OXIDATION OF XENOBIOTICS BY RECOMBINANT HUMAN CYTOCHROME P450 1B1

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ABSTRACT:
Human cytochrome P450 (P450) 1B1 (CYP1B1) has recently been shown to be an important enzyme in the activation of diverse procarcinogens such as arylarenes, nitroarenes, and arylamines to reactive metabolites that cause DNA damage in the cells. However, it is not known whether this P450 enzyme also plays roles in the oxidation of certain drugs or model substrates commonly used in P450 assays. We examined the substrate oxidation activities of recombinant human CYP1B1 in yeast microsomes and compared these activities with those catalyzed by reconstituted systems containing recombinant CYP1A1 and CYP1A2 which were isolated from membranes of Escherichia coli in which respective cDNAs have been expressed. Catalytic activities towards some of the model substrates of other human P450 enzymes including CYP2A6, 2C9, 2C19, 2D6, 2E1, and 3A4 were also determined and compared. CYP1B1 catalyzed benzo[a]pyrene 3-hydroxylation at rates lower than those of CYP1A1 but higher than those of CYP1A2. The activity towards 7-ethoxyresorufin O-deethylation catalyzed by CYP1B1 was about one-tenth of that of CYP1A1, but the Km values were lower for CYP1B1 than those for CYP1A1 and CYP1A2. CYP1B1 was also able to catalyze the oxidation of theophylline and caffeine, two prototypic substrates for CYP1A2. CYP1B1 did not oxidize other typical P450 substrates such as coumarin, tolbutamide, S-mephenytoin, chlorozoxazine, nifedipine, and testosterone, while low rates of oxidation of bufuralol and 7-ethoxycoumarin were found for CYP1B1. These results indicate that CYP1B1 has catalytic activities overlapping CYP1A1 and CYP1A2 with respect to the oxidation of drugs and model P450 substrates, although the relative catalytic roles in these three P450 enzymes differ depending upon the substrates examined. A distinct marker activity of CYP1B1 has not been identified.
are the means of duplicate or triplicate determinations; in all cases SD were less than 15% of the means. Values in human liver microsomes were means and SD of six different human samples. The values with a sign of inequality (<) showed the limit of detection of substrate oxidation activities. A blank space indicates that P450-catalytic activities were not determined.

liver microsomes (CYP2A6) or chimeric organisms in Escherichia coli (CYP2D6, 2E1, and 3A4) and yeast (CYP2C9 and 2C19).

Materials and Methods

Materials. Benzo[a]pyrene, 7-ethoxycoumarin, theophylline, caffeine, coumarin, and tolbutamide were purchased from Sigma Chemical Co. (St. Louis, MO). Other substrates, their oxidation products, and reagents used in this study were obtained from sources as described previously or were of the highest qualities commercially available (18–20).

Enzyme Preparation. The human CYP1B1 cDNA clone was introduced into Saccharomyces cerevisiae and microsomes containing CYP1B1 protein were prepared as described (8, 15). Recombinant human CYP1A1, 1A2, 2D6, 2E1, and 3A4 were purified to electrophoretic homogeneity as described previously (24). Recombinant CYP2C9 and 2C19 in yeast microsomes were purchased from Sumitomo Chemical Co. (Osaka, Japan). Rat CYP1A1 and 1A2 were purified to electrophoretic homogeneity as described previously (25). Rabbit anti-P450 antibodies were prepared and the IgG fractions were obtained from sources as described previously or were of the highest qualities commercially available (18–20).

Enzyme Assays. Standard incubation mixtures consisted of human P450 enzymes (5–50 pmol P450) with several drug substrates in a final volume of 0.25–1.0 ml of 100 mM potassium phosphate buffer (pH 7.4) containing an NADPH-generating system consisting of 0.5 mM NADP+, 5 mM glucose 6-phosphate, and 0.5 unit of glucose 6-phosphate dehydrogenase/ml (19). In the case of yeast microsomes, NADPH-P450 reductase (10–100 pmol) was added to improve catalytic activities (7). Oxidation of substrates by CYP1A1, 1A2, 2A6, and 2D6 were determined in reconstituted systems containing P450 (5–50 pmol) and NADPH-P450 reductase (10–100 pmol) with L-α-dilauroyl-sn-glycer-3-phosphocholine as described (7). In cases for reconstituted CYP2E1- and 3A4-systems, cytochrome b5 (10–100 pmol) was included in the incubation mixture, and optimal reconstitution conditions were used as described previously (30, 31). Liver microsomal incubations included microsomes (0.5 mg protein/ml) in 100 mM potassium phosphate buffer (pH 7.4) containing the NADPH-generating system and various concentrations of drug substrates (18, 19, 25). For the assay of nifedipine and testosterone oxidation activities, 30 mM MgCl2 was included and the buffer was replaced by 50 mM potassium HEPES buffer (pH 7.4) (31).

Activities of 3-hydroxylation of benzo[a]pyrene (substrate concentration, 80 μM), O-deethylation of ethoxycoumarin (10 μM), 7-hydroxylation of coumarin (50 μM), and O-deethylation of 7-ethoxycoumarin (50 μM) were assayed fluorometrically according to the methods as described (29, 32). Methods for 1-, 2-, and 3-demethylations and 8-hydroxylation of theophylline (substrate concentration, 0.5 mM) and 3-demethylation and 8-hydroxylation of caffeine (0.5 mM) have been described (33, 34). Methoxy hydroxylation of tolbutamide (substrate concentration of 2.5 mM) and 4’-hydroxylation of S-mephenytoin (0.4 mM) were determined using high-performance liquid chromatography as described (19, 35, 36). The methods used for 1’, 4-, and 6-hydroxylation and 6-methylhydroxylation of bufuralol (substrate concentration, 0.2 mM), 6-hydroxylation of chlorzoxazone (0.5 mM), oxidation of nifedipine (0.2 mM), and 6β-hydroxylation of testosterone (0.2 mM) were described previously (20, 30, 31, 37).

P450 was estimated spectrally by the methods of Omura and Sato (38). Protein concentrations were estimated by the method of Lowry et al. (39).

Kinetic parameters for the 7-ethoxycoumarin O-deethylation by human liver microsomal P450 enzymes were estimated using a nonlinear regression analysis program (Kpecat, BioMetallics, Princeton, NJ).

Results

Oxidation of Xeno-biotic Chemicals by Recombinant and Human P450 Enzymes and by Human Liver Microsomes. Catalytic activities for the oxidation of 12 substrates were determined in yeast microsomes expressing human CYP1B1 (15) and compared with those catalyzed by reconstituted systems containing CYP1A1 and 1A2 which were purified from membranes of Escherichia coli expressing the respective recombinant enzymes (table 1) (16, 17). We also determined and compared the xenobiotic-oxidation activities towards some of the model substrates by other human P450 enzymes.

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**Table 1**

<table>
<thead>
<tr>
<th>Substrate Oxidation</th>
<th>Recombinant or Liver P450 Enzymes</th>
<th>Liver Microsomes (N 6)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1A1</td>
<td>1A2</td>
</tr>
<tr>
<td>Benzo[a]pyrene 3-hydroxylation</td>
<td>929</td>
<td>23</td>
</tr>
<tr>
<td>Ethoxycoumarin O-deethylation</td>
<td>12149</td>
<td>270</td>
</tr>
<tr>
<td>Theophylline 1-demethylation</td>
<td>23</td>
<td>200</td>
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<tr>
<td>3-demethylation</td>
<td>16</td>
<td>464</td>
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<tr>
<td>8-hydroxylation</td>
<td>22</td>
<td>938</td>
</tr>
<tr>
<td>Caffeine 3-demethylation</td>
<td>37</td>
<td>188</td>
</tr>
<tr>
<td>8-hydroxylation</td>
<td>37</td>
<td>32</td>
</tr>
<tr>
<td>Coumarin 7-hydroxylation</td>
<td>&lt;50</td>
<td>&lt;50</td>
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<tr>
<td>Tolbutamide methylhydroxylation</td>
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<td>&lt;50</td>
</tr>
<tr>
<td>S-Mephenytoin 4’-hydroxylation</td>
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<td>&lt;10</td>
</tr>
<tr>
<td>Bufuralol 1’-hydroxylation</td>
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<td>4-hydroxylation</td>
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<td>6-hydroxylation</td>
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<td>7-Ethoxycoumarin O-deethylation</td>
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<td>Chlorzoxazone 6-hydroxylation</td>
<td>3560</td>
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<tr>
<td>Nifedipine oxidation</td>
<td>&lt;50</td>
<td>&lt;50</td>
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<tr>
<td>Testosterone 6β-hydroxylation</td>
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</tr>
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</table>

Substrate oxidation activities were determined by the methods as described in Materials and Methods. Values in recombinant and human liver P450 systems are the means of duplicate or triplicate determinations; in all cases SD were less than 15% of the means. Values in human liver microsomes were means and SD of six different human samples. The values with a sign of inequality (<) showed the limit of detection of substrate oxidation activities. A blank space indicates that P450-catalytic activities were not determined.
Microsomes. CYP1B1 also catalyzed bufuralol 1-hydroxylation, and 1A2, and 1B1, except for chlorzoxazone 6-hydroxylation where CYP1B1 was more active in catalyzing these substrates than those by CYP1A1, respectively, and liver microsomes of six human samples were found to be catalyzed by CYP2A6, CYP2C9, CYP2C19, CYP2E1, and CYP3A4, respectively, and testosterone. These latter substrates were oxidized most actively (table 2).

Among human P450 enzymes examined CYP1A1 had the highest activities for the 3-hydroxylation of benzo[a]pyrene, and O-deethylation of 7-ethoxyresorufin and 7-ethylcoumarin. CYP1A1 was also active for the 6-hydroxylation of chlorzoxazone, though lower than CYP2E1 did. On the other hand, CYP1A2 catalyzed efficiently 1- and 3-demethylations and 8-hydroxylation of theophylline, 3-demethylation of caffeine, and 1-, 4-, and 6-hydroxylations of bufuralol, although bufuralol 1'-hydroxylation was catalyzed most actively by CYP2D6 in reconstituted system.

CYP1B1 had considerable activities towards the 3-hydroxylation of benzo[a]pyrene, O-deethylation of 7-ethoxyresorufin, 8-hydroxylation and 1- and 3-demethylations of theophylline, 3-demethylation of caffeine, and 1', 4-, and 6-hydroxylations of bufuralol, although bufuralol 1'-hydroxylation was catalyzed most actively by CYP2D6 in reconstituted system.

CYP1B1 catalyzed bufuralol at the 4- and 6-positions. The catalytic activity also oxidized bufuralol at the 4- and 6-positions. The catalytic activities of CYP1B1 for the oxidation of bufuralol were similar to those of CYP1A1.

Kinetic Analysis of 7-Ethoxyresorufin O-Deethylation Catalyzed by Recombinant Human P450 Enzymes and by Human Liver Microsomes. Since we detected significant activities for 7-ethoxyresorufin O-deethylation by CYP1A1 (though lower than that of CYP1A1), kinetic analysis of the oxidation of 7-ethoxyresorufin was undertaken with the three recombinant P450 enzymes and with liver microsomes of human liver sample HL-16, which showed the highest catalytic activities among the six human samples examined (table 2). \( K_m \) values (about 3 \( \mu M \)) were very similar for recombinant human CYP1A1 and 1A2 and human liver microsomes, while CYP1B1 gave the lowest \( K_m \) value (about 1 \( \mu M \)). A kinetic analysis was also carried out for 7-ethoxyresorufin O-deethylation by reconstituted systems containing rat CYP1A1 and 1A2; the results showed that \( K_m \) values for these rat P450 enzymes were in the same range as those catalyzed by human CYP1A1 and 1A2 enzymes.

Immunoinhibition of 7-Ethoxyresorufin O-Deethylation Activities Catalyzed by Recombinant Human P450 Enzymes and by Human Liver Microsomes. Antibodies raised against rat CYP1A1 and 1A2 were used to examine if 7-ethoxyresorufin O-deethylation activities of recombinant CYP1A1, 1A2, and 1B1 were affected by these antibodies (Fig. 1). Anti-rat CYP1A1 IgG strongly inhibited 7-ethoxyresorufin O-deethylation catalyzed by CYP1A1 but only slightly inhibited the activities by CYP1B1 and 1A2. On the other hand, anti-CYP1A2 inhibited 7-ethoxyresorufin O-deethylation catalyzed by CYP1A2 and by human liver microsomes, but not by CYP1B1 and CYP1A1.

Effects of \( \alpha \)-Naphthoflavone on 7-Ethoxyresorufin O-Deethylation Catalyzed by Recombinant Human P450 Enzymes. Since \( \alpha \)-naphthoflavone has been reported to be a potent inhibitor for CYP1A1 and 1A2 (18,40), we determined the effects of this inhibitor on 7-ethoxyresorufin O-deethylation catalyzed by yeast microsomal CYP1B1 and by reconstituted systems containing CYP1A1 and 1A2 (table 3). The results showed that \( \alpha \)-naphthoflavone inhibited very strongly the activities of 7-ethoxyresorufin O-deethylation by CYP1B1 as well as by CYP1A1 and 1A2 at the inhibitor concentration of 10 \( \mu M \).

**Discussion**

Recently, the CYP1B1 cDNA was isolated and characterized from humans (8,41), mice (9,42), and rats (11,43). CYP1B1 proteins have been isolated from a mouse embryo fibroblast cell line and from rat adrenal microsomes (13,44,45). Both the rat and mouse enzymes have been shown to catalyze the metabolism of carcinogenic polycyclic aromatic hydrocarbons such as 7,12-dimethylbenz(a)anthracene (13,44,45). The human CYP1B1 cDNA sequence has been reported to be about 80% similar to mouse and rat counterparts and the expression of the CYP1B1 mRNA has been observed in many organs including kidney, prostate, mammary gland, pituitary, thymus, spleen, adrenal, colon, ovary, uterus, brain, heart, lung, intestine, and testis (8,41). It is, however, not known whether CYP1B1 protein is actually present in any of these tissues.
expressed in these organs, and whether there are interindividual variations in the levels of expression of CYP1B1 in humans. Although the exact roles of CYP1B1 in the metabolism of endobiotic chemicals have not been examined, the recent expression of the recombinant human CYP1B1 protein has provided evidence that this human enzyme efficiently catalyzes NADPH-dependent 4- and 2-hydroxylation of 17β-estradiol with a product ratio of 5:1 (15).

In another study of the expressed CYP1B1 enzyme (7), we have also reported that CYP1B1 is very important to the understanding of the basis of chemical carcinogenesis in humans since this P450 enzyme can catalyze the activation of diverse procarcinogenic and promutagenic chemicals to genotoxic metabolites that induce the SOS response in Salmonella tester strains (7). Of particular interest is the observation that dibenzo[a,j]pyrene-11,12-diol, 5-methylchrysene-trans-1,2-diol, and benzo[a]pyrene-trans-7,8-diol, which are suggested to be groups of the most potent inducers of mammary tumors and lung cancers in experimental animals (46–48), have been determined to be activated extensively by human CYP1B1.

CYP1A1 has also been reported to be expressed in extrahepatic organs including prostate, mammary gland, intestine, thymus, colon, adrenal, ovary, uterus, lung, and testis, while CYP1A2 is expressed primarily in microsomal fractions of the liver (19, 49, 50). Both enzymes play major roles in the metabolism of a variety of procarcinogens and promutagens, and recent studies have demonstrated that CYP1A1 and 1A2 are able to oxidize clinically used drugs such as theophylline, caffeine, bufuralol, chlorzoxazone, tamoxifen, zoxazolamine, acetaminophen, acetanilide, antipyrine, lidocaine, phenacetin, propranolol, and warfarin (19, 33, 49, 51).

The present immunoinhibition studies involving 7-ethoxyresorufin O-deethylation suggested that CYP1B1 has different immunological properties from those of CYP1A1 and 1A2 in humans. Antibodies raised against rat CYP1A1 and 1A2 did not inhibit the activities catalyzed by CYP1B1 but suppressed the reaction catalyzed by CYP1A1 and CYP1A2, respectively, in reconstituted monooxygenase systems. The distinct immunological reactivity of CYP1B1 relative to CYP1A1 or CYP1A2 is consistent with the earlier characterization of the mouse CYP1B1 protein (P450-EF) (45). In that study, anti-P450-EF was shown to only weakly (<1% of homologous protein response) recognize purified CYP1B1, 2B1, 2C7, 2E1, or 3A1 proteins. However, this antibody was found to cross-react with purified P450 2A1 (10%) and rat CYP1B1 (75%). Similarly, only anti-P450 2A1 antibody was determined to weakly cross-react with the purified P450-EF, again emphasizing the unique immunological characteristics of the CYP1B1 protein.

CYP1B1 can catalyze the oxidation of some drugs and other xenobiotic chemicals, as well as that of diverse procarcinogens and some endobiotic chemicals (7). The substrate specificities of CYP1B1 seem to resemble those of CYP1A1 and 1A2, although the catalytic activities are quantitatively different for the three P450 enzymes among the substrates determined. Human CYP1B1 has been shown to be expressed in many organs (7, 8), at least at the mRNA levels and may be very important for understanding the basis of chemical carcinogenesis (7). With respect to the drug substrates examined here, human CYP1B1 does not appear to represent a major metabolic pathway. As for model substrates of human CYP1B1, its 17β-estradiol 4-hydroxylation activity appears to be the most characteristic substrate reaction yet identified for this enzyme.

**References**


