Role of Polymorphic Human Cytochrome P450 Enzymes in Estrone Oxidation

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Abstract

Estrogen and its metabolites are believed to play important roles in breast cancer. The influence of genetic polymorphisms in the enzymes responsible for formation and disposition of estrogen on breast cancer risk may shed light on the importance of estrogen metabolites in this disease. However, for such studies to be valid, it is important to correctly identify the enzymes involved in estrogen bioactivation. Therefore, we assessed the human cytochrome P450–dependent oxidation of estrone using substrate concentrations that more closely approximate the maximum expected concentrations in breast tissue. The *in vitro* metabolism of estrone by recombinant human cytochrome P450 enzymes and human liver microsomes was studied. The formation of estrone metabolites (2-hydroxyestrone, 4-hydroxyestrone, and 16α-hydroxyestrone) was monitored by high-performance liquid chromatography. 2-Hydroxyestrone formation was catalyzed predominantly by CYP1A2, CYP1A1, and CYP1B1 enzymes; 4-hydroxyestrone formation was catalyzed predominantly by CYP1B1, CYP1A2, and CYP1A1 enzymes; and 16α-hydroxyestrone formation was catalyzed predominantly by CYP2C19, CYP1A1, and CYP3A5. This study confirms the important role of members of the CYP1 family in the 2-hydroxylation and 4-hydroxylation of estrone, but the enzymes identified as responsible for the 16α-hydroxylation of estrone are different from those previously identified. The relative importance of these enzymes *in vivo* would depend on the specific tissue expression of the enzymes. These enzymes are all known to be genetically variant in the human population, and additional studies to assess the role *CYP1A2*, *CYP2C19*, and *CYP3A5* in breast cancer risk are indicated. (Cancer Epidemiol Biomarkers Prev 2006;15(3):551–8)

Introduction

Identification of endogenous and exogenous factors influencing breast cancer is necessary for a reliable assessment of risk and for designing strategies for controlling the disease. The endogenous estrogens (E) estradiol (E_2) and estrone (E_1) contribute to breast cancer risk through their role as growth promoters (1-4). Estrogen metabolites may also contribute to the initiation and promotion of breast cancer through the formation of DNA-damaging species or activation of the estrogen receptor (1, 4-7). Two major catechol metabolites, 4-hydroxy-estrogens (4-OH-Es) and 2-hydroxy-estrogens (2-OH-Es), have been implicated in breast cancer risk. Catecholestrogens are unstable metabolites that can lead to the generation of more reactive semiquinones and quinones and produce DNA-damaging reactive oxygen species (1, 5, 8-11). The 4-OH-Es are more unstable than the 2-OH-Es (12). The 4-OH-Es are equivalent in estrogenic potency but longer-acting than the parent estrogens because of a decreased dissociation from the estrogen receptor (13, 14). Although 2-OH-Es have some inherent estrogenic activity, they are considerably less potent than the parent estrogen and may therefore function as antiestrogens *in vivo* (14-18). Thus, 4-OH-Es are thought to increase breast cancer risk, whereas 2-OH-Es are thought to be protective.

Another major oxidative pathway is the formation of 16α-hydroxyestrogens (16α-OHE). 16α-OH-estrone is generally considered to be estrogenic and is thought to be associated with an increased risk of breast cancer (19, 20). 16α-OH-estrone has been shown to be genotoxic and cause aberrant proliferation of mammary cells (21, 22) and to covalently bind to the estrogen receptor (23).

The significance of these three estrogen metabolites in human breast cancer remains controversial. The major pathways for metabolism of estradiol are through conversion to estrone and oxidation to 2-OH-estrone and 16α-OH-estrone. Therefore, the majority of attention has focused on the formation of these metabolites. Many studies have shown an association between the urinary ratio of 2-OH-estrone to 16α-OH-estrone and breast cancer risk, but other studies have not found such an association (24-27). Another approach to understanding the significance of these metabolites *in vivo* has been to assess the influence of genetic polymorphisms in the enzymes responsible for their formation and disposition on breast cancer risk. In order for these studies to be interpreted, it is important to know the enzyme specificity of estrogen hydroxylation reactions.

Several studies have explored the specificity of these reactions, but the majority of studies were done at estrogen concentrations in the range of 50 to 100 μmol/L and often used prolonged incubation times to generate metabolites for quantification (28-37). Plasma concentrations of estrogen are generally very low, ranging between 0.4 and 70 nmol/L (38). Tissue concentrations of estrogens are not directly linked to circulating estrogen concentrations as a result of local synthesis, local degradation, and other factors (38-40). Tissue concentrations of estrogens in the breast are generally much higher than plasma concentrations, with estrone and estrone sulfate concentrations as high as 3 to 5 μmol/L in breast tissue or fluids (38, 41, 42). Furthermore, up to 80% of total estrogens

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found in breast tissue are in hydroxylated forms, suggesting local metabolism (39). The endogenous concentrations of estrone, whether circulating or in tissue, are considerably below those previously used to assess the specificity of CYP-mediated estrogen oxidation. Assessment of CYP specificity at high substrate concentrations, without adequate consideration of the expected *in vivo* concentrations, may result in erroneous identification of important enzymes. Therefore, we assessed the role of human cytochrome P450 enzymes in the oxidation of estrone at lower substrate concentrations and using shorter incubation times.

Materials and Methods

Chemicals and Reagents. Estrone, 2-OH-estrone, 4-OH-estrone, and 16α-OH-estrone were purchased from Steraloids, Inc. (Newport, RI). Chloramphenicol, furafylline, ketoconazole, omeprazole, sulfaphenazole, $NADP^+$, reduced form (NADPH), and other routine chemicals were purchased from Sigma Chemical (St. Louis, MO). Ascorbic acid and high-performance liquid chromatography (HPLC)-grade acetonitrile were purchased from Fisher Scientific (Atlanta, GA). Perchloric acid was purchased from BDH Laboratory Supplies, VWR International (Missisauga, Ontario, Canada). *N*-3-benzyl-nirvanol was purchased from BD Biosciences Gentest Products and Services (Woburn, MA).

Microsomes and Recombinant Enzymes. This study was approved by the UPEI Ethics Committee. Pooled microsomes and single donor human liver microsomes were purchased from BD Biosciences Gentest Products and Services. Single donors included H006, HG23, HK27, HG30, HK31, HG42, HG43, HG56, HG89, HG93, HG103, HH2, HH9, HH31, HH48, and HH108. Microsomes were supplied at a protein concentration of 20 mg/mL in 250 mmol/L sucrose. The activities of known CYP substrates were provided by the supplier and were determined according to published protocols. Human recombinant baculovirus-expressed enzymes CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2B6, CYP2C8, CYP2C9*1, CYP2C9*2, CYP2C9*3, CYP2C18, CYP2C19, CYP2D6*1, CYP2E1, CYP2J2. CYP3A4, CYP3A4 + b5, CYP3A5, CYP3A7 + b5, CYP19, CYP4A11, and CYP4F12 were purchased from BD Biosciences Gentest Products and Services. Recombinant enzymes were suspended in 100 mmol/L Tris (pH 7.5), and the specific P450 content was provided by the supplier. Microsomes and recombinant enzymes were stored at −80°C until use.

Enzyme Assays. Microsomal incubations containing 1 mg/mL human liver microsomes, ascorbic acid (1 mmol/L), and NADPH (1 mmol/L) in PBS (pH 7.4; final volume of 250 μL) were preincubated in a shaking water at 37°C for 3 minutes. The reaction was then initiated by the addition of estrone at the specified concentrations. Estrone was prepared as a 100 mmol/L stock in DMSO and diluted so that the final concentration of solvent in the assays did not exceed 1%. The reaction was incubated in a shaking water bath for 10 minutes until terminated with the addition of 25 μL ice-cold 15% perchloric acid. The mixture was cooled on ice and then centrifuged for 10 minutes at 8,000 × *g*. The resulting supernatant was removed for HPLC analysis.

Recombinant enzyme assays were conducted in a similar fashion, except that the reaction was initiated with the addition of enzyme, rather than estrone, and the reactions were terminated after 5 minutes (or other time as specified). The concentration of recombinant enzymes used ranged from 10 to 100 pmol/mL and was adjusted as required to ensure linear formation of metabolites. Organic solvents can negatively affect the activity of cytochrome P450 enzymes (43-45). DMSO was routinely used as a solvent, but acetonitrile was employed for CYP2C19, CYP2C9, CYP3A4, and CYP3A5 to minimize effects on enzyme activity.

For inhibition studies, inhibitors were used at the concentration specified in the figures. All inhibitors were added before the addition of estrone. Furafylline was preincubated with the microsomes for 10 minutes at 37°C with NADPH before addition of estrone. Inhibition is expressed as the percent of the corresponding solvent control.

Immunoinhibition. Inhibitory monoclonal antibodies to the human cytochrome P450 1A2 (clone 26-7-5), 2C9 (clone 763-15-5), 2E1 (clone 1-73-18), 3A4/5 (clone 3-29-9), 2C19 (clone 1-7-4-8), and egg lysozyme (clone Hy-Hel-9; to serve as control) were generously provided by Dr. Harry Gelboin (National Cancer Institute, NIH, Rockville MD). Human-pooled microsomes (1 mg/mL), monoclonal antibodies (5, 10, or 20 μL), and PBS were preincubated for 5 minutes at 37°C in a shaking water bath. Ascorbic acid (1 mmol/L) and estrone (2.5 and 25 μmol/L in 0.5% ACN) were added and warmed in the shaking water bath for a further 2 minutes. The reaction was initiated with NADPH and incubated in the shaking water bath for 5 minutes. Control incubations were conducted using antibodies against egg lysozyme and antibodies not anticipated to inhibit estrone metabolism based on recombinant enzyme studies (e.g., CYP2E1 clone 1-73-18). The reaction was terminated with the addition of ice-cold 15% perchloric acid and centrifuged for 10 minutes at 8,000 × *g*. The resulting supernatant was removed for analysis by HPLC, as described below.

HPLC Analysis of Metabolites. Analysis of metabolites was done with an HPLC system (Shimadzu LC-10A series HPLC system equipped with two LC-10AT VP pumps, an SIL 10A autoinjector, an SCL-10A VP system controller, an RF-10AXL fluorescence detector, and Class-VP Chromatography Laboratory Automated Software; Shimadzu Scientific Instruments, Inc., 1998, Columbia, MD) with an Ultracarb 5-μm ODS (30) 4.6 × 150 mm analytic column (Phenomenex, Inc., Torrance, CA). Fifty microliters of the reaction supernatant was injected per run. Metabolites were separated with acetonitrile/water gradient at a flow rate of 1.25 mL/min with a linear gradient from 25% acetonitrile to 50% acetonitrile over 30 minutes. The column was then stabilized with 25% acetonitrile for 15 minutes before initiating the next run. Retention times for the estrogen standards were 16α-OH-estrone, 12.5 minutes; 2-OH-estrone, 19.0 minutes; 4-OH-estrone, 20.4 minutes; and estrone, 26.6 minutes. Metabolites were detected with a fluorescent detector with an excitation of 280 nmol/L and an emission of 445 nmol/L. Analytes were quantified against an external standard curve. Limits of quantitation were 2, 1, and 8 pmol, respectively, with coefficient of variations of 15% to 22% at the lowest concentration and <10% at higher concentrations.

Data Analysis. All incubations with recombinant enzymes were done in duplicate or triplicate, and experiments were repeated at least twice to confirm results. Experiments with >10% deviation between replicates were repeated to confirm results. As all incubations with recombinant enzymes were conducted with the same preparations, only the mean is reported. K_m and V_{max} values were derived from Hanes-Woolf plots. For experiments with human liver microsomes, a minimum of three individual livers (analyzed in duplicate or triplicate) were used and the results are presented as the mean ± SE. Correlations were assessed by Spearman's rank correlation coefficient. Effects of inhibitors were compared by one-way ANOVA to the corresponding vehicle control, with post hoc comparison with control by Bonferroni corrected *t* test. $P < 0.05$ was accepted as being statistically significant. All statistical analyses were done using GraphPad Prism version 3.03 (San Diego, CA).

Results

Initial experiments determined that the linearity with time of estrone metabolism with recombinant enzymes and human

liver microsomes was relatively short, regardless of whether NADPH or an NADPH-generating system was used. The linearity of product generation varied from 7.5 minutes up to 10 to 20 minutes, depending on the specific conditions (Fig. 1). Linearity of estrone disappearance was maintained with a loss of substrate of up to 20% at lower concentrations (2.5-5 μmol/L). Experiments with rat hepatic microsomes and recombinant human CYP enzymes revealed that NADPH-dependent metabolism of the metabolites occurred, causing a loss of metabolites with time (data not shown). This may contribute to loss of linearity.

To further assess the effect of prolonged incubation times on the results, kinetic studies were done with three recombinant enzymes and human liver microsomes using incubation times of 5 and 30 minutes (Table 1). The use of extended incubation times resulted in higher apparent K_m values and, in some cases, reduced V_{max} values. Therefore, to work as close as possible to initial rate conditions for estimation of kinetic values, incubation times were kept to 5 minutes for recombinant enzymes and to 10 minutes when performing studies with human liver microsomes.

Multiple CYP enzymes were capable of catalyzing hydroxylation of estrone. The kinetics of estrone metabolism by a suite of recombinant enzymes was determined using a substrate concentration range of 1 to 50 μmol/L and an incubation time of 5 minutes (Table 2). To allow comparison of our results with

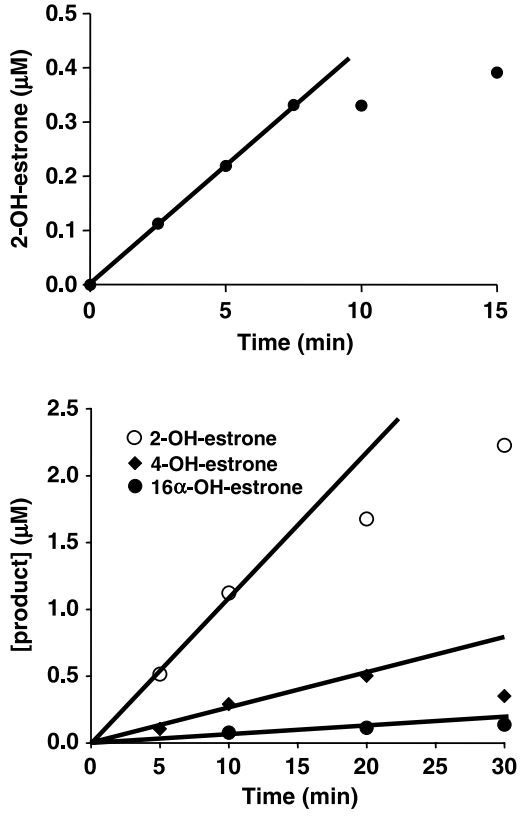

Figure 1. Linearity of estrone metabolism with time in *in vitro* incubations. Linearity of 2-OHE_1 formation from estrone by recombinant CYP2C9 (*top*; 5 μmol/L estrone, 1 mmol/L NADPH, 230 pmol/mL CYP2C9) or human liver microsomes (*bottom*; 10 μmol/L estrone, 1 mmol/L NADPH, 1 mg/mL human liver microsomes). CYP2C9 only formed 2-OH-estrone. Based on similar results with other recombinant enzymes and a variety of conditions with human liver microsomes, further reactions were restricted to 5 minutes with recombinant enzymes and a maximum of 10 minutes with human liver microsomes for inhibition studies.

Table 1. Effect of prolonged incubation times on the determination of kinetic parameters of recombinant CYP enzymes

CYP	Incubation time (min)	K_m (μmol/L)	V_{max} nmol/nmol/min)
CYP1A2	5	3.8	28
	30	5.6	27
CYP3A4	5	16	0.5
	30	39	0.6
CYP3A5	5	32	0.8
	30	76	0.5
Human liver*	5	30	2.9
	30	54	1.0

*Pooled human liver, lot 18 from BD Biosciences Gentest Products and Services.

previously published results, a table of turnover at concentrations of 1, 5, and 50 μmol/L is provided (Table 3). The CYP1 family enzymes were the most active, particularly towards 2-hydroxylations and 4-hydroxylation. The apparent K_m values for all detectable estrone hydroxylations by these enzymes were <10 μmol/L. CYP3A5 also catalyzed all three hydroxylations with apparent K_m values of ~10 μmol/L. Addition of cytochrome b_5 enhanced the activity of CYP3A5 and CYP3A4. CYP2C19 also supported the 2-hydroxylation and 16α-hydroxylation reactions.

The correlation of estrone oxidation with catalytic markers of specific CYP activities was assessed using a panel of 16 adult livers, including males and females (Table 4). The formation of 2-OH-estrone correlated with the marker activities of CYP1A2 and CYP2C19 (Fig. 2, *top*), whereas 4-OH-estrone formation correlated only with CYP2C9-mediated marker activity and with the formation of 2-OH-estrone. The formation of 16α-OH-estrone correlated with marker activities for CYP2C19 and CYP1A2 (Fig. 2, *bottom*). Interpretation of these results is confounded by a correlation between CYP2C19 and CYP1A2 marker activities (Spearman's $\rho = 0.62$, $P = 0.01$). When a substrate concentration of 2.5 μmol/L estrone was used, only 2-OH-estrone formation was identified in sufficient quantity to enable accurate correlation studies. 2-OH-estrone formation at this substrate concentration correlated with CYP1A2 ($\rho = 0.69$, $P = 0.02$) and CYP2C9 ($\rho = 0.74$, $P = 0.01$) marker activities. Furthermore, 2-OH-estrone formation was highly correlated with 4-OH-estrone formation but not 16α-hydroxylation. At a substrate concentration of 25 μmol/L, 2-hydroxylation and 16α-hydroxylation were correlated ($\rho = 0.75$, $P = 0.001$).

To further explore the contribution of specific CYP forms to estrone metabolism in the liver, chemical and immunologic inhibition studies were carried out. Immunoinhibition studies were attempted with monoclonal antibodies. Unfortunately, incubations with control antibodies led to increased metabolism of low concentrations of estrone and erratic results so that no conclusive results were obtained.

Inhibition studies done at 25 μmol/L estrone substrate concentrations (Fig. 3) showed significant inhibition of 2-OH-estrone formation by the CYP1A2 inhibitor furafylline, by the CYP3A/2C9 inhibitor ketoconazole, and by the CYP2C19/CYP2C9 inhibitor omeprazole. The effect of nirvanol, a specific CYP2C19 inhibitor, was not significant, as it varied from ~35% to <3% inhibition. However, this inhibition was inversely correlated to the activity of CYP2C19 in the liver microsomes ($r^2 = 0.99$; $P < 0.01$), suggesting that CYP2C19 did contribute when present in sufficient quantities. When the concentration of estrone was reduced to 2.5 μmol/L, furafylline reduced 2-OH-estrone formation to below detectable levels. Inhibition of the other two metabolites could not be accurately assessed at this low substrate concentration.

Table 2. Apparent K_m and V_{max} of estrone metabolism by recombinant human CYP enzymes *in vitro*

CYP	2-Hydroxylation			4-Hydroxylation			16α-Hydroxylation		
	K_m (μmol/L)	V_{max} (nmol/nmol/min)	Cl_i	K_m (μmol/L)	V_{max} (nmol/nmol/min)	Cl_i	K_m (μmol/L)	V_{max} (nmol/nmol/min)	Cl_i
CYP1A1	3.1	7.9	2.6	1.3	0.8	0.6	3.7	0.4	0.1
CYP1A2	3.8	28.4	7.5	2.1	3.8	1.8	ND	ND	ND
CYP1B1	2.0	0.7	0.3	0.6	1.8	3.0	ID	ID	ID
CYP2C9	9.8	0.9	0.09	ND	ND	ND	ND	ND	ND
CYP2C19	10	6.2	0.6	ND	ND	ND	10	0.9	0.09
CYP3A4	16	0.5	0.03	ND	ND	ND	ND	ND	ND
CYP3A4 (+b5)	26	9.8	0.4	ID	ID	ID	ID	ID	ID
CYP3A5	32	0.8	0.025	13	0.5	0.04	13	0.2	0.02
CYP3A5 (+b5)	28	0.7	0.025	22	1.4	0.06	12	0.9	0.08

Abbreviations: ND, no metabolite detected; ID, insufficient data points (see Table 3 for more data); Cl_i = relative intrinsic clearance calculated as V_{max}/K_m.

There was significant inhibition of 16α-OH-estrone formation by the CYP2C19 inhibitors nirvanol and omeprazole. The effects of ketoconazole and furafylline were not significant Fig. 3). Ketoconazole (10 μmol/L) and furafylline (10 μmol/L) both reduced formation of 4-OH-estrone to below quantifiable concentrations at a substrate concentration of 25 μmol/L in three separate livers, whereas nirvanol had no consistent effect.

Discussion

Pharmacogenetic studies designed to assess the influence of genetic polymorphisms in metabolizing enzymes on outcomes require knowledge of the specific enzymes involved in the metabolic pathways. To gain a better understanding of the role of specific CYP enzymes in estrone metabolism, we

Table 3. Metabolism of estrone by recombinant human cytochrome P450 enzymes at substrate concentrations of 1, 5, and 50 μmol/L

CYP enzyme	Estrone (μmol/L)	2-OH-estrone (nmol/nmol P450/min)	4-OH-estrone (nmol/nmol P450/min)	16α-OH-estrone (nmol/nmol P450/min)
CYP1A1	1	1.1	ND	ND
	5	4.9	0.95	0.19
	50	7.4	0.76	0.41
CYP1A2	1	1.8	0.51	ND
	5	10.9	2.5	ND
	50	20.1	4.8	ND
CYP1B1	1	0.19	0.96	ND
	5	0.47	1.8	ND
	50	0.65	1.9	ND
CYP2B6	1	ND	ND	ND
	5	0.15	ND	ND
	50	0.30	ND	ND
CYP2C9	1	ND	ND	ND
	5	0.39	ND	ND
	50	0.50	ND	ND
CYP2C19	1	0.49	ND	ND
	5	2.2	ND	0.30
	50	5.1	ND	0.78
CYP2D6	1	ND	ND	ND
	5	0.36	ND	ND
	50	0.89	ND	ND
CYP3A4	1	ND	ND	ND
	5	ND	ND	ND
	50	0.46	ND	ND
CYP3A4 (+b5)	1	0.46	ND	ND
	5	1.4	ND	ND
	50	6.6	1.21	0.32
CYP3A5	1	ND	ND	ND
	5	ND	ND	ND
	50	0.46	0.27	0.19
CYP3A5 (+b5)	1	ND	ND	ND
	5	0.12	ND	0.27
	50	0.49	0.54	0.73
CYP3A7 (+b5)	1	ND	ND	ND
	5	0.55	ND	0.34
	50	1.6	ND	0.86
Human microsomes	1	0.065	ND	ND
	5	0.23	ND	ND
	50	0.69	0.22	0.043

NOTE: Detected but below the limits of quantitation (0.15 nmol/nmol/min). All reported values were obtained with CYP concentrations of 10 to 100 pmol/L (adjusted to ensure linearity) with incubation times of 5 minutes. Enzymes not showing metabolism were tested at a concentration of at least 100 pmol/mL and as high as 500 pmol/mL to identify possible metabolites. Extended incubation times of up to 30 minutes were used to identify possible metabolites, but results were not used to determine rates. Reported rates are the mean of at least duplicate determinations. Additional enzymes assessed for which no significant turnover was detected even at elevated CYP concentrations and prolonged incubation times included CYP2A6, CYP2C8, CYP2C18, CYP2E1, CYP2J2, CYP4A11, and CYP4F12.
Abbreviation: ND, not detected.

Table 4. Spearman's correlation coefficient of estrone hydroxylations with markers of specific CYP activities

CYP enzyme	Marker activity*	2-OH-estrone	4-OH-estrone	16α-OH-estrone
CYP1A2	Phenacetin-*O*-deethylase	0.83 ($P < 0.001$)	0.37 ($P = 0.46$)	0.57 ($P = 0.03$)
CYP2C9	Diclofenac-4′-hydroxylase	0.21 ($P = 0.43$)	0.63 ($P = 0.05$)	0.20 ($P = 0.47$)
CYP2C19	(*S*)-mephenytoin-4′-hydroxylase	0.60 ($P = 0.03$)	−0.30 ($P = 0.4$)	0.69 ($P = 0.005$)
CYP3A4	Testosterone 6β-hydroxylase	0.54 ($P = 0.03$)	0.54 ($P = 0.11$)	0.43 ($P = 0.1$)
CYP3A4 protein		0.64 ($P = 0.01$)	0.31 ($P = 0.42$)	0.79 ($P = 0.001$)
CYP3A5 protein		−0.37 ($P = 0.17$)	0.28 ($P = 0.47$)	−0.43 ($P = 0.12$)
	2-OH-estrone formation		0.66 ($P = 0.04$)	0.70 ($P = 0.003$)

NOTE: Results obtained with 16 adult livers obtained from BD Biosciences Gentest Products and Services: HOO6, HG23, HK27, HG30, HK31, HG42, HG43, HG56, HG89, HG93, HG103, HH2, HH9, HH31, HH48, and HH108. Incubations were done with a substrate concentration of 25 μmol/L estrone (dissolved in DMSO), 1 mg/mL of human liver microsomes, and 1 mmol/L NADPH for 10 minutes.
*Marker activities and 3A protein content were provided by BD Biosciences Gentest Products and Services.

investigated estrone metabolism at lower estrone concentrations and shorter incubations times than had previously been employed. Using this approach, we have identified a different P450 specificity profile.

The studies with recombinant enzymes showed that multiple CYP enzymes are capable of catalyzing estrone hydroxylation. Of the enzymes evaluated, CYP1A1 and CYP1B1 are not expressed in any significant quantity in the liver. Therefore, they would not be expected to contribute to the overall systemic metabolism of estrone. However, both enzymes have been identified in breast tissue (46, 47). CYP1A1 displayed relatively high activity for all three hydroxylations, suggesting that it may play an important role in extrahepatic tissues where it is expressed. Although CYP1A1 was more active with regard to 2-hydroxylation and 4-hydroxylation, it also displayed one of the greatest 16α-hydroxylating activities of the CYP enzymes tested. CYP1B1 displayed a high affinity for estrone and preferentially catalyzed 4-hydroxylation over 2-hydroxylation. CYP1B1 did not seem to contribute to 16α-hydroxylation. These results are consistent with previously published results, where CYP1B1 was shown to preferentially catalyze 4-hydroxylation, whereas CYP1A1 was shown to preferentially catalyze 2-hydroxylation (31, 48, 49). We report here higher V_{max} and lower apparent K_m values for 2-hydroxylation and 4-hydroxylation by both enzymes compared with previous studies (despite the same source of recombinant enzymes being used in one study; ref. 31). This is likely attributable to the shorter incubation times employed in this study, as illustrated in Table 1. Huang et al. (30) reported 16α-hydroxylation by CYP1A1 but provided no kinetic assessment, whereas no activity by CYP1B1 has been reported. It has been reported that CYP1A1 can catalyze the 16α-hydroxylation of estradiol (50).

Among the CYP enzymes expressed in the liver, CYP1A2 was the most active with regard to 2-hydroxylation. When the relative intrinsic clearances of the various enzymes were corrected for the differing expression levels in the typical human liver (51, 52), the predicted contribution from CYP1A2 was eight times higher than other CYP enzymes (Table 5). The CYP2C and CYP3A forms would be expected to have a higher contribution as the substrate concentration of estrone increased. These predictions were borne out by the correlation between 2-hydroxylation of 25 μmol/L estrone and marker activities for CYP1A2, CYP2C19, and CYP3A4 (Table 4). Inhibitors of CYP1A2, CYP3A4, and CYP2C19/2C9 were all able to inhibit the formation of 2-OH-estrone, confirming the contribution of multiple enzymes to this metabolic pathway at higher estrone concentrations. One would predict that at lower substrate concentrations, CYP1A2 would be the predominant enzyme involved. This was confirmed by the ability of furafylline to reduce 2-OH-estrone formation to below detectable levels at a substrate concentration of 2.5 μmol/L, and the loss of correlation with CYP2C19 and CYP3A4 marker activities at lower concentrations. Based on the results presented here, CYP1A2 is predominantly responsible for 2-hydroxylation of estrone, but CYP2C19, CYP3A4, and, to a lesser extent, CYP2C9 contribute as the substrate concentration increases.

These results are consistent with previously published studies indicating an important role for CYP1A2 in the liver

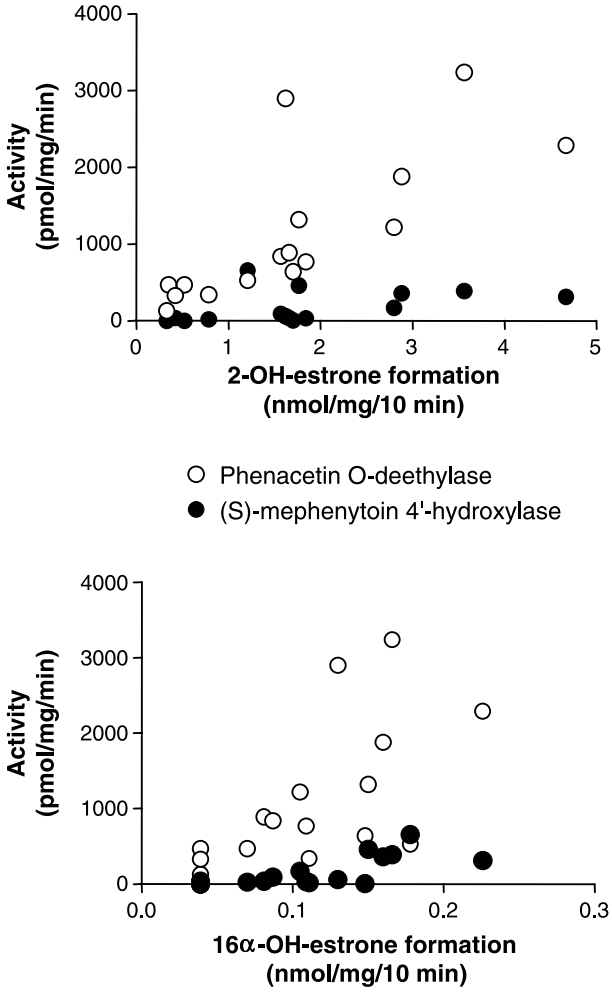

Figure 2. Correlation of estrone hydroxylations with marker activities in human liver microsomes. The formation of 2-OH-estrone (*top*) was significantly correlated with both phenacetin-*O*-deethylation (CYP1A2 marker activity; Spearman's $\rho = 0.83$, $P < 0.001$) and (*S*)-mephenytoin-4′-hydroxylation (CYP2C19 marker activity; Spearman's $\rho = 0.60$, $P = 0.03$). The formation of 16α-OH-estrone (*bottom*) was also significantly correlated with phenacetin-*O*-deethylation (Spearman's $\rho = 0.57$, $P = 0.03$) and (*S*)-mephenytoin-4′-hydroxylation (Spearman's $\rho = 0.69$, $P = 0.005$). Estrone hydroxylation was determined at a substrate concentration of 25 μmol/L, 1 mg/mL human liver microsomes, and a 10-minute incubation period.

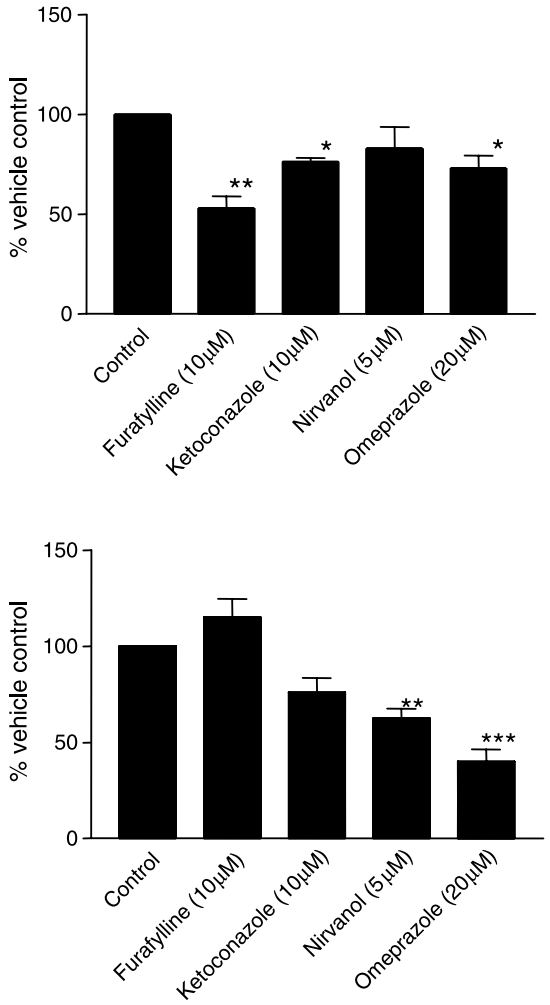

Figure 3. Inhibition of 2-OH-estrone (*top*) and 16α-OH-estrone formation by furafylline, ketoconazole, nirvanol, and omeprazole. From incubations with three to seven separate livers and expressed as percentage of the corresponding vehicle control. Inhibition was compared by one-way ANOVA and post hoc analysis with Bonferroni *t* test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

(31, 35, 36). Again, we observed higher velocities and lower apparent K_m values than previously reported. The extent of the contribution by CYP2C19 to 2-hydroxylation of estrone has been overlooked in previous studies, either as a result of not examining the recombinant enzyme or as a result of comparing activities at high substrate concentrations.

Based on the results from the recombinant enzymes, one would predict that CYP1A2 would be the major contributor at low estrone concentrations to 4-hydroxylation of estrone, but that CYP3A4/5 (see Table 3) could contribute as estrone concentrations increased. Only the CYP2C9 marker activity correlated with 4-hydroxylation of 25 μmol/L estrone in human hepatic microsomes. However, the lack of correlation with CYP1A2 marker activities may reflect the contribution of multiple enzymes at this high substrate concentration, as furafylline and ketoconazole both inhibited the formation of 4-OH-estrone. The correlation of 4-OH-estrone formation with 2-OH-estrone at low estrone (2.5 μmol/L) concentrations is consistent with a major role for CYP1A2. Furafylline and ketoconazole both inhibited the 4-hydroxylation reaction, supporting the involvement of CYP1A2 and suggesting a contribution from CYP3A4/CYP3A5 at the substrate concentration employed. Thus, our results are consistent with a predominant role of CYP1A2 in the liver and contributions from CYP3A4/CYP3A5 at higher substrate concentrations. These results are consistent with previously published results showing a dominant role for CYP1A2 (32, 35, 36). As with 2-hydroxylation, the apparent K_m was lower and the V_{max} higher than in previous studies.

Of the hepatic CYP enzymes, CYP2C19 and the CYP3A5 forms were most active in the formation of 16α-OH-estrone. Using our incubation conditions, we were not able to obtain an accurate estimate of the K_m and V_{max} for CYP3A4, because metabolism was not detected at the lower concentrations. However, it was capable of 16α-hydroxylation at higher substrate concentrations (data not shown) or longer incubation times and showed higher activity when supplemented with cytochrome b_5 (Table 3). As previously reported (32), CYP3A7 displayed 16α-hydroxylation activity (Table 3), but this enzyme is not normally expressed in significant quantities in adult tissues. From the recombinant data, CYP2C19 would be expected to be the major hepatic enzyme responsible for 16α-hydroxylation based on the relative expression of the different enzymes (Table 5). This prediction was borne out by the correlation data and the chemical inhibition studies. There was a weak correlation with CYP1A2 marker activity, but no significant inhibition by furafylline was observed; thus, the contribution of CYP1A2 was minor. In contrast, there was a significant correlation with CYP2C19 activity and a consistent inhibition by CYP2C19 inhibitors at a substrate concentration of 25 μmol/L. The weak correlation with CYP1A2 marker activity is explained by the correlation between CYP2C19 and CYP1A2. Ketoconazole showed weak, nonsignificant inhibition of 16α-OH-estrone formation, possibly indicative of a minor contribution from CYP3A4/CYP3A5 enzymes in the liver. The inhibition was not noticeably greater in livers with higher CYP3A5 expression.

Together, our results suggest that hepatic metabolism of estrone to 16α-OH-estrone is most likely mediated by CYP2C19

Table 5. Estimated contributions of different CYP enzymes to overall hepatic clearance of estrone based on expected quantity of CYP in the liver

CYP	Estimated pmol CYP/mg*	2-OH (Cl_i)	Relative hepatic clearance	4-OH (Cl_i)	Relative hepatic Clearance	16α-OH (Cl_i)	Relative hepatic clearance
CYP1A1	0	2.6	0	0.6	0	0.1	0
CYP1A2	45	7.5	338	1.8	81	—	—
CYP1B1	0	0.33	0	3.3	0	—	—
CYP2C9	96	0.09	8.6	—	—	—	—
CYP2C19	19	0.6	11.6	—	—	0.09	1.7
CYP3A4	108	0.03	3.2	—	—	—	—
CYP3A4 (+b5)	108	0.4	41	—	—	—	—
CYP3A5	1	0.02	0.02	0.01	0.01	0.02	0.02
CYP3A5 (+b5)	1	0.03	0.03	0.06	0.06	0.08	0.08

*Refs. 51 and 52.

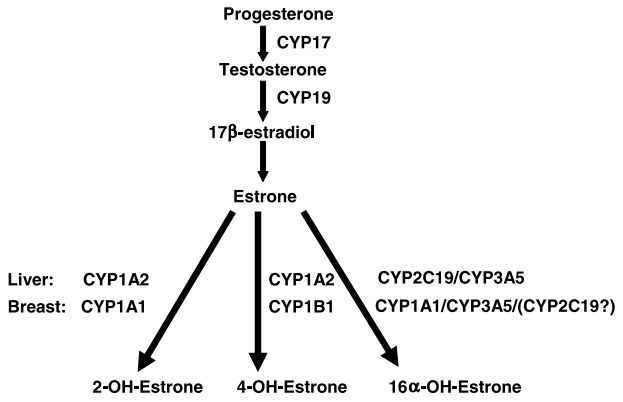

Figure 4. Postulated pathways of estrone metabolism *in vivo*. The probable contribution of different CYP enzymes to estrone metabolism *in vivo*. The question mark following CYP2C19 in breast tissue indicates that the expression of this enzyme has not been confirmed in this location.

and extrahepatic metabolism is mediated by CYP1A1, CYP2C19, and CYP3A5, depending on the relative expression of the enzymes and the endogenous estrone concentration. These conclusions are considerably different from previously reported studies that have identified CYP3A4/CYP3A5 as the predominant enzymes responsible for 16α-hydroxylation (30, 32, 35, 36). These differences may be attributed to the differing incubation conditions employed, in particular, lower substrate concentrations and shorter incubation times. The reported apparent K_m values for CYP3A4/CYP3A5 were much higher than the observed CYP3A5 K_m in this study (30, 35). Previous studies have shown that CYP2C19 can catalyze 16α-hydroxylation of estrone, but kinetic studies of CYP2C19 were not done with estrone as a substrate (30, 35, 36). CYP2C9 can also catalyze the reaction (30, 35, 36), but we only observed turnover at high substrate concentrations and with prolonged incubation times (data not shown). Lee did not observe 16α-hydroxylation with either CYP2C9 or CYP2C19 (31, 32).

These results are highly significant as numerous studies have tried to link genetic polymorphisms in CYP enzymes with breast cancer risk. Our results suggest that for estrone, the critical enzymes are CYP1A1, CYP1A2, CYP1B1, CYP2C19, and CYP3A5. Genetic polymorphisms that influence expression and activity have been identified for all five of these enzymes. However, only genetic polymorphisms in CYP1A1 and CYP1B1 have been studied in association with breast cancer risk to date (53-61), with conflicting results.

Estrone and estrone sulfate are the most abundant estrogens, particularly in postmenopausal women (40), and estradiol is predominantly cleared to estrone metabolites (62). It has also been shown that estrone sulfate can act as a direct donator of estrone for CYP-dependent metabolism (30). Therefore, oxidative metabolites of estrone are the most likely candidates to influence breast cancer risk in women, particularly postmenopausal women.

From a breast cancer perspective, it is important to consider whether or not the enzymes under study are expressed in breast tissue. Several studies have addressed this issue, with somewhat conflicting results. There is general consensus that CYP1A1 and CYP1B1 are expressed in normal and malignant breast tissue, although the percentage of normal and malignant tissue expressing the enzymes varied from 25% to 100% (46, 63-66). The expression of CYP3A4, CYP3A5, and CYP2C19 are less clear. Some studies have found a significant and consistent expression of the CYP3A enzymes (63, 64, 66, 67), whereas other studies have not found any expression (65). Nonspecific probes for CYP2C forms have identified expression of *CYP2C* mRNA in breast tissue (65) and a CYP2C protein distinct from the common liver CYP2C protein (68). However, only one study has specifically addressed the question of CYP2C19. No expression of CYP2C19 was observed at the mRNA level; thus, the presence of this enzyme in breast tissue remains unproven (64). Similarly, the expression of CYP1A2 has not been shown in the breast (64).

These data presented here suggest that CYP1A1, CYP1B1, and CYP3A5 are likely to be the most important CYP enzymes in breast tissue. In contrast, CYP1A2 and CYP2C19 are most likely to be important in the liver, whereas their role in the breast remains unknown until further studies are done to characterize their expression in breast tissue. The probable pathways of estrone metabolism *in vivo* are illustrated in Fig. 4. Based on these results, the contribution of polymorphisms in *CYP1A2, CYP2C19*, and *CYP3A5* to breast cancer risk should be assessed, in addition to the *CYP1A1* and *CYP1B1* polymorphisms previously studied. These studies are currently under way in our laboratory.

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