Effect of 17-α-Ethynylestradiol on Activities of Cytochrome P450 2B (P450 2B) Enzymes: Characterization of Inactivation of P450s 2B1 and 2B6 and Identification of Metabolites

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ABSTRACT
17-α-Ethynylestradiol (17EE) inactivated purified, reconstituted rat hepatic cytochrome P450 (P450) 2B1 and human P450 2B6 in a mechanism-based manner. Little or no inactivation was observed when P450s 2B2 or 2B4 were incubated with 17EE. The inactivation of P450s 2B1 and 2B6 was entirely dependent on both NADPH and 17EE and followed pseudo-first order kinetics. The maximal rate constants for the inactivation of P450s 2B1 and 2B6 at 30°C were 0.2 and 0.03 min⁻¹, respectively. For P450s 2B1 and 2B6 the apparent Kᵢ was 11 and 0.8 μM, respectively. Incubation of P450 2B1 with 17EE and NADPH for 20 min resulted in a 75% loss in enzymatic activity and a concurrent 20 to 25% loss of the enzyme’s ability to form a reduced CO complex. With P450 2B6, an 83% loss in enzymatic activity and a 5 to 10% loss in the CO reduced spectrum were observed. The extrapolated partition ratios for 17EE with P450 2B1 and 2B6 were 21 and 13, respectively. Simultaneous incubation of an alternate substrate together with 17EE protected both enzymes from inactivation. A 1.3:1 stoichiometry of labeling for binding of the radiolabeled 17EE to P450 2B1 and 2B6 was seen. These results indicate that 17EE inactivates P450s 2B1 and 2B6 in a mechanism-based manner, primarily by the binding of a reactive intermediate of 17EE to the apoprotein. Analysis of the 17EE metabolites showed that 2B enzymes that become inactivated differ primarily by their ability to generate two metabolites that were not produced by P450s 2B2 or 2B4.

Liver microsomal cytochromes P450 are involved in the metabolism of many drugs and carcinogens. P450 enzymes catalyze the metabolism of numerous structurally distinct substrates (Porter and Coon, 1991; Rendic and Di Carlo, 1997). The catalytic mechanism appears to be common to all P450s and involves a two-electron reduction of molecular oxygen to form a reactive oxygen intermediate and water (Porter and Coon, 1991).

Information about the critical active site amino acid residues involved in substrate binding and catalysis has come primarily from site-directed mutagenesis studies or from observations with naturally occurring mutants (Kedzie et al., 1991; Halpert, 1995). Additional insight into the active site structure has been gained from examining the crystal structures of a number of bacterial P450s (Ravichandran et al., 1993; Cupp-Vickery and Poulos, 1995) as well as a low-resolution crystal structure of mammalian P450 2C5 (Williams et al., 2000). Mechanism-based inactivators that undergo catalytic conversion to reactive intermediates that covalently bind to amino acid side chains have been used to identify peptides or critical amino acid residues within the active sites that are involved in substrate metabolism. Studies with the 2B rat and rabbit enzymes, using acetylenic compounds such as 2-ethynylnaphtalene and 9-ethynylphenanthrene (for review, see Kent et al., 2001) or secobarbital (He et al., 1996), were particularly successful in identifying such critical residues.

Relatively little is known about the physiological role of P450 2B6, the human 2B homolog, although some studies suggest that P450 2B6 was expressed at elevated levels in human breast tumor samples compared with nontumor tissue (Hellmold et al., 1998). The recent interest in 2B enzymes

ABBREVIATIONS: P450, cytochrome P450; 17EE, 17-α-ethynylestradiol; HPLC, high-performance liquid chromatography; DLPC, dilauroyl-α-phosphatidylcholine; 7-EFC, 7-ethoxy-4-(trifluoromethyl)coumarin; HFC, 7-hydroxy-4-(trifluoromethyl)coumarin; GC-MS, gas chromatography-mass spectrometry; GSH, glutathione; BSTFA, N,O-bis(trimethylsilyl) trifluoroacetamide; TMS, trimethylsilylamine; RP73401, 3-cyclopentoxy-N-(3,5-dichloro-4-pyridyl)-4-methoxybenzamide.
stems from observations that they may play a role in the activation of procarcinogens (Osborne et al., 1993). P450 2B6 comprises about 2 to 10% of the total P450s in human liver microsomes and may not be expressed in all human livers (Shimada et al., 1994). However, a recent study indicated that P450 2B6 could be induced by phenobarbital in all the human livers that were screened with a polyclonal anti-2B6 antibody (Madan et al., 1996). Recombinant P450 2B6 isolated from vaccinia- or baculovirus expression systems has been shown to metabolize a number of different substrates such as nicotine (McCracken et al., 1992), aminooxycysene and 3-methoxy-4-aminoazobenzene (Mimura et al., 1993), tamoxifen (Styles et al., 1994), 7-ethoxycoumarin (Yamazaki et al., 1996), testosterone (Ono et al., 1996), diazepam (Ono et al., 1996), and antiretroviral drugs (Hesse et al., 2001). P450 2B6 also appears to be inactivated by mechanism-based inactivators such as 9-ethynylphenathrene, n-propylxanthate, and 2-phenyl-2-(piperidinyl)propene that have also been shown to inactivate rat P450 2B1 (for review, see Kent et al., 2001). Recently, several substrates such as bupropion, RP 73401, and ketamine have been identified that appear to be metabolized exclusively by P450 2B6 (Stevens et al., 1997; Faucette et al., 2000; Yanagihara et al., 2001).

17EE, developed in 1938, is the major synthetic steroid component of many oral contraceptives (Innhoffen and Holweg, 1938). Although the acetylenic moiety increased the oral availability of 17EE, incorporation of this group into a compound metabolized by P450 enzymes can also lead to the inactivation of these enzymes (Ortiz de Montellano and Reich, 1986). It was shown that 17EE, when incubated with human liver microsomes, abolished the NADPH-dependent activity of P450 3A4 (Guengerich, 1988). Concurrently, a loss in the spectrally detectable P450 was observed (Guengerich, 1988). At least 10 metabolites of 17EE have been isolated from human urine, with the 2-hydroxy species being the major metabolite (Williams et al., 1975; Guengerich, 1990). P450 enzymes and estrogens have also been implicated in the development of certain cancers. Elevated levels of P450 1B1 and of 4-hydroxyestradiol have been linked to the occurrence of breast cancer in humans (Osborne et al., 1993). Studies by Osborne et al. (1993) also suggested that an increase in breast tissue levels of C16 α-hydroxylation of 17β-estradiol might be a biomarker of breast cancer risk.

Steroids such as testosterone also are good substrates for P450 2B enzymes (Code et al., 1997). For these reasons it was of interest to determine whether 17EE would be metabolized by 2B enzymes and to study the effects of the 17EE metabolism on the activity of some of the known P450 2B isozymes.

In this study 17EE was found to inactivate the major phenobarbital-inducible rat liver P450 2B1 and the human 2B homolog P450 2B6 by a classical mechanism-based mechanism (Silverman, 1996). Loss of enzymatic activity of P450 2B1 and 2B6 was primarily due to the binding of a reactive intermediate of 17EE to the apoprotein. P450s 2B2 and 2B4 were not significantly inactivated by 17EE. 2-Hydroxy-17α-ethynylestradiol was the major metabolite generated by all four isozymes. HPLC analysis of the 17EE metabolites revealed two peaks, C and E, that were primarily produced by P450s 2B1 and 2B6, suggesting the possibility that either or both may have been derived from a reactive intermediate of 17EE involved in the inactivation.

### Experimental Procedures

#### Materials

Dilauroyl-L-α-phosphatidylethanolamine (DLPC), NADPH, catalase, 17EE, estradiol, and estrone were purchased from Sigma Chemical (St. Louis, MO). 4-Hydroxy-α-estradiol, 2-hydroxy-α-estradiol, 16-β-hydroxy-estrone, 4-hydroxyestrone, and 2-hydroxyestrone were obtained from Steraloids (Newport, RI). 7-Ethoxy-(4-trifluoromethyl)coumarin (7-EFC) was from Molecular Probes (Eugene, OR) and 7-hydroxy-4-(trifluoromethyl)coumarin (HFC) was purchased from Enzyme Systems Products (Livermore, CA). Bicinchoninic acid reagent and Slide-A-Lyzer cassettes were from Pierce Chemical (Rockford, IL). Ultima Gold liquid scintillation cocktail was obtained from Packard (Meridian, CT). 2-Hydroxy-ethynylestradiol was a generous gift from Dr. William Slikker (Department of Health and Human Services, Food and Drug Administration, Jefferson, AR).

#### Synthesis of 17α-Carboxy-estradiol

A solution of ethylbro-acetate in sodium-dried benzene (2.16 g, 13 mmol in 20 ml) was added dropwise to a stirring mixture containing 1 g (15.3 mmol) of activated zinc and 1 g of estrone (3.7 mmol) in 15 ml of dry ether over the course of 30 min. The resulting mixture was warmed gently for 1 h and then refluxed for 5 h. After cooling to room temperature, 50 ml of ice-cold 10% sulfuric acid was added to the mixture. After transferring the mixture to a separatory funnel, the aqueous layer was removed. The benzene layer was washed twice with 50 ml of 5% sulfuric acid, once with 50 ml of 10% sodium bicarbonate followed by two 25-ml washes with water. The combined acid washes were extracted with ether. The organic phases were pooled and dried over anhydrous sodium sulfate and filtered. The filtrate was evaporated under reduced pressure. The crude product was purified by silica gel flash chromatography by using 25% ether in hexane as the eluent. The yield of extra-3,17β-diol-17α-ethyl acetate was 0.76 g (57%, melting point 66–68°C). GC-MS analysis with direct probe insertion yielded m/z (% species) 330.30 (25.1), 312.25 (7.29), 270.20 (20.83), and 213.15 (78.30). Extra-3,17β-diol-17α-ethyl acetate (0.7 g, 1.96 mmol) was added to a stirring mixture composed of 20 ml of 10% aqueous sodium hydroxide and 50 ml of ethanol. The mixture was stirred at room temperature for 2 days. The progress of the reaction was monitored using thin layer chromatography with chloroform as the solvent. Ethanol was evaporated under reduced pressure. The residue was diluted with 50 ml of water and cooled in an ice bath. The solution was acidified by slowly adding dilute sulfuric acid from a dropping funnel until the solution was acidic to Congo red paper. The solution was extracted three times with 50 ml of ether. The ether extracts were combined and dried over anhydrous sodium sulfate, filtered, and then the solvent was evaporated under reduced pressure. The residue was dissolved with 50 ml of water and cooled in an ice bath. The solution was acidified by slowly adding dilute sulfuric acid from a dropping funnel until the solution was acidic to Congo red paper. The solution was extracted three times with 50 ml of ether. The ether extracts were combined and dried over anhydrous sodium sulfate, filtered, and then the solvent was evaporated. The product was purified using preparative thin layer chromatography with chloroform as the solvent. The yield of the purified acid was 0.41 g (63%). The white solid had a melting point of 142 to 144°C. GC-MS analysis with direct probe insertion yielded the following m/z (% species): 358.30 (32.0), 340.30 (7.25), 252.20 (47.14), and 213.20 (100). Proton NMR spectra were recorded from samples dissolved in CDCl3 by using a GE omega 400-MHz FT-NMR spectrophotometer. The observed values for the estradiol 17-α-ester were 6.97 ppm (d, 1H, H-AR), 6.40 to 6.50 ppm (m, 2H, H-AR), 4.13 ppm (q, 2H, CH2-CH3), 2.81 to 2.85 ppm (m, 3H), 2.52 ppm (d, 1H, 2.24 to 2.31 ppm (m, 1H), 2.03 to 2.10 ppm (m, 1H), 1.28 to 1.76 ppm (m, 1H), and 0.95 ppm (s, 3H, CH3). For the estradiol 17-α-acetic acid the values were 6.98 ppm (d, 1H, H-AR), 6.50 to 6.60 ppm (m, 2H, H-AR), 2.80 to 2.83 ppm (m, 2H), 2.60 to 2.71 ppm (m, 2H), 2.28 to 2.32 ppm (m, 1H), 1.30 to 2.01 (m, 12H), and 0.95 ppm (s, 3H, CH3).

#### Purification of P450 and Reductase

P450 2B1 was purified from microsomes isolated from livers of fasted male Long Evans rats (175–190 g; Harlan Bioproducts for Science, Indianapolis, IN) given 0.1% phenobarbital in the drinking water for 12 days according to published procedures (Saxton and Strobel, 1981). Reductase was purified after expression in E. coli as previously described (Hanna et al., 1998b). P450s 2B2 and 2B6 were expressed in E. coli.
Inactivation of P450 2B Enzymes by 17α-Ethynylestradiol

MV1304 cells and purified as previously described (Hanna et al., 1998a, 2000). P450 2B4 was purified from livers ofphenobarbital-induced rabbits as described by Coon et al. (1978).

**Enzyme Activity Assays and Inactivation.** Purified P450 2B1 and reductase were reconstituted with lipid for 45 min at 4°C. Incubation mixtures contained 0.5 μM P450 2B1 or 0.67 μM P450 2B6, 1 μM reductase, 200 μg of DLPC/ml, 110 units of catalase/ml, 17EE, or dimethyl sulfoxide in 50 mM potassium phosphate buffer, pH 7.4. In some instances, the P450 2B6-reconstituted system also contained equimolar amounts of cytochrome b5. P450s 2B2 and 2B4 were reconstituted as described for P450 2B1 except that equimolar amounts of cytochrome b5 were also added to these isoforms. After equilibrating the reaction mixture at 30°C for 3 min, the reactions were initiated by adding NADPH to a final concentration of 1.2 mM (primary reaction mixture). The 7-EFC 17EE/7-EFC were initiated by adding NADPH to a final concentration of 1.2 mM (primary reaction mixture). The 7-EFC O-deethyl activity was measured spectrophotometrically as described by Buters et al. (1993). At the indicated times, duplicate 10-μl samples (5 pmol of P450 2B1) of the primary reaction mixture were removed and mixed with 990 μl of a secondary reaction containing 0.2 mM NADPH, 100 μM 7-EFC, and 40 μg bovine serum albumin/ml in 50 mM potassium phosphate buffer, pH 7.4, and incubated at 30°C for 5 min. For P450 2B6, duplicate 12-μl samples (8 pmol of P450 2B6) of the primary reaction mixture were mixed with 988 μl of the secondary reaction mixture and incubated for 10 min at 30°C. Enzyme activity was stopped by adding ice-cold acetonitrile to a final concentration of 25%. Fluorescence of the samples was measured directly at room temperature on an SLM-Aminco model SPF-500 C spectrophotometer (SLM-Aminco, Urbana, IL) with excitation at 410 nm and emission at 510 nm.

**Substrate Protection.** Substrate protection from 17EE-dependent inactivation of P450 2B1 was assayed by including 10 μM 17EE together with 7-EFC at molar ratios of 1:0.25, 1:0.5, 1:1, and 1:2 of 17EE/7-EFC in the primary reaction. At the indicated times duplicate 10-μl aliquots were removed and assayed for activity remaining as described above. For P450 2B6, substrate protection was assayed by including a 5-fold molar excess of ethoxycoumarin over 5 μM 17EE in the primary incubation mixture.

**Partition Ratio.** To estimate the partition ratio, P450 2B1 samples were incubated in the presence of 2.5 to 300 μM 17EE for 20 min to ensure the assay had proceeded to completion. Duplicate aliquots were removed and assayed for 7-EFC activity as described above. P450 2B6 was incubated with 5 to 350 μM 17EE, incubated for 20 min, and assayed for residual activity with 7-EFC.

**Irreversibility of Inactivation of P450 2B1 and 2B6 by 17EE.** Cytochromes P450 2B1 or 2B6 (0.5 nmol) were reconstituted and inactivated with 20 μM or 50 μM 17EE in a total volume of 138 μl as described above. Control samples were incubated with 17EE but without NADPH. After 10 min at 30°C, the samples (0.13 ml) were dialyzed overnight at 4°C against 2× 500 ml of 50 mM potassium phosphate buffer, pH 7.4, containing 20% glycerol and 0.1 mM EDTA. The dialyzed samples were reconstituted with 10 μg of lipid for 30 min on ice. Some samples also received fresh reductase. Enzymatic activity was assayed with 7-EFC as described above.

**Stoichiometry and Specificity of Binding.** The stoichiometry of binding was determined by extensively dialyzing 500-μl samples containing 1 nmol of P450 2B1 reconstituted with reductase and lipid as described above that had been incubated with 40 μM radiolabeled [3H]17EE, 10 mM GSH, and with or without NADPH for 10 min at 30°C. Aliquots were removed to measure the extent of inactivation of P450 2B1 based on the residual 7-EFC O-deethyl activity and to determine the amount of heme loss by reduced CO difference spectroscopy before dialysis (Omura and Saito, 1964). Samples were dialyzed in Slide-A-Lyzer cassettes against 4× 500 ml of 50 mM potassium phosphate, pH 7.4, containing 20% glycerol, 10 mM sodium cholate, and 0.1 mM EDTA. Aliquots were removed and the radioactivity remaining after dialysis was measured by liquid scintillation counting. Cytochrome P450 2B1 recovery was determined spectrophotometrically by measuring the reduced CO difference spectra. The stoichiometry of binding was calculated after subtracting the background counts from dialyzed samples incubated with 17EE in the absence of NADPH. P450 2B6 was incubated with 10 μM 17EE, 10 mM GSH, and with or without NADPH for 20 min. The samples were assayed for residual activity and P450 content by reduced CO difference spectroscopy before dialysis as described for P450 2B1.

**Spectrophotometric Quantitation of P450 2B1 and P450 2B6.** At the times indicated, 200-μl aliquots of the primary reaction incubation were removed and diluted with 800 μl of ice-cold 50 mM potassium phosphate, pH 7.4, containing 40% glycerol and 0.6% Tergitol Nonidet P-40. The sample was gently bubbled with CO for 60 s and the spectrum was recorded from 400 to 500 nm on a DW2 UV/VIS spectrophotometer (SLM-Aminco) equipped with an OLIS spectroscopy operating system (On-Line Instrument Systems, Bogart, GA). Dithionite was added and the reduced carbonyl spectrum was recorded (Omura and Saito, 1964). For absolute spectral determinations, P450 2B1 and reductase were reconstituted at a 1:1 ratio. The final concentration was 1 μM P450 2B1, 1 μM reductase, 200 μg of DLPC/ml, and 110 U of catalase/ml in 50 mM potassium phosphate, pH 7.4. The reference contained catalase and lipid in 50 mM potassium phosphate, pH 7.4. Spectra were recorded by scanning from 375 to 500 nm.

**Isolation of 17EE Metabolites.** P450s were reconstituted with reductase and lipid as previously described. For assays with P450 2B2, 2B4, and 2B6, cytochrome b5 was also included in equimolar amounts. Each assay contained 1 μM P450, 1 μM reductase, 1 μM cytochrome b5, 25 μg/ml DLPC, 200 μg/ml ascorbate, 110 units of catalase, 40 μM 17EE, and 50 mM potassium phosphate buffer, pH 7.4, in a total volume of 500 μl. In some instances, 17EE contained trace amounts of [3H]17EE. Reactions were initiated with 1.2 mM NADPH. P450 2B1 samples were incubated at 30°C for 30 min, whereas P450s 2B2, 2B4, and 2B6 were incubated for 60 min. The reaction mixtures were quenched with 2 μl of N₆-saturated methylene chloride. 17EE and its metabolites were extracted into the organic phase. Each sample received 3 μl of dimethyl sulfoxide and the methylene chloride was evaporated under N₂. The samples were dissolved in 100 μl of 50% solvent B (49.5% CH₃OH, 50% CH₃CN, 0.1% acetic acid) before HPLC analysis. Metabolites were chromatographed on a C18 reverse phase column (25 cm, 5 μm, 100 Å; Microsorb MV; Rainin Instruments, Woburn, MA) equilibrated with 70% solvent A (0.1% acetic acid in H₂O) and 30% solvent B at a flow rate of 1.2 ml/min. After 5 min, the concentration of solvent B was raised to 50% over 3 min, followed by a linear increase to 60% over 12 min, and then to 95% over 10 min. After 10 min at 95% solvent B, the column was brought back to initial equilibration conditions. Under these conditions 17EE and its metabolites eluted between 10 and 25 min. The retention times of the metabolites were compared with authentic standards. Metabolites were quantified by integrating the area under the peak by using the Millenium program (Waters, Milford, MA).

**Identification of 17EE Metabolites.** Fractions containing the 17EE metabolites were collected, dried, derivatized with BSTFA/TMCS, and analyzed by GC-MS essentially as described by Suchar et al. (1995). Each sample was incubated with 5 μl of redistilled pyridine and 20 μl of BSTFA, 1% TMCS for 30 min at 70°C. Each sample (4 μl) was chromatographed on a 30-m DB1 fused silica capillary column (0.32-mm i.d., 0.25-μm film coating; J&W Scientific, Folsom, CA) with a temperature gradient of 10°C/min from 80 to 320°C and analyzed over an m/z range from 45 to 750 on a JEOL JMS AX-50SH double focusing mass spectrometer coupled to a Hewlett Packard 5890J gas chromatograph via a heated interface.

**Results**

Inactivation of P450 2B1 by 17EE. Inactivation of P450s 2B1 and 2B6 in the reconstituted system showed an absolute requirement for 17EE together with NADPH. After
incubating reconstituted P450 2B1 with 50 μM 17EE and NADPH for 10 min, only 24% of the enzyme’s 7-EFC O-deethylation activity remained (Table 1). However, these samples retained most of their ability to form a reduced CO complex. Control incubations without 17EE or with 17EE but without NADPH did not lead to a substantial loss in either enzymatic activity or the ability of P450 2B1 to bind CO. Similarly, 83% of the absolute absorbance spectrum at 417 nm was retained after incubating for 10 min with 17EE and NADPH (data not shown). LC and LC-MS analysis of the incubation mixtures showed no loss in the heme peak of the inactivated sample detected by at 405 nm after HPLC separation, and no heme adduct was observed during mass analysis of the samples (data not shown). When P450 2B6 was incubated with 50 μM 17EE and NADPH for 20 min, an 83% loss in the 7-EFC O-deethylation activity was observed. With the same samples, 88% of the reduced CO complex was formed compared with control samples incubated without 17EE. P450 2B6 samples that were incubated either without 17EE or without NADPH did not show reduced activity or diminished CO binding spectra.

The inactivation of P450 2B1 by 17EE was time- and concentration-dependent (Fig. 1). Pseudo-first order inactivation kinetics were observed at 30°C with 17EE concentrations ranging from 2.5 to 50 μM. The kinetic constants describing the inactivation of P450 2B1 with 17EE were determined from the inset of Fig. 1. The maximal rate of inactivation at saturation (k_{inact}) was 0.2 min⁻¹, the concentration required for half-maximal inactivation (K_i) was 11 μM, and the time required for half the enzyme to become inactivated (t_{1/2}) was 4 min. No effect on the rates of inactivation was observed when 10 mM GSH or 1 mM dithiothreitol was added to the primary incubation mixtures (data not shown).

Figure 2 shows that the inactivation of P450 2B6 by 17EE was also time- and concentration-dependent (Fig. 2). Pseudo-first order inactivation kinetics were observed between 1 and 16 μM 17EE. The kinetic constants were determined from the inset of Fig. 2. The K_i was approximately 0.8 μM, the k_{inact} was 0.03 min⁻¹, and the t_{1/2} was 28 min. No significant change in the kinetic constants for the inactivation of P450 2B6 was seen when cytochrome b5 was included in the reconstitution mixture (K_i = 0.5 μM, k_{inact} = 0.03 min⁻¹, and t_{1/2} = 23 min). The presence of cytochrome b5 did however result in an approximately 50% increase in the enzymatic activity of P450 2B6 as measured using 7-EFC as the substrate (data not shown).

**Partition Ratio.** The number of molecules of 17EE metabolized per molecule of P450 2B1 or 2B6 inactivated (partition ratio) was estimated from Fig. 3. The P450s were incubated with different concentrations of 17EE and the inactivation reaction was allowed to go to completion. The percentage of activity remaining was plotted as a function of the molar ratio of 17EE to P450. The turnover number (partition ratio + 1) was extrapolated from the intercept between the linear regression line obtained from the lower 17EE to P450 ratios with the straight line derived from the higher 17EE to P450 ratios (Silverman, 1996). By this method the partition ratio for P450 2B1 was estimated to be 21. This indicated that approximately 21 molecules of 17EE were metabolized for every one molecule of P450 2B1 that became inactivated. For P450 2B6 the partition ratio was approximately 13.

**TABLE 1**

Effect of 17EE on the 7-EFC O-deethylation activity and cytochrome P450 content of purified P450 2B1 and 2B6

<table>
<thead>
<tr>
<th>Primary Reaction Conditions</th>
<th>Percentage of Activity Remaining</th>
<th>Percentage of P450 Remaining</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>0 min</td>
<td>10 min</td>
</tr>
<tr>
<td>P450 2B1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-17EE + NADPH</td>
<td>100</td>
<td>96 ± 4</td>
</tr>
<tr>
<td>+17EE - NADPH</td>
<td>101 ± 8</td>
<td>98 ± 9</td>
</tr>
<tr>
<td>+17EE + NADPH</td>
<td>97 ± 10</td>
<td>24 ± 5</td>
</tr>
<tr>
<td>P450 2B6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-17EE + NADPH</td>
<td>100</td>
<td>95 ± 11</td>
</tr>
<tr>
<td>+17EE - NADPH</td>
<td>106 ± 7</td>
<td>107 ± 2</td>
</tr>
<tr>
<td>+17EE + NADPH</td>
<td>100 ± 7</td>
<td>33 ± 8</td>
</tr>
</tbody>
</table>

N.D., not determined.
Irreversibility of 2B1 Inactivation by 17EE. The loss in activity of P450s 2B1 and 2B6 brought about by incubations in the presence of 17EE and NADPH was not reversible. Control samples incubated with 17EE or samples where P450 2B1 or 2B6 were inactivated with 17EE and NADPH were dialyzed extensively and tested for activity. Table 2 shows that the removal of free 17EE by dialysis did not lead to a recovery of the 7-EFC activity of 17EE-inactivated samples. No additional activity was regained when fresh reductase was added to the dialyzed samples.

Substrate Protection. Incubations of P450 2B1 with 17EE together with an alternate substrate in the primary reaction slowed the rate at which P450 2B1 was inactivated by 17EE (Fig. 4A). Virtually no inactivation was seen when the alternate substrate concentration was greater than 2-fold over that of 17EE. With P450 2B6, slightly higher concentrations of an alternate substrate were required to cause an effect. A decrease in the rate of P450 2B6 inactivation was seen when ethoxycoumarin was added to the primary incubation mixtures at a 5-fold molar excess over 17EE (Fig. 4B).

Stoichiometry of Labeling. The stoichiometry of binding of a radiolabeled 17EE metabolite to P250 2B1 and 2B6 was determined. Control samples incubated only with [3H]17EE and inactivated samples incubated with [3H]17EE and NADPH were dialyzed extensively until no more counts were detected in the dialysis buffer. Sample aliquots were assayed for recovery of P450 by reduced CO spectroscopy and for

![Table 2](image-url)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Percentage of Activity Remaining</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>17EE-Inactivated P450 2B1</td>
<td>17 ± 1</td>
</tr>
<tr>
<td>After dialysis</td>
<td>100</td>
</tr>
<tr>
<td>17EE-Inactivated P450 2B6</td>
<td>41 ± 1</td>
</tr>
<tr>
<td>After dialysis + fresh reductase</td>
<td>44 ± 1</td>
</tr>
<tr>
<td>After dialysis + fresh reductase</td>
<td>45 ± 3</td>
</tr>
</tbody>
</table>

![Fig. 2](image-url)

**Fig. 2.** Time- and concentration-dependent inactivation of the P450 2B6 7-EFC O-deethylation activity by 17EE. Aliquots were removed from the primary reaction mixture and assayed for residual activity as described under Experimental Procedures. The data shown represent the mean and standard deviation from duplicate samples of three to four separate experiments. For some points the standard deviation was smaller than the size of the symbol. The concentrations of 17EE were 0 (□), 1 (▲), 2 (△), 4 (●), 8 (○), and 16 μM (●). The inset shows the double-reciprocal plot of the rates of inactivation as a function of 17EE concentrations.

![Fig. 3](image-url)

**Fig. 3.** Loss of P450 2B1 (▲) and P450 2B6 (●) activity as a function of the ratio of 17EE to P450. P450s were incubated with different concentrations of 17EE as described under Experimental Procedures. The data shown represent the average from four experiments (2B1) or two experiments (2B6). The extrapolated partition ratio was determined from the intercept of the linear regression line from the lower ratios and the straight line obtained from the higher ratios.

![Fig. 4](image-url)

**Fig. 4.** Substrate protection against P450 2B1 and 2B6 inactivation by 17EE. Samples were removed from the primary incubation mixture at the indicated time points and assayed for activity remaining as described under Experimental Procedures. The data shown are representative of two to three separate experiments. A, primary P450 2B1 reaction mixtures contained molar ratios of 17EE/7-EFC of 0:0 (□), 1:0 (▲), 1:0.25 (△), 1:0.05 (●), 1:1 (○), and 1:2 (●). B, primary reaction mixtures contained reconstituted P450 2B6 and molar ratios of 17EE/ethoxycoumarin of 0:0 (□), 1:0 (▲), and 1:5 (●).
radioactivity by liquid scintillation counting. Background counts from control samples incubated without NADPH (5.1 \times 10^3 \pm 0.9 \times 10^3 \text{ dpm/nmol P450 2B1}) were subtracted from the counts obtained for the inactivated samples (41.8 \times 10^3 \pm 1.2 \times 10^3 \text{ dpm/nmol P450 2B1}). Determinations from two separate experiments resulted in an average stoichiometry of 1.3 mol of metabolite bound per mole of enzyme inactivated. For P450 2B6 an average stoichiometry of 1.3:1 moles of metabolite bound per mole of enzyme inactivated was obtained as well. Although GSH did not reduce the rate of inactivation by 17EE, it was necessary to include GSH and cholate in the dialysis buffer to remove unbound or tightly complexed 17EE.

Effect of 17EE on Activities of P450s 2B4 and 2B2. P450 2B4 and 2B2 were only poorly inactivated or not inactivated. For P450 2B6 an average stoichiometry of 1.3 mol of metabolite bound per mole of P450 2B1 two separate experiments resulted in an average stoichiometry of 1.3:1 mol of metabolite bound per mole of enzyme inactivated or not inactivated by 17EE (Table 3). Incubations of P450 2B4 with 90 \mu M 17EE and NADPH for 20 min resulted in only a 24\% loss in the 7-EFC activity. P450 2B2 lost virtually no activity (8\%) when incubated with 80 \mu M 17EE for 40 min in the presence of NADPH. These results suggest that 17EE either did not bind to P450s 2B2 and 2B4 or possibly bound in a different orientation such that these isoforms were unable to generate the 17EE-reactive intermediate necessary for inactivation. Alternatively, P450s 2B2 and 2B4 may not contain the critical active site amino acid residue that renders the enzyme inactive when covalently modified by a 17EE-reactive intermediate.

Metabolite Isolation and Analysis. P450s 2B1, 2B6, 2B2, and 2B4 were incubated with 17EE in the presence or absence of NADPH. 17EE and its metabolites were extracted and resolved by reverse phase HPLC as described under Experimental Procedures. Figure 5 shows representative HPLC profiles observed for the metabolites of 17EE formed by P450 2B1 when incubated in the absence (Fig. 5A) or presence of NADPH (Fig. 5B). The metabolites generated by P450 2B6 were similar, except that the amount of each metabolite generated was much less (data not shown; Table 4). Figure 5C shows a representative HPLC profile of the 17EE metabolites generated by P450 2B4 when incubated with 17EE in the presence of NADPH. The HPLC profile of 17EE metabolites generated by P450 2B2 was similar to that of P450 2B4 (Fig. 5D). The 17EE parent compound (Fig. 5A, peak F) was the primary compound observed when NADPH was omitted from the incubation mixture. A small peak that eluted at 18.8 min, and presumably corresponds to a minor \beta-estradiol contaminant of the commercial 17EE preparation, was also seen in this control sample and all samples incubated with NADPH. P450 2B1 incubated with 17EE and NADPH generated five major metabolites (Fig. 5B, peaks A–E), as did P450 2B6, albeit to a lesser extent (Table 4). Metabolism of 17EE by P450 2B2 (Fig. 5D) did not lead to the formation of peak E and the levels of A1, A2, B, and C were greatly reduced. Peaks B and C were not seen in many experiments with P450 2B2. When observed, they were present at very low levels (<1\%) and were therefore not quantitated. P450 2B4 was unable to generate peaks A1 and E and peak C was again not always observed (Fig. 5C). These observations suggested that intermediates giving rise to peaks E and/or C might be involved in the inactivation of P450s 2B1 and 2B6. Alternatively, the possibility that a reactive intermediate was produced from 17EE by all four isoforms and that a critical amino acid residue in the active site of P450s 2B2 and 2B4 is absent and therefore not modified cannot be excluded.

Individual metabolite peaks generated by P450 2B1 were collected, derivatized with BSTFA/TMCS, and subjected to GC-MS analysis as described under Experimental Procedures. Table 5 shows the HPLC retention times, the \textit{m/z} values of the unknowns and the authentic standards, when available, and the molecular masses and the predicted structures for some of the metabolites. Peak F exhibited the same HPLC retention time as the authentic \textit{m/z} ion. GC-MS analysis of peak F indicated that the total ion chromatogram (TIC) and the \textit{m/z} signals were consistent with the results obtained with the authentic 17EE standard. In addition to 17EE, GC-MS analysis of the incubation mixture in the presence of NADPH also detected approximately 5\% of a 17EE metabolite that showed an identical retention time, \textit{m/z} parent ion and \textit{m/z} fragments consistent with the estrone standard. This was probably due to carryover from peak E. The TIC of peak F also revealed trace amounts of a third component that eluted after the estrone component and before 17EE. The \textit{m/z} pattern was similar to 17EE except that the most prominent \textit{m/z} ion was at 368. It is possible that this could be a singly TMS-modified 17EE.

Peak E eluted as a broad peak from the HPLC column with retention times similar to the estrone and \beta-estradiol standards. GC-MS analysis of peak E showed one major peak and small amounts of a second peak on the TIC. The major component had a GC retention time similar to the estrone standard and also exhibited an \textit{m/z} parent ion (342) and fragment ions similar to the estrone standard. The second component had an \textit{m/z} pattern similar to the \beta-estradiol standard and presumably was derived from a minor contaminant of the commercial 17EE preparation. In addition, some of the scans also showed trace amounts of 17EE that presumably were due to carryover from peak F.

The HPLC retention time of peak D was similar to the authentic 2-OH-17EE standard. GC-MS analysis of peak D also indicated that the retention time from the TIC and the \textit{m/z} of the molecular ion (528), as well as the \textit{m/z} of the ions fragments were identical to the GC-MS results obtained for the authentic 2-OH-17EE standard.

The HPLC retention time of peak D was 14.1 min. The only standard available that eluted in this range was 16-\alpha-hydroxy-estrone (13.6 min). Two closely eluting peaks with GC retention times similar to those of 4-hydroxy-estradiol, 2-hydroxy-estradiol, and 16-\alpha-hydroxy-\beta-estradiol were seen in the TIC of sample C. However, neither the \textit{m/z} of the molecular ion nor the \textit{m/z} of the ions of peak C matched any of the

<table>
<thead>
<tr>
<th>P450</th>
<th>17EE</th>
<th>Percentage of Activity Remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td>2B1</td>
<td>50</td>
<td>11 \pm 2</td>
</tr>
<tr>
<td>2B6</td>
<td>35</td>
<td>22 \pm 2</td>
</tr>
<tr>
<td>2B4</td>
<td>90</td>
<td>73 \pm 7</td>
</tr>
<tr>
<td>2B2</td>
<td>80</td>
<td>92 \pm 1</td>
</tr>
</tbody>
</table>

Table 3: Effect of 17EE on the activities of P450s 2B

Assay conditions were as described under Experimental Procedures. Activity was assayed after 15 min (2B1), 20 min (2B6, 2B4), and 40 min (2B2) of incubation with 17EE and NADPH. The data shown represent the average of two to four separate experiments.
hydroxy-estradiol or hydroxy-estone standards tested. The primary m/z ions in sample C were 371 and 456, presumably corresponding to compounds with masses of 298 + 1 TMS, and 312 + 2 TMS, respectively. These masses would be consistent with the mass of the 17-formyl-D-homosteroid observed by Schmid et al. (1983). A potential scheme for generating this component from the metabolism of 17EE by P450s 2B is shown in Scheme 1. However, the levels of component C that were generated were too low for further characterization by NMR and conclusive identification. The second component in peak C with a mass of 312 could be the homoestrone alcohol intermediate described by Schmid et al. (1983). Elimination of water and rearrangement during the sample workup could possibly lead to a 17-methyl-homoestrone with a mass of 298.

Peak B eluted off the HPLC column at 12.8 min, between the standard peaks for estriol (12 min) and 16α-hydroxy-estrone (13.6 min). GC-MS analysis indicated that peak B was not identical to 16α-hydroxy-estrone (or 4-hydroxy-, or 2-hydroxy-estrone). The molecular ion of B corresponded to an m/z of 451 consistent with a mass of 307 + 2 TMS. Metabolite B could be the product resulting from the addition of water across an epoxide modification or addition of a ketone on ring A, or the addition of another hydroxyl moiety to the A, B, or D ring with concurrent reduction of a keto group. However, the expected parent ion consistent with three TMS modifications was not observed. Peak B was not further characterized because again it was produced in very low levels by P450 2B1.

Peak A eluted at 11.7 and 11.9 min from the HPLC column. This peak in some cases eluted as one peak and in some experiments as two very close peaks without baseline separation. The elution time of the authentic estriol standard was 11.9 min. The TIC revealed the presence of three metabolites. The second largest peak had a retention time identical to that of the estriol standard. In addition the m/z ion fragment profile was the same as that of estriol, except that the major expected ion resulting from three TMS modifications to estriol at m/z 504 was not observed. The two other molecular ions that were observed were at m/z 528 and 361 that would correspond to 17EE metabolites with masses of 312 + 3 TMS and 289 + 1 TMS modification, respectively. Because the HPLC elution time was similar to estriol the component with a mass of 312 could be similar in structure to estriol, possibly 17EE hydroxylated at the terminal ethynyl carbon. A 17EE-related component with a mass of 289 could be obtained if a modification such as oxidation of the 3-OH moiety to a ketone would lead to the loss of aromaticity of the A ring.

An additional metabolite with a HPLC retention time of 15.7 min (c') was also observed in low levels in the HPLC profiles of some P450 2B1 samples. The retention time of this metabolite corresponded to the retention time of the standard 4-hydroxy-estradiol. Because this metabolite was not

### Table 4

Differences in the 17EE metabolites generated by the P450 2B enzymes

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>P450 2B1</th>
<th>P450 2B2</th>
<th>P450 2B4</th>
<th>P450 2B6</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>13 ± 3.0</td>
<td>1.2 ± 0.4</td>
<td>2.4 ± 0.8</td>
<td>0.28 ± 0.10</td>
</tr>
<tr>
<td>B</td>
<td>2.2 ± 0.3</td>
<td>N.D.</td>
<td>3.0 ± 0.9</td>
<td>0.23 ± 0.10</td>
</tr>
<tr>
<td>C</td>
<td>2.3 ± 0.4</td>
<td>N.D.</td>
<td>N.D.</td>
<td>0.18 ± 0.02</td>
</tr>
<tr>
<td>C'</td>
<td>1.4 ± 0.4</td>
<td>3.7 ± 0.8</td>
<td>0.4 ± 0.2</td>
<td>0.38 ± 0.23</td>
</tr>
<tr>
<td>D</td>
<td>16.4 ± 4.9</td>
<td>3.9 ± 0.7</td>
<td>1.14 ± 0.23</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>7.6 ± 5.0</td>
<td>0</td>
<td>0</td>
<td>0.41 ± 0.20</td>
</tr>
<tr>
<td>F</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

N.D., not determined.

Percentage of area under the peak relative to the area under the peak of 17EE.
TABLE 5
17EE metabolites generated by P450 2B1 in the reconstituted system

<table>
<thead>
<tr>
<th>Peak</th>
<th>HPLC Retention Times</th>
<th>m/z Observed</th>
<th>m/z Standard</th>
<th>Mass</th>
<th>Name</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>A₁</td>
<td>11.7</td>
<td>528</td>
<td>N.A.</td>
<td>312</td>
<td>UK</td>
<td>Estriol</td>
</tr>
<tr>
<td></td>
<td>11.9</td>
<td>361</td>
<td>361</td>
<td>289</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A₂</td>
<td>11.9</td>
<td>451</td>
<td>N.A.</td>
<td>307</td>
<td>UK</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>12.8</td>
<td>451</td>
<td>N.A.</td>
<td>307</td>
<td>UK</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>14.1</td>
<td>456</td>
<td>N.A.</td>
<td>312</td>
<td>UK</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>16.8</td>
<td>528</td>
<td>528</td>
<td>312</td>
<td>2-OH-17EE</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>18.5</td>
<td>342</td>
<td>342</td>
<td>270</td>
<td>Estrone</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>19.5</td>
<td>440</td>
<td>440</td>
<td>296</td>
<td>17EE</td>
<td></td>
</tr>
</tbody>
</table>

N.A., not available; UK, unknown.

Discussion

This report describes the mechanism-based inactivation of P450s 2B1 and 2B6 by 17α-ethynylestradiol. 17EE was chosen for these studies because steroids are good substrates for P450 2B enzymes and the ethynyl moiety has been shown to be involved in the mechanism-based inactivation of P450 enzymes. Although both isoforms were inactivated with 17EE, little decrease in either the P450 2B1- or P450 2B6-reduced CO spectrum was observed. The combined observations from Table 1, the retention of the absolute spectrum, the HPLC heme recovery, and the lack of heme modification observed by LC-MS suggested that the inactivation by 17EE was not due to the destruction of the heme moiety but rather to a modification of the apoprotein. These observations are in contrast to studies with P450 3A4 and 17EE, where the majority of enzymatic activity loss was attributed to heme modification resulting in a loss in the P450 CO spectrum (Guengerich, 1990). Similar results were also observed using purified P450 3A4 in a reconstituted system (H.-L. Lin, U. M. Kent, and P. F. Hollenberg, unpublished data). Our data also show that inactivation by 17EE required a catalytic step because coinubcation with both 17EE and NADPH was not necessary to inactivate P450s 2B1 and 2B6.

Although the two P450s share considerable sequence similarity, an 11-fold difference between the $K_i$ of P450 2B1 (11

consistently observed with P450 2B1 and when observed it was present at low levels, it was not subjected to GC-MS analysis.
These values were similar to the $K_v (5 \mu M)$ for 17EE observed. These values were similar to the $K_v (5 \mu M)$ observed for 17EE and P450 3A4 (H.-L. Lin, U. M. Kent, and P. F. Hollenberg, unpublished data). Extensive dialysis to remove free 17EE did not lead to a recovery in enzymatic activity. The inactivation appeared to be due to a specific modification of the P450 and not the reductase because the addition of fresh reductase back to the dialyzed samples did not restore enzymatic activity to the 17EE-inactivated P450. No loss in enzymatic activity was observed when P450 2B1 was incubated with 17EE and NADPH in the presence of a 2-fold molar excess of an alternate substrate. Taken together, these observations indicated that P450 2B1 was inactivated by a reactive intermediate of 17EE that bound covalently to the P450 active site. Adding exogenous nucleophiles to the reaction mixture had no effect on the rates of inactivation. These observations further indicate that the inactivation of P450 2B1 by 17EE was due to the binding of a 17EE-reactive intermediate at the active site and not because the intermediate diffused out of the active site and bound elsewhere on the P450 molecule or the reductase. Including 10 mM GSH during the inactivation followed by dialysis against 10 mM cholate-containing buffer reduced the binding stoichiometry for radiolabeled 17EE to 1.3:1. This stoichiometry of radiolabeled 17EE metabolite to P450 2B1 also suggested that the binding occurred at the active site and that approximately one molecule of inactivator was bound per molecule of P450 2B1. Studies aimed at estimating the number of 17EE molecules metabolized per inactivation event showed that 10 to 20% of enzymatic activity remained even when the 17EE concentration exceeded that of P450 by 275- to 300-fold. Similar results have previously been obtained with other micromolar P450s and different inactivators and may indicate that these P450s either contained a subpopulation that was resistant to inactivation (for review, see Kent et al., 2001) or that 7EFC dealkylation occurred at a greatly reduced rate.

Analysis of urinary metabolites of 17EE from women have led to the identification of the 2-OH, 6a-OH, and 16ß-OH derivatives of 17EE, a minor component corresponding to 2-methoxy-17EE, and the deethynylated products estrone, estriol, estradiol, and 2-methoxy-estradiol (Williams et al., 1975). Estradiol and 2-17EE-17EE were found to be the major urinary metabolites in women. Studies with human liver microsomes showed that the P450 3A family was primarily responsible for metabolizing 17EE to the 2-17EE-17EE product (Guengerich, 1988). With P450s of the 2B family, similar metabolites were observed in the reconstituted system. For P450 2B2 the major metabolites were 2-OH, 17EE, and 4-OH-estradiol. With P450 2B4 the major metabolites were 2-OH, 17EE, metabolite B, and metabolite A. Both P450 2B1 and 2B6 were inactivated by 17EE. These two enzymes generated estranol, estrone, 2-OH, 17EE, metabolites A, and B, as well as metabolites E and C. The latter two products were either not observed or observed only in some experiments at very low levels with the P450s that were not inactivated by 17EE (2B2 and 2B4). Previous studies with hepatic microsomes from female rhesus monkeys incubated with 17EE resulted in the identification of a ß-homoestrogen product (Schmid et al., 1983). The oxidation of the ethynyl group was shown to involve a P450-dependent mechanism. Higher levels of ß-homoestrogen were also generated by microsomes isolated from phenobarbital-induced rats, suggesting an involvement of the P450 2B family. Based on the GC-MS analysis, metabolite C may be the formyl-ß-homoestrogen precursor of ß-homoestrogen. This metabolite would also be consistent with the mass increase of the 17EE-inactivated P450 2B1 apoprotein that was observed by LC-MS (U. M. Kent, D. E. Mills, K. A. Regal, M. Schrag, and P. F. Hollenberg, unpublished data).

Similar studies on mechanism-based inactivation of P450s by ethynyl compounds previously showed that the reactive intermediate was probably an ethynyl ketene (Scheme 1, pathway 1) because the corresponding carboxylic acid could be identified (for review, see Kent et al., 2001). The expected carboxylic acid of 17EE resulting from this pathway was not detected by HPLC or GC-MS. It was not surprising that small amounts of the 17EE acid would not be observed by HPLC because the 17EE acid and 17EE eluted within 0.5 min of each other under the gradient and column conditions used in these separations. The large amounts of 17EE that were present as was required for the incubation assays would have made HPLC detection of a small carboxylic acid product peak virtually impossible. However, GC-MS should have revealed the acid if it had been formed. The inability to detect the 17EE acid was not because the acid could not be extracted with our methylene chloride extraction procedure because greater than 90% of the 17EE acid was extracted from control incubations spiked with the synthesized 17EE acid (data not shown). However, if peak C is the keto-formyl homohestrone then this would be evidence for the formation of a reactive intermediate that could react with amino acid side chains in the same way a ketone would (Scheme, pathway 2). Both pathways involving either the formation of a ketene intermediate or the ring expansion would be evidence for an intermediate that is electron-deficient at the interior alkyne carbon and could be attacked by a nucleophilic group of the protein, leading to the formation of a covalent adduct.

The observation that P450s 2B2 and 2B4 were neither inactivated, or inactivated to a very minor extent by 17EE and that no metabolite E and little metabolite C were produced could also suggest that a difference in the structure of these two enzymes compared with P450s 2B1 and 2B6 either does not allow for the 17EE-reactive intermediate to be generated or to bind to a critical active site residue. P450s 2B1 and 2B2 differ by 19 residues (S363G, A321T, E322V, L327P, T335S, S344T, S360A, V363A, V367L, S367T, N417D, A419T, H463P, L463V, K479R, P482Q, T483V, S489L, and R491H). A 17EE-modified P450 2B1 peptide corresponding to amino acids P347-M376 was isolated (U. M. Kent, D. E. Mills, K. A. Regal, M. Schrag, and P. F. Hollenberg, unpublished data). Because neither P450 2B2 nor P450 2B4 is inactivated efficiently by 17EE, the residues that differ between these two proteins in the 347 to 376 region may not be important for the inactivation by 17EE. Analogously, because P450 2B6 is inactivated by 17EE, the residues that are the same between P450s 2B2, 2B4, and 2B6 in this region presumably do not play a critical role in the inactivation process by 17EE.
lation pathway during the metabolism of lidocaine (Hanna et al., 1998b). Similarly, residues 363 and 367 have been shown to play a role in the differential hydroxylation of androstenedione and testosterone by P450s 2B1 and 2B2 (Strobel and Halpert, 1997). Due to its low reactivity

\[ V_{\text{max}} \]

probably does not serve as a target for addition, making S\textsubscript{366} a more likely candidate for modification by 17EE-reactive intermediate.

In summary, this report demonstrates that 17EE is an effective mechanism-based inactivator for both P450s 2B1 and 2B6. The data indicate that the reactive intermediate generated during the metabolism of 17EE is covalently bound to the P450 2B1 and 2B6 active sites. 17EE is a major component in many oral contraceptives and the apparent elevated occurrence of P450 2B6 in tumor tissue as well as the finding that P450 2B6 plays an important and pivotal role in the metabolism of a number of drugs in humans (Stevens et al., 1997; Fauchette et al., 2000; Yanagihara et al., 2001) underscore the potential importance of this mechanism in vivo. Metabolite analysis demonstrated that only P450s 2B1 and 2B6 generated metabolites C and E.

Acknowledgments

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References


