Oxidation of 7-dehydrocholesterol and desmosterol by human cytochrome P450 46A1

Sandeep Goyal,* Yi Xiao,* Ned A. Porter,† Libin Xu,‡† and F. Peter Guengerich†,*

Department of Biochemistry and Center in Molecular Toxicology,* Vanderbilt University School of Medicine, Nashville, TN 37232; and Department of Chemistry and Vanderbilt Institute of Chemical Biology,† Vanderbilt University, Nashville, TN 37235

Abstract Cytochrome P450 (P450 or CYP) 46A1 is expressed in brain and has been characterized by its ability to oxidize cholesterol to 24S-hydroxycholesterol. In addition, the same enzyme is known to further oxidize 24S-hydroxycholesterol to the 24,25- and 24,27-dihydroxy products, as well as to catalyze side-chain oxidations of 7α-hydroxycholesterol and cholesterol. As precursors in the biosynthesis of cholesterol, 7-dehydrocholesterol has not been found to be a substrate of P450 46A1 and desmosterol has not been previously tested. However, 24-hydroxy-7-dehydrocholesterol was recently identified in brain tissues, which prompted us to reexamine this enzyme and its potential substrates. Here we report that P450 46A1 oxidizes 7-dehydrocholesterol to 24-hydroxy-7-dehydrocholesterol and 25-hydroxy-7-dehydrocholesterol, as confirmed by LC-MS and GC-MS. Overall, the catalytic rates of formation increased in the order of 24-hydroxy-7-dehydrocholesterol < 24-hydroxycholesterol < 25-hydroxy-7-dehydrocholesterol from their respective precursors, with a ratio of 1:2.5:5. In the case of desmosterol, epoxidation to 24S,25-epoxycholesterol and 27-hydroxylation was observed, at roughly equal rates. The formation of these oxysterols in the brain may be of relevance in Smith-Lemli-Opitz syndrome, desmosterolosis, and other relevant diseases, as well as in signal transduction by lipids.—Goyal, S., Y. Xiao, N. A. Porter, L. Xu, and F. P. Guengerich. Oxidation of 7-dehydrocholesterol and desmosterol by human cytochrome P450 46A1. J. Lipid Res. 2014. 55: 1933–1943.

Supplementary key words 24-hydroxy-7-dehydrocholesterol • 25-hydroxy-7-dehydrocholesterol • 24S,25-epoxycholesterol • 27-hydroxydesmosterol

Cytochrome P450 (P450 or CYP) 46A1, or cholesterol 24-hydroxylase, is a brain-specific enzyme that converts cholesterol to 24S-hydroxycholesterol, which can be further oxidized to 24,25- and 24,27-dihydroxycholesterols by the same enzyme (1, 2) (Fig. 1). 24S-hydroxycholesterol is an important brain oxysterol that regulates cholesterol homeostasis by interacting with liver X receptors (LXRs) and the SREBP2 pathway (3–5). It has been reported that P450 46A1 can oxidize a broad range of substrates with diverse chemical structures, including sterols, Δ4-steroids, and some drugs (1).

7-Dehydrocholesterol is a biosynthetic precursor to previtamin D3 in human skin and to cholesterol in tissues (6, 7). Although the level of 7-dehydrocholesterol is normally low in human tissues and fluids, it is significantly elevated in a number of human disorders, including Smith-Lemli-Opitz syndrome (SLOS) and cerebrotendinous xanthomatosis (7–9). SLOS is caused by mutations in the gene encoding the enzyme 3β-hydroxysterol-Δ7-reductase (DHCR7), which reduces 7-dehydrocholesterol to cholesterol (7, 10, 11). In a previous study, we identified 24-hydroxy-7-dehydrocholesterol in brain tissue of a rat model for SLOS, and we speculated that this oxysterol was derived from the oxidation of 7-dehydrocholesterol by P450 46A1 (12). Although it was previously reported that 7-dehydrocholesterol is not a substrate of P450 46A1 (13), the existence of 24-hydroxy-7-dehydrocholesterol in vivo warranted reexamination of this possibility.

The additional double bond at C7 renders 7-dehydrocholesterol highly reactive toward free radical oxidation (14), a process that leads to a number of biologically active oxysterols (12, 15–19). In addition, we recently reported that the Δ7-double bond also makes 7-dehydrocholesterol an unusual substrate for P450 7A1, which directly converts 7-dehydrocholesterol to 7-ketocholesterol, with the 7,8-epoxide being a minor product (20). Elevated levels of these two oxysterol products have been observed in tissues and/or fluids of an animal model and patients affected with Smith-Lemli-Opitz syndrome.

Abbreviations: APCI, atmospheric pressure chemical ionization; HPβCD, 2-hydroxypropyl-β-cyclodextrin; LXR, liver X receptor; NOE, nuclear Overhauser effect; P450 (or CYP), cytochrome P450; SLOS, Smith-Lemli-Opitz syndrome.

1To whom correspondence should be addressed.

e-mail: libin.xu@vanderbilt.edu (L.X.); fguengerich@vanderbilt.edu (F.P.G.)

The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of eight figures.
SLOS and/or cerebrotendinous xanthomatosis (12, 21), providing support for such enzymatic transformation in vivo. Desmosterol, an alternate immediate precursor of cholesterol, contains a double bond at C24 and we hypothesized that, analogous to the reactions of 7-dehydrocholesterol and P450 7A1, desmosterol could be a substrate for P450 46A1, leading to 24,25-epoxycholesterol, 24-ketocholesterol, and/or some side-chain hydroxylated product.

The present work was designed to study the metabolic reactions of 7-dehydrocholesterol and desmosterol by cytochrome P450 46A1. In addition to cholesterol, both 7-dehydrocholesterol and desmosterol were found to be substrates of CYP46A1. Both hydroxylated and epoxidized products (but no ketone) were identified from desmosterol, and only hydroxylated products were identified from 7-dehydrocholesterol by LC-MS, NMR, and GC-MS studies (Fig. 1). The rates of formation of these products were compared with cholesterol oxidation to 24-hydroxycholesterol.

MATERIALS AND METHODS

**Materials**

7-Dehydrocholesterol, cholesterol, 2-hydroxypropyl-β-cyclo-dextrin (HPβCD), N,N-bis(trimethylsilyl)trifluoroacetamide, and dimethylformamide were purchased from Sigma-Aldrich. Desmosterol and a racemic mixture of 24S,25-epoxycholesterol and 24R,25-epoxycholesterol were purchased from Avanti Polar Lipids (Alabaster, AL). 24S,25-epoxycholesterol and L-α,β-dilauroyl-sn-glycero-3-phosphocholine were purchased from Enzo Life Sciences. 24S-hydroxycholesterol was purchased from Abcam Biochemicals (Cambridge, UK). All organic solvents (Fisher) were of HPLC grade.

**Codon optimization and choice of vector**

Codon optimization for P450 46A1 was done using on-line software available through Integrated DNA Technologies (Coralville, IA). A DNA containing an optimized coding sequence (supplementary Fig. I) and a His6 tag were synthesized by Genewiz (South Plainfield, NJ) and ligated into a pCW vector (Coralville, IA). A DNA containing an optimized coding sequence (supplementary Fig. I) and a His6 tag were synthesized by Genewiz (South Plainfield, NJ) and ligated into a pCW vector (Coralville, IA). A fresh overnight culture was prepared by adding 20 μl of a glycerol stock of *Escherichia coli* (DH5α) cells containing the plasmid for the P450 46A1 cDNA, plus a plasmid containing the gene for the *E. coli* molecular chaperone GroEL/ES, to 100 ml of Luria-Bertani medium containing ampicillin (100 μg/ml) and kanamycin (50 μg/ml). This starter culture was grown overnight at 37°C and 220 rpm. Large-scale expression of P450 46A1 was done with 6 l of Terrific broth medium supplemented with the same antibiotics (see above), trace elements (250 μl/1 of culture) (25), 1 mM NaCl, 1 mM thiamine, 2 ml of glycerol per liter of culture, and an overnight starter culture (at a dilution of 1:100, v/v). Cultures were incubated for at least 6 h with shaking at 37°C and 220 rpm, until the optical density at 600 nm reached 0.7. The expression of P450 46A1 was induced by the addition of 1.0 mM isopropyl β-D-1-thiogalactopyranoside, 1.0 mM 5-aminolevulinic acid, and 1.0 mM arabinose. Cultures were incubated for another 42 h at 27°C and 225 rpm in a Multifors incubator, followed by harvesting of cells by centrifugation at 3,500 g for 15 min (Sorvall RC 3B Plus centrifuge, H6000A/HBB6 rotor).

**Purification of human P450 46A1**

All operations were done at 4°C. The recovered cell pellets (from 6 l of culture) were resuspended in 650 ml of TES buffer [100 mM Tris-acetate (pH 7.5) containing 0.5 M sucrose and 0.5 mM EDTA] per liter of cell culture. Cells were mixed with lysozyme (2 mg/l of cell culture) and kept on ice for 60 min. Spheroplasts were obtained by centrifuging the cell suspension at 3,500 g for 15 min. The resulting spheroplasts were resuspended in 50 ml of sonication buffer [100 mM potassium phosphate (pH 7.5) containing 16% glycerol (v/v), 9 mM magnesium acetate, 100 μM dithiothreitol, 1.0 mM phenylmethylsulfonyl fluoride, and two protease inhibitor tablets (Roche) per liter of cell culture]. After sonication on ice, the material was centrifuged at 12,000 g for 15 min, and the recovered supernatant was further centrifuged at 1.4 × 10^7 g for 60 min. The pellet obtained after ultracentrifugation was collected, homogenized, and solubilized in 600 ml of solubilization buffer [100 mM potassium phosphate buffer (pH 7.5) containing 20% glycerol (v/v), 0.1 mM EDTA, 10 mM β-mercaptoethanol, 0.5 M KCl, and 1.0% sodium cholate (w/v)]. The resulting material was centrifuged at 1.4 × 10^7 g for 60 min, and the supernatant was loaded on a 1.5 × 8 cm Nitrilotriacetic acid column, which had been pre-equilibrated with 100 mM potassium phosphate buffer (pH 7.5) containing 20% glycerol (v/v), 10 mM β-mercaptoethanol, 0.5 M KCl, and 1.0% sodium cholate (w/v). The column was washed with the same buffer containing 20 mM imidazole, and P450 46A1 was eluted with the same buffer containing 200 mM imidazole. The eluted fractions containing P450 were dialyzed against 2 l of 100 mM potassium phosphate buffer (pH 7.5) containing 20% glycerol (v/v, 24 h, three buffer changes). The concentration of P450 was measured spectrally (26) with an OLIS/Amino DW2 spectrophotometer (On-Line Equipment Systems, Bogart, GA). A typical yield of P450 46A1 from 6 l of culture was 130 nmol after purification.

**Expression of NADPH-P450 reductase**

The rat enzyme was expressed in *E. coli* and purified as described elsewhere (27).

**LC-MS**

LC-MS was done using a Waters Acquity UPLC system connected to a Thermo LTQ mass spectrometer (see below for LC conditions). Analyte (10 μl, otherwise stated) was injected into the system with a partial loop in the needle overfill mode. An atmospheric pressure chemical ionization (APCI)’ source was used in all cases with the following parameters: discharge voltage, 4.1 kV; discharge current, 5.1 μA; vaporizer temperature, 450°C; sheath gas flow rate, 50 arbitrary units; auxiliary gas flow rate, 5 arbitrary units; sweep gas flow rate, 5 arbitrary units; capillary voltage, 18 V; capillary temperature, 275°C; and tube lens voltage, 80 V.

**GC-MS**

GC-MS analysis was performed using a gas chromatograph (Agilent 6890 GC) coupled with a mass selective detector (Agilent MSD 5973). An Agilent-J and W Scientific GC capillary column (DB-5MS; 25 m length, 0.2 mm inside diameter, 0.33 μm film thickness) was used. Helium was used as the carrier gas, maintained at a constant flow rate of 1.5 ml/min. The GC temperature program was as follows: initial temperature 70°C for 1 min; program from 70 to 130°C at a rate of 50°C/min, hold for 1 min at 130°C; program...
from 130 to 300°C at a rate of 15°C/min, hold at 300°C for 15 min. Analyte was injected in a splitless mode. MS conditions were as follows: electron impact mode at an ionization energy of 70 eV, transfer line and ion source temperature at 280 and 250°C, respectively. Mass spectra were recorded from m/z 50–700.

NMR
A 600 MHz Bruker NMR spectrometer was used to acquire NMR spectra. The NMR spectrometer was equipped with a 5-mm Z-gradient TCI cryo-probe and Topspin software was used to analyze the data. CDCl3 was used as a solvent. The peak at (δ) 7.26 ppm for CHCl3 was used as a reference in 1H NMR. 2D NMR was obtained with an optimized 90° pulse width.

Enzymatic assays
Enzymatic assays with 7-dehydrocholesterol and desmosterol were carried out in a 0.5 ml reaction volume containing 1.0 μM P450 46A1, 2.0 μM NADPH-P450 reductase, 100 mM potassium phosphate buffer (pH 7.5), 150 μM L-α,2,3-dilauroyl-sn-glycero-3-phosphocholine, and (unless indicated otherwise) a final substrate concentration of 15 μM. The stock solutions of substrates were prepared in 310 mM HPßCD [45% HPßCD (w/v)]. A 5 μl aliquot of stock solution of substrate was added to the 500 μl final reaction volume. After preincubation for 15 min at 37°C, enzymatic reactions were initiated by the addition of 150 μl of an NADPH-generating system (final concentrations: 20 mM glucose 6-phosphate, 1 mM NADP+, and 2 U/ml yeast glucose 6-phosphate dehydrogenase) (28) and incubated for 15 min at 37°C. The reaction was quenched with CH2Cl2 (2 ml), and the products were extracted twice with 2 ml of CH2Cl2 and centrifuged at 2,000 g for 10 min (23°C). The organic layers were combined and dried under a nitrogen stream. The dried sample was dissolved in CH2CN:CH3OH (80:20, v/v) and centrifuged as above to precipitate the suspended particles; the organic layer was collected carefully and subjected to purification. Purification was done with a Waters Acquity UPLC system using an ACQUITY UPLC BEH octadeclsilane (C18) column (1.7 μm, 2.1 mm × 100 mm) at 35°C with a flow rate of 0.6 ml/min and an injection volume of 15 μl. Because of the lack of chromophore in the analyte, LC-MS was used for detection, with an 8:2 (v/v) splitter into the mass spectrometer. Mobile phase A consisted of 95:5 (v/v) water and CH3CN with 0.1% HCO2H (v/v); mobile phase B consisted of 5:95 (v/v) water and CH3CN with 0.1% HCO2H (v/v). LC methods were as follows. For 7-dehydrocholesterol products: 0–8 min, 70% mobile phase B (v/v); 8–9 min, 70% mobile phase B to 100% mobile phase B (v/v); 9–20 min, 100% mobile phase B; 20–21 min, 100% mobile phase B to 70% mobile phase B (v/v); 21–30 min, 70% mobile phase B (v/v). For desmosterol products: 0–15 min, 80% mobile phase B (v/v); 15–16 min, 80% mobile phase B to 100% mobile phase B (v/v); 16–30 min, 100% mobile phase B; 30–31 min, 100% mobile phase B to 80% mobile phase B (v/v); 31–40 min, 80% mobile phase B (v/v).

Identification of reaction products
The various products obtained from 7-dehydrocholesterol, desmosterol, and cholesterol assays with P450 46A1 were confirmed either by comparison with authentic standards, by LC-MS and GC-MS (of TMS ethers), or by LC-MS and NMR. The stereochemistry of 24,25-epoxycholesterol was confirmed by comparison with standards of 24R,25- and 24S,25-epoxycholesterol separated on a Waters Alliance (2695) HPLC system that was coupled to a Thermo LTQ mass spectrometer using a chiral column [CHIRALPAK AD-H column; 5 μm, 4.6 mm × 250 mm; solvent condition: 5% CH3OH in hexanes (v/v); flow rate, 1.0 ml/min; temperature, 25°C]. The MS conditions were the same as described above.

Trimethylsilylation of enzymatic products for GC-MS analysis
The oxidative products of 7-dehydrocholesterol were converted into TMS ether derivatives for GC-MS analysis. The fragmentation patterns of TMS ether derivatives of the products were analyzed for structure elucidation. Derivatization was done using N,O-bis(trimethylsilyl)trifluoroacetamide-diethylformamide in a 1:1 ratio (v/v) at 25°C for 60 min, and reactions were directly subjected to GC-MS analysis.

Large-scale incubation of P450 46A1 with desmosterol for NMR
NMR was used to identify the structure of the other unknown oxidized product of desmosterol. In order to obtain a sufficient amount of the products for NMR analysis, a large-scale incubation was performed. P450 46A1 (50 nmol) was incubated with 100 μM desmosterol in 50 ml of 100 mM potassium phosphate buffer (pH 7.5) containing 50 nmol of NADPH-P450 reductase and 150 μM L-α,2,3-dilauroyl-sn-glycero-3-phosphocholine. After initial incubation for 30 min at 37°C, the enzymatic reaction was initiated by the addition of 15 ml of an NADPH-generating system (28) and incubated for 2 h at 37°C. The reaction was quenched with CH2Cl2 and product was extracted twice with 80 ml of CH2Cl2, with centrifugation at 2,000 g for 10 min. The organic layers were combined and dried under a nitrogen stream. The dried sample was dissolved in CH2CN:CH3OH (80:20, v/v) and centrifuged as above to precipitate the suspended particles; the organic layer was collected carefully and subjected to purification. Purification was done with a Waters Acquity UPLC system using an ACQUITY UPLC BEH octadeclsilane (C18) column (1.7 μm, 2.1 mm × 100 mm) at 35°C with a flow rate of 0.6 ml/min and an injection volume of 15 μl. Because of the lack of chromophore in the analyte, LC-MS was used for detection, with an 8:2 (v/v) splitter into the mass spectrometer. Mobile phase A consisted of 95:5 (v/v) water and CH3CN with 0.1% HCO2H (v/v); mobile phase B consisted of 5:95 (v/v) water and CH3CN with 0.1% HCO2H (v/v). The LC method was as follows: 0–4.0 min, 80% mobile phase B (v/v); 4.0–4.5 min, 80% mobile phase B (v/v) to 100% mobile phase B; 4.5–8.0 min, 100% mobile phase B; 8.0–8.5 min, 100% mobile phase B to 80% mobile phase B (v/v); 8.5–12.5 min, 80% mobile phase B (v/v). Purified fractions were combined, extracted into CH2Cl2, dried under nitrogen, and redissolved in CDCl3 for NMR analysis.

Kinetic assays
Kinetic assays with 7-dehydrocholesterol, desmosterol, and cholesterol were performed as described above but with substrate concentrations varying from 1 μM to 100 μM [substrates were dissolved in 310 mM HPßCD [45% HPßCD (w/v)], and 5 μl of stock substrate solution was added to a 500 μl final reaction volume]. The same LC method was used for cholesterol products as in the case of 7-dehydrocholesterol. A product at m/z 385 was detected in the cholesterol assay (24S-hydroxycholesterol). The kcat and Km values were calculated using the program Dynafit (29).

Binding assays
For binding studies, a series of stock solutions of varying concentrations (of each substrate) were prepared in 310 mM HPßCD [45% HPßCD (w/v)]. Binding of various substrates was done in a final volume of 0.33 ml containing 100 mM potassium phosphate (pH 7.5) and 2 μM P450 46A1, and substrate was varied from 1–100 μM (3.3 μl of stock solution of substrate was added). The same volume of cyclodextrin without substrate (3.3 μl) was added to a reference cuvette, and spectra were recorded from 350 to 500 nm. The ΔAmax-A285, ΔAmax-A195, and ΔAmax-A225 differences for 7-dehydrocholesterol, desmosterol, and cholesterol (respectively) were plotted against increasing concentrations of substrate and binding dissociation constants (Kd) were estimated by using the program Dynafit (29).
DNA sequence optimized for *E. coli* expression (supplementary Fig. I). The purified protein showed a band at 450 nm in the reduced-CO difference spectrum (Fig. 2). The Soret band was observed at 417 nm (data not shown), a characteristic feature of ferric low-spin P450. SDS-PAGE gel showed a single major band at 50 kDa, accounting for >94% of the protein as judged by densitometry of the Coomassie staining (Fig. 3).

Oxidation of 7-dehydrocholesterol, desmosterol, and cholesterol

Incubation of 7-dehydrocholesterol with P450 46A1 yielded two products, *t* 5.5 and 6.4 min (Fig. 4B). Incubation

**RESULTS**

**Protein expression and purification**

P450 46A1 was expressed in *E. coli* cells. We were unsuccessful in the expression of the native sequence and did not obtain good expression in this system until we used a DNA sequence optimized for *E. coli* expression (supplementary Fig. 1). The purified protein showed a band at 450 nm in the reduced-CO difference spectrum (Fig. 2). The Soret band was observed at 417 nm (data not shown), a characteristic feature of ferric low-spin P450. SDS-PAGE gel showed a single major band at 50 kDa, accounting for >94% of the protein as judged by densitometry of the Coomassie staining (Fig. 3).

Oxidation of 7-dehydrocholesterol, desmosterol, and cholesterol

Incubation of 7-dehydrocholesterol with P450 46A1 yielded two products, *t* 5.5 and 6.4 min (Fig. 4B). Incubation
P450 46A1 oxidation of 7-dehydrocholesterol and desmosterol

or desmosterol plus one oxygen atom). The product eluted at $t_R$ 6.4 min in the 7-dehydrocholesterol assay was identified as 24-hydroxy-7-dehydrocholesterol by comparison with an authentic standard (Fig. 4C). Further, the fragmentation pattern obtained by collision-induced dissociation for 24-hydroxy-7-dehydrocholesterol matched that of the standard (i.e., $MS^2$ at $m/z$ 383.3 and $MS^3$ at $m/z$ 365.3, respectively) (Fig. 6). The product 24-hydroxy-7-dehydrocholesterol was further confirmed by GC-MS (supplementary Fig. II). Similarly, the product at $t_R$ 6.4 min from the desmosterol assay was identified as 24,25-epoxycholesterol by comparison with a commercial standard (Fig. 5B, C). The stereochemistry of 24,25-epoxycholesterol was confirmed to be $24S$ by comparing it with standards available using a chiral column (supplementary Fig. III). The fragmentation pattern of the product matched with that of a 24S,25-epoxycholesterol standard (data not shown). The desmosterol product ($t_R$ 3.9 min) was not 24-ketocholesterol, as judged by comparison with an authentic standard. The reaction with cholesterol yielded only one product, 24-hydroxycholesterol, confirmed with commercially available standard (supplementary Fig. IV). The identification of the unknown products of 7-dehydrocholesterol and desmosterol is presented below.

**Characterization of a second product from 7-dehydrocholesterol**

In order to characterize the unknown peak eluting at $t_R$ 5.5 min, the collected product was converted to a TMS ether derivative and subjected to GC-MS analysis. GC-MS
The nuclear Overhauser effect (NOE) spectroscopy experiment (supplementary Fig. VD), there was a strong NOE between H-24 and a proton in the terminal methylene group, but no NOE between H-24 and the terminal methyl group, indicating the product is 27-hydroxydesmosterol (terminal methylene group is syn to H-24 to produce the NOE correlated spectroscopy signal).

**Kinetic analysis**

The calculated $k_{cat}$ and $K_M$ values for the formation of 24-hydroxy-7-dehydrocholesterol (from 7-dehydrocholesterol) were 24 ± 1 pmol·nmol$^{-1}$·min$^{-1}$ and 2.8 ± 0.02 μM, respectively (corrected for the binding to HPβCD) (Table 1, supplementary Fig. VI). Because a 25-hydroxy-7-dehydrocholesterol standard was not available, the rate for its formation was estimated relative to the rate for 24-hydroxy-7-dehydrocholesterol, assuming an equal MS response, to be 110 pmol·nmol$^{-1}$·min$^{-1}$. The $k_{cat}$ and $K_M$ values for 24S,25-epoxycholesterol (from desmosterol) were 33 ± 1 pmol·nmol$^{-1}$·min$^{-1}$ and 9.4 ± 1.0 μM, respectively (corrected for the binding to HPβCD) (Table 1, supplementary Fig. VI). Again, because a 27-hydroxydesmosterol standard was not available, the rate for this was estimated relative to the rate for 24S, 25-epoxycholesterol, assuming an equal MS response, to be 44 pmol·nmol$^{-1}$·min$^{-1}$.

**Binding analysis**

The calculated $K_d$ values for 7-dehydrocholesterol, desmosterol, and cholesterol were 1.4 ± 0.1 μM, 1.0 ± 0.4 μM,
machinery in them. Alternatively, the expression levels of added P450s are relatively low in such cells, and the conversion rate might have been too low for detection. A third possibility is that the oxidation products of 7-dehydrocholesterol might have been further metabolized by other enzymes in the HEK293 cells.

The binding of all substrates showed typical "type I" difference spectra (supplementary Fig. VII). The estimated dissociation constant (K_d) for 7-dehydrocholesterol was less than that for cholesterol, indicating stronger binding to HPβCD (supplementary Figs. VII, VIII).

and 2.2 ± 0.4 μM, respectively (corrected for the binding to HPβCD) (supplementary Figs. VII, VIII).

DISCUSSION

This study demonstrates that 7-dehydrocholesterol and desmosterol are substrates of P450 46A1, leading to 24-hydroxy- and 25-hydroxy-7-dehydrocholesterol and to 24S,25-epoxycholesterol and 27-hydroxydesmosterol as the products, respectively. The rate of formation (k_cat) of 24-hydroxy-7-dehydrocholesterol was about one-third that of 24S-hydroxycholesterol, but the formation of 25-hydroxy-7-dehydrocholesterol was a much more favored reaction (Table 1). The enzyme efficiency (k_cat/K_m) was similar for 7-dehydrocholesterol compared with cholesterol but 2–3 times higher than that of desmosterol. We note that although previous work by Björkhem et al. (13) did not find 7-dehydrocholesterol as a substrate of P450 46A1; their work was carried out in HEK293 cells, which presumably would metabolize 7-dehydrocholesterol to cholesterol efficiently with intact cholesterol biosynthesis machinery in them. Alternatively, the expression levels of added P450s are relatively low in such cells, and the conversion rate might have been too low for detection. A third possibility is that the oxidation products of 7-dehydrocholesterol might have been further metabolized by other enzymes in the HEK293 cells.

The binding of all substrates showed typical "type I" difference spectra (supplementary Fig. VII). The estimated dissociation constant (K_d) for 7-dehydrocholesterol was less than that for cholesterol, indicating stronger binding to HPβCD. The K_d for desmosterol was similar to that of 7-dehydrocholesterol, but it was found to be a less efficient substrate compared with 7-dehydrocholesterol.

Under normal physiological conditions, the cholesterol product 24S-hydroxycholesterol is the dominant oxysterol in the adult brain (32–35), modulating cholesterol homeostasis through activation of LXRα and LXRβ (5) and inhibition of the SREBP2 pathway by binding INSIG (5). A reduced level of 24S-hydroxycholesterol has been associated with aging and severe neurological diseases (36, 37). A decreased level of 24S-hydroxycholesterol has also been reported in plasma of SLOS patients (13), likely due to
observed in the urine of SLOS patients, suggesting that 7-dehydrocholesterol is also a substrate of sterol side-chain cleavage enzyme (P450scc, P450 11A1) (41, 42).

The formation of 24S,25-epoxycholesterol directly from desmosterol may have significant biological and physiological consequences. Until now, squalene 2,3;22,23-diepoxide was the only known biosynthetic precursor to 24S,25-epoxycholesterol (43), but our study reveals a novel pathway for the formation of this biologically important oxysterol. 24S,25-epoxycholesterol was found to be the most potent and abundant oxysterol ligand of LXRs in developing mouse brain (significantly higher than the level of 24S-hydroxycholesterol), and it promotes neurogenesis and neuronal survival in an LXR-dependent fashion (44, 45).

Notably, the levels of desmosterol are also high in developing brains (up to 30% of total brain sterols) (46–48) and deficiency in cholesterol. We have previously identified 24-hydroxy-7-dehydrocholesterol in the brain tissues of SLOS rodent models (12, 38). However, to what extent the 24-hydroxy-7-dehydrocholesterol could compensate for the lack of 24S-hydroxycholesterol in terms of its biological functions remains to be elucidated.

It is not entirely unexpected to identify 7-dehydrocholesterol as a substrate of P450. In addition to our findings on this sterol being a substrate of P450 7A1 (20) and P450 46A1 (current study), it has also been reported that P450 27A1 catalyzes the metabolism of 7-dehydrocholesterol to 25-hydroxy-7-dehydrocholesterol and 26/27-hydroxy-7-dehydrocholesterol (13, 39, 40). Interestingly, both oxysterols were found to be activators of LXRs, slightly less potent than their cholesterol analogs (39, 40). Furthermore, 7-dehydrocholesterol-derived steroids have been observed in the urine of SLOS patients, suggesting that 7-dehydrocholesterol is also a substrate of sterol side-chain cleavage enzyme (P450scc, P450 11A1) (41, 42).

The formation of 24S,25-epoxycholesterol directly from desmosterol may have significant biological and physiological consequences. Until now, squalene 2,3;22,23-diepoxide was the only known biosynthetic precursor to 24S,25-epoxycholesterol (43), but our study reveals a novel pathway for the formation of this biologically important oxysterol. 24S,25-epoxycholesterol was found to be the most potent and abundant oxysterol ligand of LXRs in developing mouse brain (significantly higher than the level of 24S-hydroxycholesterol), and it promotes neurogenesis and neuronal survival in an LXR-dependent fashion (44, 45). Notably, the levels of desmosterol are also high in developing brains (up to 30% of total brain sterols) (46–48) and

![GC-MS fragmentation pattern of TMS ether product of unknown peak (tR 5.5 min) in LC-MS profile of 7-dehydrocholesterol assay (Fig. 4B). The fragment at m/z 413 confirmed the product to be 25-hydroxy-7-dehydrocholesterol.](image)

**Table 1.** Kinetic parameters measured for 7-dehydrocholesterol, desmosterol, and cholesterol

| Substrate          | Product                        | kcat (pmol-nmol⁻¹-min⁻¹) | Km (µM)  | kcat/Km (mM⁻¹-min⁻¹) |
|--------------------|-------------------------------|--------------------------|-----------|----------------------|
| 7-Dehydrocholesterol | 24-hydroxy-7-dehydrocholesterol | 24 ± 1                   | 2.8 ± 0.02 | 8.5 ± 0.4            |
|                    | 25-hydroxy-7-dehydrocholesterol | ~110                     | —         | —                    |
| Desmosterol        | 24S,25-epoxycholesterol       | 33 ± 1                   | 9.4 ± 1   | 3.5 ± 0.4            |
|                    | 27-hydroxydesmosterol         | ~44                      | —         | —                    |
| Cholesterol        | 24-hydroxycholesterol         | 59 ± 6                   | 7 ± 1     | 8.4 ± 1.5            |
low in the adult brain (~0.1% of cholesterol) (49). Thus, our finding suggests a possible link between desmosterol and the production of 24S,25-epoxycholesterol during brain development. Interestingly, Griffiths and coworkers recently reported that the level of 24S,25-epoxycholesterol was greatly decreased in the brain of Cyp46a1-knockout mice (one-fifth of the level in wild-type mice), providing support for the participation of P450 46A1 in the production of this oxysterol in vivo (50). It is of additional interest that 24S,25-epoxycholesterol was found to be an inhibitor of 3β-hydroxysterol-Δ24-reductase (DHCR24), leading to the accumulation of desmosterol in a number of cell lines (51).

The substrate selectivity of P450 46A1 is not as limited as once thought. Cholesterol 24S-hydroxylation has been the main reaction ascribed to P450 46A1. However, several other sterols are also substrates, including 24S-hydroxycholesterol (25- and 27-hydroxylations), 7α-hydroxycholesterol, cholestanol, progesterone, and testosterone (1). Some drugs are also substrates (1). Here we have shown that recombinant human P450 46A1 catalyzes the 24- and 25-hydroxylation of 7-dehydrocholesterol and the epoxidation and hydroxylation of desmosterol. Interestingly, P450 46A1 binds and oxidizes a number of drugs (1, 52). This is an unusual phenomenon in that most of the P450s that appear to be specialized for oxidation of endogenous substrates do not use xenobiotic chemicals as substrates. The overall in vivo contribution of P450 46A1 to the metabolism of these drugs, even in brain, is unknown. Furthermore, P450 46A1 activity (toward cholesterol) is stimulated by binding to some drugs (e.g., efavirenz, acetaminophen, mirtazapine, galantamine), and the in vivo relevance of this effect has been shown in a mouse model (53). We have not examined the effects of any of these drugs on the new reactions we characterized here.

In conclusion, we have identified 7-dehydrocholesterol and desmosterol as substrates of P450 46A1. The oxysterols derived from these enzymatic pathways could have significant biological consequences in related physiological or pathological conditions where the levels of these two cholesterol precursors are high, such as in SLOS (7-dehydrocholesterol) (7, 8), desmosterolosis (desmosterol) (7, 54), and developing brain (desmosterol) (46–48). The direct conversion of desmosterol to 24S,25-epoxycholesterol by P450 46A1 represents a distinctly new pathway for the formation of this potent oxysterol ligand of LXRs (4, 44, 45).

The authors thank Dr. Steven J. Fliesler, Departments of Ophthalmology (Ross Eye Institute) and Biochemistry, University at Buffalo-State University of New York and State University of New York Eye Institute, Buffalo, NY, for providing brain samples from AYA9944-treated rats for the isolation of 24-hydroxy-7-dehydrocholesterol. The authors also thank Kathy Trisler for assistance in preparation of the manuscript.

REFERENCES

1. Mast, N., R. Norcross, U. Andersson, M. Shou, K. Nakayama, I. Björkhem, and I. A. Pikuleva. 2003. Broad substrate specificity of human cytochrome P450 46A1 which initiates cholesterol degradation in the brain. Biochemistry. 42:14284–14292.
2. Pikuleva, I. A. 2006. Cholesterol-metabolizing cytochromes P450. Drug Metab. Dispos. 34:513–520.
3. Janowski, B. A. P. J. Willy, T. R. Devi, J. R. Falek, and D. J. Mangeldsdorf. 1996. An oxysterol signalling pathway mediated by the nuclear receptor LXR alpha. *Nature*. **383**:729–731.

4. Janowski, B. A. M. J. Grogan, S. A. Jones, G. B. Wisely, S. A. Kliewer, E. J. Corey, and D. J. Mangeldsdorf. 1999. Structural requirements of ligands for the oxysterol liver X receptors LXalpaha and LXbeta. *Proc. Natl. Acad. Sci. USA*. **96**:266–271.

5. Radhakrishnan, A. Y. Ikeda, H. J. Kwon, M. S. Brown, and J. L. Goldstein. 2007. Sterol-regulated transport of SREBP3s from endoplasmic reticulum to Golgi: oxysterols block transport by binding to Insig. *Proc. Natl. Acad. Sci. USA*. **104**:6511–6516.

6. Holick, M. F., J. E. Frommer, S. C. McNeill, N. M. Richtand, J. W. Henley, and J. T. Potts, Jr. 1977. Photometabolism of 7-dehydrocholesterol to previtamin D3 in skin. *Biochem. Biophys. Res. Commun.* **76**:107–114.

7. Porter, F. D., and G. E. Herman. 2011. Malformation syndromes caused by disorders of cholesterol synthesis. *J. Lipid Res.* **52**:6–34.

8. Kelley, R. L., and R. C. Hennekam. 2000. The Smith-Lemli-Opitz syndrome. *J. Med. Genet.* **37**:321–335.

9. de Sain-van der Velden, M. G., A. Verrips, B. H. Prinsen, M. de Beeck, M. G. Jonsson, K. Tallman, H. Schirmer, L. Bomme Ousager, P. J. Crick, and F. W. Dahlquist. 1989. Expression and nitrogen-15 labeling of proteins for proton and nitrogen-15 nuclear magnetic resonance. *Methods Enzymol.* **177**:44–73.

10. Sigal, E., and M. R. Waterman. 1991. Expression and enzymatic activity of recombinant cytochrome P450 17a-hydroxylase in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA*. **88**:5597–5601.

11. Barnes, H. J. 1996. Maximizing expression of eukaryotic cytochrome P450s in *Escherichia coli*. *Methods Enzymol.* **272**:5–14.

12. Sandhu, T., B. B. and F. P. Guengerich. 1993. Expression of modified cytochrome P450 2C10 (2C9) in *Escherichia coli*, purification, and reconstitution of catalytic activity. *Arch. Biochem. Biophys.* **306**:443–450.

13. Omura, T., and R. Sato. 1964. The carbon monoxide-binding pigment of liver microsomes. I. evidence for its hemoprotein nature. *J. Biol. Chem.* **239**:2570–2578.

14. Hanna, I. H., J. F. Feiber, K. L. Kokones, and P. F. Hohenberg. 1998. Role of the alanine at position 363 of cytochrome P450 2B2 in influencing the NAPDH- and hydroperoxide-supported activities. *Arch. Biochem. Biophys*. **350**:324–332.

15. Guengerich, F. P., and C. J. Bartleson. 2007. Analysis and characterization of enzymes and nucleic acids. In Principles and Methods of Toxicology. A. W. Hayes, editor. CRC Press, Boca Raton, FL. 1981–2048.

16. Kuzmic, P. 1996. Program DYNAFIT for the analysis of enzyme kinetic data: application to HIV protease. *Anal. Biochem*. **237**:260–273.

17. Shinkyo, R., and F. P. Guengerich. 2011. Cytochrome P450 7A1 cholesterol 7a-hydroxylation: individual reaction steps in the catalytic cycle and rate-limiting ferric iron reduction. *J. Biol. Chem*. **286**:4643–4653.

18. Mast, N., and I. A. Pikuleva. 2005. A simple and rapid method to measure cholesterol binding to P450s and other proteins. *J. Lipid Res*. **46**:1561–1568.

19. Björkhem, I., D. Lutjohann, U. Diczfalusy, L. Stahle, G. Ahlberg, and J. Wahren. 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.

20. McDonald, J. G., B. M. Thompson, E. C. McCrum, and D. W. Russell. 2007. Extraction and analysis of sterols in biological matrices by high performance liquid chromatography electrospray ionization mass spectrometry. *Methods Enzymol.* **432**:145–170.

21. Karu, K., M. Hornshaw, G. Wolfendin, K. Bodin, M. Hamberg, G. Alvelius, J. Sjovall, J. Turton, Y. Wang, and W. J. Griffiths. 2007. Liquid chromatography-mass spectrometry utilizing multi-stage fragmentation for the identification of oxysterols. *J. Lipid Res*. **48**:976–987.

22. Griffiths, W. J., and Y. Wang. 2009. Analysis of neurosteroids by GC-MS and LC-MS/MS. *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci*. **877**:2778–2805.

23. Lütjohann, D., O. Breuer, G. Ahlberg, I. Nennenso, A. Sidén, U. Diczfalusy, and I. Björkhem. 1996. Cholesterol homeostasis in human brain: evidence for an age-dependent flux of 24S-hydroxycholesterol from the brain into the circulation. *Proc. Natl. Acad. Sci. USA*. **93**:9780–9784.

24. Bretilion, L., A. Sidén, L. O. Wahlund, D. Lütjohann, L. Minthon, M. Crisby, J. Hillert, C. G. Groth, U. Diczfalusy, and I. Björkhem. 2000. Plasma levels of 24S-hydroxycholesterol in patients with neurological diseases. *Neurosci. Lett*. **293**:87–90.

25. Korade, Z., L. Xu, K. Mirnics, and N. A. Porter. 2013. Lipid biomarkers of oxidative stress in a genetic mouse model of Smith-Lemli-Opitz syndrome. *J. Inherit. Metab. Dis.* **36**:113–122.

26. Endo-Umeda, K., K. Yasuda, K. Sugita, A. Honda, M. Ohta, M. Ishikawa, Y. Hashimoto, T. Sakaki, and M. Makishima. 2014. 7-dehydrocholesterol metabolites produced by sterol 27-hydroxylase (CYP27A1) modulate liver X receptor activity. *J. Steroid Biochem. Mol. Biol.* **140**:7–16.

27. Wassif, C. A., J. J. Cui, F. D. Porter, and N. B. Javitt. 2003. 27-Hydroxylation of 7- and 8-dehydrocholesterol in Smith-Lemli-Opitz syndrome: a novel metabolic pathway. *Stereoids*. **68**:497–502.

28. Shackleton, C., E. Roitman, L. W. Guo, W. K. Wilson, and F. D. Porter. 2002. Identification of 7(8) and 8(9) unsaturated adrenal steroid metabolites produced by patients with 7-dehydrosterol-delta7-reductase deficiency (Smith-Lemli-Opitz syndrome). *J. Steroid Biochem. Mol. Biol.* **82**:225–229.

29. Marcos, J., L. W. Guo, W. K. Wilson, F. D. Porter, and C. Shackleton. 2004. The implications of 7-dehydrosterol-7-reductase deficiency (Smith-Lemli-Opitz syndrome) to neurosteroid production. *Steroids*. **69**:51–60.

30. Nelson, J. A., S. R. Steckbeck, and T. A. Spencer. 1981. Biosynthesis of 24,25-epoxycholesterol from squalene 2,3,22,23-dioxy. *J. Biol. Chem*. **256**:1067–1068.
44. Meljon, A., S. Theofilopoulos, C. H. Shackleton, G. L. Watson, N. B. Javitt, H. J. Knolker, R. Saini, E. Arenas, Y. Wang, and W. J. Griffiths. 2012. Analysis of bioactive oxysterols in newborn mouse brain by LC/MS. *J. Lipid Res.* **53**:2469–2483.

45. Theofilopoulos, S., Y. Wang, S. S. Kitambi, P. Sacchetti, K. M. Sousa, K. Bodin, J. Kirk, C. Salto, M. Gustafsson, E. M. Toledo, et al. 2013. Brain endogenous liver X receptor ligands selectively promote midbrain neurogenesis. *Nat. Chem. Biol.* **9**:126–133.

46. Dennick, R. G., P. D. Dean, and D. A. Abramovich. 1973. Desmosterol levels in human foetal brain—a reassessment. *J. Neurochem.* **20**:1293–1294.

47. Fumagalli, R., and R. Paoletti. 1963. The identification and significance of desmosterol in the developing human and animal brain. *Life Sci.* **5**:291–295.

48. Quan, G., C. Xie, J. M. Dietschy, and S. D. Turley. 2003. Ontogenesis and regulation of cholesterol metabolism in the central nervous system of the mouse. *Brain Res. Dev. Brain Res.* **146**:87–98.

49. Lütjohann, D., A. Brzezinka, E. Barth, D. Abramowski, M. Staufenbiel, K. von Bergmann, K. Beyreuther, G. Multhaup, and T. A. Bayer. 2002. Profile of cholesterol-related sterols in aged amyloid precursor protein transgenic mouse brain. *J. Lipid Res.* **43**:1078–1085.

50. Meljon, A., Y. Wang, and W. J. Griffiths. 2014. Oxysterols in the brain of the cholesterol 24-hydroxylase knockout mouse. *Biochem. Biophys. Res. Commun.* **446**:768–774.

51. Zerenuruk, E. J., I. Kristiana, S. Gill, and A. J. Brown. 2012. The endogenous regulator 24(S),25-epoxycholesterol inhibits cholesterol synthesis at DHCR24 (Seladin-1). *Biochim. Biophys. Acta.* **1821**:1269–1277.

52. Mast, N., C. Charvet, I. A. Pikuleva, and C. D. Stout. 2010. Structural basis of drug binding to CYP46A1, an enzyme that controls cholesterol turnover in the brain. *J. Biol. Chem.* **285**:31783–31795.

53. Mast, N., Y. Li, M. Linger, M. Clark, J. Wiseman, and I. A. Pikuleva. 2014. Pharmacologic stimulation of cytochrome P450 46A1 and cerebral cholesterol turnover in mice. *J. Biol. Chem.* **289**:3529–3538.

54. Clayton, P., K. Mills, J. Keeling, and D. FitzPatrick. 1996. Desmosterolosis: a new inborn error of cholesterol biosynthesis. *Lancet.* **348**:404.