24-Hydroxycholesterol Sulfation by Human Cytosolic Sulfotransferases: Formation of Monosulfates and Disulfates, Molecular Modeling, Sulfatase Sensitivity, and Inhibition of Liver X Receptor Activation^[S]

Ian T. Cook, Zofia Duniec-Dmuchowski, Thomas A. Kocarek, Melissa Runge-Morris, and Charles N. Falany

Department of Pharmacology and Toxicology, University of Alabama at Birmingham, Birmingham, Alabama (I.T.C., C.N.F.); and Institute of Environmental Health Sciences, Wayne State University, Detroit, Michigan (Z.D.-D., T.A.K., M.R.-M.)

Received March 13, 2009; accepted July 6, 2009

ABSTRACT:

24-Hydroxycholesterol (24-OHChol) is a major cholesterol metabolite and the form in which cholesterol is secreted from the brain. 24-OHChol is transported by apolipoprotein E to the liver and converted into bile acids or excreted. In both brain and liver, 24-OHChol is a liver X receptor (LXR) agonist and has an important role in cholesterol homeostasis. 24-OHChol sulfation was examined to understand its role in 24-OHChol metabolism and its effect on LXR activation. 24-OHChol was conjugated by three isoforms of human cytosolic sulfotransferase (SULT). SULT2A1 and SULT1E1 sulfated both the 3- and 24-hydroxyls to form the 24-OHChol-3, 24-disulfate. SULT2B1b formed only 24-OHChol-3-sulfate. The 3-sulfate as a monosulfate or as the disulfate was hydrolyzed by human placental steroid sulfatase, whereas the 24-sulfate was resistant. At physiological 24-OHChol concentrations, SULT2A1 formed the 3-monosulfate and the 3, 24-disulfate as a result of a

high affinity for sulfation of the 3-OH in 24-OHChol-24-sulfate. Molecular docking simulations indicate that 24-OHChol-24-sulfate binds in an active configuration in the SULT2A1 substrate binding site with high affinity only when the SULT2A1 homodimer structure was used. 24-OHChol is an LXR activator. In contrast, the 24-OHChol monosulfates were not LXR agonists in a fluorescence resonance energy transfer coactivator recruitment assay. However, both the 24-OHChol-3-sulfate and 24-sulfate were antagonists of LXR activation by *N*-(2,2,2-trifluoroethyl)-*N*-[4-[2,2,2-trifluoro-1-hydroxy-1-(trifluoromethyl)ethyl]phenyl]-benzenesulfonamide (T0901317) with an IC₅₀ of 0.15 and 0.31 μ M, respectively. Inhibition of LXR activation by the 24-OHChol monosulfates at low nanomolar concentrations indicates that sulfation has a role in LXR regulation by oxysterols.

24(*S*)-Hydroxycholesterol (24-OHChol) is the major metabolite of cholesterol in human brain and the form in which cholesterol is secreted across the blood-brain barrier (Björkhem et al., 1998; Mast et al., 2003). Regulation of cholesterol levels in the brain is vital in maintaining proper neuronal activity. 24-OHChol is synthesized by the brain-specific cytochrome P450 46A isoform and is transported out of the brain by the organic anion transporter (OAT) 2 (Björkhem,

2006). It is subsequently carried in the circulation by apolipoprotein E (ApoE) to the liver, where it can be hydroxylated by CYP7A and converted into bile acids (Björkhem et al., 2001; Fujiyoshi et al., 2007). However, conversion to bile acids accounts for only 40 to 50% of 24-OHChol elimination, leaving a large fraction of 24-OHChol metabolism and excretion unresolved (Björkhem et al., 2001). Conjugation with either sulfonate or glucuronic acid has been implicated as important for biliary excretion (Björkhem et al., 2001).

Although 24-OHChol represents a mechanism for cholesterol secretion in the central nervous system, it also has a role in the regulation of cholesterol mobilization. Several oxysterols, including 24-OHChol, are endogenous ligands for activation of the liver X receptors (LXRs) (Janowski et al., 1999). In brain, activation of LXR β by 24-OHChol is associated with increased mobilization of cholesterol for use in myelin formation and synaptic plasticity (Koudinov and Koudinova, 2002). LXR β activation also induces expression of OAT2 and ApoE, which are involved in the increased secretion of

This work was supported in part by the National Institutes of Health National Institute of General Medical Sciences [Grant GM38953]; the National Institutes of Health National Institute of Environmental Health Sciences [Grant ES05823]; the National Institutes of Health National Heart, Lung, and Blood Institute [Grant HL050710]; and the Environmental Health Sciences Center [Grant ES06636].

Article, publication date, and citation information can be found at <http://dmd.aspetjournals.org>.

doi:10.1124/dmd.108.025759.

[S] The online version of this article (available at <http://dmd.aspetjournals.org>) contains supplemental material.

ABBREVIATIONS: 24-OHChol, 24(*S*)-hydroxycholesterol; OAT, organic anion transporter; ApoE, apolipoprotein E; LXR, liver X receptor; SULT, sulfotransferase; PAPS, 3'-phosphoadenosine-5'-phosphosulfate; STS, steroid sulfatase C; DHEA, dehydroepiandrosterone; E2, β -estradiol; TLC, thin-layer chromatography; T0901317, *N*-(2,2,2-trifluoroethyl)-*N*-[4-[2,2,2-trifluoro-1-hydroxy-1-(trifluoromethyl)ethyl]phenyl]-benzenesulfonamide; TR-FRET, time-resolved fluorescence resonance energy transfer; BFE, binding free energy; MS/MS, tandem mass spectrometry; DMSO, dimethyl sulfoxide; LC, liquid chromatography.

24-OHChol from the brain and its transport to the liver (Björkhem et al., 1998). In rats, LXR α activation reportedly results in the induction of hepatic CYP7A, leading to increased 7 α -hydroxylation of 24-OHChol and its conversion into bile acids (Lehmann et al., 1997); however, CYP7A1 expression may not be induced in human hepatocytes (Chen et al., 2002). Although oxysterols may activate LXRs, auto-oxidized cholesterol sulfates have been reported to function as LXR antagonists (Song et al., 2001). The ability of 24-OHChol monosulfates or the 3, 24-disulfate to either activate or inhibit LXR has not been reported.

Sulfation of oxysterols and 24-OHChol by selected isoforms of human cytosolic sulfotransferase (SULT) has recently been reported (Javitt et al., 2001; Fuda et al., 2007). Sulfation involves the transfer of the sulfonate group of the obligate sulfonate donor 3'-phosphoadenosine-5'-phosphosulfate (PAPS) to an acceptor compound, usually possessing a hydroxyl group, to form a sulfate ester. The reaction is catalyzed by a family of cytosolic SULTs involved with the conjugation of therapeutic drugs, xenobiotics, and small endogenous compounds, including hydroxysteroids, thyroid hormones, estrogens, bile acids, cholesterol, and oxysterols (Falany, 1997; Glatt et al., 2000). The isoforms of human SULT responsible for the formation of 24-OHChol monosulfates and disulfate, as well as the kinetics of these reactions, have not been well described.

Conjugation of a compound with a sulfonate group is generally considered to be an inactivation reaction. However, the parent compound in many cases can be regenerated by hydrolysis by sulfatase activity (Iwamori, 2005). Steroid sulfatase C (STS) is expressed in human liver and is responsible for the hydrolysis of many estrogen and hydroxysteroid sulfates, including dehydroepiandrosterone (DHEA)-sulfate and β -estradiol (E2)-3-sulfate (Hernandez-Guzman et al., 2001; Iwamori, 2005). However, the sulfates of some hormonally active drugs are resistant to STS. The 3 α -sulfate of tibolone is resistant to STS, whereas the 3 β -sulfate of tibolone is hydrolyzed (Falany and Falany, 2007). In addition, the benzothiophene sulfate of raloxifene is hydrolyzed by STS, whereas the phenolic sulfate of raloxifene is resistant (Falany and Falany, 2007). The sensitivity of the 24-OHChol monosulfates and disulfate to STS has not been reported. Selective hydrolysis of the 3- or 24-sulfates could lead to the formation and accumulation of selective 24-OHChol metabolites.

In this report, the major SULT isoforms responsible for the formation of the 24-OHChol monosulfates and disulfate are identified. The kinetic properties of the reactions are described, as well as the molecular docking of 24-OHChol and the monosulfates in the active sites of SULT2A1, to better understand the mechanism by which the different sulfated products are generated. 24-OHChol-24-sulfate, which is rapidly converted to the 3, 24-disulfate, can only be modeled in an apparently active configuration when the published SULT2A1 homodimer structure was used (Protein Data Bank 1efh). Sensitivity of the 24-OHChol sulfates to hydrolysis by STS is described. The 24-OHChol monosulfates were not LXR activators; however, the ability of the 24-OHChol monosulfates to inhibit activation of LXR α at physiologically relevant concentrations was shown. This information is important for developing a better understanding of the metabolism and the role of 24-OHChol and its sulfated metabolites in the regulation of LXR activation.

Materials and Methods

24-OHChol was purchased from BIOMOL Research Laboratories (Plymouth Meeting, PA). LK6DF 60-Å silica gel thin-layer chromatography (TLC) plates with a layer thickness of 250 μ m were obtained from Whatman (Clifton, NJ). PAPS was purchased from Dr. Sanford Singer (University of Dayton, Dayton, OH). [35 S]PAPS (2.2 Ci/mmol) and [1,2,6,7- 3 H(N)]DHEA-sulfate (60

Ci/mmol) were purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). C-18 Sep-Pak cartridges were purchased from Waters (Milford, MA). The pQE-31 vector and Ni-NTA resin were purchased from QIAGEN (Valencia, CA). Con A Sepharose and DEAE-Sepharose Cl-6B were from GE Healthcare (Little Chalfont, Buckinghamshire, UK). T0901317 was obtained from Sigma-Aldrich (St. Louis, MO). The LanthaScreen time-resolved fluorescence resonance energy transfer (TR-FRET) LXR α Coactivator Assay kit was from Invitrogen (Carlsbad, CA). All other materials were purchased from Thermo Fisher Scientific (Waltham, MA).

Preparation of SULT Isoforms and STS. The native forms of SULT1E1 and SULT2A1 were expressed in *Escherichia coli* DH5 α and purified by DEAE-Sepharose Cl-6B chromatography as described previously (Falany et al., 1994). The amount of the individual SULT isoforms in the preparations was estimated by comparison with known standards during immunoblot analysis.

Because SULT2B1b cannot be expressed in an active native form in *E. coli* (Meloche and Falany, 2001; Falany et al., 2006), the enzyme was subcloned into pQE-31 and expressed in *E. coli* DH5 α with a cleavable 6xHis-tag. The expressed His-SULT2B1b protein was purified on a Ni-NTA affinity column. To recover native SULT2B1b, the His-tag was cleaved by rTev protease, and the preparation was passed through the Ni-NTA affinity column again to remove the His-tag from the pure SULT2B1b. This generated an active but relatively unstable preparation of native SULT2B1b (Meloche and Falany, 2001; Falany et al., 2006).

STS was purified from human full-term placenta using the method of Hernandez-Guzman et al. (2001) as described previously (Falany and Falany, 2007). In brief, fresh term human placental tissue was obtained from the Tissue Procurement Service of the University of Alabama at Birmingham Comprehensive Cancer Center. The placental tissue was homogenized in 67 mM phosphate buffer, pH 7.4, containing 0.5 mM dithiothreitol and 0.24 M sucrose and stored at -80°C until use. For STS purification, placental homogenate was thawed on ice and diluted with 10 mM potassium phosphate, pH 7.4, 20% glycerol, 0.5 μ M androstenedione, and 0.1 mM EDTA and then centrifuged at 105,000g for 60 min. The pellet was then homogenized in the same buffer, adjusted to 0.3% Na cholate and 0.3% Emulgen 911, stirred at 4°C for 30 min, and then centrifuged at 105,000g for 1 h. The supernatant fraction was diluted with an equal volume of the same buffer and then loaded on a 60-ml DE-52 anion exchange column equilibrated in the same buffer. The column was washed with buffer containing 0.15% Emulgen 911 and then with 20 mM Tris-HCl, pH 7.4, containing 0.1% Triton X-100. STS activity was then eluted with 0.15 M NaCl in the same buffer in 3-ml fractions. Fractions containing STS activity were pooled and adjusted to contain 5 mM CaCl_2 , 5 mM MgCl_2 , 5 mM MnCl_2 , and 1 M NaCl. The pool was stirred for 30 min at 4°C and then centrifuged at 105,000g for 30 min. STS was further purified from the supernatant fraction on a 5-ml Con A Sepharose column equilibrated in 20 mM Tris-HCl, pH 7.4, containing 0.1% Triton X-100, 5 mM CaCl_2 , 5 mM MgCl_2 , 5 mM MnCl_2 , and 1 M NaCl. Subsequent to the wash step, STS was eluted with 10% α -mannopyranoside, and fractions containing STS activity were pooled. The α -mannopyranoside was removed by repeated concentration and dilution using a Centricon 30,000-molecular weight cutoff membrane. The purified STS was aliquoted and stored at -80°C .

SULT and STS Assays. 24-OHChol sulfation assays were carried out using a standard procedure for the sulfation of nonradiolabeled sulfonate acceptor substrates (Meloche and Falany, 2001). In brief, reactions contained 75 mM Tris-HCl, pH 7.4, 5 mM MgCl_2 buffer, and either 24-OHChol in ethanol or an equal volume of ethanol alone. Enzyme was added to the mixture and equilibrated for 2 min at 37°C . [35 S]PAPS was then added to the mixture to start the reaction. The reactions were terminated by chloroform extraction, and the sulfated products in the aqueous phase were resolved by TLC on silica gel TLC plates using a solvent system of methylene chloride, methanol, and ammonium hydroxide (84:16:5). The plates were dried and exposed to autoradiograph film, and then the radioactive spots were scraped for quantitation by scintillation counting.

To determine the sensitivity of the 24-OHChol sulfates to STS hydrolysis, the 24-OHChol-3-sulfate was generated using SULT2B1b, and the 24-OHChol-3-monosulfate and disulfate were synthesized with SULT2A1 and SULT1E1. The reactions were terminated by chloroform extraction and the aqueous phase containing the sulfates divided into two fractions. One fraction

was analyzed by TLC for quantification of 24-OHChol sulfation. The other fraction was incubated at 37°C for 2 min to vaporize the residual chloroform; then 1.0 μ g of purified STS was added, and the reaction was incubated for 15 min. After hydrolysis of the 24-OHChol sulfates with STS, the products were analyzed by TLC and autoradiography.

STS activity was determined using reactions containing 20 μ M [3 H]DHEA-sulfate as substrate in 0.1 M Tris-maleate buffer, pH 7.4. STS was added to start the reaction, and the reactions were stopped by the addition of 0.25 M Tris-HCl, pH 8.7, to alkalize the reaction, followed by extraction with chloroform. Radioactivity was determined in an aliquot of the chloroform phase by scintillation counting to quantify the generation of desulfated [3 H]DHEA.

Molecular Docking Simulations. For the molecular simulations of the docking of 24-OHChol and its sulfates into the substrate binding sites of SULT2A1, the 24-OHChol Protein Data Bank file was generated from a cholesterol Protein Data Bank file (Protein Data Bank code 1lri) (Lascombe et al., 2002), and an OH moiety was added manually at the 24-position. The 24-OHChol sulfates were generated by the addition of the appropriate sulfonates, and the structures were energetically minimized with Autodock (The Scripps Research Institute, La Jolla, CA). The crystal structure used for the docking studies was SULT2A1 solved with PAP in the binding site (Protein Data Bank code 1efh) (Pedersen et al., 2000). A SULT2A1 structure solved with DHEA in the binding site (Protein Data Bank code 1j99) was used for initial orientation of the hydroxyl groups of the 24-OHChol compounds (Rehse et al., 2002). The positioning of either of the nucleophilic hydroxyl groups of 24-OHChol and the sulfated metabolites was based on the position of the 3-hydroxyl of DHEA. Protons were added to the structure, pH 7, and electrostatic potentials and charges were assigned by Autodock, which includes terms for sulfur and phosphorus. An energetically minimized configuration was calculated for each of the 24-OHChol models with Autodock (Huey et al., 2007) using a Lamarckian search with a population of 150 initial test orientations and a minimum of 2.5×10^6 iterations. The binding free energy (BFE) was calculated for the lowest energy configuration.

Mass Spectroscopy. To identify 24-OHChol and the sulfated reaction products, nonradioactive reactions were run in parallel with reactions containing [3 S]PAPS that were monitored by TLC. The nonradioactive reactions were extracted with chloroform, and the water phase was loaded onto a Sep-Pak cartridge, washed with water, and eluted with MeOH. The eluates were concentrated by evaporation under a stream of N_2 . Identification of the 24-OHChol mono- and disulfates was carried out by high-performance liquid chromatography/mass spectroscopy using an Applied Biosystems/MDS Sciex (Concord, ON, Canada) API-4000 Triple Quadrupole mass spectrometer with two PerkinElmer Life and Analytical Sciences series 200 micropumps and a Synergi Fusion (100 $\times$ 2 mm i.d.; Phenomenex, Torrance, CA) analytical C-18 column with a C-18 guard column. The mobile phases were 10 mM ammonium acetate (A) and 100% acetonitrile/10 mM ammonium acetate (B). The gradient profile was 0 to 5 min, 100% A; 5 to 10 min, 20% A (linear); 10 to 15 min, 20% A; and 15 to 20 min, 100% A (step). Mass spectroscopy results were analyzed with the Analyst 1.4 software (Applied Biosystems, Foster City, CA). Identification of the 24-OHChol monosulfates and disulfate was carried out by tandem mass spectrometry (MS/MS) to generate the parent 24-OHChol and sulfate ions. MS/MS analysis of the putative 24-OHChol monosulfates was attempted with increasing collision energies (30–120 eV). Fragmentation other than the parent and sulfate ions was observed only with the 24-OHChol-24-monosulfate.

TR-FRET LXR α Coactivator Recruitment Assay. A sample of 25 μ M 24-OHChol-3-sulfate in ethanol was evaporated under nitrogen and redissolved in dimethyl sulfoxide (DMSO) to a concentration of 250 μ M. A sample of 5 μ M 24-OHChol-24-sulfate in 50% ethanol was evaporated under nitrogen and redissolved in DMSO/methanol (50:50) to a concentration of 50 μ M. Then 1:2 dilutions of each sterol sulfate were prepared in DMSO. The synthetic LXR agonist, T0901317 (dissolved in DMSO), and the parental oxysterol, 24-OHChol (dissolved in ethanol), were included as positive control LXR ligands. The Lanthascreen TR-FRET LXR α Coactivator Assay was used according to the manufacturer's instructions (Invitrogen). To test the ability of an agent to bind to LXR α as an agonist, a dilution series was prepared at 100 $\times$ and then diluted to 2 $\times$ with Complete TR-FRET Coregulator buffer H. A 10- μ l aliquot of each dilution was transferred to the wells of a 384-well plate in triplicate,

FIG. 1. Sulfated products of 24-OHChol generated by SULT2A1 and SULT2B1b, and sensitivity to hydrolysis by STS. A, the structure of 24-OHChol is shown, and the arrows identify the 3- and 24-hydroxyl groups that are sulfated. B, the formation of 24-OHChol monosulfate and disulfate is shown, as well as their sensitivity to STS hydrolysis. 24-OHChol (20 μ M) was incubated with SULT2A1 or SULT2B1b in the presence of 10 μ M [3 S]PAPS for 30 min at 37°C. The reactions were terminated by extraction with chloroform, and an aliquot was applied to a silica gel TLC plate. For treatment with STS, an aliquot of the reaction after chloroform extraction was incubated with 1 μ g of STS for 30 min at 37°C. The STS reaction was extracted with chloroform, and an aliquot of the aqueous phase was spotted on the TLC plate. The plates were developed as described under *Materials and Methods* and exposed to autoradiograph film to identify 3 S-containing bands. [3 S]PAPS does not move from the loading zone.

and 5 μ l of 20 nM LXR α -ligand binding domain was added to each well, followed by 5 μ l of a mixture containing 1 μ M fluorescein-labeled TRAP220/DRIP-2 peptide and 40 nM terbium-conjugated anti-glutathione *S*-transferase antibody. The plate was sealed, protected from light, incubated with gentle shaking for 2 h at room temperature, and read with a Victor³ Multilabel Reader (PerkinElmer Life and Analytical Sciences), with an excitation wavelength of 340 nm, emission wavelengths of 520 and 490 nm, a delay time of 100 μ s, and an integration time of 200 μ s. Emission ratios were calculated as the quotients of the emission values at 520 nm divided by the emission values at 490 nm. Binding curves were generated using the log(agonist) versus response-variable slope function of Prism 5 (GraphPad Software Inc., San Diego, CA). To test the ability of an agent to bind to LXR α as an antagonist, assays were performed as described above, except that each binding reaction also contained 100 nM T0901317, and binding curves were generated using the log(inhibitor) versus response-variable slope function of Prism 5.

Results

24-OHChol Sulfation. Seven isoforms of human SULT were tested for their ability to sulfate 24-OHChol, including SULT1A1, SULT1A3, SULT1B1, SULT1C1, SULT1E1, SULT2A1, and SULT2B1b. Three of the SULT isoforms were capable of sulfating 24-OHChol (SULT1E1, SULT2A1, and SULT2B1b), whereas the other isoforms displayed no detectable activity. Figure 1 shows that SULT2A1 was capable of forming both 24-OHChol monosulfates and the 3, 24-disulfate, whereas SULT2B1b formed only a 24-OHChol monosulfate. SULT1E1 was also capable of forming both 24-OHChol monosulfates and the disulfate (supplemental data).

Hydrolysis by STS. The sensitivity of the different 24-OHChol sulfates to hydrolysis by placental STS was examined. Figure 1 also shows that the single 24-OHChol monosulfate formed by SULT2B1b was completely hydrolyzed by STS treatment. In contrast, the 3, 24-disulfate formed by SULT2A1 was hydrolyzed to an STS-resistant monosulfate. Therefore, SULT2B1b formed a single 24-OHChol monosulfate that was sensitive to STS hydrolysis, whereas in the disulfate formed by SULT2A1 only one of the possible monosulfates was

FIG. 2. Sulfation of 24-OHChol by SULT2A1. Increasing concentrations of 24-OHChol were sulfated by SULT2A1 (2.6 ng) in the presence of 10 μ M [35 S]PAPS. Reactions were run for 10 min at 37°C and then terminated by extraction with chloroform. The aqueous phase of duplicate reactions was then treated with STS for 30 min and stopped by chloroform extraction. Aliquots of the reactions were loaded onto silica gel TLC plates and developed as described under *Materials and Methods*. The plates were then exposed to autoradiograph film, and the radioactive bands were scraped into vials for scintillation counting. A, the formation of 24-OHChol monosulfate and disulfate at low 24-OHChol concentrations. B, the levels of 24-OHChol monosulfate and disulfate formation with increasing 24-OHChol concentrations, as well as the residual 24-OHChol monosulfate present after STS treatment.

cleaved. The remaining STS-resistant monosulfate indicated that the monosulfate formed by SULT2B1b and the STS-sensitive monosulfate synthesized by SULT2A1 were identical.

24-OHChol Sulfation by SULT2A1. Figure 2A shows that at low 24-OHChol concentrations the overall rate of disulfate formation was more rapid than monosulfate formation. To understand the mechanism for the rapid 3, 24-disulfate synthesis, the rates of monosulfate and disulfate formation were examined. Figure 2B shows the relationship between the formation of 24-OHChol monosulfates and disulfate, as well as their sensitivity to STS hydrolysis. Treatment of the sulfated products with STS resulted in the formation of an STS-resistant monosulfate in equal amounts to the amount of disulfate present in the initial reaction. These data indicate that the monosulfate present in the reaction mixture was sensitive to STS hydrolysis, whereas the disulfate is hydrolyzed to an STS-resistant monosulfate. Very little of the STS-resistant monosulfate was present in the reaction mixture before STS treatment.

24-OHChol Sulfation by SULT1E1. SULT1E1 was also capable of forming detectable amounts of 24-OHChol disulfate, although at significantly lower rates than SULT2A1 (Fig. 3). In contrast to SULT2A1, SULT1E1 formed primarily monosulfates at low 24-OHChol concentrations. The amount of 24-OHChol disulfate generated in these reactions was approximately 20% of the monosulfate formed. At concentrations greater than 7 μ M, 24-OHChol monosulfate formation showed apparent substrate inhibition, a feature frequently observed with the SULTs (Falany et al., 1994; Zhang et al., 1998). The major monosulfate formed by SULT1E1 was also sensi-

FIG. 3. Sulfation of 24-OHChol by SULT1E1. Increasing concentrations of 24-OHChol were sulfated by SULT1E1 in the presence of 10 μ M [35 S]PAPS. Reactions were run for 20 min at 37°C and then terminated by extraction with chloroform. The aqueous phase of duplicate reactions was then treated with STS for 30 min and stopped by chloroform extraction. Aliquots of the reactions were loaded onto silica gel TLC plates and developed as described under *Materials and Methods*. The levels of 24-OHChol monosulfate and disulfate formation with increasing 24-OHChol concentrations, as well as the residual 24-OHChol monosulfate present after STS treatment, are shown.

tive to hydrolysis by STS. Very little of the STS-resistant monosulfate was present in the initial reaction because the amounts of STS-resistant monosulfate present after STS treatment are similar to the levels of 24-OHChol disulfate present in the initial reaction (Fig. 3).

24-OHChol Sulfation by SULT2B1b. Of the three SULT isoforms capable of sulfating 24-OHChol, SULT2B1b was the only isoform that formed only 24-OHChol monosulfates. Figure 4 shows that SULT2B1b required higher 24-OHChol concentrations for activity compared with SULT1E1 and SULT2A1, although the rates of sulfation were also generally greater. Figure 1 also shows that the single 24-OHChol monosulfate formed by SULT2B1b is the STS-sensitive monosulfate.

Liquid Chromatography MS/MS Analysis of the Sulfation of 24-OHChol. The sulfation of 24-OHChol by SULT2A1 and SULT2B1b was analyzed by liquid chromatography (LC)/MS/MS to identify the monosulfates and the products of STS hydrolysis. Figure 5A shows the elution of the 24-OHChol monosulfates generated by SULT2A1. The major peak eluting at 9.0 min is the 3-monosulfate, and the smaller peak at 8.3 min is the 24-monosulfate. Treatment of the initial reaction products with STS resulted in the total loss of only the 9.0 min or 3-monosulfate peak. This result is consistent with the selectivity of STS for the hydrolysis of 3 β -sulfates compared with 3 α -sulfates on steroidal compounds (Falany and Falany, 2007). Figure 5B shows the elution of the 24-OHChol-3, 24-disulfate formed by SULT2A1. Hydrolysis of the disulfate with STS generated only the 8.3 min or 24-monosulfate peak (Fig. 5C), indicating that 24-OHChol-24-sulfate is the STS-resistant monosulfate. Figure 5D shows that SULT2B1b formed only the 3-monosulfate and was apparently incapable of forming either the 24-sulfate or the disulfate, consistent with the selectivity of SULT2B1b for conjugation of 3 β -hydroxysteroids (Falany et al., 2006). The 24-OHChol-3-monosulfate formed by SULT2B1b was also completely hydrolyzed by STS.

To define the sites of sulfate conjugation, an LC/MS/MS approach was used. The analysis was complicated by the tendency for the loss of the sulfate group before fragmentation of the ring system. Using a range of electron volt potentials for the fragmentation of the putative 24-OHChol-24-sulfate molecule generated a fragment possessing the 3 β -OH moiety and portions of the A ring (Fig. 6). This is consistent with sulfate conjugation at the 24-position, supporting its identity as

FIG. 4. Formation of 24-OHChol sulfate by SULT2B1b. Increasing concentrations of 24-OHChol were converted to a monosulfate by SULT2B1b in the presence of 10 μ M [35 S]PAPS. Each point represents the mean of three assays $\pm$ S.D. The error bars are essentially contained within the data points.

24-OHChol-24-sulfate. LC/MS/MS analysis of the putative 24-OHChol-3-sulfate under similar conditions generated only the parent and sulfate ions. However, the LC/MS/MS identification of the 24-OHChol-24-sulfate and 24-OHChol-3-sulfate is consistent with their anticipated STS sensitivities and expected syntheses, especially the formation of the 3-monosulfate by SULT2B1b that is highly selective for the sulfation of 3 β -hydroxysteroids (Meloche and Falany, 2001).

Metabolism of the 24-OHChol Monosulfates to the Disulfate by SULT2A1. Analysis of the products of the sulfation of 24-OHChol by SULT2A1 indicates that the major products formed are the 3-monosulfate and the disulfate. Very little of the 24-monosulfate was detected (Fig. 5). To examine whether one of the monosulfates was the preferred substrate for conversion to the disulfate, both of the 24-OHChol monosulfates were purified and used in assays with SULT2A1 to form the disulfate. Figure 7 shows the effect of increasing concentrations of the separate monosulfates on their conversion to 24-OHChol disulfate. The conversion of the 24-sulfate to the disulfate

FIG. 5. LC/MS/MS analysis of the 24-OHChol sulfates formed by SULT2A1 and SULT2B1b. A, the monosulfated products generated by sulfation of 24-OHChol with SULT2A1. MS/MS analysis of the peaks identified the more abundant peak as the 3-sulfate (9.0 min) and the smaller peak (8.3 min) as the 24-sulfate. B, the 24-OHChol disulfate eluted at 7.3 min, consistent with its more charged nature. C, STS treatment of the disulfate generates only the STS-resistant 24-sulfate eluting at 8.3 min. D, sulfation of 24-OHChol with SULT2B1b results in the formation of only the 3-monosulfate.

occurs at much lower concentrations and with a higher rate than sulfation of the 3-monosulfate.

Kinetics of 24-OHChol Sulfation. Table 1 presents the kinetic parameters determined for the formation of the different sulfated metabolites of 24-OHChol catalyzed by SULT2A1, SULT1E1, and SULT2B1b. SULT2B1b showed the highest K_m (23.5 μ M) and a K_{cat} of 218/min for the formation of 24-OHChol-3-sulfate, its only product. SULT1E1 had a K_m of 0.9 μ M for the synthesis of the 3-monosulfate with a K_{cat} of 55/min. For the overall metabolism of 24-OHChol to a disulfate, SULT1E1 had a relatively high K_m of 4.9 μ M and the lowest K_{cat} (4/min) of the reactions analyzed. SULT2A1 showed a K_m of 3.7 μ M for the synthesis of the 3-monosulfate with a K_{cat} equivalent to that of SULT2B1b (218/min). The overall conversion of 24-OHChol to the disulfate by SULT2A1 had a K_m of 1.5 μ M and a K_{cat} of 133/min, consistent with the favored formation of the disulfate by SULT2A1 at low 24-OHChol concentrations.

The detection of low amounts of 24-OHChol-24-monosulfate during the sulfation of 24-OHChol by SULT2A1 suggested that the 24-monosulfate was more rapidly converted to the disulfate than the 3-monosulfate. Table 1 shows that the K_m for the conversion of 24-OHChol-24-sulfate to the disulfate by SULT2A1 was 0.1 μ M and the K_{cat} is 2410/min, whereas the conversion of the 3-sulfate to the disulfate possessed a 40-fold higher K_m and 20-fold lower K_{cat} . These data indicate that SULT2A1 forms both 24-OHChol monosulfates at low concentrations; however, because of its higher affinity the 24-monosulfate is rapidly converted to the disulfate. Therefore, the K_m and V_{max} for the overall conversion of 24-OHChol to the disulfate are a good estimate for the formation of the 24-monosulfate.

Molecular Modeling of the 24-OHChol Monosulfates in the Active Site of SULT2A1. The sulfation of 24-OHChol results in the formation of the 3-monosulfate and the 3, 24-disulfate. Lack of detection of the 24-monosulfate in the reactions was apparently caused by the high affinity of the 24-monosulfate for conversion to the disulfate (Table 1). To examine the molecular interactions associated with the rapid formation of 24-OHChol disulfate, both the 24-OHChol monosulfates were docked in the active site of SULT2A1. First, the affinity of 24-OHChol with either the 3-hydroxyl or 24-hydroxyl positioned for catalysis in the substrate binding site of SULT2A1 was analyzed using Autodock. The BFE of 24-OHChol with either the 3-hydroxyl or 24-hydroxyl in the active site was -6.0 and -6.1 kcal/mol, respectively, indicating a similar affinity for sulfation at both hydroxyls. The nonsulfated hydroxyl groups of both compounds were positioned appropriately in relation to PAP and the active site His99 residue for catalysis. The first three preferred binding orientations for sulfation of both the 3-OH and 24-OH positions in 24-OHChol and the second and third preferred orientations for both the 24-OHChol monosulfates are included as supplemental data.

The conjugation of compounds with a bulky charged sulfonate group generally limits their further metabolism. To assess the mechanism for the high affinity of 24-OHChol-24-sulfate for sulfation to the 3, 24-disulfate, the binding of both the 3- and 24-monosulfates in the active site of SULT2A1 was analyzed. Figure 8A depicts the optimal orientation of 24-OHChol-3-sulfate in the binding pocket of SULT2A1 using the Autodock program to minimize the BFE. 24-OHChol-3-sulfate orients in the binding pocket of SULT2A1 in association with PAP and the active site His99 residue with a BFE of -3.5 Kcal/mol. In contrast, 24-OHChol-24-sulfate did not bind in the substrate binding pocket in a catalytically competent orientation when the docking experiment used the SULT2A1 monomer structure. Figure 8B shows the optimal binding orientation of the 24-OHChol-24-sulfate in the SULT2A1 monomer. The 24-sulfate group strongly interacts with Lys138, and the 3-hydroxyl group hydrogen bonds with

FIG. 6. Identification of 24-OHChol-24-sulfate by LC/MS/MS analysis. The site of sulfate conjugation in the putative 24-OHChol-24-sulfate was analyzed by LC/MS/MS after fragmentation with increasing energies as described under *Materials and Methods*. After fragmentation at 60 eV, the major ions detected were the parent ion 24-OHChol (481 Da), the sulfate moiety (96.9 Da), and a fragment derived from the A-ring containing the 3-OH (59.1 Da). The presence of the 3-OH indicates the sulfate is conjugated at the 24-position.

FIG. 7. Conversion of the 24-OHChol monosulfates to the disulfate by SULT2A1. The two 24-OHChol monosulfates were synthesized as described under *Materials and Methods* and used in reactions to assay their conversion to 24-OHChol disulfate by SULT2A1. The reactions used nonradiolabeled monosulfates and [^{35}S]PAPS to monitor formation of the disulfate using the TLC assay described under *Materials and Methods*. A, the conversion of 24-OHChol-3-sulfate to the disulfate. B, the sulfation of 24-OHChol-24-sulfate to the disulfate. The data represent three sets of reactions done in duplicate.

Ser80, preventing entry of 24-OHChol-24-sulfate into the binding pocket. The BFE of this orientation is -10.0 Kcal/mol. The 3-OH group of 24-OHChol-24-sulfate is positioned approximately 0.9 nm

from the active site His99 residue. Because the BFE and orientation of the 24-sulfate in the substrate binding pocket of SULT2A1 are inconsistent with the high affinity and reactivity of the 24-sulfate to the disulfate observed in the kinetic experiments, the docking experiments were carried out using the dimer structure of SULT2A1 (Protein Data Bank code 1efh). Figure 8C shows that when using the homodimer structure of SULT2A1, 24-OHChol-24-sulfate orients in the binding pocket with the 3-hydroxyl group in a position close to PAP and the active site His99 residue consistent with efficient catalysis. The BFE is -7.9 Kcal/mol, indicating that the 24-OHChol-24-sulfate is a better substrate for a second sulfation and conversion to a disulfate than the 3-monosulfate. The ability of 24-OHChol-24-sulfate to efficiently bind with the 3-hydroxyl in a catalytically competent orientation results from an interaction of Glu73 from the second SULT2A1 molecule in the homodimer structure blocking access for the 24-sulfate group of 24-OHChol-24-sulfate to interact with Lys138. Because the 24-sulfate moiety cannot bind to Lys138 in the SULT2A1 homodimer as a result of the presence of the Glu73 residue, the 24-OHChol-24-sulfate can enter into the binding pocket and situate closer to PAP and the His99 residue.

Agonist and Antagonist Properties of the 24-OHChol Sulfates in the Activation of LXR α . Oxysterols, including 24-OHChol, are considered physiological agonists of the LXRs (Lehmann et al., 1997; Janowski et al., 1999). To test for interaction of 24-OHChol and its sulfated metabolites with LXR α , a FRET-based coactivator recruitment assay was used. Figure 9A indicates that the synthetic LXR agonist T0901317 and 24-OHChol were capable of activating LXR α with EC $_{50}$ s of 0.30 and 1.99 μM , respectively. Both 24-OHChol-3-sulfate and the 24-sulfate were tested for LXR activation activity, and no ability to activate LXR α was observed. Although neither of the 24-OHChol monosulfates activated LXR α , both monosulfates were tested for their ability to antagonize the activation activity of T0901317. Figure 9B shows that both 24-OHChol monosulfates were efficient antagonists of LXR α activation by 100 nM T0901317. The IC $_{50}$ s of 24-OHChol-3-sulfate and 24-sulfate were 0.15 and 0.31 μM , respectively, indicating that the monosulfates were better inhibitors of LXR activation in the coactivator assay than the parent 24-OHChol was an agonist.

TABLE 1

Kinetic parameters for the sulfation of 24-OHChol by expressed SULT isoforms

The sulfation reactions were carried out as described under *Materials and Methods*. Kinetic parameters were calculated using the Enzyme Kinetics program. $V_{\max}$ is expressed as micromoles of sulfated product per minute per nanomole of enzyme, and K_{cat} is per minute.

SULT	Substrate	Product	K_m	$V_{\max}$	K_{cat}
			μM	$\mu\text{mol}/\text{min}/\text{nmol}$	/min
2B1b	24-OHChol	3-Monosulfate	23.5	5113	218
1E1	24-OHChol	3-Monosulfate	0.9	50	55
1E1	24-OHChol	Disulfate	4.9	14	4
2A1	24-OHChol	3-Monosulfate	3.7	807	218
2A1	24-OHChol	Disulfate	1.5	199	133
2A1	24-OHChol 24-S	Disulfate	0.1	265	2410
2A1	24-OHChol 3-S	Disulfate	4.0	452	113

Discussion

Synthesis of 24-OHChol is the major mechanism for the removal of cholesterol from the brain, and after transport to the liver it is a substrate for the synthesis of bile acids (Bjorkhem et al., 2001; Bjorkhem, 2006). As an oxysterol, 24-OHChol is also considered an important mediator of the activity of nuclear receptors, including the LXRs (Lehmann et al., 1997). In brain, 24-OHChol is synthesized by CYP46 (Mast et al., 2003) and acts to increase cholesterol mobilization for membrane synthesis and synaptic remodeling via LXR β activation (Cartagena et al., 2008). LXR activation also induces OAT2 and ApoE synthesis, resulting in the transport of 24-OHChol from the brain into the circulation (Abildayeva et al., 2006). 24-OHChol circulates bound to ApoE and is taken up by the liver for metabolism and excretion. In liver and macrophages, 24-OHChol is involved in the activation of LXR α and stimulates reverse cholesterol transport and the retention of fatty acids (Naik et al., 2006; Calpe-Berdiel et al., 2008).

24-OHChol is synthesized in neurons in the central nervous system, secreted from brain, and carried to the liver. In the liver, approximately 50% of the 24-OHChol is converted to bile acids following 7 α -hydroxylation catalyzed by either CYP39 or CYP7A (Bjorkhem et al., 2001; Bjorkhem, 2007). Bjorkhem et al. (2001) reported that 24-OHChol is also secreted in the bile as sulfate and glucuronide conjugates and that sulfate/glucuronide diconjugates are also formed. In ileocecal fluid from patients, sulfates represented 3 to 45% of the 24-OHChol detected, whereas 40 to 50% was present as glucuronide conjugates, although this fraction included the sulfate/glucuronide diconjugates. The relative positions of the sulfates and glucuronides in the diconjugate were not reported. These authors did not detect the presence of 24-OHChol disulfates that are formed at low 24-OHChol concentrations by SULT2A1, which is one of the two most abundant SULT isoforms in human liver (Falany, 1997; Glatt et al., 2000). Sulfated 24-OHChol has been detected in the brain and in the circulation. Considerable amounts of 5-cholestene-3 β ,24S,27-triol were also detected in ileocecal fluid, suggesting the hydroxylation of 24-OHChol by CYP27, and this metabolite was also readily conjugated (Bjorkhem, 2007). Therefore, sulfation represents a major pathway for the metabolism of 24-OHChol in the brain, circulation, and liver, although many of the details of the metabolism of 24-OHChol in different human tissues remain to be identified.

Of the major SULT isoforms tested, only three showed activity in sulfating 24-OHChol: SULT2A1, SULT1E1, and SULT2B1b. These are the major isoforms associated with steroid sulfation in human tissues. Consistent with its selective activity in conjugating 3 β -hydroxysteroids (Falany et al., 2006), SULT2B1b formed only 24-OHChol-3-monosulfates, although with the lowest affinity and highest $V_{\max}$ of the three isoforms (Table 1). SULT2B1b also readily sulfates cholesterol (Javitt et al., 2001), and in human keratinocytes

the expression of SULT2B1 was inducible by LXR activation (Jiang et al., 2005). SULT2B1b is widely expressed in human tissues, including brain, although its expression was not detectable in liver (Geese and Raftogianis, 2001; Falany et al., 2006). The regulation of SULT2B1b expression in brain tissues by LXR activation has not been described.

Identification of the 24-OHChol monosulfates was accomplished using several methods. LC/MS/MS was used to identify the 24-OHChol monosulfates and disulfate by selective ion monitoring and loss of the sulfate moieties. The monosulfates also showed different elution times. The 3-sulfate was assigned by its sensitivity to STS hydrolysis that is specific for the 3-position of sterols (Hernandez-Guzman et al., 2001; Iwamori, 2005; Falany and Falany, 2007), differential formation by SULT2A1 and SULT2B1b, and MS/MS analysis. SULT2B1b is highly selective for the 3-position of sterols and docking affinities of 24-OHChol in the active sites of SULT2A1 and SULT2B1b. Both the 3-OH and 24-OH positions orient in the active site of SULT2A1 in apparently catalytically active orientations (Supplemental Figs. 1 and 2), whereas in SULT2B1b only the 3-OH of 24-OHChol is positioned in an active configuration (Supplemental Fig. 6). These approaches are consistent with SULT2A1 conjugating both hydroxyls and SULT2B1b forming only the 3-sulfate. Identification of 24-OHChol monosulfate was achieved by MS/MS analysis via detection of a fragment containing the 3-OH moiety (Fig. 6). In contrast, MS/MS analysis of the putative 24-OHChol-3-monosulfate only gave rise to the parent 24-OHChol and sulfate ions.

SULT1E1 has a K_m for E2 sulfation of 4 nM and is an important mechanism for the inactivation of E2 in human tissues (Zhang et al., 1998; Kotov et al., 1999). SULT1E1 also sulfates hydroxysteroids (Falany et al., 1994, 1995), although with an affinity similar to that for the formation of 24-OHChol-3-sulfate (Table 1). SULT1E1 displayed a low rate of 24-OHChol disulfate formation, indicating that the 24-hydroxyl of 24-OHChol was not an efficient site for sulfation. Similar to SULT2B1b, SULT1E1 displayed a strong preference for the formation of the 3-monosulfate, although with a better affinity but lower activity. SULT1E1 is not as widely expressed in tissues as SULT2B1b being detected primarily in estrogen-responsive tissues, although low levels are present in liver (Duanmu et al., 2006).

SULT2A1 is the major SULT expressed in liver and has a relatively broad substrate reactivity being capable of conjugating 3 α - and 3 β -hydroxysteroids, estrogens, and aliphatic hydroxyls in xenobiotics (Falany et al., 1995). It is capable of conjugating the D-ring hydroxyls of androgens, as well as therapeutic drugs such as tibolone (Falany et al., 2004). At low 24-OHChol concentrations that represent physiological levels, SULT2A1 forms approximately equal amounts of the 3-sulfate and the 3, 24-disulfate. Formation of the 3, 24-disulfate results from the high affinity of SULT2A1 for the sulfation of the 3-OH position in 24-OHChol-24-sulfate.

FIG. 8. Molecular simulation of the orientation of the 24-OHChol monosulfates in the active site of SULT2A1. The two monosulfates of 24-OHChol were molecularly oriented into the active site of SULT2A1 (Protein Data Bank code 1efh) using the Autodock program to allow sulfonation of the free hydroxyl to allow disulfate formation. A, the orientation of 24-OHChol-3-sulfate in the substrate binding site in the SULT2A1 monomer. The BFE is -3.5 Kcal/mol, and the 24-hydroxyl group is close to Ser97 (0.35 nm) and His99 (0.60 nm). B, in the SULT2A1 monomer structure, 24-OHChol-24-sulfate does not readily bind in the pocket but prefers to bind to Lys138 and Ser80 in an apparent noncatalytic orientation. The BFE of the 24-OHChol-24-sulfate in this orientation is -10.0 Kcal/mol. C, the orientation of 24-OHChol-24-sulfate in the SULT2A1 dimer structure. In the SULT2A1 dimer structure, Glu73 from the other monomer inhibits access of the 24-sulfate to Lys138 and allows for docking in the active site in an apparent catalytically active conformation. The BFE is -7.9 Kcal/mol, and the 3-hydroxyl group is 0.4 nm from His99.

Molecular docking simulations indicate that 24-OHChol has a similar BFE with either the 3-OH or 24-OH groups positioned for efficient catalysis, suggesting that both monosulfates are formed at similar rates. Because of the high affinity of SULT2A1 for conjugation of the 3-OH of 24-OHChol-24-sulfate, the 24-sulfate is not readily detected. The 24-OH of 24-OHChol-3-sulfate is not readily

FIG. 9. In vitro interactions of 24-OHChol sulfate metabolites with LXR α . The Lanthascreen TR-FRET LXR α coactivator assay was used to evaluate the abilities of 24-OHChol-3-sulfate and 24-OHChol-24-sulfate to interact with LXR α , either as agonists (A) or antagonists (B). T0901317 and 24-OH-Chol were included as positive control LXR α agonists, and antagonist activities were determined in the presence of 100 nM T0901317. Binding curves were generated using Prism 5. The EC $_{50}$ s for T0901317 and 24-OHChol were 0.030 and 1.99 μ M, respectively, and there was no detectable activation for the sulfated metabolites. The IC $_{50}$ s of the sulfated metabolites were 0.149 μ M for 24-OHChol-3-sulfate and 0.309 μ M for 24-OHChol-24-sulfate.

conjugated so the 3-sulfate accumulates along with the disulfate. Molecular docking simulations were used to examine the preference of SULT2A1 for sulfation of the 24-sulfate relative to the 3-sulfate. In these studies the 3-sulfate bound similarly in both the SULT2A1 monomer and in the homodimer. In contrast, the 24-sulfate did not bind in the substrate binding pocket when modeled with the SULT2A1 monomer but did orient the 3-hydroxyl properly in the substrate binding pocket when modeled in the SULT2A1 homodimer. The BFE of the 24-OHChol-24-sulfate in the SULT2A1 homodimer was 3.9 Kcal/mol lower than the 3-sulfate consistent with their K_m values and the increased metabolism of the 24-sulfate to the 3, 24-disulfate.

The necessity of using the SULT2A1 homodimer (Protein Data Bank code 1efh) to obtain a docking orientation with 24-OHChol-24-sulfate that is consistent with its observed sulfation activity supports the previous identification of the dimerization sites in this structure. Petrotchenko et al. (2001) have reported that the "KTVE" domain (amino acids 255–264 in SULT2A1) is the dimerization interface used in the SULTs. In contrast, in the SULT2A1 structures the dimerization interface involves amino acids M16-R19, S68-P70, and M228-Y238 (Zhou et al., 2001; Rehse et al., 2002). The reported dimer structure of SULT2A1 (Protein Data Bank code 1efh) is more consistent with the high affinity sulfation of 24-OHChol-24-sulfate because interaction with Lys138 is blocked by Glu73 in the second SULT2A1 subunit in the homodimer. If the dimerization domain is assumed to be

the KTVE domain, this interaction is not inhibited and 24-OHChol-24-sulfate is not anticipated to be an efficient substrate for conversion to the 3, 24-disulfate. These observations suggest that the different SULT isoforms may use different dimerization domains, although there is considerable conservation of structure in the monomeric forms of the enzymes (Allali-Hassani et al., 2007).

Many sulfate esters are sensitive to hydrolysis by STS activity allowing regeneration of the parent compounds in tissues. STS is primarily responsible for the hydrolysis of estrogen sulfates and many hydroxysteroid sulfates, including DHEA-3-sulfate. The 3 β -sulfate of 24-OHChol was readily hydrolyzed by STS, whereas the 24-sulfate was resistant. In addition, in the 3, 24-disulfate only the 3-sulfate was hydrolyzed. Therefore, 3-sulfation of 24-OHChol represents a reversible reaction allowing regeneration of the parent compound by STS activity. Formation of either the 24-sulfate or 3, 24-disulfate is a terminal reaction in that the 24-sulfate is not hydrolyzed. The formation of the stable 24-sulfate will occur primarily in tissues with high levels of SULT2A1 activity such as liver, stomach, and the gastrointestinal tract. Whether sulfation inhibits hydroxylation by CYP7A and conversion into bile acids has not been reported, although this may account for the difference in 24-OHChol synthesis and its conversion into bile acids (Björkhem et al., 2001).

Although oxysterols are considered physiological activators of the LXRs (Janowski et al., 1999), the properties of the oxysterol sulfates in nuclear receptor activation have not been extensively studied. In the FRET coactivator recruitment assay, both of the 24-OHChol monosulfates were significantly better inhibitors of LXR activation than 24-OHChol was an agonist. In human liver that has high levels of SULT2A1 activity, the rapid synthesis of 24-OHChol-3-sulfate and the 3, 24-disulfate would possibly result in the generation of a potent stable LXR antagonist, 24-OHChol-24-sulfate. Liver possesses STS activity indicating that the 3-sulfate in the 3, 24-disulfate may be hydrolyzed, regenerating 24-OHChol; however, the 24-sulfate formed from 3, 24-disulfate would be stable because of its resistance to STS hydrolysis. High levels of SULT2A1 activity that favor generation of inhibitory 24-OHChol sulfates are limited to the adrenal cortex, gastrointestinal tract, and liver in humans (Falany et al., 2004). SULT2B1b, which forms only the 3-sulfate, is widely expressed in tissues and has been reported to be regulated by LXR in human keratinocytes (Jiang et al., 2005). The induction of SULT2B1b by LXR activation suggests a feedback mechanism by which high levels of 24-OHChol could activate LXR and induce SULT2B1b in tissues such as breast (Dumas et al., 2008), resulting in the increased synthesis of inhibitory 24-OHChol-3-sulfate. STS would then serve to hydrolyze the inhibitory 3-sulfates, allowing regeneration of 24-OHChol. In support of the LXR-modulating potential of SULT2B1b-catalyzed oxysterol sulfation, the transactivation of an LXR α reporter gene in human embryonic kidney 293 cells by a naturally occurring oxysterol, 24,25-epoxycholesterol, was substantially impaired by the cotransfection of mouse cholesterol sulfotransferase (SULT2B1b) (Chen et al., 2007). In addition, when mice were infected with a mouse SULT2B1b adenoviral expression vector, the expression of endogenous LXR α -activated hepatic target genes was decreased. The regulatory properties of the oxysterols and their sulfated metabolites with LXR activation or inhibition in different tissues remain to be elucidated.

The sulfation of 24-OHChol is catalyzed by at least three of the major human cytosolic SULT isoforms with different affinities for the formation of the 24-OHChol monosulfates and disulfate. SULT2B1b forms only the 3-sulfate, whereas SULT1E1 forms primarily the 3-sulfate but also a small amount of the 24-sulfate and disulfate. SULT2A1 preferentially catalyzes the formation of the disulfate at relatively low 24-OHChol concentrations as a result of a high affinity for sulfation of the 24-OHChol-24-sulfate to the disulfate. Only the

3-sulfate was hydrolyzed by human STS, allowing for the accumulation of the 24-sulfate in tissues. This may occur primarily in liver as a result of the abundant expression of SULT2A1. The formation of the 24-OHChol sulfates may be physiologically relevant because both the 24-OHChol monosulfates were 5- to 6-fold better antagonists of LXR activation than 24-OHChol was an agonist. Elucidating the sulfation of 24-OHChol in human tissues is important for understanding its multiple properties, regulation, and metabolism in different human tissues, including liver, brain, and breast.

References

- Abildayeva K, Jansen PJ, Hirsch-Reinshagen V, Bloks VW, Bakker AH, Ramaekers FC, de Vente J, Groen AK, Wellington CL, Kuipers F, and Mulder M (2006) 24(S)-Hydroxycholesterol participates in a liver X receptor-controlled pathway in astrocytes that regulates apolipoprotein E-mediated cholesterol efflux. *J Biol Chem* **281**:12799–12808.
- Allali-Hassani A, Pan PW, Dombrovski L, Najmanovich R, Tempel W, Dong A, Lopnau P, Martin F, Thornton J, Thontong J, et al. (2007) Structural and chemical profiling of the human cytosolic sulfotransferases. *PLoS Biol* **5**:e97.
- Björkhem I (2006) Crossing the barrier: oxysterols as cholesterol transporters and metabolic modulators in the brain. *J Intern Med* **260**:493–508.
- Björkhem I (2007) Rediscovery of cerebrosterol. *Lipids* **42**:5–14.
- Björkhem I, Andersson U, Ellis E, Alvelius G, Ellegård L, Diczfalussy U, Sjövall J, and Einarsson C (2001) From brain to bile. Evidence that conjugation and omega-hydroxylation are important for elimination of 24S-hydroxycholesterol (cerebrosterol) in humans. *J Biol Chem* **276**:37004–37010.
- Björkhem I, Lütjohann D, Diczfalussy U, Ståhle L, Ahlberg G, and Wahren J (1998) Cholesterol homeostasis in human brain: turnover of 24S-hydroxycholesterol and evidence for a cerebral origin of most of this oxysterol in the circulation. *J Lipid Res* **39**:1594–1600.
- Calpe-Berdiel L, Rotllan N, Fiévet C, Roig R, Blanco-Vaca F, and Escolà-Gil JC (2008) Liver X receptor-mediated activation of reverse cholesterol transport from macrophages to feces in vivo requires ABCG5/G8. *J Lipid Res* **49**:1904–1911.
- Cartagena CM, Ahmed F, Burns MP, Pajooesh-Ganji A, Pak DT, Faden AI, and Rebeck GW (2008) Cortical injury increases cholesterol 24S hydroxylase (Cyp46) levels in the rat brain. *J Neurotrauma* **25**:1087–1098.
- Chen JY, Levy-Wilson B, Goodart S, and Cooper AD (2002) Mice expressing the human CYP7A1 gene in the mouse CYP7A1 knock-out background lack induction of CYP7A1 expression by cholesterol feeding and have increased hypercholesterolemia when fed a high fat diet. *J Biol Chem* **277**:42588–42595.
- Chen W, Chen G, Head DL, Mangelsdorf DJ, and Russell DW (2007) Enzymatic reduction of oxysterols impairs LXR signaling in cultured cells and the livers of mice. *Cell Metab* **5**:73–79.
- Duanmu Z, Weckle A, Koukouritaki SB, Hines RN, Falany JL, Falany CN, Kocarek TA, and Runge-Morris M (2006) Developmental expression of aryl, estrogen, and hydroxysteroid sulfotransferases in pre- and postnatal human liver. *J Pharmacol Exp Ther* **316**:1310–1317.
- Dumas NA, He D, Frost AR, and Falany CN (2008) Sulfotransferase 2B1b in human breast: differences in subcellular localization in African American and Caucasian women. *J Steroid Biochem Mol Biol* **111**:171–177.
- Falany CN (1997) Enzymology of human cytosolic sulfotransferases. *FASEB J* **11**:206–216.
- Falany CN, Comer KA, Dooley TP, and Glatt H (1995) Human dehydroepiandrosterone sulfotransferase. Purification, molecular cloning, and characterization. *Ann NY Acad Sci* **774**:59–72.
- Falany CN, He D, Dumas N, Frost AR, and Falany JL (2006) Human cytosolic sulfotransferase 2B1: isoform expression, tissue specificity and subcellular localization. *J Steroid Biochem Mol Biol* **102**:214–221.
- Falany CN, Krasnykh V, and Falany JL (1995) Bacterial expression and characterization of a cDNA for human liver estrogen sulfotransferase. *J Steroid Biochem Mol Biol* **52**:529–539.
- Falany CN, Wheeler J, Oh TS, and Falany JL (1994) Steroid sulfation by expressed human cytosolic sulfotransferases. *J Steroid Biochem Mol Biol* **48**:369–375.
- Falany JL and Falany CN (2007) Interactions of the human cytosolic sulfotransferases and steroid sulfatase in the metabolism of tibolone and raloxifene. *J Steroid Biochem Mol Biol* **107**:202–210.
- Falany JL, Macrina N, and Falany CN (2004) Sulfation of tibolone and tibolone metabolites by expressed human cytosolic sulfotransferases. *J Steroid Biochem Mol Biol* **88**:383–391.
- Fuda H, Javitt NB, Mitamura K, Ikegawa S, and Strott CA (2007) Oxysterols are substrates for cholesterol sulfotransferase. *J Lipid Res* **48**:1343–1352.
- Fujiyoshi M, Ohtsuki S, Hori S, Tachikawa M, and Terasaki T (2007) 24S-Hydroxycholesterol induces cholesterol release from chorioid plexus epithelial cells in an apical- and apoE isoform-dependent manner concomitantly with the induction of ABCA1 and ABCG1 expression. *J Neurochem* **100**:968–978.
- Geese WJ and Raftogiannis RB (2001) Biochemical characterization and tissue distribution of human SULT2B1. *Biochem Biophys Res Commun* **288**:280–289.
- Glatt H, Engelke CE, Pabel U, Teubner W, Jones AL, Coughtrie MW, Andrae U, Falany CN, and Mehl W (2000) Sulfotransferases: genetics and role in toxicology. *Toxicol Lett* **112**:113–341–348.
- Hernandez-Guzman FG, Higashiyama T, Osawa Y, and Ghosh D (2001) Purification, characterization and crystallization of human placental estrone/dehydroepiandrosterone sulfatase, a membrane-bound enzyme of the endoplasmic reticulum. *J Steroid Biochem Mol Biol* **78**:441–450.
- Huey R, Morris GM, Olson AJ, and Goodsell DS (2007) A semiempirical free energy force field with charge-based desolvation. *J Comput Chem* **28**:1145–1152.
- Iwamori M (2005) Estrogen sulfatase. *Methods Enzymol* **400**:293–302.
- Janowski BA, Grogan MJ, Jones SA, Wisely GB, Kliewer SA, Corey EJ, and Mangelsdorf DJ (1999) Structural requirements of ligands for the oxysterol liver X receptors LXRalpha and LXRBeta. *Proc Natl Acad Sci U S A* **96**:266–271.
- Javitt NB, Lee YC, Shimizu C, Fuda H, and Strott CA (2001) Cholesterol and hydroxycholesterol

- sulfotransferases: identification, distinction from dehydroepiandrosterone sulfotransferase, and differential tissue expression. *Endocrinology* **142**:2978–2984.
- Jiang YJ, Kim P, Elias PM, and Feingold KR (2005) LXR and PPAR activators stimulate cholesterol sulfotransferase type 2 isoform 1b in human keratinocytes. *J Lipid Res* **46**:2657–2666.
- Kotov A, Falany JL, Wang J, and Falany CN (1999) Regulation of estrogen activity by sulfation in human Ishikawa endometrial adenocarcinoma cells. *J Steroid Biochem Mol Biol* **68**:137–144.
- Koudinov AR and Koudinova NV (2002) Cholesterol's role in synapse formation. *Science* **295**:2213.
- Lascombe MB, Ponchet M, Venard P, Milat ML, Blein JP, and Prangé T (2002) The 1.45 Å resolution structure of the cryptogeiin-cholesterol complex: a close-up view of a sterol carrier protein (SCP) active site. *Acta Crystallogr D Biol Crystallogr* **58**:1442–1447.
- Lehmann JM, Kliewer SA, Moore LB, Smith-Oliver TA, Oliver BB, Su JL, Sundseth SS, Winegar DA, Blanchard DE, Spencer TA, et al. (1997) Activation of the nuclear receptor LXR by oxysterols defines a new hormone response pathway. *J Biol Chem* **272**:3137–3140.
- Mast N, Norcross R, Andersson U, Shou M, Nakayama K, Bjorkhem I, and Pikuleva IA (2003) Broad substrate specificity of human cytochrome P450 46A1 which initiates cholesterol degradation in the brain. *Biochemistry* **42**:14284–14292.
- Meloche CA and Falany CN (2001) Expression and characterization of the human 3 beta-hydroxysteroid sulfotransferases (SULT2B1a and SULT2B1b). *J Steroid Biochem Mol Biol* **77**:261–269.
- Naik SU, Wang X, Da Silva JS, Jaye M, Macphee CH, Reilly MP, Billheimer JT, Rothblat GH, and Rader DJ (2006) Pharmacological activation of liver X receptors promotes reverse cholesterol transport in vivo. *Circulation* **113**:90–97.
- Pedersen LC, Petrotchenko EV, and Negishi M (2000) Crystal structure of SULT2A3, human hydroxysteroid sulfotransferase. *FEBS Lett* **475**:61–64.
- Petrotchenko EV, Pedersen LC, Borchers CH, Tomer KB, and Negishi M (2001) The dimerization motif of cytosolic sulfotransferases. *FEBS Lett* **490**:39–43.
- Rehse PH, Zhou M, and Lin SX (2002) Crystal structure of human dehydroepiandrosterone sulphotransferase in complex with substrate. *Biochem J* **364**:165–171.
- Song C, Hiipakka RA, and Liao S (2001) Auto-oxidized cholesterol sulfates are antagonistic ligands of liver X receptors: implications for the development and treatment of atherosclerosis. *Steroids* **66**:473–479.
- Zhang H, Varlamova O, Vargas FM, Falany CN, Leyh TS, and Varmalova O (1998) Sulfuryl transfer: the catalytic mechanism of human estrogen sulfotransferase. *J Biol Chem* **273**:10888–10892.
- Zhou M, Rehse P, Chang HJ, Luu-The V, and Lin SX (2001) Crystallization and preliminary crystallographic results of apo and complex forms of human dehydroepiandrosterone sulfotransferase. *Acta Crystallogr D Biol Crystallogr* **57**:1630–1633.

Address correspondence to: Charles N. Falany, Department of Pharmacology and Toxicology, 1670 University Blvd., Volker Hall G133M, University of Alabama at Birmingham, Birmingham, AL 35294. E-mail: cfalany@uab.edu