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Characterization of the Oxidative Metabolites of 17β-Estradiol and Estrone Formed by 15 Selectively Expressed Human Cytochrome P450 Isoforms

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We systematically characterized the oxidative metabolites of 17β-estradiol and estrone formed by 15 human cytochrome P450 (CYP) isoforms. CYP1A1 had high activity for 17β-estradiol 2-hydroxylation, followed by 15α-, 6α-, 4-, and 7α-hydroxylation. However, when estrone was the substrate, CYP1A1 formed more 4-hydroxyestrone than 15α- or 6α-hydroxyestrone, with 2-hydroxyestrone as the major metabolite. CYP1A2 had the highest activity for the 2-hydroxylation of both 17β-estradiol and estrone, although it also had considerable activity for their 4-hydroxylation (9–13% of 2-hydroxylation). CYP1B1 mainly catalyzed the formation of catechol estrogens, with 4-hydroxyestrogens predominant. CYP2A6, 2B6, 2C8, 2C9, 2C19, and 2D6 each showed a varying degree of low catalytic activity for estrogen 2-hydroxylation, whereas CYP2C18 and CYP2E1 did not show any detectable estrogen-hydroxylating activity. CYP3A4 had strong activity for the formation of 2-hydroxyestradiol, followed by 4-hydroxyestradiol and an unknown polar metabolite, and small amounts of 16α- and 16β-hydroxyestrogens were also formed. The ratio of 4- to 2-hydroxylation of 17β-estradiol or estrone with CYP3A4 was 0.22 or 0.51, respectively. CYP3A5 had similar catalytic activity for the formation of 2- and 4-hydroxyestrogens. Notably, CYP3A5 had an unusually high ratio of 4- to 2-hydroxylation of 17β-estradiol or estrone (0.53 or 1.26, respectively). CYP3A4 and 3A5 also catalyzed the formation of nonpolar estrogen metabolite peaks (chromatographically less polar than estrone). CYP3A7 had a distinct catalytic activity for the 16α-hydroxylation of estrone, but not 17β-estradiol. CYP4A11 had little catalytic activity for the metabolism of 17β-estradiol and estrone. In conclusion, many human CYP isoforms are involved in the oxidative metabolism of 17β-estradiol and estrone, with a varying degree of catalytic activity and distinct regioselectivity. (*Endocrinology* 144: 3382–3398, 2003)

THE ENDOGENOUS ESTROGENS, such as 17β-estradiol (E_2) and estrone (E_1), undergo extensive oxidative metabolism (namely, hydroxylation and keto formation) at various positions catalyzed by enzymes present in liver as well as in extrahepatic estrogen target organs (reviewed in Refs. 1 and 2). Members of various cytochrome P450 (CYP) families are the major enzymes that are responsible for the nicotinamide adenine dinucleotide phosphate, reduced form (NADPH)-dependent oxidative metabolism of endogenous estrogens to various metabolites (1, 2). Liver usually expresses high levels of multiple CYP isoforms (such as CYP3A4 and CYP1A2), but extrahepatic target tissues or cultured cells derived from estrogen target organs often also express significant amounts of additional CYP isoforms with estrogen-metabolizing activity (3–20). Many different CYP isoforms (some are known to metabolize E_2 and E_1) were found to be present in mammary tissues of female rats (16) and humans (17–20), as well as in other extrahepatic tissues such as certain regions of brain (4–8). Notably, certain CYP isoforms (*e.g.* CYP1B1) with selective catalytic activity for regiospecific hydroxylation of endogenous estrogens are among the major CYP isoforms that are present in estrogen target cells but are essentially not expressed in the liver. Moreover, the expression of some of these CYP isoforms in estrogen target cells or the ovary appeared to be tightly regulated under different physiological or pathophysiological conditions.

Several earlier studies have examined the catalytic activity of a number of CYP isoforms from animals and humans for the NADPH-dependent oxidative metabolism of E_2 and E_1 (21–30). In most of these studies, usually only a few estrogen metabolites (*e.g.* products of estrogen 2-, 4-, and 16α-hydroxylation) were determined. However, it is now known that a large number of hydroxylated or keto metabolites of E_2 and/or E_1 are present in biological samples (*e.g.* urine, tissues, and blood) or formed during *in vitro* incubations of estrogen substrates with microsomal enzymes from either animals or humans (1). Some of the estrogen metabolites (such as 4-OH-E_2, 15α-OH-E_2, 16α-OH-E_1, and 2-methoxyestradiol) may have unique biological functions that are not associated with their parent hormones E_2 and E_1 (1, 31–33). It is, therefore, important to characterize the complete profiles of the hydroxylated or keto metabolites of endogenous E_2 and E_1 that are formed by each of the human CYP isoforms present in liver and particularly those selectively present in estrogen target tissues or cells, and also to identify the potential biological functions associated with these estrogen metabolites. Detailed knowledge on this would be useful for our better understanding of their biological functions in different target cells in which some of these CYP isoforms are

Abbreviations: CYP, Cytochrome P450; GC/MS, gas chromatography/mass spectrometry; E_1, estrone; E_2, 17β-estradiol; NADPH, nicotinamide adenine dinucleotide phosphate, reduced form; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TMS, trimethylsilyl.

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selectively expressed under certain physiological or pathological conditions.

Recently, we have characterized a large number of oxidative metabolites of E_2 and E_1 formed by human liver (34, 35) and human term placenta (36). We describe here our data on the systematic characterization of the NADPH-dependent metabolites of E_2 and E_1 formed by 15 human CYP isoforms. These human CYP isoforms were selectively expressed in insect cells that were transfected with a baculovirus expression system containing the cDNA for each of the desired human CYP isoforms. Our data demonstrate that a variety of human CYP isoforms are catalytically active for the NADPH-dependent oxidative metabolism of E_2 and E_1 to multiple metabolites, and many of them have distinct regioselectivity for the catalysis of metabolic reactions.

Materials and Methods

Chemicals

E_2, E_1, NADPH, and ascorbic acid were purchased from the Sigma Chemical Co. (St. Louis, MO). 7β-OH-E_2 was a generous gift from Dr. I. Yoshizawa of Hokkaido College of Pharmacy (Hokkaido, Japan). The sources of 6β-OH-E_1, 7α-OH-E_2, 12β-OH-E_2, 12-keto-E_2, 14-OH-E_1, 14-OH-E_2, 15α-OH-E_2, and 15β-OH-E_2 were described in an earlier paper (37). Several hydroxy-E_1 metabolites, including 6α-OH-E_1, 7α-OH-E_1, 7β-OH-E_1, 12β-OH-E_1, 15α-OH-E_1, 15β-OH-E_1, and 16β-OH-E_1, were biosynthetically prepared in our laboratory from their respective hydroxy-E_2 metabolites through incubations with human liver microsomes in the presence of NAD^+ as cofactor. Each of the products formed was extracted with ethyl acetate and then isolated by HPLC (described in *HPLC analysis of [^{3}H]E_2 or [^{3}H]E_1 metabolites*). The reference compounds for all other estrogen metabolites used in this study were obtained from Steraloids, Inc. (Newport, RI). *N,O*-bis(Trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane was obtained from Pierce Chemical Co. (Rockford, IL). Most of the organic solvents used in this study were of HPLC grade or better, and they were obtained from Fisher Scientific (Atlanta, GA).

[2,4,6,7,16,17-^{3}H]E_2 and [2,4,6,7-^{3}H]E_1 (numerically labeled, specific radioactivity of 123 and 100 Ci/mmol, respectively) were purchased from Perkin-Elmer Life Sciences (Boston, MA). There was no published information available that would enable us to ascertain whether each of the designated positions was evenly labeled. However, a comparison of several tritium-labeled E_2 and E_1 products such as [6,7-^{3}H]E_2, [2,4,6,7,-^{3}H]E_2, and [2,4,6,7,16,17-^{3}H]E_2 prepared by the same company showed that their highest specific activities (curies per millimole) increased almost proportionally with increasing positions labeled with tritium, which suggested that each position likely was quite evenly labeled. In addition, it should be noted that when we compared the estrogen metabolites formed by various human CYP isoforms with 20 μM of [2,4,6,7,16,17-^{3}H]E_2 or [4-^{14}C]E_2 as substrate, the overall profiles of the major E_2 metabolites formed were found to be highly similar, but most of the quantitatively minor metabolites of E_2 could not be detected with [4-^{14}C]E_2 because of its low specific radioactivity.

Selectively expressed human CYP isoforms

The selectively expressed human CYP isoforms were obtained from BD Gentest Co. (Woburn, MA). These human CYP isoforms were expressed in insect cells that were selectively transfected with a baculovirus (*Autographa californica*) expression system containing the cDNA for each of the desired human CYP isoforms. The total microsomal protein concentration, CYP content, CYP reductase activity, and cytochrome b_5 content, and the specific catalytic activity for the marker substrate for each expressed CYP isoform in the microsomes are summarized in Table 1.

Assay of the NADPH-dependent metabolism of [^{3}H]E_2 or [^{3}H]E_1 by human CYP isoforms

For the *in vitro* metabolism, the reaction mixture consisted of microsomes (at 70 or 140 pmol of CYP/ml), a desired concentration of E_2 or E_1 (containing ~3 μCi of [^{3}H]E_2 or [^{3}H]E_1), 2 mM NADPH, and 5 mM ascorbic acid in a final volume of 0.5 ml of 0.1 M Tris-HCl buffer (pH 7.4)

TABLE 1. Microsomes prepared from baculovirus-infected insect cells that selectively expressed a desired human CYP isoform[a]

Microsomes from baculovirus-infected insect cells expressing a human CYP isoform	Protein content (mg/ml)	Total CYP content (pmol/ml)	Reductase activity (nmol/mg of protein/min)	Catalytic activity for selective probe substrates (pmol product formed/pmol of CYP/min)
Control	5.0	N.D.	38	
CYP1A1	12.9	1000	1950	36.3 (7-Ethoxyresorufin *O*-deethylase activity)
CYP1A2	4.9	1000	2080	28.3 (Phenacetin deethylase activity)
CYP1B1	7.4	1000	230	5.4 (7-Ethoxyresorufin *O*-deethylase activity)
CYP2A6	18.6	2000	120	8.8 (Coumarin 7-hydroxylase activity)
CYP2B6	9.3	1000	2620	5.2 (7-Ethoxy-4-trifluoromethylcoumarin deethylase activity)
CYP2C8	3.6	2000	1350	4.4 (Paclitaxel 6α-hydroxylase activity)
CYP2C9[b]	3.2	2000	575	16.5 (Diclofenac 4′-hydroxylase activity)
CYP2C18	4.3	1000	250	0.5 (Diclofenac 4′-hydroxylase activity)
CYP2C19	2.3	1000	860	2.6 (*S*-Mephenytoin 4′-hydroxylase activity)
CYP2D6[c]	6.8	1000	2590	30 ([+/−]-Bufuralol 1′-hydroxylase activity)
CYP2E1 + b_5[d]	4.5	2000	1690	9.3 (*p*-Nitrophenol hydroxylase activity)
CYP3A4	2.5	2000	770	7.0; 4.7[g] (Testosterone 6β-hydroxylase activity)
CYP3A4 + b_5[e]	5.1	1000	2510	110 (Testosterone 6β-hydroxylase activity)
CYP3A5	3.6	2000	720	3.6; 4.4[g] (Testosterone 6β-hydroxylase activity)
CYP3A7 + b_5[f]	7.7	1000	1100	0.84 (Testosterone 6β-hydroxylase activity)
CYP4A11	7.1	1000	1910	38 (Lauric acid ω-hydroxylase activity)

N.D., Not determined.

[a] Information on microsomal protein concentration, total CYP content, reductase activity, and catalytic activity for selective probe substrates were provided by the BD Gentest.

[b] Wild-type CYP2C9.

[c] Wild-type CYP2D6.

[d] Cytochrome b_5 content, 420 pmol/mg of protein.

[e] Cytochrome b_5 content, 1020 pmol/mg of protein.

[f] Cytochrome b_5 content, 170 pmol/mg of protein.

[g] A different batch of CYP3A4 and CYP3A5 with different overall catalytic activity was used for the determination of the K_M and V_{MAX} values.

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containing 0.05 M HEPES. The presence of 5 mM ascorbic acid in the incubation mixture has previously been shown to protect catechol estrogen metabolites from oxidative degradation without significantly altering the enzyme activity (4). The enzymatic reaction was initiated by addition of microsomes, and the incubations were carried out at 37 C for 20 min with mild shaking. The microsomal reaction was arrested by placing test tubes on ice and was then immediately extracted with 8 ml of ethyl acetate. The organic supernatants were transferred to another set of test tubes and dried under a stream of nitrogen. The resulting residues were redissolved in 100 μl of methanol, and a 40-μl aliquot was injected into the HPLC for analysis of estrogen metabolite composition.

It should also be noted that all of the glass test tubes used in our study were silanized with 5% (vol/vol) dimethyldichlorosilane in toluene for 10 min, followed by rinses in pure toluene once and pure methanol twice. The test tubes were allowed to dry at room temperature and then were thoroughly rinsed with distilled water. Our earlier analyses of the NADPH-dependent $[^3H]E_2$ metabolism by human and rat liver microsomes using seven different types of unsilanized glass test tubes obtained from three different manufacturers showed that even under exactly the same incubation, extraction, and HPLC analytical conditions, the results were very different for each of the hydroxylated $[^3H]E_2$ metabolites detected. Based on measuring the radioactivity associated with $[^3H]$2-OH-E_2 and $[^3H]$4-OH-E_2 peaks, their overall recoveries with unsilanized test tubes were only 30–67% of the recoveries with the silanized test tubes. The reason for the increased recoveries of hydroxyestrogen metabolites with silanized glass tubes likely was because pretreatment of the glassware surface with dimethyldichlorosilane deactivated the active chemical groups, thereby reducing physical adsorption of the hydroxylated estrogen metabolites to the test tubes (38).

HPLC analysis of $[^3H]E_2$ or $[^3H]E_1$ metabolites

Analysis of $[^3H]E_2$ and $[^3H]E_1$ metabolites was performed with an HPLC system coupled with in-line UV and radioactivity detections as described earlier (37). The HPLC system consisted of a Waters 2690 separation module (Milford, MA), a Waters UV detector (model 484), an IN/US β-RAM radioactivity detector (Tampa, FL), and an Ultracarb 5 ODS column (150 × 4.60 mm, Phenomenex, Torrance, CA). The solvent system for separation of E_2, E_1, and their metabolites consisted of acetonitrile (solvent A), 0.1% acetic acid in water (solvent B), and 0.1% acetic acid in methanol (solvent C). The solvent gradient (solvent A/solvent B/solvent C) used for eluting estrogen metabolites was as follows: 8 min of isocratic at an initial composition of 16:68:16, 7 min of a concave gradient (curve number 9) to 18:64:18, 13 min of a concave gradient (curve number 8) to 20:59:21, 10 min of a convex gradient (curve number 2) to 22:57:21, 13 min of a concave gradient (curve number 8) to 58:21:21, followed by a 0.1-min step to 92:5:3 and a 8.9-min isocratic period at 92:5:3. The gradient was then returned to the initial composition (16:68:16) and held for 10 min before analysis of the next sample.

The HPLC retention times for all authentic estrogen metabolites were determined by using in-line UV detection, whereas the $[^3H]E_2$ or $[^3H]E_1$ metabolite peaks formed with selectively expressed human CYP isoforms were determined by using in-line radioactivity detection. The calculation of the amount of each estrogen metabolite formed was based on the amount of radioactivity detected for each corresponding metabolite peak. Here, it should also be noted that CYP isoform-mediated formation of hydroxylated or keto metabolites of $[^3H]E_2$ or $[^3H]E_1$ at any of their ^{3}H-labeled positions (namely, 2, 4, 6, 7, 16, and 17 for $[^3H]E_2$ and 2, 4, 6, and 7 for $[^3H]E_1$) was known to remove tritium from the substrate, resulting in the formation of $[^3H]H_2O$. In the present study, therefore, the calculated final rates for the formation of hydroxylated or keto metabolites at the ^{3}H-labeled positions were adjusted according to the estimated loss of radioactivity in each of these products. The rate for the formation of an estrogen metabolite by an expressed CYP isoform was expressed as "picomoles of the estrogen metabolite formed per nanomole of CYP isoform per minute," abbreviated as "pmol/nmol·min."

Structural identification of E_2 or E_1 metabolites formed by selectively expressed human CYP isoforms

The identity of most of E_2 or E_1 metabolites formed by selectively expressed CYP isoforms was confirmed through comparisons of their HPLC retention times, gas chromatography/mass spectrometry (GC/MS) retention times, and mass fragmentation spectra with all authentic reference compounds. We had a total of 49 authentic metabolites of E_2 and E_1, which included: 2-OH-E_1, 2-OH-E_2, 2-OH-E_3, 2-methoxy-E_1, 2-methoxy-E_2, 4-OH-E_1, 4-OH-E_2, 4-methoxy-E_1, 4-methoxy-E_2, 6α-OH-E_1, 6α-OH-E_2, 6β-OH-E_1, 6β-OH-E_2, 6-keto-E_1, 6-keto-E_2, 6-keto-E_3, 6-dehydro-E_1, 6-dehydro-E_2, 7α-OH-E_1, 7α-OH-E_2, 7β-OH-E_1, 7β-OH-E_2, 7-dehydro-E_1, 7-dehydro-E_2, 9(11)-dehydro-E_2, 11α-OH-E_1, 11α-OH-E_2, 11β-OH-E_1, 11β-OH-E_2, 11-keto-E_1, 12β-OH-E_1, 12β-OH-E_2, 12-keto-E_2, 14-OH-E_1, 14-OH-E_2, 15α-OH-E_1, 15α-OH-E_2, 15α-OH-E_3, 15β-OH-E_1, 15β-OH-E_2, 16α-OH-E_1, 16α-OH-E_2, 16α-OH-17α-E_2, 16β-OH-E_1, 16β-OH-E_2, 16β-OH-17α-E_2, 16-keto-E_1, 6-keto-E_2, and 17α-E_2. For the purpose of comparison, the mass spectrum for each trimethylsilylated reference compound was obtained with our GC/MS system under the same analytical conditions for metabolically formed estrogen metabolites.

Experimentally, the collected HPLC fractions containing suspected estrogen metabolite(s) were first evaporated to dryness under a stream of nitrogen gas and then incubated at 60–65 C for 30 min in the presence of 50 μl of *N*,*O*-bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane. A Hewlett Packard model-5890 gas chromatograph and a model-5970 mass spectrometer (Hewlett Packard, Palo Alto, CA) were used with an RTX-5MS capillary column (0.25 mm × 30 m; 0.25-μm film thickness; Restek Corp., Bellafonte, PA), with helium as the carrier gas. The mass spectrometer was operated in the electron impact mode (70 eV), and the mass abundance was determined by scanning masses from 50–600 *m/z* at 1.4 times/sec. The injector and detector temperatures were 260 and 280 C, respectively. During analysis, the column temperature was increased from 180–260 C at a rate of 4 C/min and then maintained isothermal at 260 C for the remainder of the run. For spectrum match-up between the metabolically formed E_2 or E_1 metabolites and the authentic standards, we used both the built-in library search function of our GC/MS system and the manual comparison of their mass spectra. Notably, selected ion monitoring method was also used in some cases for the identification of certain quantitatively minor estrogen metabolites.

Results

It is of note that we initially conducted all the assays using ^{14}C-labeled E_2 and E_1 (specific activity, 55.1 and 56.4 mCi/mmol, respectively) as substrates. Two concentrations (20 and 50 μM, triplicate measurements) of each estrogen substrate were assayed with the selectively expressed human CYP isoforms. Some of the data from this initial effort were described in a meeting abstract earlier (39). Because of the relatively low radioactive specificity of the ^{14}C-labeled E_2 and E_1, and also because of the relatively low total catalytic activity of several expressed human CYP isoforms, the resolution for a number of quantitatively minor estrogen metabolites was not satisfactory. Recently, we reanalyzed a total of 15 human CYP isoforms using ^{3}H-labeled E_2 or E_1 (specific activity, 123 and 100 Ci/mmol, respectively). Our results showed that the overall profiles of the estrogen metabolites detected with ^{14}C-labeled or ^{3}H-labeled substrates were very similar. However, owing to the high radioactive specificity of the ^{3}H-labeled E_2 and E_1, the sensitivity of detection for minor estrogen metabolites was greatly enhanced. For brevity, the data described here were based on our recent analyses using the ^{3}H-labeled E_2 and E_1. Notably, for most of the CYP isoforms, only one substrate concentration (at 20 μM) was reanalyzed because our earlier assays using 20 and 50 μM $[^{14}C]E_2$ or $[^{14}C]E_1$ showed very similar overall profiles for various estrogen metabolites formed.

Blank microsomes without the expressed human CYP isoforms

When 20 μM of $[^3H]E_2$ or $[^3H]E_1$ was incubated with microsomes prepared from the insect cells infected with the

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wild-type baculovirus without a human *CYP* gene, several very small baseline radioactive peaks were detected by our HPLC system (data not shown). Notably, when some of the baseline metabolite peaks were collected from the HPLC column and further analyzed by GC/MS after trimethylsilyl (TMS) derivatization, we found that a few of them did not consistently match any of the 49 authentic E_2 or E_1 metabolites on the basis of their HPLC retention times, GC/MS retention times, and mass fragmentation spectra. Notably, the blank control microsomes also had weak but detectable activity for catalyzing the interconversions between E_2 and E_1, likely due to the presence of weak 17β-hydroxysteroid dehydrogenase activity in these microsomes. When we quantified each of the estrogen metabolites formed with the expressed human CYP isoforms, its metabolite peak was compared against the metabolite profile of blank microsomes, and usually only the metabolite peak with its radioactivity substantially above the control baseline peak was considered and quantified.

CYP1 family isoforms

CYP1A1. When 20 μM $[^3H]E_2$ was used as substrate, CYP1A1 had high catalytic activity for 2-OH-E_2 formation (2523 ± 208 pmol/nmol·min), followed by 15α-OH-E_2 formation (927 ± 45 pmol/nmol·min) (Fig. 1). 4-OH-E_2, 6α-OH-E_2, and 7α-OH-E_2 were also formed in significant quantities. Notably, because 7α-OH-E_2 was coeluted with or very near 6α-OH-E_2 and 15α-OH-E_2 on our HPLC system, its identification was based on further GC/MS analysis. We analyzed the collected HPLC fraction from 8–13 min (corresponding to 6α-OH-E_2, 7α-OH-E_2, and 15α-OH-E_2) and identified 7α-OH-E_2 as a metabolite (Fig. 2). The estimated ratio between 6α-OH-E_2, 7α-OH-E_2, and 15α-OH-E_2 was approximately 4.7:1:7.1. Small amounts of 6β-OH-E_2 were detected on the HPLC (Fig. 1), which was further confirmed by GC/MS analysis. Two radioactive HPLC peaks, with their retention times matched for 16α-OH-E_2 (E_3) and *y*-OH-E_2,[1] were also detected. In addition, substantial amounts of E_1 as well as small amounts of a few hydroxy-E_1 metabolites (2-OH-E_1, 4-OH-E_1, and 15α-OH-E_1) were also detected. It is likely that these hydroxy-E_1 metabolites were largely formed through 17β-oxidation of their corresponding hydroxy-E_2 metabolites. The ratio of E_2 4-hydroxylation to 2-hydroxylation was approximately 7% with CYP1A1 (Table 2). Notably, when $[^3H]E_2$ was the substrate, large peaks D (eluted right before E_2) were detected on the HPLC for all three CYP1 family enzymes. Further GC/MS analysis of these radioactive peaks (from 43–47 min) formed by CYP1A1 showed the presence of 6- dehydro-E_2 and a few unknown compounds (Fig. 3, *top*). The quantification of the dehydroestrogen metabolites formed was not attempted in the present study, largely because smaller amounts of them were also formed with blank microsomes, and more importantly, because we did not have various dehydroestrogens as standards.

At 20 μM $[^3H]E_1$, CYP1A1 also had high catalytic activity for the formation of 2-OH-E_1 (2027 ± 112 pmol/nmol·min), followed by 4-OH-E_1 (378 ± 16 pmol/nmol·min), 15α-OH-E_1 and 7α-OH-E_2 (combined rate of 172 ± 11 pmol/nmol·min), 6α-OH-E_1 (168 ± 5 pmol/nmol·min), and 16α-OH-E_1 (101 ± 6 pmol/nmol·min) (Fig. 1). Again, small amounts of 7α-OH-E_1 were found to be coeluted with 15α-OH-E_1 on the HPLC, and the structures for both metabolites were further confirmed by GC/MS analysis (data not shown). The ratio of E_1 4-hydroxylation to 2-hydroxylation was 19% (Table 2), which is more than twice as high as the ratio with E_2 as substrate. Similarly, small amounts of several hydroxy-E_2 metabolites (2-OH-E_2, 6α-OH-E_2, and 15α-OH-E_2) were also detected during the incubation with E_1 as substrate. Notably, although the dehydro-E_2 metabolites (peak D) were readily detected with E_2 as substrate, the corresponding dehydro-E_1 peaks were not identified with E_1. Because we found that 6-dehydro-E_1 and 7-dehydro-E_1 (two standards we have) were not clearly separated from E_1 on the HPLC, it is thus very likely that some of those dehydro-E_1 metabolites formed were not identified because they were not separated from E_1 and thus were not visible on the HPLC traces.

CYP1A2. At 20 μM $[^3H]E_2$, CYP1A2 had the highest catalytic activity for 2-hydroxylation (4065 ± 156 pmol/nmol·min), and it also had high absolute activity for 4-hydroxylation (343 ± 24 pmol/nmol·min) (Fig. 1 and Table 2). The ratio of 4-OH-E_2 formation to 2-OH-E_2 formation by this isoform was 9% (Table 2). Two other radioactive HPLC peaks with their retention times matched for *y*-OH-E_2 and 6β-OH-E_2 were detected at very small quantities (<70 pmol/nmol·min). In addition, the formation of considerable amounts of 2-OH-E_1 and 4-OH-E_1 (357 ± 14 and 138 ± 3 pmol/nmol·min, respectively) was also detected, but the rate for the conversion of E_2 to E_1 with CYP1A2 was not significantly different from that with control microsomes.

When 20 μM $[^3H]E_1$ was the substrate, CYP1A2 had the highest catalytic activity for E_1 2-hydroxylation (8503 ± 317 pmol/nmol·min). Notably, the rate of E_1 2-hydroxylation by CYP1A2 was more than twice the rate of E_2 2-hydroxylation under the same reaction conditions. Large amounts of 4-OH-E_1 (1109 ± 34 pmol/nmol·min) were also formed. The ratio of E_1 4- to 2-hydroxylation was approximately 13%, somewhat higher than the ratio with E_2 as substrate (Table 2). In addition, small amounts of E_2, 2-OH-E_2, and 4-OH-E_2 were also detected, likely due to metabolic conversion from E_1 or their corresponding E_1 metabolites.

CYP1B1. At 20 μM $[^3H]E_2$, CYP1B1 had a distinct selectivity for the formation of 4-OH-E_2 (Fig. 1). Although the rate of 4-OH-E_2 formation was 371 ± 16 pmol/nmol·min, the rate of 2-OH-E_2 formation was only 108 ± 3 pmol/nmol·min, giving a ratio of E_2 4- to 2-hydroxylation of 3.4 (Table 2). Very small metabolite peaks with retention times matched for 15α-OH-E_2, 6β-OH-E_2, and 16β-OH-E_2 were also detected with E_2 as substrate. In addition, substantial amounts of E_1 as well as small amounts of 4-OH-E_1 (99 ± 11 pmol/nmol·min) were

[1] The structures for *y*-hydroxylated E_2 and E_1 metabolites (*y*-OH-E_2 and *y*-OH-E_1, respectively) described in this study are still unidentified. Notably, in our recent studies (34, 35), we suggested that the *y*-hydroxylated estrogens might be 1-, 8-, 9-, 12α-, or 18-hydroxyestrogens on the basis of the HPLC and GC/MS analyses of these two unknown metabolite peaks with all available estrogen standards.

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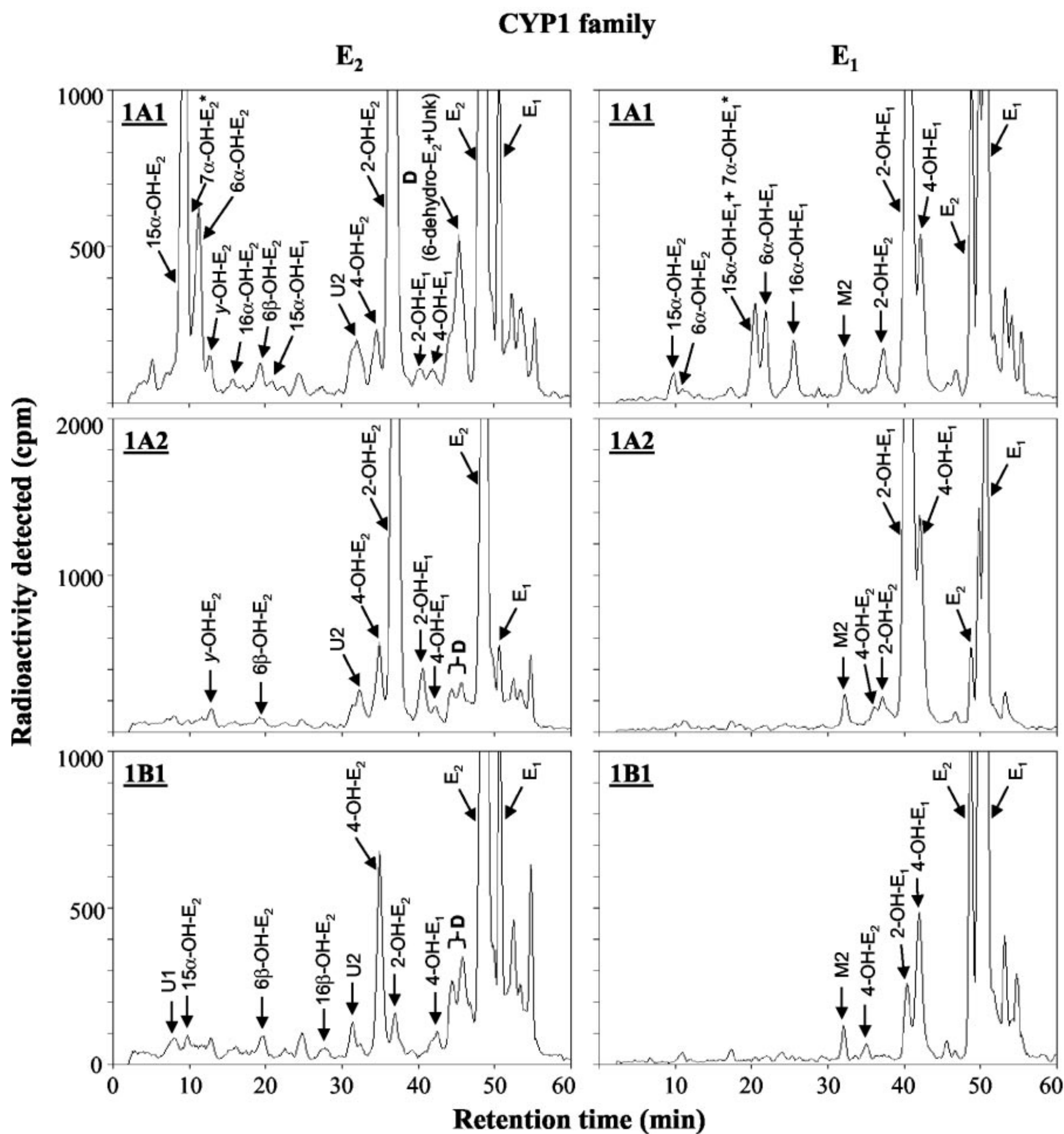

FIG. 1. Representative HPLC traces for the NADPH-dependent metabolism of $[^3H]E_2$ or $[^3H]E_1$ by human CYP1 family enzymes. The human CYP enzymes were selectively expressed in insect cells infected with a baculovirus expression system containing the desired cDNA (purchased from Gentest). The incubation mixture consisted of 20 μM E_2 or E_1 (containing 3 μCi of $[^3H]E_2$ or $[^3H]E_1$), 70 or 140 pmol of P450/ml, 2 mM NADPH, and 5 mM ascorbic acid in a final volume of 0.5 ml of Tris-HCl/HEPES buffer [100 mM/50 mM (pH 7.4)]. The incubation was at 37 C for 20 min with mild shaking. The method for the HPLC separation of the estrogen metabolites was described under *Materials and Methods*. Peaks U1, U2, M1, and M2 in Figs. 1, 4, and 5 are the unidentified radioactive metabolite peaks and were discussed in our earlier reports (33, 34). *, Coeluted metabolites.

also detected, likely due to the 17β-oxidation of E_2 and 4-OH-E_2.

At 20 μM E_1, CYP1B1 had similar but slightly lower catalytic activity for the formation of 4-OH-E_1 and 2-OH-E_1 compared with that with E_2 as substrate. The rates of the 4- and 2-hydroxylation of E_1 were 366 ± 25 and 149 ± 28 pmol/nmol·min, respectively, giving a ratio of E_1 4- to 2-hydroxylation of 2.5 (Table 2). In addition, substantial amounts of E_2 as well as small amounts of 4-OH-E_2 and 2-OH-E_2 were also detected.

CYP2 family isoforms

CYP2A6. At 20 μM $[^3H]E_2$ as the substrate, CYP2A6 had no detectable activity for the formation of catechol estrogen metabolites, but a small peak with retention time matched for 16β-OH-E_2 (58 ± 4 pmol/nmol·min) was detected (Fig. 4). CYP2A6 had substantial activity for the conversion of E_2 to E_1 and to metabolites less polar than E_1. In addition, a few radioactive peaks (collectively labeled as D) eluted shortly before E_2 were detected with CYP2A6. Notably, it appeared

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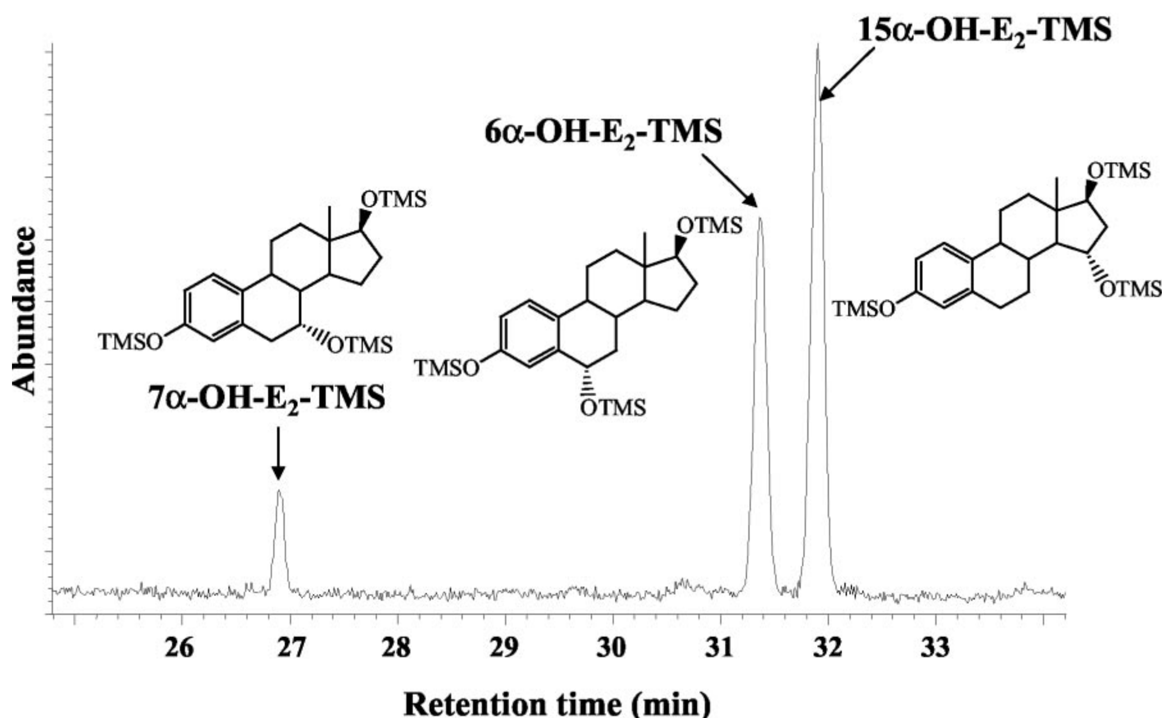

FIG. 2. GC/MS separation and identification of the TMS derivatives of 6α-, 7α-, and 15α-OH-E_2 formed from E_2 by human CYP1A1. The collected HPLC fraction (8–13 min in Fig. 1, *top left panel*) was first converted to TMS derivatives before analysis by GC/MS. The methods for the derivatization and GC/MS analysis were described under *Materials and Methods*.

TABLE 2. The rate of formation and the ratio of estrogen 4- to 2-hydroxylation for several selectively expressed human CYP isoforms

CYP isoforms	20 μM E_2 as substrate			20 μM E_1 as substrate		
	4-OH-E_2 (pmol/nmol·min)	2-OH-E_2 (pmol/nmol·min)	Ratio of 4-OH-E_2/2-OH-E_2 (%)	4-OH-E_2 (pmol/nmol·min)	2-OH-E_2 (pmol/nmol·min)	Ratio of 4-OH-E_1/2-OH-E_1 (%)
CYP1A1	163 ± 22	2523 ± 208	7	378 ± 16	2027 ± 112	19
CYP1A2	343 ± 24	4065 ± 156	9	1109 ± 34	8503 ± 317	13
CYP1B1	371 ± 16	108 ± 3	344	366 ± 25	149 ± 28	246
CYP2C9	16 ± 2	114 ± 21	14	59 ± 8	137 ± 11	43
CYP3A4	78 ± 11	355 ± 41	22	159 ± 14	312 ± 6	51
CYP3A4 + b_5	497 ± 27	3093 ± 91	16	962 ± 34	2829 ± 131	34
CYP3A5	67 ± 5	125 ± 22	53	84 ± 8	67 ± 11	126
CYP3A7 + b_5	55 ± 3	146 ± 17	38	33[a]	335[a]	10[a]

[a] The combined rate for the formation of 4-OH-E_1 and 2-OH-E_1 (calculated according to the radioactivity detection of the HPLC peaks) was 368 ± 18 pmol product/nmol of CYP·min. The assigned individual rate for E_1 2- and 4-hydroxylation as well as the ratio for 2- to 4-hydroxylation was estimated on the basis of further GC/MS analysis of the collected fraction from HPLC.

that all other CYP2 family isoforms tested also had a varying degree of catalytic activity for the formation of these metabolite peaks. Further GC/MS analysis of the isolated HPLC fractions (from 43–51 min) that were generated with CYP2A6 showed the presence of 6-dehydro-E_2, 9(11)-dehydro-E_2, and a few unknown compounds (Fig. 3, *middle*).

At 20 μM [^{3}H]E_1, CYP2A6 had no appreciable activity for the oxidative metabolism of E_1, but it had some catalytic activity for the conversion of E_1 to E_2 and to metabolites less polar than E_1 (Fig. 4).

CYP2B6. At 20 μM [^{3}H]E_2 or [^{3}H]E_1, CYP2B6 only had weak detectable activity for the formation of 2-OH-E_2 and 2-OH-E_1 (data not shown), at rates of 98 ± 3 and 133 ± 10 pmol/nmol·min, respectively. CYP2B6 had little or no catalytic activity for the formation of 4-hydroxylated E_2 or E_1. In addition, CYP2B6 had some catalytic activity for the interconversion between E_1 and E_2 and for the formation of metabolites less polar than E_1.

CYP2C8. At 20 μM [^{3}H]E_2, CYP2C8 showed a weak activity for the formation of 2-OH-E_2 (51 ± 3 pmol/nmol·min) (Fig. 4). Interestingly, small radioactive HPLC peaks with retention times matched for 16α-OH-E_2 and 16β-OH-E_2 were also detected (26 ± 3 and 38 ± 3 pmol/nmol·min, respectively). This isoform had no detectable activity for the 4-hydroxylation of E_2 or E_1. Formation of E_1 and metabolites less polar than E_1 was also observed.

At 20 μM [^{3}H]E_1, CYP2C8 had a weak activity for the formation of 2-OH-E_1 (29 ± 3 pmol/nmol·min) and 16α-OH-E_1/16β-OH-E_1 (two coeluted metabolites, with a combined rate of 31 ± 2 pmol/nmol·min) (Fig. 4). However, the structures of 16α-OH-E_1 and 16β-OH-E_1 were not further confirmed by GC/MS analysis in this case because of their

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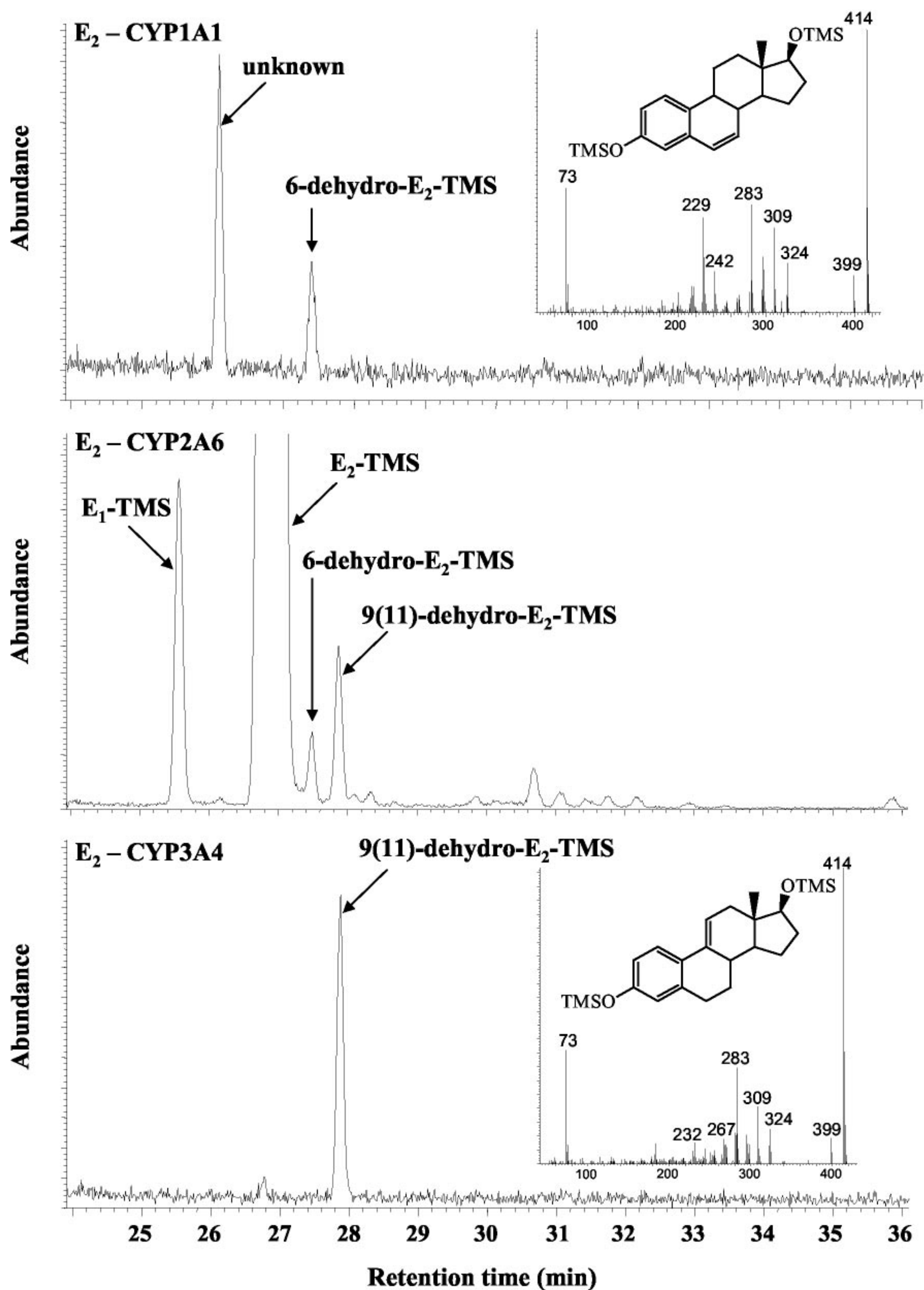

FIG. 3. GC/MS identification of the TMS derivatives of 6-dehydro-E_2 and 9(11)-dehydro-E_2 formed from E_2 by human CYP1A1 (*top*), CYP2A6 (*middle*), or CYP3A4 (*bottom*). The collected HPLC fractions (43–47 min for CYP1A1 and CYP3A4; 43–51 min for CYP2A6) were first converted to TMS derivatives before analysis by GC/MS. The methods for the derivatization and GC/MS analysis were described in *Materials and Methods*.

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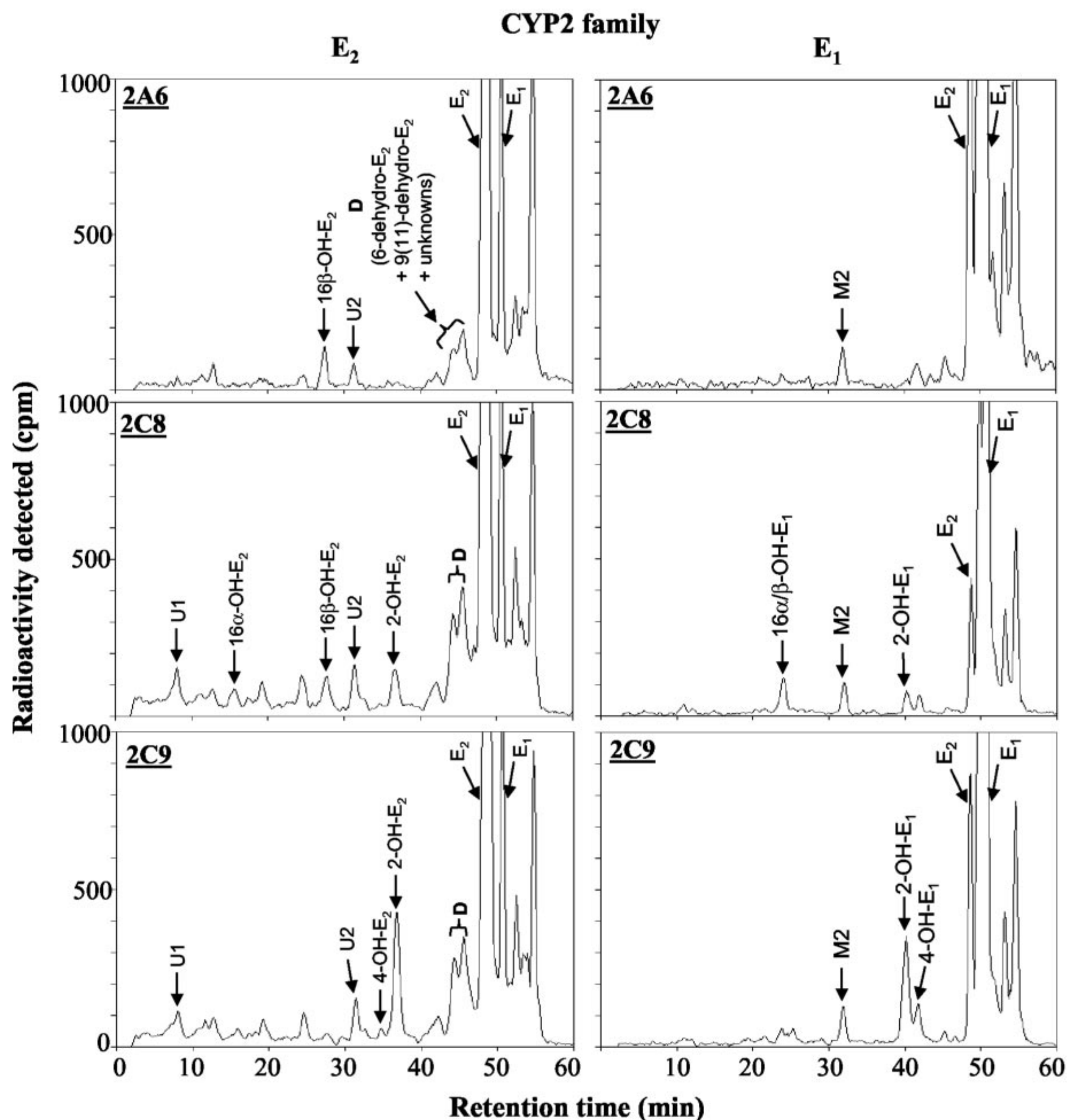

FIG. 4. Representative HPLC traces for the NADPH-dependent metabolism of $[^3H]E_2$ or $[^3H]E_1$ by human CYP2A6, CYP2C8, and CYP2C9. The experimental procedures were the same as described in the legend to Fig. 1.

relatively small quantities. Formation of E_2 and metabolites less polar than E_1 were also observed.

CYP2C9. At 20 μM of $[^3H]E_2$ or $[^3H]E_1$, CYP2C9 showed mainly 2-hydroxylase activity at the rate of 114 ± 21 or 137 ± 11 pmol/nmol·min, respectively (Fig. 4). Notably, this estrogen 2-hydroxylation activity was considered to be very high among all the CYP2 family isoforms tested. CYP2C9 also had a weak but detectable activity for the 4-hydroxylation of E_2 and E_1 (16 ± 2 and 59 ± 8 pmol/nmol·min, respectively). In addition, CYP2C9 had some catalytic activity for the interconversion between E_1 and E_2.

CYP2C18. At 20 μM $[^3H]E_2$ or $[^3H]E_1$, CYP2C18 had little or no catalytic activity for the oxidative metabolism of estrogens, but had a weak activity for the interconversion between E_2 and E_1 (data not shown). Metabolites less polar than E_1 were also observed.

CYP2C19. At 20 μM $[^3H]E_2$ or $[^3H]E_1$, CYP2C19 had a weak activity for their 2-hydroxylation, 123 ± 33 or 64 ± 25 pmol/nmol·min, respectively (data not shown). CYP2C19 also had a weak activity for the conversion of E_2 to E_1, but the activity for the conversion of E_1 to E_2 was negligible. Metabolites less polar than E_1 were also observed.

CYP2D6. At 20 μM $[^3H]E_2$ or $[^3H]E_1$, CYP2D6 showed weak 2-hydroxylase activity, at the rate of 113 ± 17 or 131 ± 14 pmol/nmol·min, respectively (data not shown). Some inter-

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conversion between E_2 and E_1 and the formation of metabolites less polar than E_1 were also observed.

CYP2E1 + b_5. At 20 μM [^{3}H]E_2 or [^{3}H]E_1, CYP2E1 had little or no detectable catalytic activity for the hydroxylation of estrogens, but it had a weak activity for the conversion of E_2 to E_1 and the formation of metabolites less polar than E_1 (data not shown).

CYP3A family isoforms

CYP3A4. At 20 μM [^{3}H]E_2, CYP3A4 had high catalytic activity for the formation of multiple hydroxylated estrogen metabolites (Fig. 5). 2-OH-E_2 (355 ± 41 pmol/nmol·min) was the major hydroxy-E_2 metabolite formed, followed by *y*-OH-E_2 and 4-OH-E_2 (98 ± 10 and 78 ± 11 pmol/nmol·min, respectively). Several other hydroxylated metabolites (6α-OH-E_2, 6β-OH-E_2, 12β-OH-E_2, 15α-OH-E_2, 16α-OH-E_2, and 16β-OH-E_2) were also formed from E_2 with CYP3A4. The structures of these metabolites were confirmed by GC/MS analyses of the isolated fractions from the HPLC (data not shown). In addition, GC/MS analysis of the peak(s) labeled as D in Fig. 5 showed that 9(11)-dehydro-E_2 was a major component (Fig. 3, *bottom*). In addition, large amounts of nonpolar metabolites (collectively labeled as X in Fig. 5) were formed during incubations of E_2 with CYP3A4.

We also determined the kinetic parameters (K_M and V_{MAX}) for CYP3A4-mediated formation of 2-OH-E_2, 4-OH-E_2, and other major metabolites. Under the same conditions for *in vitro* metabolic reactions, different concentrations of [^{3}H]E_2 (at 5, 10, 25, 50, 75, 100, and 150 μM) were incubated with CYP3A4 (a separate batch) in the presence of 2 mM NADPH. The K_M and V_{MAX} values for the formation of 2-OH-E_2 were 52.2 μM and 1020 pmol/nmol·min, respectively, and those for the formation of 4-OH-E_2 were 53.9 μM and 449 pmol/nmol·min, respectively (Fig. 6).

At 20 μM [^{3}H]E_1 as the substrate, CYP3A4 had a similar catalytic activity for the formation of several hydroxylated estrogen metabolites (Fig. 5). 2-OH-E_1 was the major hydroxy-E_1 metabolite formed, followed by 4-OH-E_1 and *y*-OH-E_1. Quantitatively, the rates for the formation of 2-OH-E_1 and 4-OH-E_1 were 312 ± 6 and 159 ± 14 pmol/nmol·min, respectively, which gave the ratio of E_1 4- to 2-hydroxylation approximately 51% (Table 2). The K_M and V_{MAX} values determined for the formation of 2-OH-E_1 by CYP3A4 were 7.7 μM and 168 pmol/nmol·min, respectively, and those for the formation of 4-OH-E_1 were 7.2 μM and 80 pmol/nmol·min, respectively (Fig. 6).

Several other hydroxy-E_1 metabolites (6α-OH-E_1, 6β-OH-E_1, 16α-OH-E_1, and 16β-OH-E_1) and 6-keto-E_1 were also formed in substantial quantities by CYP3A4, and their structures were confirmed by GC/MS analyses. In addition, large amounts of nonpolar metabolites (collectively labeled as X in Fig. 5) were formed during incubations of E_1 with CYP3A4.

Notably, we also analyzed estrogen metabolism by microsomes with either CYP3A4 alone or CYP3A4 combined with cytochrome b_5 expression. We found that the overall profiles of estrogen metabolites formed with CYP3A4 plus cytochrome b_5-expressed microsomes were almost the same as those formed with CYP3A4-expressed microsomes, but the rate for the formation of each metabolite was much higher with the CYP3A4/cytochrome b_5 microsomes (Fig. 5). This observation showed that the presence of higher levels of cytochrome b_5 accelerated the rate of estrogen metabolism under the same reaction conditions, although it did not significantly alter the overall profile of estrogen metabolites formed.

CYP3A5. At 20 μM [^{3}H]E_2, CYP3A5 catalyzed the formation of several hydroxylated estrogen metabolites. The overall profile formed with this CYP isoform was very similar to that formed with CYP3A4 (Fig. 5). 2-OH-E_2 was the major hydroxy-E_2 metabolite formed (at the rate of 125 ± 22 pmol/nmol·min), followed by *y*-OH-E_2 (82 ± 7 pmol/nmol·min) and 4-OH-E_2 (67 ± 5 pmol/nmol·min). The ratio of E_2 4- to 2-hydroxylation was 0.53 (Table 2). The K_M and V_{MAX} values determined for the formation of 2-OH-E_2 by CYP3A5 (a separate batch) were 52.5 μM and 627 pmol/nmol·min, respectively, and those for the formation of 4-OH-E_2 were 46.0 μM and 298 pmol/nmol·min, respectively (Fig. 6).

In addition, several other hydroxy-E_2 metabolites (6β-OH-E_2, 16α-OH-E_2, and 16β-OH-E_2) were also formed by CYP3A5. The formation of all these metabolites was confirmed by GC/MS analyses of the isolated fractions from the HPLC (data not shown). Considerable amounts of a few dehydro-E_2 metabolites (labeled as D) and nonpolar metabolites (collectively labeled as X in Fig. 5) were also detected during incubations of E_2 with CYP3A5.

At 20 μM [^{3}H]E_1, CYP3A5 had a slightly different profile for E_1 metabolism than did CYP3A4 (Fig. 5). CYP3A5 at the same molar concentration formed much less catechol estrogen metabolites than CYP3A4. However, 4-OH-E_1 became the major hydroxyestrogen metabolite with CYP3A5, followed by 2-OH-E_1 and *y*-OH-E_1. Quantitatively, the rates for the formation of 2-OH-E_1 and 4-OH-E_1 were 67 ± 11 and 84 ± 8 pmol/nmol·min, respectively, giving a ratio of 4- to 2-hydroxylation of 1.26 (Table 2). Further enzyme kinetic analysis showed that the K_M and V_{MAX} values for the formation of 2-OH-E_1 were 15.0 μM and 103 pmol/nmol·min, respectively, and those for the formation of 4-OH-E_1 were 27.8 μM and 156 pmol/nmol·min, respectively (Fig. 6).

In addition, several other hydroxy-E_1 metabolites (6β-OH-E_1, 16α-OH-E_1, and 16β-OH-E_1) were also formed in substantial quantities by CYP3A5 (Fig. 5). The formation of these metabolites was confirmed by GC/MS analyses of the isolated fractions from the HPLC (data not shown). Large amounts of nonpolar metabolites (collectively labeled as X in Fig. 5) were formed during incubations of E_1 with CYP3A5.

CYP3A7 + b_5. At 20 μM [^{3}H]E_2, CYP3A7 (coexpressed with cytochrome b_5) had a weak catalytic activity for E_2 2-hydroxylation (146 ± 17 pmol/nmol·min). In addition, small metabolite peaks with retention times matched for 6β-OH-E_2, 16α-OH-E_2, and *y*-OH-E_2 as well as the dehydro-E_2 metabolites (peak D) were detected (Fig. 5). Appreciable amounts of E_1 and metabolites less polar than E_1 were also formed.

At 20 μM [^{3}H]E_1, CYP3A7 had a modest catalytic activity for the formation of 2-OH-E_1 and 4-OH-E_1, with a combined rate of 368 ± 18 pmol/nmol·min (Fig. 5). Further GC/MS analysis showed that the ratio of E_1 4- to 2-hydroxylation was

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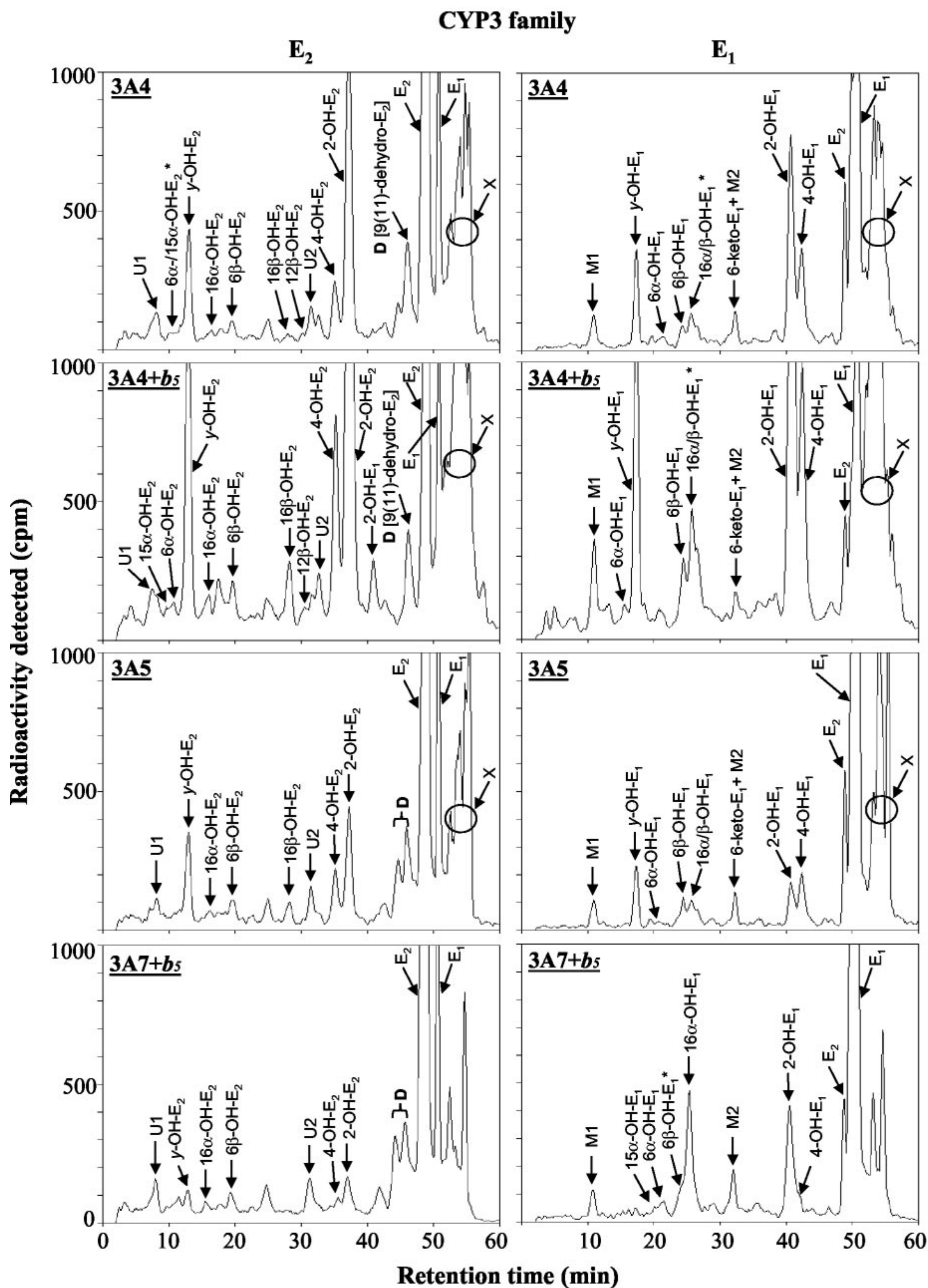

FIG. 5. Representative HPLC traces for the NADPH-dependent metabolism of $[^3H]E_2$ or $[^3H]E_1$ by human CYP3 family enzymes. The experimental procedures were the same as described in the legend to Fig. 1. *, Coeluted metabolites.

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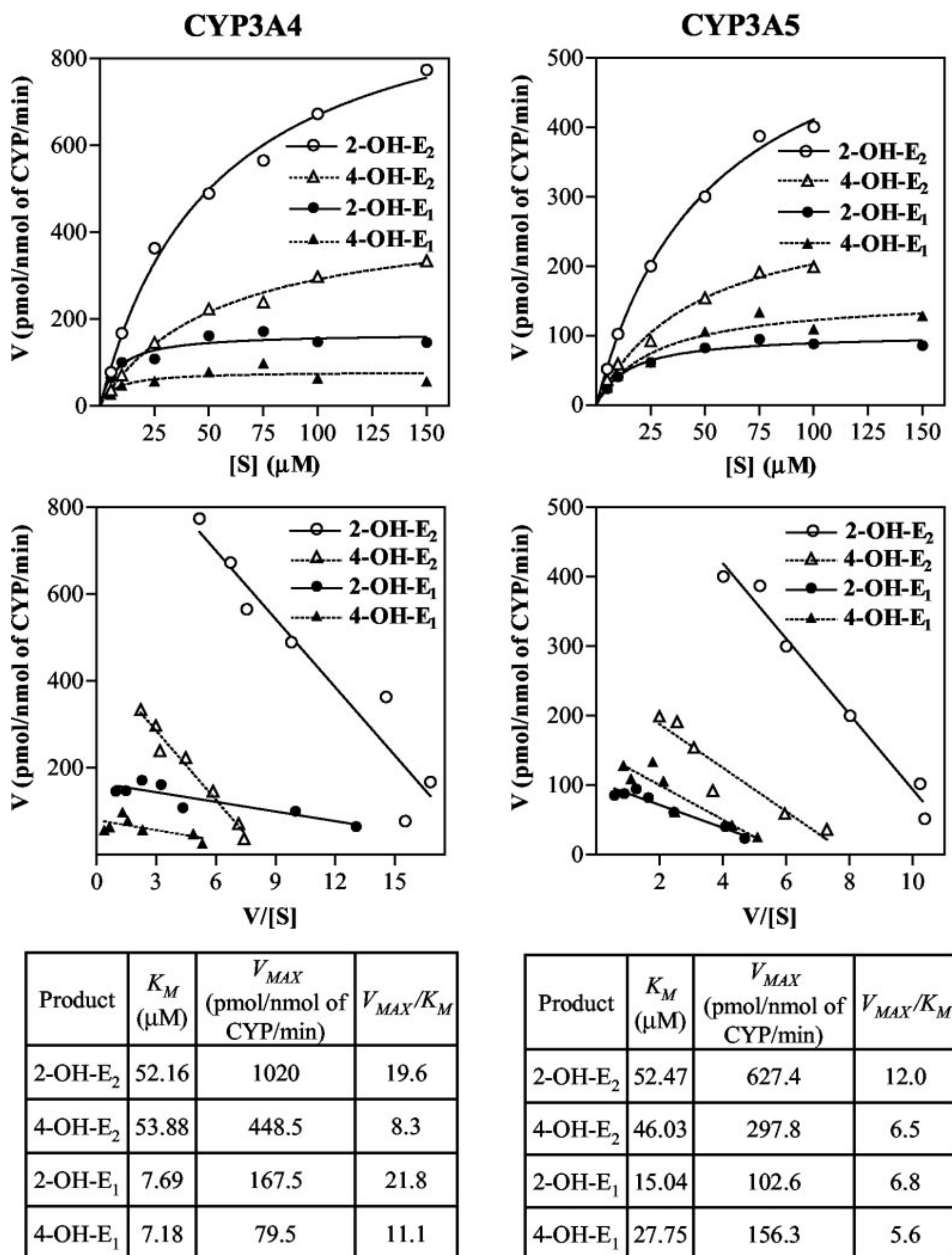

Product	K_M (μM)	V_{MAX} (pmol/nmol of CYP/min)	V_{MAX}/K_M
2-OH-E_2	52.16	1020	19.6
4-OH-E_2	53.88	448.5	8.3
2-OH-E_1	7.69	167.5	21.8
4-OH-E_1	7.18	79.5	11.1

Product	K_M (μM)	V_{MAX} (pmol/nmol of CYP/min)	V_{MAX}/K_M
2-OH-E_2	52.47	627.4	12.0
4-OH-E_2	46.03	297.8	6.5
2-OH-E_1	15.04	102.6	6.8
4-OH-E_1	27.75	156.3	5.6

FIG. 6. Michaelis-Menten curves (*top*), Eadie-Hofstee plots (*middle*), and the calculated kinetic parameters (*bottom*) for the 2- and 4-hydroxylation of E_2 or E_1 by human CYP3A4 (*left*) and CYP3A5 (*right*). Under the same conditions for the *in vitro* metabolic reactions, different concentrations of $[^3H]E_2$ or $[^3H]E_1$ (from 5–150 μM) were incubated with CYP3A4 or CYP3A5. The K_M and V_{MAX} values were obtained by nonlinear regression using Prism software (GraphPad Software, Inc., San Diego, CA).

10% (Table 2). Notably, CYP3A7 plus cytochrome b_5 had the highest catalytic activity for the formation of 16α-OH-E_1 and 6β-OH-E_1 (two coeluted metabolites with a combined rate of 346 ± 3 pmol/nmol·min) (Fig. 6). GC/MS analysis showed that 16α-OH-E_1 accounted for more than 90%.

CYP4 family isoform

CYP4A11. At 20 μM $[^3H]E_2$ or $[^3H]E_1$, CYP4A11 did not have detectable catalytic activity for the oxidative metabolism of the estrogens (data not shown). When E_2 was the substrate, substantial amounts of E_1 and metabolites less polar than E_1 were formed. Similarly, E_2 and metabolites less polar than E_1 were formed from E_1 as substrate.

Discussion

In the present study, we systematically characterized the profiles of the NADPH-dependent oxidative metabolites of E_2 and E_1 formed by 15 human CYP isoforms. Our results

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showed that almost all of the human CYP isoforms are actively involved in the oxidative metabolism of E_2 and E_1, yet with a varying degree of catalytic activity and distinct regioselectivity. The role of each of these CYP isoform families in the NADPH-dependent oxidative metabolism of endogenous estrogens is briefly discussed below.

CYP1 family isoforms

CYP1A1. Human CYP1A1 is essentially expressed in extrahepatic tissues, and the level of its expression is readily inducible by exposure to environmental chemicals or toxins, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and polycyclic aromatic hydrocarbons (40). The results of our present study showed that CYP1A1 catalyzed the formation of several hydroxylated estrogen metabolites from E_2 or E_1. When E_2 was the substrate, CYP1A1 had high catalytic activity for its 2-hydroxylation, followed by 15α-, 6α-, and 4-hydroxylation. The formation of small amounts of 6β-OH-E_2, 7α-OH-E_2, and 16α-OH-E_2 was also detected. In addition, CYP1A1 also catalyzed the formation of 6-dehydro-E_2.

When E_1 was the substrate, CYP1A1 had high catalytic activity for the formation of 2-OH-E_1, 4-OH-E_1, 15α-OH-E_1, 6α-OH-E_1, and 16α-OH-E_1. Small amounts of 7α-OH-E_1 were also formed. Notably, although the ratio of 4- to 2-hydroxylation with CYP1A1 was approximately 7% when E_2 was the substrate, this ratio was increased almost three times (19%) with E_1 as substrate.

Although there is little information in the literature on CYP1A1-mediated metabolism of E_1, the results of our present study on its metabolism of E_2 are in agreement with several earlier studies (14, 24, 30). Spink *et al.* (14) reported earlier that exposure of MCF-7 human breast cancer cells to TCDD markedly increased the microsomal formation of 2-OH-E_2, 4-OH-E_2, 15α-OH-E_2, and 6α-OH-E_2 from E_2 as a substrate. Additional studies by these authors using transfected human *CYP1A1* gene confirmed that CYP1A1 catalyzed the 2-, 4-, 6α-, and 15α-hydroxylation of E_2 (24). In a recent study, it was also reported that CYP1A1 had high activity for E_2 2-hydroxylation but lower activity for E_2 4- and 16α-hydroxylation (30).

It is of note that among the 15 human CYP isoforms analyzed in the present study, CYP1A1 was the only CYP isoform that had a distinctly high catalytic activity for the 15α-hydroxylation of estrogens. When E_2 was the substrate, 15α-OH-E_2 was the second major hydroxy-E_2 metabolite formed (next to 2-OH-E_2). In comparison, the rate for the 15α-hydroxylation of E_1 by CYP1A1 was markedly lower than the rate for the 15α-hydroxylation of E_2. This difference in the rate of 15α-hydroxylation of E_2 *vs.* E_1 likely is due to the differential influence of the 17β-hydroxyl group (in the case of E_2) and the 17-keto group (in the case of E_1) on the CYP1A1-mediated 15α-hydroxylation.

It is of interest to note that the 15α-hydroxylated estrogen metabolites are known to be formed in large amounts during human pregnancy (41), and the amount of urinary 15α-hydroxyestriol excretion by the expectant mother was found to be a reliable indicator for fetal well-being (42, 43). However, it is yet to be determined whether CYP1A1 is the major or sole CYP isoform that is responsible for the 15α-hydroxylation of endogenous estrogens during human pregnancy.

It has been suggested for many years that 16α-OH-E_1 plays an important role in mammary carcinogenesis (31, 44, 45). This estrogen metabolite is not only hormonally active but also chemically reactive and may bind covalently to the estrogen receptor, possibly resulting in sustained hormonal stimulation of the ERα-positive target cells (31, 46). Our recent studies showed that human liver microsomes form only very minute amounts of 16α-OH-E_2 and 16α-OH-E_1 when $[^3H]E_2$ or $[^3H]E_1$ was used as the substrate (34, 35). However, the results of our present study showed, for the first time, that human CYP1A1 (an extrahepatic isoform) has relatively high catalytic activity for the 16α-hydroxylation of E_1, but not so with E_2. Although CYP1A1 has a preference for the 15α-hydroxylation of E_2 over E_1, its opposite preference for the 16α-hydroxylation of E_1 over E_2 may also be due to a different influence of the 17-keto or 17β-hydroxyl groups to the neighboring C16α-positions.

CYP1A2. CYP1A2 constitutes approximately 13% of the total CYP enzymes contained in human liver (47), and it metabolizes E_2 and/or E_1 to 2-hydroxylated metabolites (22, 27, 29, 30). The results of our present study showed that among all human CYP isoforms tested, CYP1A2 had the highest 2-hydroxylase activity for E_2 and E_1 (4065 or 8503 pmol/nmol·min, respectively), with E_1 2-hydroxylation more than twice as fast as E_2 2-hydroxylation. The rate of 4-hydroxylation of E_2 and E_1 by CYP1A2 was 9 and 13%, respectively, of the rate of their 2-hydroxylation (Table 2). It should be noted that similar high rates for the 2-hydroxylation of E_2 and E_1 by a cDNA-expressed human CYP1A2 were also reported earlier (22, 27, 29, 30).

Notably, an earlier study suggested that human CYP1A2 had weak activity for the 16α-hydroxylation of E_2 but not E_1 (29). In addition, it was suggested that the 16α-hydroxylation of E_2 by human liver microsomes (which contained CYP1A2) was partially inhibited by anti-CYP1A2 antibody, whereas the 16α-hydroxylation of E_1 was not affected by anti-CYP1A2 antibody (29). However, the results of our present study clearly showed that human CYP1A2 had little or no catalytic activity for the formation of 16α-hydroxylated metabolites of E_2 or E_1.

CYP1B1. CYP1B1 is another extrahepatic CYP1 family isoform that is chemically inducible and often is overexpressed in tumor tissues (48). This CYP isoform is known to play an important role in the 4-hydroxylation of estrogens. Hayes *et al.* (26) reported earlier that the elevated E_2 4-hydroxylase activity in TCDD-treated MCF-7 breast cancer cells was, in part, due to an elevated expression of the *CYP1B1* gene. In addition, selective expression of human *CYP1B1* gene in *Saccharomyces cerevisiae* produced an enzyme that predominantly catalyzed E_2 4- and 2-hydroxylation (26). The ratios of E_2 4- to 2-hydroxylation for the expressed proteins of four human *CYP1B1* gene variants were reported earlier to range from 1.8–3.6 (49). Our results also showed a predominant hydroxylase activity for CYP1B1 in the C4-positions of E_2 and E_1, giving the highest ratios (3.4 and 2.5) for estrogen 4- to 2-hydroxylation among the 15 CYP isoforms tested (Table 2).

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However, despite a distinct activity for catechol estrogen formation and a high ratio for estrogen 4- to 2-hydroxylation, it should be pointed out that the absolute amounts of 4-OH-E_2 and 4-OH-E_1 formed with CYP1B1 were not high when compared with the amounts formed with some other CYP isoforms, such as CYP1A1 and 1A2.

In summary, whereas CYP1A1 had high catalytic activity for the 2-hydroxylation of E_2 and E_1 (with 4-hydroxylation a minor metabolic pathway), this isoform had a highly distinct activity for the *D*-ring hydroxylation of estrogens at the C15α-positions (for E_2 and E_1) and at the C16α-position (for E_1). CYP1A2 was catalytically most active for the 2-hydroxylation of E_2 and E_1. CYP1B1 had a distinct, selective activity for the 4-hydroxylation of E_2 and E_1 when compared with the 2-hydroxylation of these steroids.

CYP2 family isoforms

CYP2A6. Our results showed that CYP2A6 had no detectable activity for catechol estrogen formation from E_2 or E_1, which is in agreement with an earlier suggestion that CYP2A6 did not have estrogen-metabolizing activity (28). Interestingly, our results showed that a few dehydro-E_2 metabolites, such as 6-dehydro-E_2 and 9(11)-dehydro-E_2, were formed with this CYP isoform. All other CYP2 family isoforms also formed varying amounts of the peak D (which presumably also contained dehydro-E_2 metabolites). Notably, the peak D was earlier suggested to be impurities from the radioactive E_2 (37). We indeed noted that a small amount of the peak D was contained in the radioactive [^{3}H]E_2 substrate, and incubation of [^{3}H]E_2 with control blank microsomes also formed small amounts of the peak D. When microsomes with most of the selectively expressed CYP 2 family isoforms were tested, the formation of peak D appeared to be increased to varying degrees.

CYP2B6. CYP2B6 was reported to be present in 12 of 17 human liver microsomes tested, ranging from 3–74 pmol/mg microsomal protein (50). An earlier study suggested that CYP2B6 did not form any significant amounts of estrogen metabolites (27). Our data showed that CYP2B6 had a moderate activity for the 2-hydroxylation of E_2 and E_1 (98 and 133 pmol/nmol·min, respectively), but this CYP isoform had no detectable activity for estrogen 4-hydroxylation. The discrepancy between our data and an earlier report may be attributable to the greatly improved sensitivity of our *in vitro* assay system.

CYP2C8. Several earlier studies suggested that CYP2C8 had no detectable activity for the oxidative metabolism of estrogens (22, 27). Our data showed that CYP2C8 had a weak but detectable activity for the 2-hydroxylation of E_2 and E_1. In addition, small amounts of estrogen 16α/16β-hydroxylation products were also detected with CYP2C8.

CYP2C9. Earlier studies suggested that human CYP2C9 had a moderate catalytic activity for catechol estrogen formation from E_2 (22, 29) or E_1 (27). Our results showed that CYP2C9 had a relatively high activity for the 2-hydroxylation of E_2 and E_1 among the CYP2 family isoforms tested, and it also had a weak but detectable activity for estrogen 4-hydroxylation. Notably, an earlier study showed that the presence of anti-CYP2C9 antibody or sulfaphenazole did not significantly affect liver microsomal hydroxylation of E_2 or E_1 (29), likely suggesting the relatively minor contribution of CYP2C9 to the overall estrogen metabolism in human liver.

Although it was also suggested earlier that CYP2C9 had a weak activity for the 16α-hydroxylation of E_2 (29), little or no estrogen 16α-hydroxylase activity was detected in the present study.

CYP2C18. Our results showed that CYP2C18 had little or no detectable activity for the oxidative metabolism of E_2 and E_1.

CYP2C19. CYP2C19 has been suggested to have a weak catalytic activity for the 16α-hydroxylation of E_2 (29). Our results showed that CYP2C19 had a relatively high 2-hydroxylase activity for E_2 or E_1, but the formation of 16α-hydroxylated estrogen metabolites was not detected.

CYP2D6. An earlier study suggested that CYP2D6 had no detectable activity for the formation of estrogen metabolites (22). We found that CYP2D6 had a modest activity for the formation of 2-OH-E_2 and 2-OH-E_1 (at rates of 113 and 131 pmol/nmol·min).

CYP2E1 + b_5. Although an earlier study suggested that CYP2E1 was among the important hepatic CYP isoforms for estrogen 2-hydroxylation (23), most other studies (22, 25, 27) have reported that CYP2E1 had no detectable activity for the formation of estrogen metabolites. Our results also showed that CYP2E1 had no detectable activity for the hydroxylation of E_2 or E_1.

In summary, the results of our present study showed that CYP2 family isoforms overall did not have distinctly high activity for the oxidative metabolism of E_2 and E_1, but a varying degree of estrogen 2-hydroxylase activity was detected with several of the isoforms. Because the CYP2 family isoforms account for approximately 30% of human hepatic CYP enzymes (with 2C subfamily isoforms predominant; Ref. 47), their collective contribution to the catechol estrogen formation (particularly 2-hydroxyestrogen) may still be of considerable importance. In addition, CYP2 family isoforms showed a varying degree of catalytic activity for the formation of certain dehydro-E_2 metabolites from E_2.

CYP3 family isoforms

CYP3A4. CYP3A family enzymes are the most abundant CYP isoforms present in human liver (51, 52), and it was estimated that CYP3A4 accounts for approximately 30% of total CYP content (47). Several earlier studies showed that CYP3A4 is involved in the 2- and 4-hydroxylation of E_2 by human liver microsomes (22, 25, 29, 30, 34, 53).

Our results showed that CYP3A4 had high catalytic activity for the formation of multiple hydroxylated metabolites of E_2 and E_1. The catechol estrogen metabolites were the major metabolites formed from 20 μM E_2 and E_1, with apparent ratios of 4- to 2-hydroxylation of 0.22 and 0.51, respectively. Notably, the ratio of E_1 4- to 2-hydroxylation was almost twice as high as the ratio for E_2. Further enzyme kinetic analysis showed that although the capacity (V_{MAX}) of the CYP3A4-mediated 2- and 4-hydroxylation of E_2 was ap-

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proximately eight times higher than its catalytic capacity for E_1, its affinity ($1/K_M$) for the 2- and 4-hydroxylation of E_2 was only approximately 20% of its affinity for E_1. Hence, although CYP3A4 has a lower affinity but a higher capacity for the 2- and 4-hydroxylation of E_2, it has a higher affinity but a lower capacity for the 2- and 4-hydroxylation of E_1. The causes for the opposite kinetic features of CYP3A4-mediated 2- and 4-hydroxylation of E_2 *vs.* E_1 are not known. Interestingly, despite the dramatic differences in the V_{MAX} and K_M values for the 2- and 4-hydroxylation of E_2 and E_1, the V_{MAX}/K_M ratios of CYP3A4-mediated 2- and 4-hydroxylation of these two estrogens were very similar. This likely would make the CYP3A4-mediated metabolism of physiological concentrations of E_2 or E_1 *in vivo* proceed at comparable rates.

Notably, our results with radioactive E_2 and E_1 as substrates showed that CYP3A4 only had a very weak catalytic activity for the 16α-hydroxylation of E_1 and E_2. This observation is in contrast to a few earlier studies that showed that CYP3A4 had high catalytic activity for the 16α-hydroxylation of E_2 and E_1, based on the detection of nonradioactive estrogen metabolites (27–30).

Besides 2-, 4-, and 16α-hydroxy-E_2 metabolites, we also showed that several other hydroxy-E_2 metabolites (*y*-OH-E_2, 6α-OH-E_2, 6β-OH-E_2, 12β-OH-E_2, 15α-OH-E_2, and 16β-OH-E_2) and a dehydroestrogen metabolite, 9(11)-dehydro-E_2, were also formed from E_2 by CYP3A4. Similarly, several other hydroxy-E_1 metabolites (*y*-OH-E_1, 6α-OH-E_1, 6β-OH-E_1, and 16β-OH-E_1) were formed from E_1 in substantial quantities. In addition, our results showed that large amounts of nonpolar metabolites (collectively labeled as X in Fig. 5) were formed during incubations of CYP3A4 with either E_2 or E_1 as substrate.

Lastly, it is of interest to note that when a higher level of cytochrome b_5 was present, CYP3A4 showed a markedly enhanced activity for the formation of multiple hydroxylated estrogen metabolites, but it did not alter the overall estrogen metabolite profiles. Mechanistically, because cytochrome b_5 (a mediator of second electron from NADPH) has also been shown to stimulate CYP-mediated metabolism of certain other substrates (54, 55), this stimulation likely is due to a nonspecific mechanism, such as the increased availability of the second electron required for the reactions. In light of our observations, it should be noted that when the metabolic rates determined in this study are used for comparing the rate of each CYP isoform-mediated metabolism of E_2 or E_1, it is under the assumption that the cytochrome b_5 content as well as other supporting factors (such as CYP reductase) in the expressed microsomes were the same. Because their variability was almost unavoidable, it is thus advised to take into careful consideration their possible influence when making definitive comparisons of the absolute metabolic rates for each of the CYP isoforms.

CYP3A5. Our results showed that when E_2 was the substrate, CYP3A5 formed estrogen metabolites with a similar profile as that of CYP3A4, with 2-OH-E_2 as the major hydroxy-E_2 metabolite, followed by *y*-OH-E_2 and 4-OH-E_2. In comparison, CYP3A5 formed much less catechol estrogen metabolites from E_1 than did CYP3A4, but 4-OH-E_1 became the major hydroxyestrogen metabolite formed by CYP3A5, followed by 2-OH-E_1 and *y*-OH-E_1. Accordingly, although the ratio of E_2 4- to 2-hydroxylation by CYP3A5 was 0.53, its ratio for E_1 4- to 2-hydroxylation was 1.26, which was the second highest ratio for E_1 (next to CYP1B1) among the 15 isoforms tested.

Our enzyme kinetic analysis showed that the capacity (V_{MAX}) of CYP3A5-mediated 2- and 4-hydroxylation of E_2 was approximately 6 and 2 times higher, respectively, than its catalytic capacity for E_1, but its affinity ($1/K_M$) for the 2- and 4-hydroxylation of E_2 was significantly lower than its affinity for E_1. Therefore, similar to CYP3A4, CYP3A5 had a relatively lower affinity but a higher capacity for the 2- and 4-hydroxylation of E_2, but it had a relatively higher affinity but a lower capacity for the 2- and 4-hydroxylation of E_1. The V_{MAX}/K_M ratio of CYP3A5-mediated 2-hydroxylation of E_2 was higher than that for the 2-hydroxylation of E_1, but the V_{MAX}/K_M ratios for the 4-hydroxylation of E_2 and E_1 were similar.

Our results showed that CYP3A5 also formed a few other hydroxy-E_2 metabolites (6β-OH-E_2, 16α-OH-E_2, and 16β-OH-E_2) from E_2. Similarly, several other hydroxy-E_1 metabolites (6β-OH-E_1, 16α-OH-E_1, and 16β-OH-E_1) were formed from E_1 in substantial quantities. In addition, we found that large amounts of nonpolar metabolites (collectively labeled as X in Fig. 5) were also formed during incubations of CYP3A5 with E_2 or E_1 as substrate. It is noteworthy that CYP3A4 and CYP3A5 had much higher activity for the formation of these nonpolar metabolite peaks than the other CYP isoforms.

Notably, a recent study suggested that hepatic CYP3A5 is expressed in approximately one third of Caucasians and approximately 60% of African-Americans tested (56). Quantitatively, CYP3A5 has been found in 10–30% of hepatic samples and estimated to be 10–30% of the CYP3A4 content (57), and in certain individuals it might account for as high as approximately 50% of the total CYP3A content (56). Because it is known that CYP3A5 in human liver has a polymorphic distribution (57, 58), this CYP isoform likely is one of the genetic factors that may affect the interindividual and interracial differences in the hepatic formation of catechol estrogens, particularly the 4-hydroxylated estrogens. In light of the mounting evidence for an important etiological role of 4-hydroxyestrogen metabolites in estrogen-induced cancer (32), we believe that it would be of considerable interest to determine whether this polymorphism correlates with the amounts of 4-hydroxylated estrogens produced in humans as well as the risk for estrogen-associated human cancers.

CYP3A7 + b_5*.* CYP3A7 was originally found in human fetal liver where it accounted for 30–50% of the total CYP content (59, 60). Additional studies also suggested the presence of constitutive or induced expression of CYP3A7 in adult human liver (61–63), although quantitative data on its level is still not available, partly due to the lack of specific antibodies.

Our results showed that CYP3A7 (coexpressed with cytochrome b_5) had a very different metabolite profile from those of CYP3A4 and 3A5. When E_2 was the substrate, CYP3A7 had moderate activity for its 2-hydroxylation (146 pmol/nmol·min), and small amounts of 6β-OH-E_2, 16α-OH-E_2, and *y*-OH-E_2 were also formed. However, with E_1 as

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Regioselective Hydroxylation of E_2 and E_1 by Human CYP Isoforms

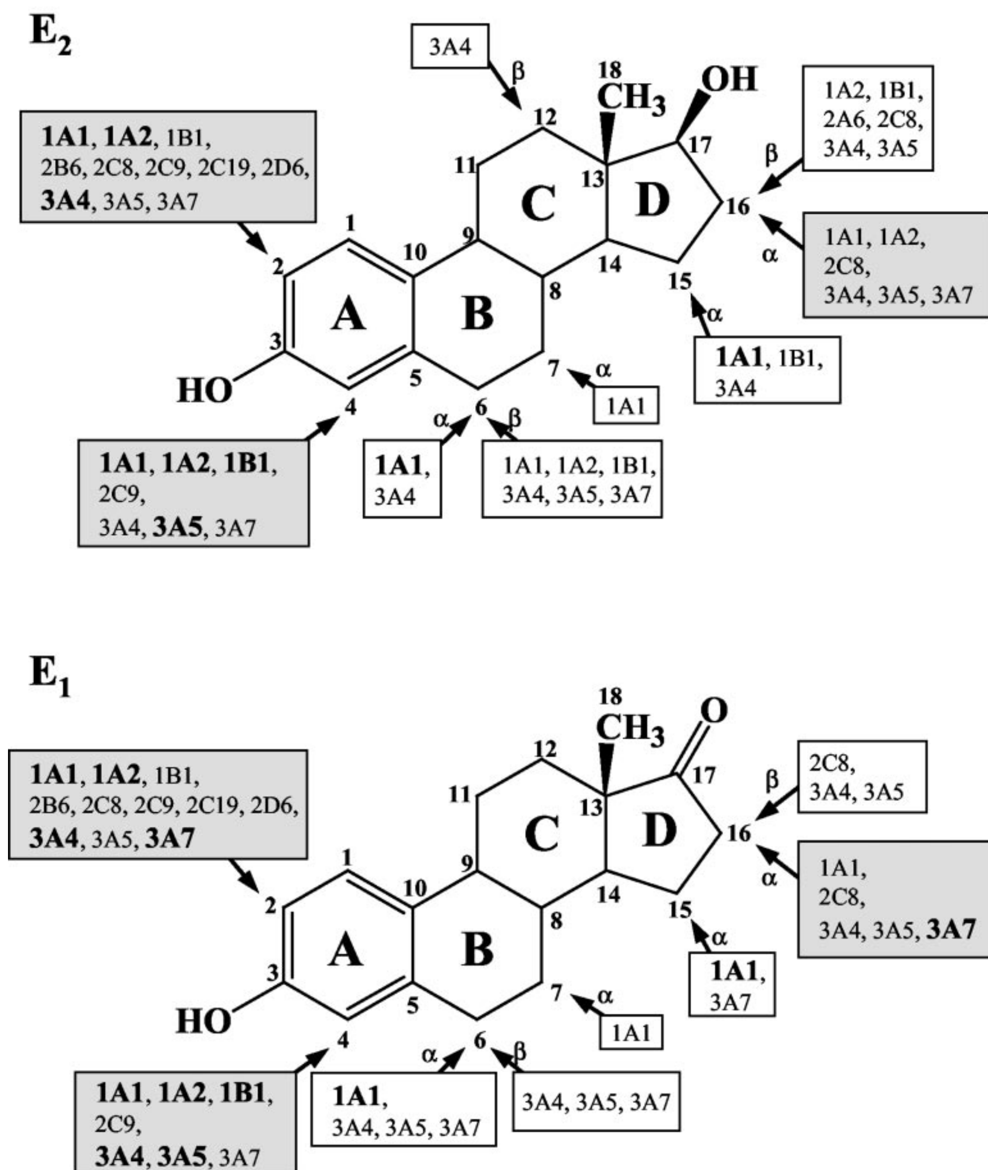

FIG. 7. A summary of the regioselective hydroxylation of E_2 (*top*) and E_1 (*bottom*) by 15 human CYP isoforms tested in the present study. Note that a *larger boldface font* is used to denote the CYP isoform that has either a uniquely high activity or a distinct regioselectivity for catalyzing a given metabolic reaction.

substrate, CYP3A7 had modest catalytic activity for the formation of 2-OH-E_1 (335 pmol/nmol·min), but it had the highest catalytic activity for the formation of two coeluted metabolites 16α-OH-E_1 and 6β-OH-E_1 (with the former accounting for >90%) at a combined rate of 346 pmol/nmol·min. This is the first demonstration that human CYP3A7 has a distinct high activity for the 16α-hydroxylation of E_1, but this was not observed with E_2.

Our data showed that although E_1 was a very good substrate for CYP3A7-mediated 16α-hydroxylation, E_2 was essentially not 16α-hydroxylated by this CYP isoform. We hypothesize that the presence of a 17-keto group in the steroid is essential for it to be a suitable substrate for CYP3A7. In support of this hypothesis, an earlier study also showed that although CYP3A7 catalyzed the 16α-hydroxylation of dehydroepiandrosterone and its 3-sulfate (both have a 17-keto group), it did not catalyze the 16α-hydroxylation of testosterone or cortisol (both lack a 17-keto group) (54, 64).

It is of interest to point out that the long-held view that 16α-hydroxylation only occurred with E_1 as the substrate (65) appears to be largely true in the case of CYP3A7 as catalyst. However, it is also worth noting that when the 16α-hydroxylation of E_2 or E_1 was recently analyzed with 33 adult human liver microsomes (34, 35), we found that the average rates for

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the 16α-hydroxylation of these two estrogens were very low and similar, thus suggesting that the contribution of CYP3A7 to the overall hepatic estrogen metabolism in humans is very minor. Nonetheless, because CYP3A7 is known to be also expressed in human uterine endometrium and placenta (66), the extent of its contribution to the *in situ* as well as systematic formation of 16α-OH-E_1 remains to be determined. In this context, it is also of note that because CYP3A7 expression in human liver and intestine appears to have a polymorphic distribution, with an estimated approximately 11% of Caucasians belonging to a distinct subgroup of high expression phenotype (67), it may be of interest to determine whether individuals with the high expression phenotype produce more 16α-OH-E_1 and whether they have an increased risk for breast cancer.

In summary, CYP3A4 and 3A5 catalyzed the metabolism of E_2 to various hydroxylated estrogens, generally with 2-hydroxyestrogens as the predominant metabolites. However, when E_1 was substrate, CYP3A5 formed more 4-hydroxyestrone than 2-hydroxyestrone, giving an unusually high ratio of E_1 4- to 2-hydroxylation (1.26). CYP3A4 and 3A5 also catalyzed the formation of large amounts of nonpolar metabolites from E_2 and E_1. CYP3A7 had moderate activity for the 2-hydroxylation of E_2 and E_1, but it had distinct catalytic activity for the 16α- and 6β-hydroxylation of E_1.

CYP4A11

CYP4A11 did not have detectable activity for the oxidative metabolism of E_2 or E_1.

Conclusions

The results of our present study showed that a variety of human CYP isoforms were catalytically active for the NADPH-dependent oxidative metabolism of E_2 and E_1, with a varying degree of catalytic activity and distinct regioselectivity (summarized in Fig. 7). For the 2-hydroxylation of E_2 and E_1, CYP1A2 had the highest catalytic activity, but CYP1A1, 3A4, and 3A5 also had relatively high catalytic activity. Several CYP2 family enzymes also had modest activity for the 2-hydroxylation of estrogens. For the 4-hydroxylation of E_2 and E_1, CYP1B1 had selective activity, but CYP3A5 also had distinct activity for the 4-hydroxylation of estrogens (E_1 in particular). CYP1A1, 1A2, and 3A4 had a modest activity for estrogen 4-hydroxylation. Except for CYP1B1 and CYP3A7, most of CYP enzymes had a higher ratio of 4- to 2-hydroxylation with E_1 as substrate than with E_2. For the 15α-hydroxylation of E_2 and E_1, CYP1A1 was the only isoform that had high catalytic activity, although very small amounts of 15α-OH-E_2 were also formed with CYP1B1 and 3A4. For the 16α-hydroxylation of E_1, CYP3A7 had distinct high activity, but it only had very weak activity for the 16α-hydroxylation of E_2. CYP1A1, 3A4, and 3A5 showed a weak activity for the 16α-hydroxylation of both E_2 and E_1. In addition to the quantitatively major metabolic pathways at the C-2, C-4, C-15α, and C-16α positions with certain CYP isoforms studied here, E_2 and E_1 were also oxidatively metabolized at several other positions (such as C-6α, C-6β, C-7α, C-16β) by some of the CYP isoforms (summarized in Fig. 7). Given the fact that some of the estrogen metabolites (such as 4-OH-E_2, 15α-OH-E_2, 16α-OH-E_1, and 2-methoxyestradiol) may have unique biological functions that are not associated with their parent hormones E_2 and E_1 (1, 31–33), we believe that the detailed knowledge provided in this study regarding the complete profiles of estrogen metabolites formed by various human CYP isoforms would be very helpful to the understanding of their biological functions in different target cells that are known to preferentially express certain CYP isoforms.

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