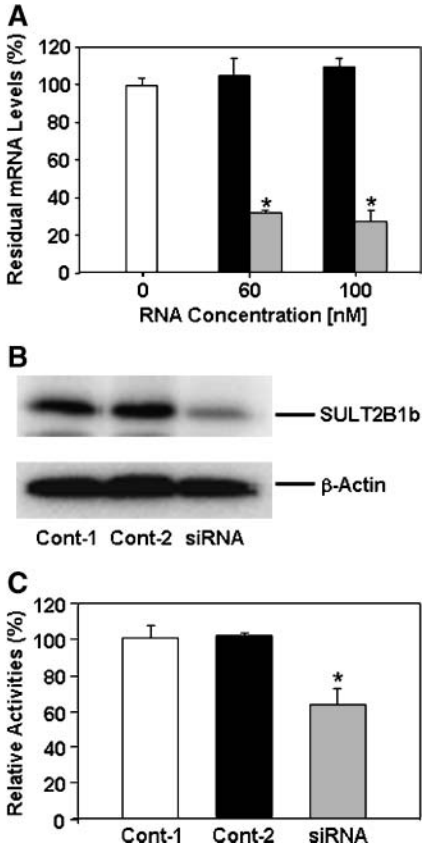

Fig. 6. Effects of specific small interfering RNAs on SULT2B1b expression and activities. The cells were harvested at 48 h after small interfering RNA transfection. A: Real-time RT-PCR analysis of SULT2B1b mRNA levels. B: Western blot analysis of SULT2B1b proteins. C: Sulfotransferase activities in the cells after transfection. Open bars represent control cells (without the addition of any RNA; Cont-1); closed bars represent cells with the addition of scrambled RNA (Cont-2); shaded bars represent cells with the addition of small interfering RNAs. The data points represent means of three experiments ± SD. * $P < 0.01$.

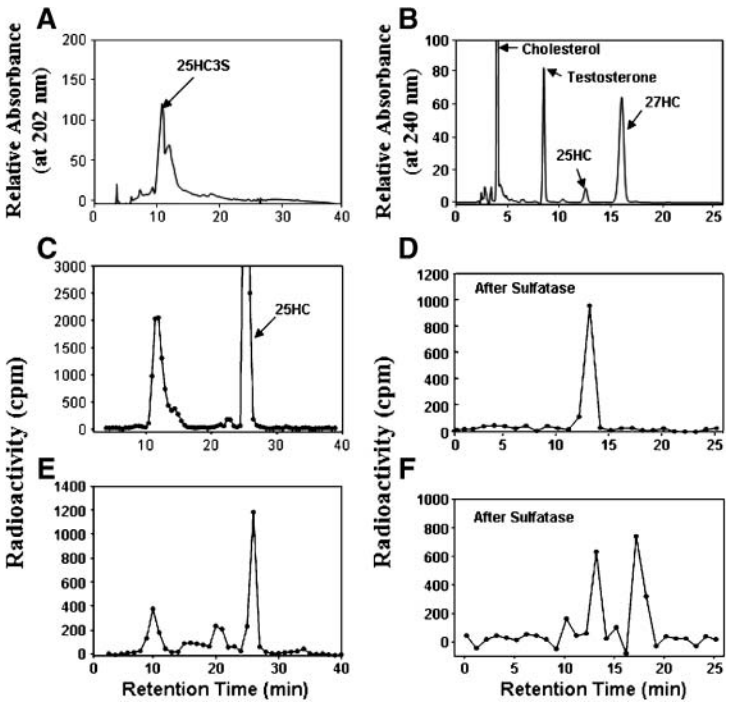

Fig. 7. Detection of the synthesis of 25HC3S in hepatocytes. A, B: HPLC elution profiles of 25HC3S; 25HC and 27HC were used as standards. C: HPLC analysis of the products after incubation of the cytosol with [^{3}H]25HC in the presence of 3′-phosphoadenosyl 5′-phosphosulfate (PAPS). E: After incubation of the cytosol and mitochondria with [^{14}C]cholesterol in the presence of PAPS. D, F: HPLC analysis of the products after treatment of the collected products with sulfatase: [^{3}H]25HC derivative digest (D) and [^{14}C]cholesterol derivatives (F). The data represent typical results from one of three independent experiments.

In vitro synthesis of 25HC3S

After incubation with rat liver cytosol in the presence of PAPS as a sulfate donor, ~20–40% of [^{3}H]25HC was converted to a hydrophilic product that had the same retention time as that of chemically synthesized 25HC3S on HPLC elution profiles (**Fig. 7A, C**). To confirm that this peak corresponded to 25HC3S, the collected product was treated with sulfatase, extracted with hexane, and characterized by HPLC analysis. The results showed that the sulfatase-treated product had the same retention time as 25HC (Fig. 7B, D), confirming that the original product was 25HC3S. TLC analysis showed that the sulfatase-treated cholesterol metabolite migrated with the same Rf value as 25HC (data not shown).

To confirm the route of synthesis of 25HC3S from cholesterol, [^{14}C]cholesterol was incubated with mitochondrial and cytosolic fractions in the presence of PAPS. After incubation, the products were analyzed as described in Materials and Methods. One of the major [^{14}C]cholesterol derivatives (~1% of added [^{14}C]cholesterol) had the same retention time as the 25HC3S standard (Fig. 7E). Treatment of this product with sulfatase also gave rise to 25HC (~50% of the products) (Fig. 7F).

DISCUSSION

Based on the chemical structure of 25HC3S, two biochemical steps are required for its synthesis. In the present study, we provide evidence that CYP27A1 is responsible for the mitochondrial synthesis of 25HC and cytosolic SULT2B1b is responsible for the synthesis of 25HC3S in hepatocytes.

CYP27A1 has long been thought to play a role in the regulation of cholesterol homeostasis (7, 13). In peripheral tissues, metabolites generated by CYP27A1 have been shown to play roles in the maintenance of cholesterol homeostasis (7, 9, 11, 12, 29–31). Recently, we identified 25HC3S in hepatocyte nuclei and found that this novel oxysterol plays an important role in the maintenance of intracellular lipid homeostasis (15). The pres-

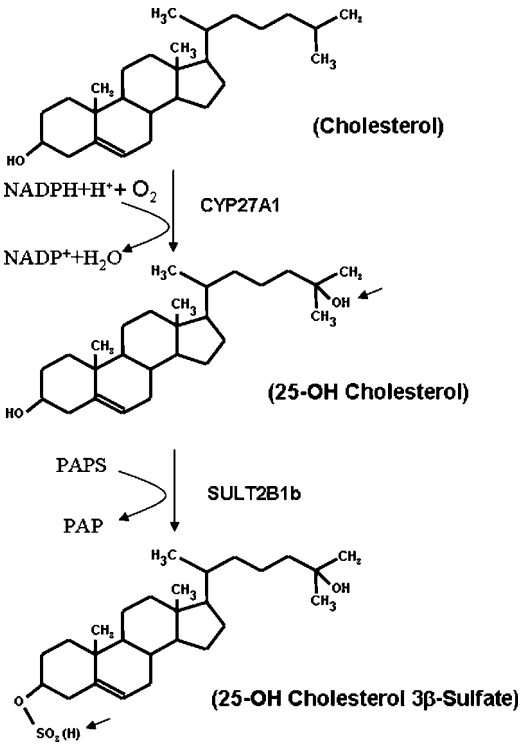

Fig. 8. Summary of the biosynthetic pathway for 25HC3S. Cholesterol is hydroxylated by CYP27A1 in the mitochondria to 25HC, which is subsequently sulfated at the 3β-position by SULT2B1b in the cytosol to form 25HC3S.

ent study shows that 25HC is synthesized by CYP27A1 in liver mitochondria and provides evidence that 25HC can be sulfated by the insulin-regulated cytosolic enzyme, SULT2B1b.

The present study shows that two SULTs, SULT2B1a and SULT2B1b, were detected in human hepatocytes and that SULT2A2 and SULT2B1b were detected in rat liver at mRNA levels. Their expressions are highly regulated (Fig. 3). These enzymes are all capable of transferring a sulfate group from PAPS to hydroxysteroids (32). Enzymatic characterization of SULT2B1b indicated that this isozyme is selective for the sulfation of 3β-hydroxysteroids such as cholesterol and hydroxycholesterols and shows little reactivity with 3α-hydroxysteroids of the 3-phenolic hydroxyl group of estrogens (33). This selective reactivity for the 3β-hydroxyl configuration of hydroxysteroids is in contrast to the broader reactivity of SULT2As, which conjugate both 3α- and 3β-hydroxysteroids as well as estrogens (34, 35).

Previous reports have shown that SULT2B1b is expressed in hormone-responsive organs such as prostate, placenta, breast, skin, and lung but not in liver (19, 32). As 25HC3S has the sulfate group at the 3β-position, 25HC is likely to be sulfated specifically by SULT2B1b in liver. In support of this notion, the present study shows that the expression of SULT2B1b is highly regulated: low levels in fresh liver and hepatocytes; high levels after the cells were cultured for 48 h; upregulated by insulin, although its physiological significance is unknown at present; downregulated by the product, 25HC3S, but not by the substrate, 25HC. Based on the product feedback regulation mechanism, the results strongly suggest that SULT2B1b is most likely responsible for the synthesis of 25HC3S in the liver. The 25HC3S biosynthetic pathway in liver hepatocytes is summarized in **Fig. 8**.

Two isoenzymes, SULT2A2 and SULT2B1b, were detected in rat liver and primary rat hepatocytes, whereas SULT2A1 and SULT2B1b were detected in human liver, primary human hepatocytes, and HepG2 cells (Fig. 3). Whether SULT2A2 in rat hepatocytes functions like SULT2A1 in human hepatocytes is not clear at present. It is interesting that SULT2A2 and SULT2B1b are expressed at different stages: when SULT2A2 expression is high, SULT2B1b is low; when SULT2A2 expression is low, SULT2B1b is high (Fig. 3C, D). These results imply that these two enzymes may play different physiological roles in hepatic sterol metabolism.

The acid pathway has been traditionally believed to be an alternative pathway for primary bile acid synthesis. However, many intermediates in this pathway, including 25HC and 27HC, have been implicated in the regulation of lipid metabolism. Cholesterol delivery into mitochondria, and its 25-hydroxylation by CYP27A1 followed by 3β-sulfation, may serve to initiate a new regulatory pathway. For example, when cellular cholesterol levels are increased in certain circumstances, cholesterol may be delivered by the StarD1 protein to mitochondria, where it is hydroxylated to 25HC, sulfated, and translocated to nuclei. We hypothesize that there it may alter gene expression and affect cellular lipid metabolism.

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