Broad Substrate Specificity of Human Cytochrome P450 46A1 Which Initiates Cholesterol Degradation in the Brain†

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Received August 22, 2003; Revised Manuscript Received October 7, 2003

ABSTRACT: The known activity of cytochrome P450 46A1 (P450 46A1) is 24(*S*)-hydroxylation of cholesterol. This reaction produces biologically active oxysterol, 24(*S*)-hydroxycholesterol, and is also the first step in enzymatic degradation of cholesterol in the brain. We report here that P450 46A1 can further metabolize 24(*S*)-hydroxycholesterol, giving 24,25- and 24,27-dihydroxycholesterols in both the cell cultures transfected with P450 46A1 cDNA and the in vitro reconstituted system with recombinant enzyme. In addition, P450 46A1 was able to carry out side chain hydroxylations of two endogenous C27-steroids with and without a double bond between C5−C6 (7α-hydroxycholesterol and cholestanol, respectively) and introduce a hydroxyl group on the steroid nucleus of the C21-steroid hormones with the C4−C5 double bond (progesterone and testosterone). Also, P450 46A1 was found to metabolize xenobiotics carrying out dextromethorphan O- and N-demethylations, diclofenac 4′-hydroxylation, and phenacetin O-deethylation. Thus, substrate specificities of P450 46A1 are not limited to cholesterol and include a number of structurally diverse compounds. Activities of P450 46A1 suggest that, in addition to the involvement in cholesterol homeostasis in the brain, this enzyme may participate in metabolism of neurosteroids and drugs that can cross the blood−brain barrier and are targeted to the central nervous system.

The central nervous system (CNS)[1] accounts for only 2% of the entire body mass but contains almost one-fourth of the unesterified cholesterol present in the whole individual (*1*). For many years, the study of brain cholesterol metabolism had been relatively ignored compared with the study of peripheral cholesterol metabolism because the brain does not appear to contribute significantly to the whole-body cholesterol balance (*1*). However, the recent findings that cholesterol plays an essential role in synaptic plasticity and that disturbances in brain cholesterol metabolism lead to neuronal degeneration and severe neurological disorders such as Alzheimer's disease (AD), Smith−Lemli−Opitz syndrome, and Niemann−Pick type C disease have provided a strong impetus for further studies of CNS cholesterol metabolism (*2*−*9*). Cholesterol within the brain is derived almost entirely through in situ synthesis because the blood−brain barrier impedes cholesterol exchange with circulating lipoproteins (*10*). Under normal conditions, removal of brain cholesterol in humans appears to be accomplished mainly through the enzymatic conversion to 24(*S*)-hydroxycholesterol, catalyzed by cytochrome P450 46A1 (*11*−*13*). In contrast to cholesterol, 24(*S*)-hydroxycholesterol can enter the circulation through the blood−brain barrier and be delivered to the liver for further degradation. P450 46A1-dependent cholesterol 24-hydroxylation also constitutes a major pathway for cholesterol elimination from the brain in rodents, as demonstrated by the studies of the normal and knockout animals with an introduced mutation that abolishes expression of P450 46A1 (*14*, *15*). These studies, however, suggested that rodents have an additional unknown mechanism that contributes to the CNS cholesterol turnover (*14*, *15*).

Three genetic studies investigated whether there is an association between intronic polymorphisms in the P450 *46A1* gene (*CYP46A1*) and AD (*16*−*18*). Two of these studies established such an association (*17*, *18*), whereas the third one did not (*16*). Although the relationship between the *CYP46A1* polymorphisms and AD is unclear, experimental evidence indicates that elevated levels of cholesterol in the brain increase the production of the amyloid-β protein, a small peptide, which currently is believed to play a causal role in the development of AD (*4*−*6*). Accumulation of the amyloid-β protein causes neurodegeneration with subsequent destruction of cholesterol-rich cell membranes, release of cholesterol within the CNS, and conversion to 24(*S*)-

† This work was supported by U.S. Public Health Service Grant GM62882 (to I.A.P.), Center Grant ES06676, and Howard Hughes Medical Institute Grant 53000266 and by grants from the Swedish Medical Research Council and the Swedish Heart-Lung Foundation (to I.B.).

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[1] Abbreviations: CNS, central nervous system; P450, cytochrome P450; AD, Alzheimer's disease; LXR, liver X receptor; PCR, polymerase chain reaction; KP_i, potassium phosphate buffer; HPLC, high-pressure liquid chromatography; GC-MS, combined gas chromatography−mass spectrometry; TLC, thin-layer chromatography; TMS, trimethylsilyl.

10.1021/bi035512f CCC: $25.00
Published on Web 11/11/2003

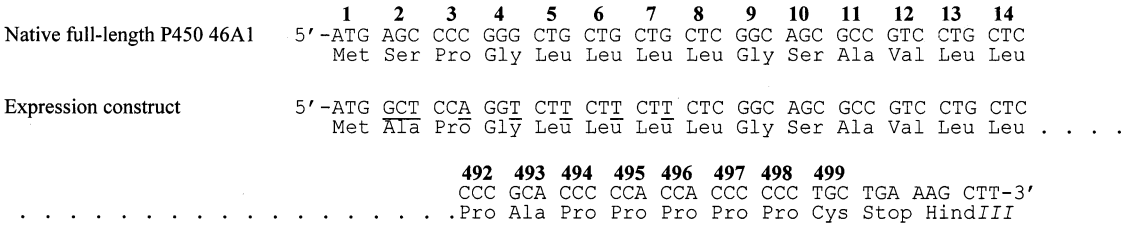

FIGURE 1: Nucleotide and amino acid sequences at the 5′- and 3′-ends of the expression construct for full-length P450 46A1.

hydroxycholesterol by P450 46A1 (*19*). Levels of 24(*S*)-hydroxycholesterol were found to be elevated in cerebrospinal fluid of patients with early onset Alzheimer's disease, whereas the plasma levels were reduced at an advanced stage of the disease, most probably due to loss of neuronal cells and P450 46A1 (*20*). 24(*S*)-Hydroxycholesterol was shown to be neurotoxic toward different cell types, including nerve cells (*21*), and the possibility has been discussed that increased levels of this oxysterol may be an additional risk factor in neurodegenerative disorders. 24(*S*)-Hydroxycholesterol is also a ligand for the liver X receptor (LXR) that activates the transcription of many genes involved in lipid metabolism (*22*), and absence of 24(*S*)-hydroxycholesterol or reduction of its levels as a result of *CYP46* polymorphisms may cause a corresponding reduction in the LXR activity (*13*).

P450 46A1 is a recently cloned enzyme (*13*), and it has not yet been characterized at a biochemical level. Here we describe previously unreported catalytic activities of P450 46A1 that provide further insight into metabolism of cholesterol in the brain and indicate that this enzyme may play a role in the metabolism of steroid hormones and drugs as well.

EXPERIMENTAL PROCEDURES

Materials. Unlabeled and tritium-labeled 24(*S*)-hydroxycholesterols were either purchased from Steraloids (Newport, RI) and American Radiolabeled Chemicals (St. Louis, MO), respectively, or prepared as described previously (*23*). [26,26,26,27,27,27-$^2H_6$]Cholesterol was from Medical Isotopes (Concord, NH), and [1,2-^{3}H]cholesterol was from PerkinElmer Life and Analytical Sciences (Boston, MA). Diclofenac, cortisone, flufenamic acid, dextromethorphan, dextrorphan, propranolol, 4′-hydroxybutyranilide, testosterone, and NADPH were purchased from Sigma (St. Louis, MO). Bufuralol, 1′-hydroxybufuralol, 4-hydroxydiclofenac, and 3-methoxymorphinan were obtained from Ultrafine Chemicals (Manchester, U.K.). 6β-, 16α-, 16β-, 2α/2β-, and 15α-hydroxytestosterone, 17α- and 6β-hydroxyprogesterone, progesterone, cortisol, and 6β-hydroxycortisol were from Steraloids Inc. (Wilton, NH). Methoxyresorufin and resorufin were purchased from Gentest (Woburn, MA).

Incubations with HEK293 Cells Transfected with CYP46A1. Transfection of human embryonic kidney 293 cells (ATCC CRL 1573) with cDNA for P450 46A1 (obtained from Dr. D. Russell, University of Texas Southwestern Medical Center) in pcDNA 3.1 vector and treatment with 20 mg/mL 2-hydroxypropyl-β-cyclodextrin (Sigma, St. Louis, MO) to reduce the cholesterol content of the membranes were performed as described (*13*, *23*). A part of endogenous cholesterol was removed to increase the percent of metabolism of the exogenous, labeled or unlabeled, steroid compounds that CYP46A1-transfected HEK293 cells were then incubated with. Charcoal-stripped, delipidized calf serum was used for incubations of approximately 5×10^6 cholesterol-depleted cells with different steroid substrates for 48 h at 37 °C in an atmosphere of 5% CO_2. The compounds used were trace amounts of [^{3}H]cholesterol, to monitor separation of the products by thin-layer chromatography (TLC); 20 μg of 24(*S*)-hydroxycholesterol, to confirm that this oxysterol is the substrate for P450 46A1; and 50 μg each of [^{2}H]-cholesterol, 7α-hydroxycholesterol, cholestanol, cholesteryl sulfate, and sitosterol, to test whether the latter three steroids are metabolized by P450 46A1 and, if so, to compare the rates of product formation. It was established in a separate experiment that the product pattern and the rates of hydroxylation were very similar in the incubations with unlabeled and deuterated cholesterol (not shown). The cells, the medium, or combined cells and medium were then extracted according to the Folch procedure as described (*23*).

Analysis of Steroid Extracts by TLC and Radioscanning. This was carried out using toluene/ethyl acetate (3:7 v/v) as a moving phase followed by scanning of the plates with a Berthold Tracemaster 20 TLC scanner. In some cases radioactive spots were scraped from the plates and eluted with methanol for identification by combined gas chromatography−mass spectrometry (GC-MS).

Analysis by GC-MS. Lipid extracts were converted into trimethylsilyl (TMS) ethers and analyzed by GC-MS using a Hewlett-Packard 5973 quadropole instrument (Palo Alto, CA) equipped with a Hewlett-Packard 19891A-102 column (25 m $\times$ 200 μm) (*23*).

Expression of P450 46A1 in Escherichia coli. The cDNA for full-length human P450 46A1 in pUC18 vector was used as a polymerase chain reaction template (PCR). The 5′-primer was designed to introduce a *Nde*I restriction site that contains the initiator methionine codon, GCT as the second codon, and silent mutations within codons 3−7 to increase the AT richness of the region and replace codons unfavorable for *E. coli*: 5′-GGGGGGCATATGGCTCCAGGTCTTCTTCT-TCTCGGCAGCGCC-3′ (changed nucleotides are underlined) (Figure 1). The antisense 3′-primer encoded a *Hin*dIII restriction site, followed by the termination codon TGA and a sequence complementary to the last five P450 46A1 codons: 5′-CCCCCCAAGCTTTCAGCAGGGGGGTGG-TGG-3′. The PCR product was digested with *Nde*I and *Hin*dIII and subcloned into the pCW expression vector digested previously with the same restriction enzymes. The correct sequence of the PCR-amplified fragment and the junction regions has been confirmed by DNA sequencing. The expression construct was transformed into *E. coli* DH5α F′IQ competent cells (Gibco BRL, Grand Island, NY), and expression conditions were optimized with respect to time (48 h), temperature (26 °C), aeration (200 rpm), and

concentrations of isopropyl 1-thio-β-D-galactopyranoside (1 mM), δ-aminolevulinic acid (0.5 mM), and chloramphenicol (1 μg/mL), as described for P450s 11A1, 27A1, and 7A1 (*24*−*26*).

Partial Purification of Recombinant P450 46A1. Harvested *E. coli* cells were suspended in 10% of the original culture volume in 100 mM potassium phosphate buffer (KP_i), pH 7.2, containing 20% glycerol, and incubated with lysozyme (0.2 mg/mL) for 30 min on ice. After the addition of 1% sodium cholate, 0.5 mM phenylmethanesulfonyl fluoride, 0.5 μg/mL leupeptin, 2 μg/mL aprotinin, 1 μg/mL pepstatin, and 0.5 mg/mL DNase, the cell suspension was sonicated on ice using six 20 s pulses at 1 min intervals and subjected to high-speed centrifugation at 106000*g* for 60 min. After ultracentrifugation, KCl was added to a final concentration of 1 M to the supernatant, and the solution was applied to an octyl-Sepharose column equilibrated with 100 mM KP_i (pH 7.2), 20% glycerol, 0.3% sodium cholate, and 1 mM EDTA. The column was washed with 5 column volumes of the equilibrating buffer, and P450 46A1 was eluted with the same buffer but containing 0.2% polyoxyethylene 10 lauryl ether. The fractions displaying a 450 nm CO-difference spectrum were pooled, diluted twice with 20% glycerol, and applied to a hydroxylapatite (HAP) column equilibrated with 20 mM KP_i (pH 7.2), 20% glycerol, 0.2% sodium cholate, and 1 mM EDTA. The column was washed with 10 column volumes of the equilibrating buffer, and P450 46A1 was eluted with 400 mM KP_i (pH 7.2), 20% glycerol, 0.2% sodium cholate, and 1 mM EDTA. After dialysis against 40 mM KP_i (pH 7.2) containing 20% glycerol, 0.2% sodium cholate, and 1 mM EDTA, P450 46A1 was aliquoted and frozen at −70 °C.

Incubations with Recombinant P450 46A1. Nine compounds at the following concentrations were tested to determine whether they are substrates for P450 46A1: 30 μM 24(*S*)-hydroxycholesterol, 30 μM cortisol, 0.5 μM progesterone, 50 μM phenacetin, 30 μM bufuralol, 4 μM dextromethorphan, 100 μM diclofenac, 2 μM methoxyresorufin, and 100 μM testosterone. The reconstituted system contained 0.5 μM partially purified P450 46A1, 10 μM NADPH−cytochrome P450 reductase, substrate, 0.2% sodium cholate, 40 mM potassium phosphate, pH 7.2, and 1 mM NADPH in a total volume of 1 mL. The reaction time was 30 min. The same assay conditions were used to determine the turnover number for cholesterol 24-hydroxylation. The concentration of cholesterol was 30 μM cholesterol. When 24(*S*)-hydroxycholesterol was used as a substrate, smaller amounts of P450 46A1 (0.1 μM) and NADPH−cytochrome P450 reductase (2 μM) were used in the assay, and the reaction time was decreased to 10 min. The enzymatic assays were carried out at 37 °C and terminated by addition of 2−3 mL of either CH_2Cl_2, CH_3OH, or CH_3CN. The CH_2Cl_2 extracts from the incubations with cholesterol or 24(*S*)-hydroxycholesterol were then evaporated, dissolved in methanol, and analyzed by HPLC, as described previously for the cholesterol 27-hydroxylase assay (*25*). The apparent K_m and k_{cat} values for hydroxylation of 24(*S*)-hydroxycholesterol were determined as described previously for P450 27A1 (*27*) using substrate concentrations ranging from 2 to 50 μM. The product formation was linear with time and enzyme concentration and did not exceed 15% of total metabolism. Kinetic data were analyzed with the Graph-Pad Prism 3.0 software (San Diego, CA) using the Michaelis−Menten equation. Incubations with the other eight probe substrates were extracted with acetonitrile or methanol containing the respective internal standards and subjected to centrifugation to precipitate protein. The supernatants obtained were used for liquid chromatography/mass spectrometry/mass spectrometry for metabolite identification and quantitation, as described previously (*28*).

Spectral Binding Assay. The assay was performed in 1 mL of 40 mM potassium phosphate, pH 7.2, containing 0.2% sodium cholate. The concentration of P450 varied, depending on the substrate used, and was 0.5 and 0.1 μM for cholesterol and 24(*S*)-hydroxycholesterol, respectively. Steroids were added from a stock solution in 45% aqueous 2-hydroxypropyl-β-cyclodextrin. After each experiment, the P450 content was quantified by the reduced CO-difference spectrum (*29*) to confirm that there was no enzyme denaturation during titration. Apparent binding constants (K_d) of P450 46A1 for cholesterol and 24(*S*)-hydroxycholesterol were determined from a plot of a spectral change, ΔA, versus substrate concentration added, [S]. Two equations were used to analyze the data by Graph-Pad Prism software: $\Delta A = (\Delta A_{max}[S])/(K_d + [S])$ and $\Delta A = 0.5\Delta A_{max}(K_d + [E] + [S] - ((K_d + [E] + [S])^2 - 4[E][S])^{1/2})$, where ΔA_{max} is the maximal amplitude of the spectral response and [E] is the enzyme concentration. The former is used when K_d is higher than the enzyme concentration, whereas the latter, the quadratic, is applied when K_d is lower than the enzyme concentration assuming 1:1 stoichiometry. The values of apparent binding constants appeared to be essentially the same when calculated by using two different equations.

RESULTS

Metabolism of Cholesterol by HEK293 Cells Transfected with CYP46A1. CYP46A1-expressing HEK293 cells were found to metabolize a part of endogenous cholesterol remaining after treatment with 2-hydroxypropyl-β-cyclodextrin as indicated by gas chromatographic analysis (Figure 2A). Cholesterol metabolites were separated as two peaks. The first peak was identified as 24(*S*)-hydroxycholesterol by mass spectrometry. The second peak corresponded to a metabolite(s) of 24-hydroxycholesterol with an additional hydroxyl group in the side chain. Because the second peak was suspected to contain more than one metabolite, CYP46A1-expressing HEK293 cells were incubated with trace amounts of [^{3}H]cholesterol, and the lipid extract from this incubation was separated by TLC (Figure 2B) followed by the GC-MS analysis of radioactive spots. The mobilities of the product peaks corresponded to those of 24-hydroxycholesterol and monohydroxylated metabolites of this steroid (peaks 1 and 2). Peak 1 had a mass spectrum consistent with that of 24,27-dihydroxycholesterol (*23*) with peaks at *m*/*z* 544 (M − 90), *m*/*z* 503 (loss of $CH_3-CH-CH_2OTMS$ from the side chain), *m*/*z* 413 (M − 131 − 90), and *m*/*z* 233 (loss of C23−C27 together with two TMS groups from the side chain) (Figure 3A). Peak 2 contained the same ions except the ion at *m*/*z* 131 dominated (corresponds to the terminal isopropyl group + TMS) (Figure 3B). This fragmentation pattern is consistent with the presence of an OTMS group at the 25-position and thus corresponds to 24,25-dihydroxycholesterol. To exclude with certainty the possibility that the fragment at *m*/*z* 131 could have some other origin, we also recorded

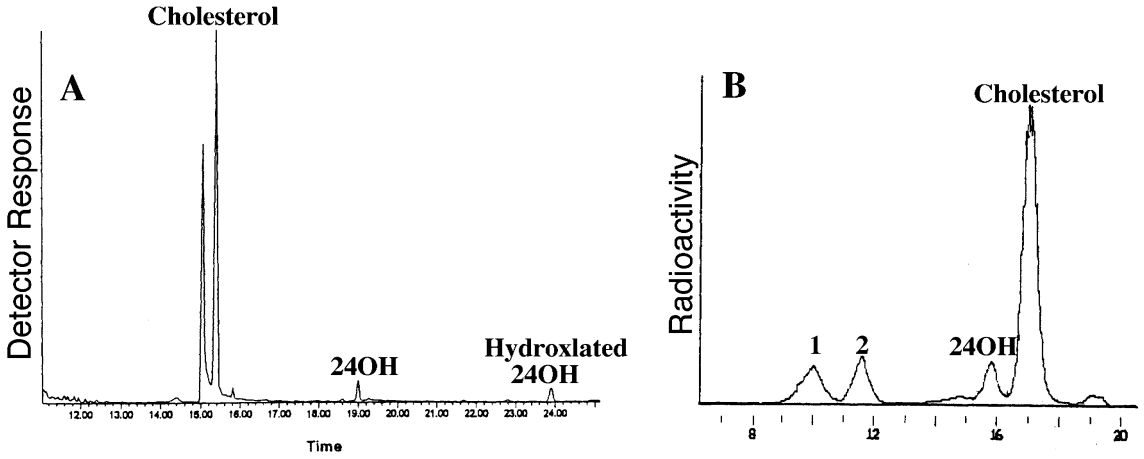

FIGURE 2: Gas chromatographic separation of the lipid extract from CYP46A1-transfected HEK293 cells that were partially depleted of endogenous cholesterol and then cultured for 48 h in the absence of cholesterol in the culture medium (A). TLC separation of the peak "hydroxylated 24OH" in panel A after CYP46A1-expressing HEK293 cells were incubated for 48 h with trace amounts of [^{3}H]cholesterol (B). Abbreviations: 24OH, 24(*S*)-hydroxycholesterol; hydroxylated 24OH, a mixture of 24,25- and 24,27-dihydroxycholesterols. The unlabeled peak in panel A corresponds to an endogenous contaminant of nonsteroid nature.

a mass spectrum of the same product formed from hexadeuterated cholesterol with the six deuterium atoms in the two methyl groups. As expected, the base peak at *m*/*z* 131 had shifted to *m*/*z* 137 in the mass spectrum of that product (not shown).

Metabolism of 24(S)-Hydroxycholesterol and Other Cholesterol Derivatives by HEK293 Cells Transfected with CYP46A1. To test whether 24,25- and 24,27-dihydroxycholersterols are indeed the products of P450 46A1 activities, unlabeled 24(*S*)-hydroxycholesterol was incubated with both nontransfected and CYP46A1-transfected HEK293 cells. Transfected cells metabolized about 80% of 24(*S*)-hydroxycholesterol, as assessed by the appearance of peaks A−D in Figure 4 during gas chromatographic separation of the lipid extract. These peaks were never observed in the incubations with the nontransfected cells. Peaks B and D (Figure 4) were always seen in chromatograms, whereas peaks A and C were not always present when incubations with 24(*S*)-hydroxycholesterol were repeated. As expected, the product peak D contained both 24,25- and 24,27-dihydroxycholesterols as indicated by mass spectrometry. The product peak B had a very prominent peak at *m*/*z* 131 as expected for 25-hydroxylated steroid. Minor peaks were seen at *m*/*z* 632, *m*/*z* 501, *m*/*z* 411, *m*/*z* 253, and *m*/*z* 233 (not shown). This fragmentation is consistent with 24,25-dihydroxycholesterol with an extra hydroxyl group in the steroid nucleus. The product peak A gave a mass spectrum with prominent peaks at *m*/*z* 632 (M − 90), *m*/*z* 501 (M − 90 − 131), *m*/*z* 253 (steroid nucleus with three double bonds), and *m*/*z* 233 (not shown). This fragmentation is consistent with 24,27-dihydroxycholesterol with an additional hydroxyl group in the steroid nucleus. The product peak C mass spectrum is consistent with 24(*S*)-hydroxycholesterol with an extra hydroxyl group in the steroid nucleus. Peaks were seen at *m*/*z* 634 (M), 544 (M − 90), 501 (M − 90 − 43), *m*/*z* 411 (M − 2 × 90 − 43), *m*/*z* 253 (steroid nucleus with three double bonds), and *m*/*z* 145 (C24−C27 with an OTMS function at C24) (not shown).

CYP46A1-transfected and nontransfected HEK293 cells were also incubated with 7α-hydroxycholesterol, cholestanol, cholesteryl sulfate, and sitosterol. In a parallel experiment, the transfected cells metabolized 7α-hydroxycholesterol at a six times higher rate than they 24-hydroxylated [^{2}H]-cholesterol. The single product in the incubations with 7α-hydroxycholesterol had a mass spectrum identical to that of 7α,24-dihydroxycholesterol (*30*) with prominent peaks at *m*/*z* 544 (M − 90), *m*/*z* 501 (M − 90 − 43), and *m*/*z* 145 (not shown). 24-Hydroxylation was also observed in the incubations with cholestanol, but the rate was less than 10% of that with [^{2}H]cholesterol. The mass spectrum of the product contained the expected fragment *m*/*z* 415 (M − 90 − 43), but the low yield prevented a more detailed characterization. No significant substrate conversion was detected in the incubations with cholesteryl sulfate and sitosterol.

Expression and Partial Purification of P450 46A1. Experiments with CYP46A1-transfected HEK293 cells strongly suggested that activities of P450 46A1 are not limited to 24-hydroxylation of cholesterol. We developed an *E. coli* expression system and partially purified recombinant P450 46A1 to test whether similar activities could be observed in the in vitro reconstituted system. The resulting level of P450 46A1 expression in *E. coli* was 150 nmol/L of culture, as assessed by the reduced CO-difference spectrum of the *E. coli* cells resuspended in buffer. Partial purification resulted in the enzyme with a specific heme content of 3.8−4.2 nmol/mg of protein that did not contain the denatured P420 form. As assessed by the SDS−PAGE (not shown), a rather low heme content is likely due to contamination with other bacterial proteins rather than an apoprotein formation. This is the first successful heterologous expression of the full-length human P450 46A1. Thus, a basis was created for detailed biochemical studies of the important metabolic enzyme, P450 46A1.

Metabolism of Cholesterol and 24(S)-Hydroxycholesterol by Full-Length Recombinant P450 46A1. We began with optimization of the assay conditions for cholesterol and 24-(*S*)-hydroxycholesterol hydroxylations: P450 and steroid concentrations, reaction time, and ratio of P450 reductase to P450. The following additives were also tested either separately or in combination: dithiothreitol (5-fold molar excess over P450), cytochrome b_5 (5-fold molar excess over P450; kindly provided by Dr. J. Halpert, University of Texas Medical Branch), two detergents (0.05−0.4% sodium cholate and 0.05−0.2% Tween-20), and two phospholipids (20−100

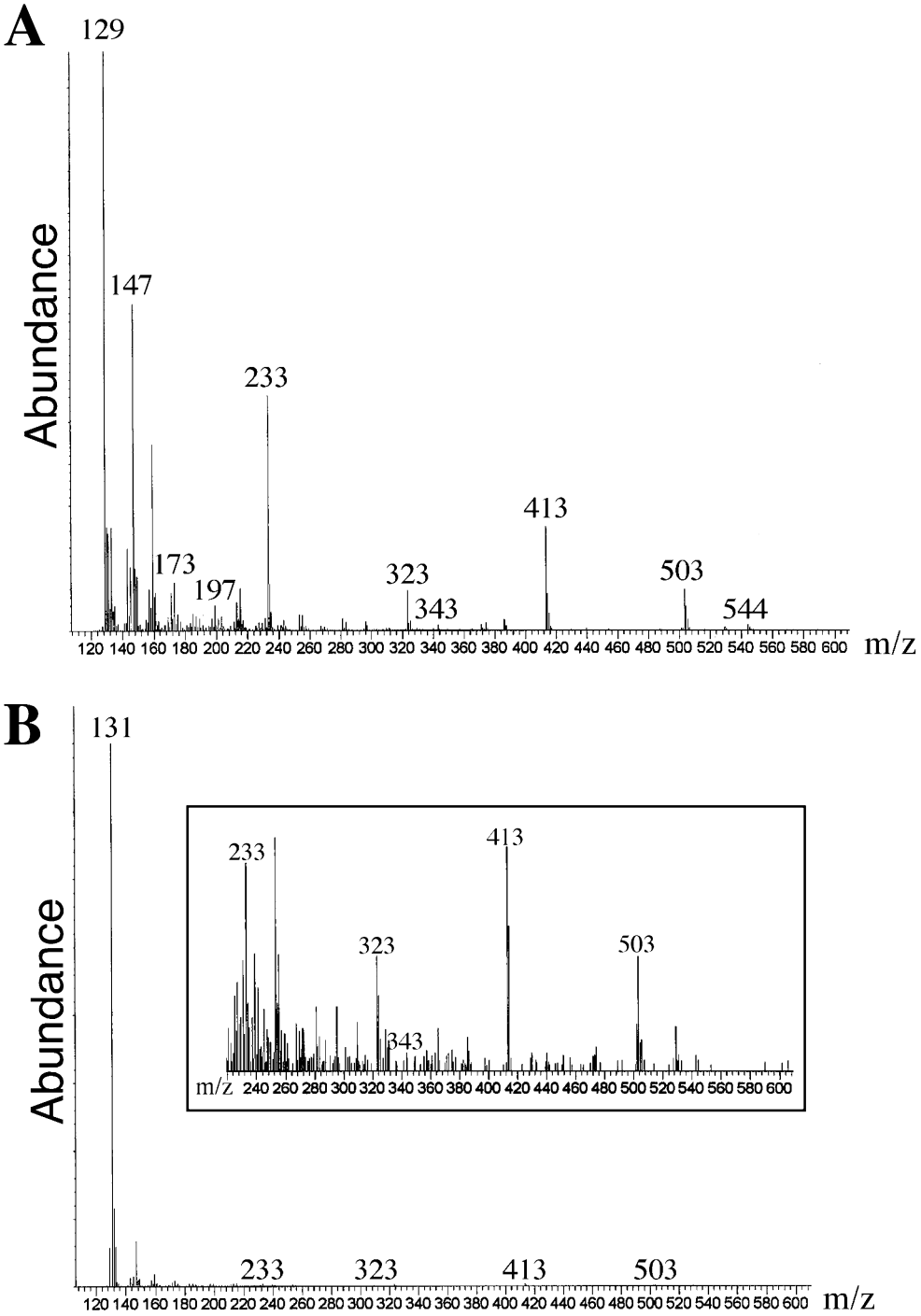

FIGURE 3: Mass spectra of TMS ethers of cholesterol metabolites produced by both CYP46A1-transfected HEK293 cells and by recombinant P450 46A1 in the in vitro reconstituted system that are consistent with 24,27-dihydroxycholesterol (A) and 24,25-dihydroxycholesterol (B). The inset in panel B shows the enlarged portion (*m*/*z* 233−600) of the mass spectrum.

μg/mL phosphatidylcholine and 20−100 μg/mL phosphatidylethanolamine). Only sodium cholate and Tween-20 at concentrations up to 0.2% and 0.1%, respectively, were found to stimulate cholesterol and 24(*S*)-hydroxycholesterol hydroxylase activities. During optimization of the reaction conditions, we saw two peaks, at 4 and 10 min (Figure 5A), that were not present when either NADPH or P450 reductase was omitted from the reconstituted system. The peak at 10 min corresponded to the elution time of 24(*S*)-hydroxycholesterol and at 4 min to the elution time of 24,25- and 24,27-dihydroxycholesterols that were not separated under the conditions of HPLC. The ratio between the peaks at 4 and 10 min varied under different conditions, indicating that the products at 4 min are metabolites of 24(*S*)-hydroxycholesterol. P450 46A1 was then incubated directly with 24-(*S*)-hydroxycholesterol, and the peaks at 4 and 12 min were isolated by HPLC (Figure 5B) and analyzed by TLC and GC-MS. The peak at 4 min represented a 4-to-1 molar mixture of 24,25- and 24,27-dihydroxycholesterols. The peak at 12 min had a mass spectrum consistent with 24-oxocholesterol with one extra hydroxyl group in the steroid nucleus [peaks at *m*/*z* 560 (M), *m*/*z* 470 (M − 0), 380 (M − 2 × 90), *m*/*z* 129 (3β-hydroxy-Δ5 structure), and *m*/*z* 253 (one double bond and two hydroxyl groups in the nucleus]

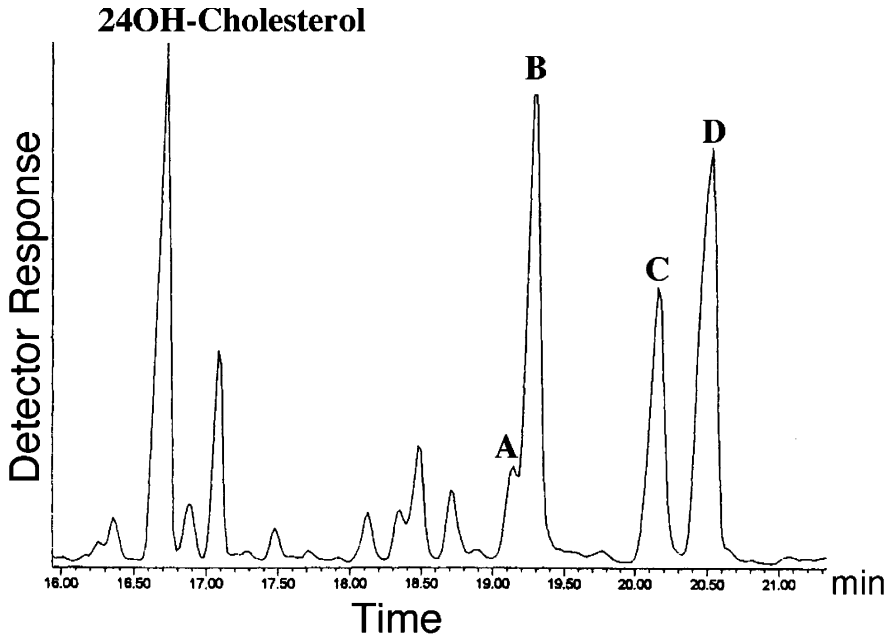

FIGURE 4: Gas chromatographic separation of the lipid extract from the CYP46A1-transfected HEK293 cells that were cultured for 46 h with 20 μg of 24(*S*)-hydroxycholesterol. Peaks: A, 24,27-dihydroxycholesterol with an extra hydroxyl group in the steroid nucleus; B, 24,25-dihydroxycholesterol with an extra hydroxyl group in the steroid nucleus; C, 24-hydroxycholesterol with an extra hydroxyl group in the steroid nucleus; D, a mixture of 24,25- and 24,27-dihydroxycholesterols.

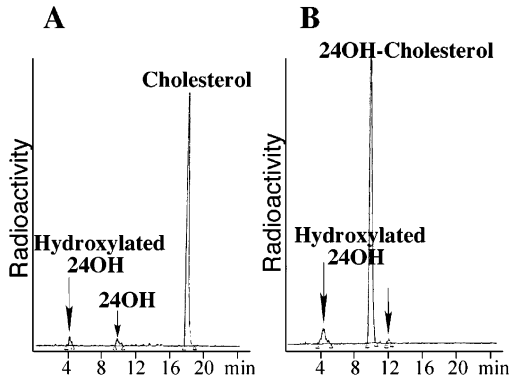

FIGURE 5: HPLC separation of products formed in the incubations of recombinant P450 46A1 with cholesterol (A) and 24(*S*)-hydroxycholesterol (B). Abbreviations: 24OH, 24(*S*)-hydroxycholesterol; hydroxylated 24OH, a mixture of 24,25- and 24,27-dihydroxycholesterols. The arrow at 12 min in panel B indicates 24-oxocholesterol with one extra hydroxyl group in the steroid nucleus.

(not shown). This metabolite was not seen in the incubations with CYP46A1-transfected HEK293 cells.

Catalytic properties of recombinant P450 46A1 against cholesterol and 24(*S*)-hydroxycholesterol were studied under optimized conditions when only one product peak was seen during HPLC separation. When cholesterol was used as a substrate, this product peak corresponded to a single metabolite, 24(*S*)-hydroxycholesterol. When 24(*S*)-hydroxycholesterol was used as a substrate, the product peak corresponded to a mixture of 24,25- and 24,27-dihydroxycholesterols. Cholesterol 24-hydroxylase activity appeared to be low, and we determined the turnover number only (Table 1). The rates of 24(*S*)-hydroxycholesterol metabolism were ~30 times higher, allowing determination of the kinetic parameters (Table 1).

The apparent binding constants of P450 46A1 for cholesterol and 24(*S*)-hydroxycholesterol were also measured by using a spectral binding assay (Table 1). 24(*S*)-Hydroxycholesterol was found to bind almost 80 times more tightly to the enzyme than did cholesterol.

Metabolism of Drugs by Recombinant Full-Length P450 46A1. We examined whether some of the drugs that are metabolized by P450s 1A2, 2D6, 2C9, and 3A4 (Table 2, Figure 6) could be the substrates for P450 46A1 as well. Enzyme activity was measured under the conditions optimal for cholesterol 24-hydroxylation at a substrate concentration close to the K_m value of each probe substrate for the major human P450 that metabolizes this compound in vivo. It is thus possible that the turnover numbers of P450 46A1 toward the substrates tested could be even higher than those shown in Table 2, if the assay conditions were optimized for each drug individually. P450 46A1 was inactive in methoxyresorufin O-dealkylation and insignificant toward bufuralol 1′-hydroxylation. Dextromethorphan O-demethylation (formation of 3-methoxydextrorphanin), dextromethorphan N-demethylation (formation of dextrorphan), diclofenac 4′-hydroxylation, and phenacetin O-deethylation activities of P450 46A1 were low but comparable with the turnover number for cholesterol 24-hydroxylation. The enzyme was capable of hydroxylating the testosterone molecule at different positions, leading to the formation of 6β-, 2α/β-, and 16β-hydroxytestosterones. The rates of testosterone 6β- and 2α/2β-hydroxylations by P450 46A1 were much higher than those with nonsteroid substrates and progesterone, but they were lower than those of P450 3A4 (3.2 and 7.6 nmol min^{-1} nmol^{-1}, respectively) (*28*). It is noteworthy that P450 3A4 does not produce 16β-hydroxytestosterone. Another steroid hormone, cortisol, was also found to be metabolized by P450 46A1 to an unknown product.

DISCUSSION

The results obtained in this study indicate that the enzyme that initiates cholesterol degradation in the brain, P450 46A1, has a broad substrate specificity and is able to metabolize a number of endogenous and exogenous compounds other than

Table 1: Catalytic and Substrate-Binding Properties of P450 46A1 Against Cholesterol and 24(*S*)-Hydroxycholesterol[a]

substrate	metabolite	turnover, pmol min^{-1} nmol^{-1}	K_m, μM	k_{cat}, pmol min^{-1} nmol^{-1}	K_d, μM
cholesterol	24(*S*)-hydroxycholestrol	40 ± 10	ND[b]	ND	8.58 ± 1.51
24(*S*)-hydroxycholestrol	24,25- and 24,27-dihydroxycholesterols	see k_{cat}[c]	2.2 ± 0.1	1170 ± 150	0.11 ± 0.01

[a] Experiments were carried out as described under Experimental Procedures. The results are the mean ± standard deviation of three to four measurements. [b] ND, not determined. [c] k_{cat} is also known as the turnover number, and these two parameters are essentially the same when the enzymatic reaction fits the Michaelis−Menten model (35), as it does in the case of cholesterol and 24(*S*)-hydroxycholesterol hydroxylations by P450 46A1.

Table 2: Metabolism of Different Endogenous and Exogenous Compounds by P450 46A1[a]

substrate	substrate concn, μM	metabolite	metabolite formation, pmol min^{-1} nmol^{-1}	marker assay for P450
methoxyresorufin	2	resorufin	0	1A/1A2
bufuralol	30	1′-hydroxybufuralol	1	2D6
progesterone	0.5	17α-hydroxyprogesterone	12	17A
dextromethorphan	4	3-methoxydextrorphanin	17	3A4
		dextrorphan	27	2D6
cortisol	30	unknown peak	37	
diclofenac	100	4′-hydroxydiclofenac	57	2C9
phenacetin	50	acetaminophen	58	1A2
		unknown peak	55	
testosterone	100	6β-hydroxytestosterone	884	3A4
		2α/2β-hydroxytestosterones[b]	1310	3A4
		16β-hydroxytestosterone	2000	46A1

[a] Enzyme activities were measured as described under Experimental Procedures. [b] 2α- and 2β-isomers are not separated under the conditions of liquid chromatography.

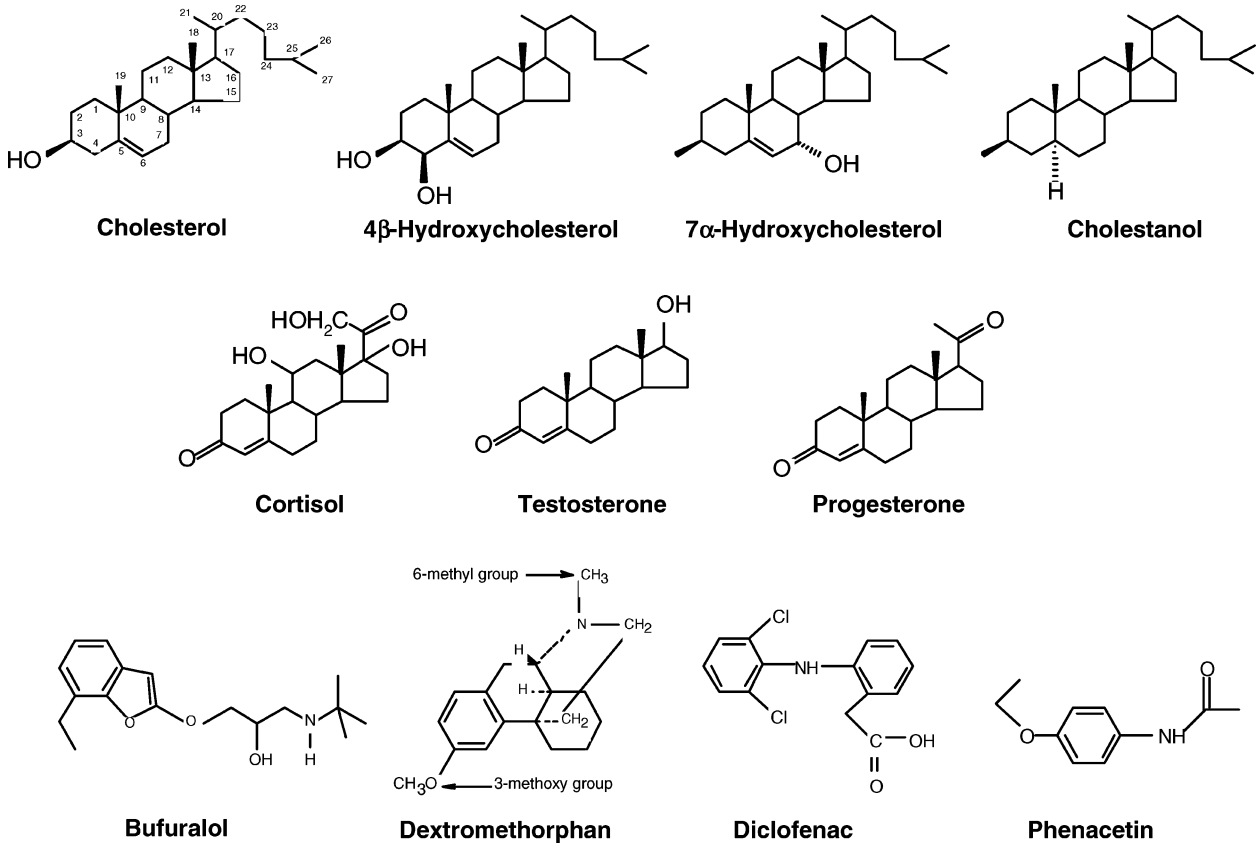

FIGURE 6: Some of the compounds that are metabolized by P450 46A1.

cholesterol. A major finding is that P450 46A1 can sequentially hydroxylate cholesterol, initially giving 24(*S*)-hydroxycholesterol and then 24,25- and 24,27-dihydroxycholesterols. The three metabolites were formed from cholesterol in the incubations with both CYP46A1-transfected HEK293 cells and the in vitro reconstituted system with recombinant P450 46A1. 24(*S*)-Hydroxycholesterol was seen during either gas chromatographic or HPLC separations, indicating that at least part of this intermediate is released from the enzyme active site during catalysis and then reenters the substrate binding pocket for further hydroxylations. Subsequent spectral binding and enzyme activity data demonstrated that in vitro 24-

(*S*)-hydroxycholesterol is even a better substrate for P450 46A1 than cholesterol. P450 46A1 was found to have an 80-fold higher affinity for 24(*S*)-hydroxycholesterol than cholesterol, and the former was hydroxylated 30 times faster than the latter. Low cholesterol 24-hydroxylase activity of P450 46A1 likely reflects physiological requirements for cholesterol metabolism in the brain: the half-life of cholesterol in the brain is 6 months, with the rate of turnover being only 1% of that in peripheral tissues (*31*). P450 46A1 appears to be the enzyme that was designed by nature to produce only small quantities of the biologically active product, 24-(*S*)-hydroxycholesterol.

When 24(*S*)-hydroxycholesterol was used as the substrate, 24,25- and 24,27-dihydroxycholesterols were the major products in both cell cultures and the in vitro reconstituted system. In addition to these metabolites, 24,25-dihydroxycholesterol with an extra hydoxyl group in the steroid nucleus was reproducibly present in the incubations with CYP46A1-transfected HEK293 cells, whereas in the incubations with recombinant P450 46A1, the additional metabolite was 24-oxocholesterol with an extra hydoxyl group in the steroid nucleus. Thus, P450 46A1 has the capacity to hydroxylate the steroid nucleus, although it is not clear at present why some differences were observed in the product pattern between the cell culture and the in vitro system.

The finding of the present study that a single enzyme can catalyze both the formation and further metabolism of the biologically active oxysterol 24(*S*)-hydroxycholesterol raises a question about its physiological relevance. What is currently known is that sulfate and glucuronide conjugates of 24,27-dihydroxycholesterol were found in the ileocecal fluid from the patients with ileostomy (*23*) and that attempts to detect the 24,25- and 24,27-dihydroxycholesterols in blood from the jugular vein of a healthy volunteer were not successful.[2] Clearly, further research is needed to establish the metabolic fate of 24(*S*)-hydroxycholesterol in vivo. The present work provides some directions for these future studies.

P450 46A1 was also found to hydroxylate endogenous steroids that differ in the length of the steroid side chain, position of the double bond, and substitutions in the steroid nucleus and the side chain (Figure 6). In previous studies, we have shown that 4β-hydroxycholesterol, a steroid with an extra hydroxyl group at C4, is 24-hydroxylated by P450 46A1 (32), whereas 7-dehydrocholesterol, a steroid with an extra C7−C8 double bond, was not a substrate for the enzyme (*33*). In the present study, we tested two C27-steroids and three C21-steroids. The C27-steroids were 7α-hydroxycholesterol (contains an extra hydroxyl group at C7) and cholestanol (has no double bond in the nucleus). The C21-steroids had a C4−C5 double bond (cholesterol has a C5−C6 double bond), a keto group at C3, and different functionalities in the side chain (cortisol, testosterone, and progesterone). 7α-Hydroxycholesterol and cholestanol were hydroxylated at the C24, cortisol was metabolized to an unknown product, and testosterone and progesterone were hydroxylated onto different positions of the steroid nucleus. The latter two activities may have physiological significance because the brain is a steroidogeneic organ, in which steroid hormones (neurosteroids) such as progesterone and testosterone are synthesized either de novo from cholesterol or from blood-born steroidal precursors (*34*). Neurosteroids play a critical role in mediating many brain functions; however, pathways of neurosteroidogenesis and degradation are not completely understood (*34*). Our studies provide insight into which enzymes, besides the classical steroidogenic ones, could be involved in metabolism of steroids in the brain.

We also demonstrated that recombinant P450 46A1 metabolizes a number of drugs that are commonly used as marker substrates for individual human P450s (Table 2). The role of P450 46A1 in the metabolism of xenobiotics in the brain remains to be further elucidated, especially with respect to antiseizure, antipsychotic, and antidepressant agents as well as drugs that are used to treat neurodegenerative disorders. Knowledge of the chemical structures that are hydroxylated by P450 46A1 derived from the present work provides a better understanding as to which compounds should be tested as potential substrates for P450 46A1. Our studies also indicate that the substrate-binding pocket of P450 46A1 is large and can accommodate structurally diverse substrates of different size. This knowledge will be utilized in our future structure/function studies aimed at defining the architecture of the enzyme active site and identifying the functionally important amino acid residues.

In summary, the present work reveals novel and hitherto unsuspected activities of important, but poorly characterized, P450 46A1. Our studies establish the enzymatic basis for production of the initial intermediates in the catabolic degradation of cholesterol in the brain, provide insight into the metabolic fate of biologically active endogenous compounds such as 24(*S*)-hydroxycholesterol, progesterone, testosterone, and cortisol, and also indicate that P450 46A1 could be involved in biotransformation of drugs. This is also the first report on *E. coli* expression, purification, and biochemical characterization of P450 46A1.

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BI035512F