SPECIFIC DEHYDROGENATION OF 3-METHYLINDOLE AND EPOXIDATION OF NAPHTHALENE BY RECOMBINANT HUMAN CYP2F1 EXPRESSED IN LYMPHOBLASTOID CELLS

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ABSTRACT:

3-Methylindole (3MI) is a naturally occurring pulmonary toxin that requires metabolic activation. Previous studies have shown that 3MI-induced pneumotoxicity resulted from cytochrome P-450-catalyzed dehydrogenation of 3MI to an electrophilic methylene imine (3-methyleneindolenine), which covalently bound to cellular macromolecules. Multiple cytochrome P-450s are capable of metabolizing 3MI to several different metabolites, including oxygenated products. In the present study, the role of human CYP2F1 in the metabolism of 3MI was examined to determine whether it catalyzes dehydrogenation rather than hydroxylation or ring oxidation. Metabolism was examined using microsomal fractions from human lymphoblastoid cells that expressed the recombinant human CYP2F1 P-450 enzyme. Expression of CYP2F1 in the lymphoblastoid cells proved to be an appropriate expression system for this enzyme. Products were analyzed using HPLC and the mercapturic acid, 3-[[N-acetylcytstein-S-yl]methyl]indole, of the reactive intermediate was identified and quantified. Product analysis showed that human CYP2F1 efficiently catalyzed the dehydrogenation of 3MI to the methylene imine without detectable formation of indole-3-carbinol or 3-methylindoxil. High substrate concentrations of 3MI strongly inhibited production of the dehydrogenated product, a result that may indicate the existence of mechanism-based inhibition of CYP2F1 by 3MI. Recombinant CYP2F1 demonstrated remarkable selectivity for the bioactivation of 3MI to the putative dehydrogenated reactive electrophile. Bioactivation of naphthalene to its pneumotoxic epoxide by CYP2F1 was also demonstrated.

Certain compounds can selectively cause lung damage after systemic circulation (Yost, 1997). 3-Methylindole (3MI)1 is a prototypical example of a highly organ-specific pneumotoxicant. 3MI is a fermentation product of tryptophan that is formed in the rumen of cattle and goats and in the large intestine of humans. Previous studies have demonstrated that 3MI toxicity is species-, organ-, and cell-selective (Yost, 1989). Ruminants are the most susceptible species and exhibit lung toxicity to type I alveolar and Clara cells. At least three reactive intermediates have been implicated as ultimate electrophiles (Fig. 1). These intermediates are 3-methyleneindolenine, 3-hydroxy-3-methylindolenine, and 2,3-epoxy-3-methylindoline (Skiles and Yost, 1996; Skordos et al., 1998a,b). The selective expression of certain cytochrome P-450 enzymes in pulmonary tissue is a likely mechanism for the organ-selective toxicity. Metabolic bioactivation by P-450 enzymes to electrophilic derivatives generally leads to covalent binding at the nucleophilic sites of protein, DNA, and RNA. Three cytochrome P-450 2F subfamily genes were identified by cDNA cloning in the last several years. All of the genes are transcribed and/or expressed in the lung, and transcripts are also observed in liver tissues of mice and rats (Ritter et al., 1991; Ramakanth et al., 1994; Buckpitt et al., 1995; Wang et al., 1998). CYP2F1 cDNA from human lung (Nhamburo et al., 1990) was the first to be identified. Since that time CYP2F2 (Ritter et al., 1991) and CYP2F3 (Wang et al., 1998) were identified in mouse and goat lung, respectively. The most direct evidence for cytochrome P-450 participation in the bioactivation of 3MI comes from the studies done in vaccinia virus-expressed human, rat, and rabbit cytochrome P-450s. The results indicated that human CYP2F1, CYP2A6, CYP3A4, and CYP2C8, rat CYP1A2, and rabbit CYP4B1 were able to metabolize 3MI to a covalent binding intermediate (Thornton-Manning et al., 1991). Incubation of 3MI with vaccinia virus-expressed cytochrome P-450s demonstrated that human P-450s turn over 3MI to several metabolites such as indole-3-carbinol, 3-methylindoxil, and the methylene imine. However, different products were formed by the various enzymes. Among all of the vaccinia-expressed human enzymes, human CYP2F1 showed the highest enzymatic activity toward the dehydrogenation pathway to form the methylene imine-reactive intermediate (Thornton-Manning et al., 1996). However, substantial incubation periods (6 h) were required to permit the detection of 3MI metabolites due to the relatively low levels of cDNA-driven expression in hepatoma cells (Thornton-Manning et al., 1996). This limitation precluded kinetic analyses of 3MI metabolism. In addition, the results indicated that CYP2F1 catalyzed not only the dehydrogenation of 3MI, but also

1 Abbreviations used are: 3MI, 3-methylindole; NAC, N-acetylcysteine; 3MINAC, 3-[[N-acetylcytstein-S-yl]methyl]indole.
the oxygenation of 3MI to 3-methyloxindole and indole-3-carbinol. The production of the oxygenated products by a CYP2F enzyme was not consistent with studies that showed only the dehydrogenated product formed by CYP2F3 (Wang et al., 1998). Therefore, the CYP2F1 cDNA was expressed in the lymphoblast system (Crespi et al., 1993) to permit determination of the enzyme kinetics and product specificity.

Naphthalene is another excellent example of a compound that causes lung damage after systemic circulation and subsequent bioactivation. Clara cells are highly susceptible to naphthalene-induced cytotoxicity. The stereoselective formation of the 1R,2S-oxide of naphthalene by murine CYP2F2 in Clara cells appears to be the primary mechanism for the cell-selective toxicity of naphthalene (Chichester et al., 1994; Buckpitt et al., 1995). We have demonstrated (Wang et al., 1998) that the goat lung enzyme, CYP2F3, also bioactivates naphthalene to its epoxide. The bioactivation of naphthalene to its 1,2-epoxide by human lung microsomes has been shown by Buckpitt and Bahnson (1986). However, to our knowledge, formation of the naphthalene epoxide by a human pulmonary P-450 enzyme has not been described previously. Therefore, we included naphthalene as a test substrate to evaluate the hypothesis that the human CYP2F1 can bioactivate this pneumotoxicant in addition to 3MI.

Materials and Methods

Chemicals. 3MI, ammonium acetate, NADPH, N-acetylcysteine (NAC), naphthalene, glutathione, and alkaline phosphatase-conjugated anti-rabbit IgG were purchased from Sigma Chemical Company (St. Louis, MO). 3-Phenyl-oxindole was purchased from Aldrich Chemical Company (Milwaukee, WI). Glutathione-S-transferase was a gift from Dr. Alan Buckpitt (University of California, Davis, CA). Restriction enzymes and linkers were obtained from New England Biolabs (Beverly, MA). Anti-mouse CYP2F2 antibody was the gift of Dr. Kenneth Korzekwa (University of Pittsburgh, Pittsburgh, PA). CYP2F1-containing microsomes and control microsomes were produced by Gentest Corporation (Woburn, MA). All other chemicals and reagents were of the highest purity available from local vendors.

Cell Line and Microsome Preparation. Full length, CYP2F1 cDNA (Nhamburo et al., 1990) was excised with EcoRI, modified by the addition of XhoI linkers, and introduced into the SalI site of the pHSR vector (Penman et al., 1994). Cells were transfected, vector-bearing cells were selected, and clonally derived populations were isolated (Penman et al., 1994; Crespi et al., 1997). CYP2F1-containing human lymphoblastoid cell microsomes were produced as reported previously by Crespi et al. (1991).

Immunoblot Analysis. Samples were prepared by addition of an equal volume of 2X SDS sample buffer (60 mM Tris, pH 6.8, 2% SDS, 20% glycerol, 2% 2-mercaptoethanol, 0.001% bromophenol blue) followed by heating for 5 min at 100°C. Samples were loaded on a SDS-polyacrylamide gel electrophoresis and electrophoresed on a 10% polyacrylamide minigel at 120 V for 2 h in a Hoefer (San Francisco, CA) electrophoresis apparatus. Proteins were transferred to nitrocellulose (0.45 µm pore size) by electroblotting at room temperature for 1 h at 160 mA in 192 mM glycine, 25 mM Tris, and 20% methanol using a Hoefer Semiphor semidry transfer unit. The nitrocellulose was incubated 4% powdered nonfat milk, 0.1% Tween 20, 25 mM Tris (pH 7.5), and 150 mM NaCl (blocking buffer) for 1 h at room temperature. The nitrocellulose was incubated with rabbit anti-mouse CYP2F2 diluted 1:500 in blocking buffer for 1 h at room temperature. The nitrocellulose was rinsed three times with blocking buffer for 5 min each wash and was then incubated with alkaline phosphatase-conjugated mouse anti-rabbit IgG at 1:500 in blocking buffer for 1 h at room temperature. The nitrocellulose was rinsed three times with blocking buffer, then two times with 0.1% Tween 20, 25 mM Tris (pH 7.5), and 150 mM NaCl for 5 min each wash. The nitrocellulose was developed with nitroblue tetrazolium and 5-bromo-3-chloro-3-
indolylphosphate developing solution at room temperature until bands appeared (5–15 min).

3MI Incubations. A standard reaction mixture consisted of microsomes containing 240 pmol P-450, sodium phosphate buffer (0.05 M, pH 7.4), 4 mM NADPH, 4 mM NAC, and 0.25 mM 3MI in a final volume of 2 ml. Once mixed, the sample was incubated at 37°C for 30 min. The reaction was stopped by addition of an equal volume (2 ml) of ice-cold acetonitrile, centrifuged at 3000 g for 20 min, and 20 μl of 1 mM 3-phenyloxindole were added to the supernatant to serve as an internal standard. The sample supernatant was then dried to a volume of 200 μl. The supernatant was analyzed directly by HPLC.

In two separate experiments, similar incubations were performed; in one set, the 3MI concentrations were varied; in the other set, the incubation times were varied. All determinations were repeated at least four times.

HPLC for Analysis of 3MI Metabolites. HPLC was performed on a Beckman (Berkeley, CA) system composed of dual 114 M solvent pumps, a 412A controller, and a 210A injector connected to a Hewlett-Packard (Palo Alto, CA) 1040A diode-array UV detector. The 3MI metabolites were separated on a 4.6 × 250 mm, 5 μm reversed phase Ultemex C18 column (Phenomenex, Torrance, CA) using a gradient solvent system that began at 10% acetonitrile and 90% 0.1 M ammonium acetate buffer (pH 6.0) and then changed to 50% acetonitrile over 5 min, then changed to 55% acetonitrile over another 5 min, followed by a final change to 95% acetonitrile over 5 min using a flow rate of 1 ml/min. The chromatograms were monitored by ultraviolet absorption at 254 and 280 nm. The data was analyzed on a model 9000 Series 300 Hewlett-Packard computer using HP79995A operating software. The mercapturate adduct 3-[N-acetylcystein-S-yl)methyl]indole (3MINAC) had a retention time of approximately 6.8 min and a UV spectrum identical with a synthetic standard. Peak ratios were used with a standard curve to determine amounts of the mercapturate.

Naphthalene Incubations. The following reaction components were pre-incubated for 5 min at room temperature: 1 mM NADPH, sodium phosphate buffer (0.1 M, pH 6.5), 5 U glutathione-S-transferase, and 1.5 μM glutathione. This was followed by addition of 0.5 mM naphthalene and microsomes containing 240 pmol P-450. The mixture was incubated in an air-right tube for 8 min at 37°C. The reaction was stopped by the addition of 2 volumes of ice-cold methanol. The sample was then centrifuged at 14,000g to pellet the precipitated protein. The supernatant was evaporated to dryness and then resuspended in 200 μl water. Fifty microliters of the sample were analyzed directly by HPLC.

Analysis of Glutathione Adducts of Naphthalene. HPLC was performed on a Waters Associates (Milford, MA) system connected to a Hewlett-Packard 1050 detector. The metabolites were separated on a 4.6 × 220 mm, 5 μm Phase Sep ODS 2 column (Phenomenex, Torrance, CA). Solvent A was 0.06% triethylamine in which phosphoric acid was added to adjust the pH to 3.1. Solvent B was a mixture of 50% solvent A and 50% acetonitrile. The linear gradient system used began at 90% solvent A where it stayed until 75 min later when solvent A was decreased to 85%. Five minutes later solvent A was decreased to 10% and then brought back up again to 90% over the next 5 min.

Proteins were separated by electrophoresis on a 10% polyacrylamide gel and then transferred to a nitrocellulose membrane and probed with rabbit anti-mouse CYP2F2 antibodies. Lane 1 is control microsomes (50 μg protein) and lane 2 is CYP2F1 microsomes (30 μg protein).

Fig. 2. Immunoblot analysis of control and CYP2F1-expressing human lymphoblast microsomes.

CYP2F1 microsomes were incubated with 0.25 mM 3MI for times ranging from 10 to 120 min. Each time point represents four incubations. The supernatant from each incubation was analyzed directly by HPLC.

Fig. 3. Time dependence of 3MINAC formation from 3MI.

CYP2F1 microsomes were incubated with varying amounts of 3MI for 30 min each. Each data point represents four incubations. The supernatant from each incubation was analyzed directly by HPLC.

Fig. 4. Substrate dependence of 3MINAC formation from 3MI.
The flow rate was 1 ml/min. Peak integration was done on a Waters Maxima workstation.

Results

CYP2F1 Expression. Microsomes prepared from human lymphoblastoid cells containing human CYP2F1 exhibited immunodetectable CYP2F1 protein with an apparent molecular mass of 55 kDa. This band was not detectable in control microsomal preparations (Fig. 2). The level of CYP2F1 expression, approximately 20 pmol/mg microsomal protein, as determined by CO-difference spectra, was stable for 2 months of continuous cell culture.

Determination of Time and Substrate Dependence. The rate of 3MI mercapturate formation was essentially linear for approximately 30 min, although it appears that the rate becomes nonlinear at about 30 min (Fig. 3). Results from the varied 3MI concentration incubations showed that the CYP2F1 enzyme seemed to be saturated at a concentration of 0.25 mM and that inhibition occurred at higher 3MI concentrations (Fig. 4). A possible explanation for the lower rates at high substrate concentrations is that 3MI is a mechanism-based inhibitor of CYP2F1. No detectable 3MI metabolism was observed in control microsomes from cells that contained the vector without the CYP2F1 cDNA (Fig. 5B).

Identification and Quantification of 3MI Metabolites. Figure 5A shows a representative chromatogram from a CYP2F1 microsomal
incubation. 3MINAC was not formed when NADPH was omitted (data not shown), nor was it formed when complete incubations were performed with lymphoblast microsomes produced by transfecting the vector without the CYP2F1 cDNA insert (Fig. 5B). Careful analysis of the products of 3MI oxidation by CYP2F1 showed exclusive dehydrogenation without detectable hydroxylation or ring oxidation during the 30-min incubation. Incubations performed with 0.25 mM 3MI and microsomes containing 240 pmol 2F1 produced a total of 9.1 nmol 3MINAC. Using a minimal signal-to-noise ratio of 3:1 at 254 nm, we determined that the lower limit of detection of indole-3-carbinol by HPLC analysis was 0.3 nmol in the same incubation volume. Thus, although indole-3-carbinol might have been formed by CYP2F1 and might have been detected in these incubations with a more sensitive analytical technique, the molar amount of the hydroxylated product, indole-3-carbinol, was at least 30 times less than the molar amount of the dehydrogenated product, 3-methylenedioine.

The results of these incubations, which demonstrate exclusive formation of 3-methylenedioine without production of the oxygenated metabolite 3-methyloxindole, disagree somewhat with our earlier studies with the vaccinia expression system (Thornton-Manning et al., 1996). In the previous work recombinant CYP2F1 produced 3-methyloxindole in long incubations (6 h) that were necessary due to low enzyme expression. The 2F1-containing microsomes from the lymphoblastoid cells in the current studies do not produce 3-methyloxindole at incubation times up to 2 h. We cannot explain this inconsistency except that it is possible that the vaccinia expression system might have been slightly contaminated with another P-450 enzyme that slowly formed the oxygenated metabolite.

Other peaks in the HPLC chromatogram are background contaminants that were present in chromatograms from control incubations that excluded 3MI (data not shown). The UV spectrum of the mercapturate is shown as an inset in Fig. 5A; this was identical with a spectrum that excluded 3MI (data not shown). The UV spectrum of the mercapturates that were present in chromatograms from control incubations was identical with a spectrum that excluded 3MI (data not shown). All three conjugates were formed by CYP2F1 and the quan-

<table>
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<th>Enzymes</th>
<th>( V_{\text{max}} )</th>
<th>( K_m )</th>
<th>( V_{\text{max}}/K_m )</th>
<th>Other Substrates</th>
<th>cDNA Sources</th>
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<td>Goat lung microsomes</td>
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<td>Naphthalene</td>
<td>Human lung</td>
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<tr>
<td>CYP2F1</td>
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<td>Naphthalene</td>
<td>Mouse lung</td>
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<tr>
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<td>0.34</td>
<td>1.6</td>
<td>Naphthalene</td>
<td>Goat lung</td>
</tr>
</tbody>
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\( a \) Values from Skiles and Yost, 1996.
\( b \) These are apparent kinetic constants, based on rates observed at substrate concentrations less than 0.3 mM.
\( c \) Naphthalene oxidation demonstrated in Ritter et al., 1991.
\( d \) Values from Wang et al., 1998.

Identification and Quantification of Glutathione Adducts of Naphthalene. The glutathione conjugate peaks had retention times of 24.9, 26.9, and 29.3 min, respectively. They were determined to be the peaks of interest by comparison with synthesized standards. A standard curve was used to determine the amount of each conjugate present. All three conjugates were formed by CYP2F1 and the quantities were determined to be 13.2 pmol/nmol P-450/min for conjugate 1, 4.1 pmol/nmol P-450/min for conjugate 2, and 18.2 pmol/nmol P-450/min for conjugate 3. Glutathione conjugates 1 and 3 are formed from naphthalene 1,2R-oxide, whereas conjugate 2 is formed from the enantiomeric naphthalene 1R,2S-oxide (Buckpitt et al., 1995). The mouse lung CYP2F2 forms almost exclusively conjugate 2 from naphthalene 1R,2S-oxide (Buckpitt et al., 1995). Thus, human CYP2F2 appears to predominately form the opposite enantiomeric epoxide (naphthalene 1S,2R-oxide) from the mouse CYP2F2 enzyme.

Discussion

Many studies have demonstrated the importance of cytochrome P-450 bioactivation in the production of pneumotoxic intermediates in several animal species (Yost, 1989; 1997; Buckpitt et al., 1995; Wang et al., 1998). However, although human lung microsomes bioactivate 3MI to a covalent binding intermediate (Ruangyuttikarn et al., 1991), the capability of human lung P-450 enzymes to bioactivate 3MI or naphthalene has not been extensively documented except for our previous work (Thornton-Manning et al., 1991; 1996) and the current studies. The results presented here have demonstrated the importance of the human cytochrome P-450 2F1 in the bioactivation of two prototypical pneumotoxins, 3MI and naphthalene, to their putative reactive intermediates. CYP2F1 catalyzes the epoxidation of naphthalene to a reactive epoxide, although the stereoselectivity of the human enzyme appears to be almost the opposite of the mouse CYP2F2 enzyme. Previous studies with human lung microsomes (Buckpitt and Bahnson, 1986) also showed a lack of stereoselective preference for formation of the 1R,2S-oxide, which is the predominant epoxide from CYP2F2 catalysis.

CYP2F1 also demonstrates a unique specificity for the bioactivation of 3MI to the toxic 3-methylenedioine intermediate, and this bioactivation occurs through a highly selective dehydrogenation mechanism, without concomitant formation of the oxygenated metabolites, indole-3-carbinol and 3-methyloxindole. The preference for dehydrogenation appears to be a common trait of at least two members of the CYP2F subfamily of P-450 enzymes, CYP2F1 and goat CYP2F3 (Wang et al., 1998). We had reported previously (Thornton-Manning et al., 1996) that 3-methyloxindole is efficiently formed by 2F1 using the vaccinia expression system. In the current studies we can observe none of this ring-oxidized product. The reason for this discrepancy may be that the incubation times were very long (6 h) with the vaccinia expression system in HepG2 cell lysates and a contaminating P-450 enzyme may have produced 3-methyloxindole during this time period.

The apparent \( V_{\text{max}}/K_m \) of this human P-450 enzyme for the dehydrogenation of 3MI is approximately 40-fold higher than the \( V_{\text{max}}/K_m \) of CYP2F3, the goat lung P-450 enzyme that participates in the bioactivation of 3MI in this highly susceptible species (Table 1). The \( V_{\text{max}}/K_m \) of CYP2F1 was about half of the total microsomal \( V_{\text{max}}/K_m \) from goat lung. It would appear, therefore, that human lung cells that contain CYP2F1 might be highly susceptible to 3MI-induced damage. In addition, 3MI may also be an interesting mechanism-based inhibitor of CYP2F1. Studies are underway to evaluate the mechanism of inhibition.

In summary, these studies have shown that a P-450 enzyme that is selectively expressed in lung tissues of humans efficiently catalyzes the formation of the putative toxic electrophilic intermediate of 3MI, 3-methylenedioine. The catalytic properties of this interesting enzyme were shown to include a unique propensity for dehydrogenation of 3MI without formation of the expected hydroxylated product, indole-3-carbinol, or the ring-oxidized product, 3-methyloxindole. Thus, it appears that the selective expression of CYP2F1 in human respiratory cells may significantly increase the susceptibility of these cells to toxic insult by 3MI. Because 3MI is produced in humans...
through colonic fermentation of tryptophan by anaerobic bacteria and is also found in reasonably high concentrations in cigarette smoke, it is likely that humans may be susceptible to 3MI-mediated lung injury through bioactivation by CYP2F1.

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References