Metabolic Capabilities of CYP2F2 with Various Pulmonary Toxicants and Its Relative Abundance in Mouse Lung Subcompartments

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ABSTRACT
The tissue- and species-selective toxicity of a number of pulmonary toxicants has been attributed to the presence and distribution of activating enzymes with high \( k_{cat} \) in target airways of susceptible species. The mouse is especially sensitive to a variety of metabolically activated lung toxicants. Recombinant CYP2F2 (mouse) was recently shown to effectively metabolize the species-selective pulmonary toxicant naphthalene. Here we show that the pulmonary toxicants 1-nitronaphthalene and 2-methylnaphthalene are metabolized readily with high \( k_{cat} \) values (17.1 and 67.6 min\(^{-1}\), respectively) to potentially cytotoxic intermediates at biologically relevant \( K_m \) values (21.5 and 3.7 \( \mu \)M, respectively). Additionally, anthracene and benzo[a]pyrene are both metabolized by CYP2F2 (0.14 ± 0.04 and 0.04 ± 0.00 nmol/nmol/min, respectively), albeit at much lower rates. The levels of total CYP in mouse airways are considerably higher than those in parenchyma and trachea, and this is consistent with much higher rates of naphthalene metabolism in microsomal preparations from airways compared with the other subcompartments. The data suggest that CYP2F2 is a prominent cytochrome P450 in mouse lung that metabolizes a number of pulmonary toxicants. The presence of CYP2F2 may be important in the susceptibility of the mouse to metabolically activated pulmonary toxicants.

Numerous studies with laboratory animals have demonstrated the importance of respiratory epithelial cells as targets for both inhaled and ingested chemicals. Specifically, nonciliated bronchiolar epithelial (Clara) cells are very sensitive to toxicants, and this appears to be due to the metabolic capabilities of the Clara cell (Plopper, 1993; Gram, 1997). With several lung toxicants such as trichloroethylene, nitronaphthalene, 3-methylindole, and naphthalene (for review, see Yost, 1997), metabolism by cytochrome P450 is an obligate step in the formation of toxicologically active derivatives. Of the CYPs identified to date, a total of eight has been shown to be present in lung tissues of a variety of species, including CYP1A1, 1B1, 2B, 2E1, 2F, 2J, 3A, and 4B1/2. The catalytic activities of a few of these enzymes have been reported using either purified proteins or recombinant enzymes (Willey et al., 1996; for review, see Buckpitt and Cruikshank, 1997; Yost, 1997). The roles of each of the pulmonary cytochrome P450s in substrate turnover, with a few exceptions, have not been assessed fully. The expression levels of these CYPs in the lung and their metabolic capabilities with various substrates may determine the susceptibility of the lung to metabolically activated toxicants.

The primary sources of human exposure to naphthalene, 2-methylnaphthalene, and closely related congeners are through mainstream and sidestream tobacco smoke as well as industrial processes that use naphthalene as a synthetic intermediate (ATSDR, 1995). In general, combustion processes generate polycyclic compounds, which include naphthalene, anthracene, and benzo[a]pyrene. Nitronaphthalenes and methyl-nitronaphthalenes, which are gas phase reaction products of the appropriate parent compound and \( N_2O_5 \), account for a major portion of the mutagenicity associated with ambient air samples (Gupta et al., 1996).

The tissue- and species-selective toxicity of a number of pulmonary toxicants has been attributed to the presence and distribution of activating enzymes with high \( k_{cat} \) in target airways of susceptible species (Boyd et al., 1978; Wolf et al., 1982; Csanydy et al., 1992). The mouse, unlike the rat, is susceptible to a variety of metabolically activated lung toxicants. In particular, parenteral administration of naphth...
lence, 2-methylnaphthalene, dichloro- and trichloroethylene, coumarin, and styrene results in Clara cell necrosis in mice, but not in rats (Plopper, 1993; Gadberry et al., 1996; Born et al., 1998). Previous work with naphthalene has demonstrated a correlation between the presence of CYP2F2 in mouse Clara cells with the stereoselective epoxidation of naphthalene and the cytotoxicity of this chemical in murine lung (Buckpitt et al., 1995).

We have recently expressed CYP2F2 using the baculovirus expression system and have characterized the catalytic activities of this protein with naphthalene under optimal conditions (Shultz et al., 1999). This enzyme readily metabolizes naphthalene with a high degree of stereoselectivity to the 1R,2S-naphthalene oxide at a ratio of 66:1 with a \(K_{\text{cat}}\) of 3 \(\mu M\) and \(k_{\text{cat}}\) of 104 min\(^{-1}\). The stereoselectivity of naphthalene metabolism by CYP2F2 is similar to that observed in mouse lung microsomes (10:1) as well as mouse airway explants (>20:1). Thus, the localization of CYP2F2 to mouse Clara cells and the high turnover rates by CYP2F2 are consistent with the possibility that this protein plays a role in the sensitivity of murine Clara cells to naphthalene-induced injury.

The eventual outcome from exposure of a particular tissue or cell appears to depend on a complex interplay among the exposure levels, the rates of formation and detoxication of electrophilic intermediates, and the sensitivity of the cell to the biologically active derivative. There are no striking differences in either epoxide hydrolase or glutathione S-transferase (GST)-mediated detoxification pathways that would account for the unusual susceptibility to naphthalene oxide toxicity in the mouse compared with the rat (Lorenz et al., 1984). This would indicate that the metabolic activation step is a very important factor in the eventual toxicity of compounds such as naphthalene. Our hypothesis is that the species differences in susceptibility are due to the presence of a constitutively expressed pulmonary CYP isozyme (CYP2F2) in the mouse that shows high catalytic activity with naphthalene and possibly to a number of other metabolically activated lung toxicants.

The objective of this work was to examine the possible importance of CYP2F2 in rendering the mouse susceptible to metabolically activated pulmonary toxicants by fully characterizing the catalytic activities of CYP2F2 toward 1-nitronaphthalene and 2-methylnaphthalene, as well as determining whether two larger polyaromatic hydrocarbons, anthracene and benzo(a)pyrene, are metabolized by this enzyme. For a specific CYP to be of importance in the unique susceptibility of mouse lung to a number of toxicants, it would have to be able to catalyze the metabolism of various substrates efficiently and, just as importantly, would have to be present at significant levels. Heavy staining present in the Clara cells with anti-CYP2F2, although not quantitative, suggests that CYP2F2 is a prominent isozyme in the Clara cell of mouse lung (Buckpitt et al., 1995). The current work provides additional data on the relative levels of CYP2F2 present in mouse airways, parenchyma, and trachea.

### Experimental Procedures

**Materials and Reagents.** Recombinant mouse CYP2F2 was expressed using the baculovirus expression system and microsomes were prepared as described by Shultz et al. (1999). Mouse NADPH-cytochrome P450 oxidoreductase (reductase) was purified from mouse liver using published procedures (Strobel and Dignam, 1978). The specific activity of the purified reductase preparation was 13.8 U/mg of protein (1 U = 1 \(\mu\)mol of cytochrome c reduced/min) as quantified by the standard cytochrome c reduction assay (Guengerich, 1994). Conversions from cytochrome c units to molar concentrations are based on the assumption that pure reductase reduces 55 \(\mu\)mol of cytochrome c/min/mg of protein under the assay conditions described above (Grueneke et al., 1995). GSTs from mouse liver cytosol were purified using affinity column chromatography (Simons and Vander Jagt, 1981) and activities were assessed with 1-chloro-2,4-dinitrobenzene as the substrate (1 U = 1 \(\mu\)mol of 1-chloro-2,4-dinitrobenzene-glutathione (GSH) conjugate/min; Habig et al., 1974). Recombinant human cytochrome b\(_5\) was a generous gift from Dr. M. Shet and Dr. R. Estabrook, University of Texas, Dallas, TX.

Benzo[a]pyrene-4,5- and -7,8-dihydropyrenoids (±) were purchased from the NCI Chemical Carcinogen Reference Standard Repository at the Midwest Research Institute (Kansas City, MO). GSH conjugates were synthesized separately from 7.45 \(\mu\)mol of 7,8- and 7,45 \(\mu\)mol of 4,5-epoxides by incubating with GSH (5 mM) and GST (10 U/ml) in 10 ml of 0.1 M Na\(_2\)HPO\(_4\), pH 7.4, while stirring at room temperature for 2 h. The reaction was terminated by acidification to pH 3 with glacial acetic acid added dropwise. Conjugates, precipitated from solution, were partially purified by centrifugation and resuspended to saturation in 50:50 v/v acetonitrile/water. Unless otherwise stated, all other reagents were purchased from commercial vendors and were of "reagent/analytical grade".

**Radioactive Chemicals.** 8-[\(^{14}\text{C}\)]2-Methylnaphthalene (specific activity 8.2 mCi/mmol), (1,2,3,4,4A,9A)-[\(^{14}\text{C}\)]anthracene (specific activity 20.6 mCi/mmol), and 7-[\(^{14}\text{C}\)]benzo[a]pyrene (specific activity 26.6 mCi/mmol) were all purchased from Sigma Chemical Co. (St. Louis, MO). 1-[\(^{14}\text{C}\)]1-Nitronaphthalene (specific activity 6.1 mCi/mmol) was prepared in the laboratory by the nitration of 1-[\(^{14}\text{C}\)]naphthalene with trifluoroacetic acid anhydride and ammonium nitrate and purified by normal phase HPLC (Watt et al., 1999).

**Experimental Animals.** Male Swiss-Webster mice (20–30 g of body weight) were purchased from Charles River Laboratories (Hollister, CA). Animals were housed over inert bedding in cages within HEPA-filtered laminar airflow cabinets. They were allowed free access to food and filtered deionized water, and kept on a 12-h light/dark cycle in facilities at the University of California at Davis, which are certified by the American Association for the Accreditation of Laboratory Animal Care. They were used no sooner than 7 days after receipt from the supplier.

**Measurement of 1-Nitronaphthalene Metabolism.** Incubations with 1-nitronaphthalene (3.75–500 \(\mu\)M) were carried out in 250-\(\mu\)l volumes containing 2.5 \(\mu\)mol of recombinant CYP2F2 (quantity determined spectrophotometrically), NADPH cytochrome P450 reductase (quantity determined by cytochrome c reducing activity), 0.4 mM CHAPS, NADPH regenerating system (0.25 \(U\) of glucose-6-phosphate dehydrogenase, 14 mM glucose 6-phosphate, 2.18 mM NADP, and 1 mM MgCl\(_2\)), 1 mM GSH, and 2.5 \(U\) of GST in 0.1 M Na\(_2\)HPO\(_4\), pH 7.4. Incubations were performed in the presence of GSH and GST for the trapping of any reactive epoxides as glutathione conjugates. CYP2F2 and reductase were preincubated on ice in a 50-\(\mu\)l volume of 0.1 M Na\(_2\)HPO\(_4\), pH 7.4, containing 2 mM CHAPS for 2 h before use. Incubations were performed in a shaking water bath at 37°C for 10 min. Reactions were quenched on ice by the addition of 2 volumes of methanol. Protein was removed by centrifugation and the supernatants were evaporated under reduced pressure. Glutathione conjugates were separated on a Phase Sep C\(_{18}\) reversed-phase column (25 cm \(\times\) 4.6 mm i.d.; 5-\(\mu\)m particle) as described previously (Watt et al., 1999) with a mobile phase of 0.06% triethylamine phosphate in water (pH 3.1) and acetonitrile. Samples were run at a flow rate of 1 ml/min with a linear increase from 5 to 16% acetonitrile over 60 min. Peaks were monitored at 256 nm using a Waters Dual Wavelength 2487 UV detector and quantified with the use of 1-[\(^{14}\text{C}\)]nitronaph-
thalone (5000 dpm/nmol) in separate incubations to obtain UV area unit/nmol ratios. Fractions were collected at 0.5-min intervals and counted using a Beckman LS5000TD scintillation counter.

**Measurement of 2-Methylnaphthalene Metabolism.** The rate of 2-methylnaphthalene metabolism was assessed in incubations containing recombinant CYP2F2, reductase, CHAPS, and NADPH regeneration system as described above for 1-nitronaphthalene. The amounts of CYP2F2 and reductase varied between experiments, and these are listed in the figure legends. Substrate concentrations varied from 2.5 to 500 μM. GSH and GST were included for the trapping of reactive epoxides as stable glutathione conjugates. Incubations were performed in a shaking water bath at 37°C for 5 or 10 min as specified in the figure legends. Reactions were stopped and samples prepared for analysis as with 1-nitronaphthalene (see above). Glutathione conjugates were separated by reversed-phase HPLC using the same mobile phase components as used for the separation of GSH conjugates of 1-nitronaphthalene except for the gradient. Acetonitrile concentrations were increased linearly from 5 to 11% over 20 min, followed by 20 min at 11% and a linear increase to 58% over the last 30 min. An altered gradient was used for the separation of more nonpolar 2-methylnaphthalene metabolites, which consisted of increasing acetonitrile from 15 to 20% over the first 15 min, followed by an increase to 30% over the next 25 min and a subsequent increase to 35% over the last 30 min. Peaks were monitored using a Waters Dual Wavelength 2487 UV detector at 260 and 223 nm and quantified by using 2-[14C]methylcyclohexane (5000 dpm/nmol) in separate incubations as described above.

**Mouse Liver Microsomal 2-Methylnaphthalene Metabolism and Sample Preparation for Mass Spectrometry.** Microsomes were prepared from mouse liver by differential centrifugation at 10,000g for 20 min followed by centrifugation of the supernatant at 100,000g for 70 min at 4°C. A 100-ml incubation containing 500 μM 2-methylnaphthalene, mouse liver microsomes (no more than 2 mg/ml), NADPH regeneration system, GSH, and GST were performed at 37°C for 30 min. The incubation was stopped with 2 volumes of methanol on ice. Precipitated protein was removed by centrifugation. The supernatant was evaporated to near dryness under vacuum to remove all traces of methanol, acidified to pH 3 to 4 with acetic acid, and the extract was applied to a C18 prepacked cartridge (Varian Bond Elut, 6 ml) equilibrated with 1% acetic acid. Glutathione conjugates were eluted with 50% acetonitrile, lyophilized, and the residue was resuspended in water for reversed-phase HPLC on a Rainin Dynamax C18 prep column [60 Å, 21.2 cm × 250 mm i.d.; 5-μm particle (Woburn, MA)] under the conditions described above for 2-methylnaphthalene conjugate separation. In preparation for mass spectrometry, isolated metabolites were purified by an additional chromatography step to remove traces of salts.

**Mass Spectrometry.** Metabolites of 2-methylnaphthalene were analyzed on a Finnigan LCQ (Finnigan Corp., San Jose, CA) ion trap mass spectrometer with a 2000 atomic mass unit mass range using 50:50 v/v acetonitrile/water as the mobile phase. A Varian 9010 mass spectrometer with a 2000 atomic mass unit mass range using the method of Lowry et al. (1951). The trachea was exposed, cannulated, and the lungs removed and inflated with 1% agarose in phosphate-buffered saline, which was then allowed to cool in ice-cold phosphate-buffered saline for 30 min. Trachea, parenchyma, and airways from five to seven mice were pooled separately and treated as one sample. Pooled tissue was homogenized in 0.02 M Tris, 1.15% KCl, 0.2 mM EDTA, and 0.5 mM dithiothreitol buffer (pH 7.4) containing 15% glycerol using glass-class homogenizers on ice. Microsomes were prepared by differential centrifugation as described previously and the pellet was resuspended in 100 μl of 0.1 M Na2HPO4, pH 7.4, containing 20% glycerol.

**Total Cytochrome P450 Determination.** Total cytochrome P450 and protein content were measured for each of the microsomal samples. Cytochrome P450 levels were determined by obtaining difference spectra of sodium dithionite-reduced versus CO-bubbled samples at 500 to 400 nm according to Omura and Sato (1964). Protein content was determined from the same samples by the method of Lowry et al. (1951).

**Microsomal Metabolism of Naphthalene.** Microsomes, prepared as described above for airway, trachea, and parenchyma, were used to assess the metabolism of naphthalene in the presence of GSH and GST. Measurement of naphthalene glutathione conjugates 1 to 4 was used as an index of the formation of the 1R,2S- and 1S,2R-naphthalene oxides. The rates of naphthalene metabolism were assessed in incubations containing no more than 2 mg of microsomal protein/ml, NADPH regenerating system, 1 mM GSH, and 2.5 U of GST in 0.1 M Na2HPO4, pH 7.4. Incubations were performed in a shaking water bath at 37°C for 10 min. Reactions were quenched on ice by the addition of 2 volumes of methanol. Protein was removed by centrifugation and the supernatants were evaporated under reduced pressure. Glutathione conjugates were separated by reversed-phase HPLC and were quantified by peak areas at 260 nm as described previously (Shultz et al., 1999). Glutathione conjugate standards were prepared by synthesis from naphthalene oxide and GSH, and were purified by preparative HPLC.

**Statistical Methods.** Cytochrome P450 levels from mouse airways, trachea, and parenchyma were expressed as nanomoles of P450 per milligram of microsomal protein (mean ± S.D.). Compari-
Results

Metabolism of 1-Nitronaphthalene by Recombinant CYP2F2. Recombinant CYP2F2 metabolized 1-nitronaphthalene to both the 5,6- and 7,8-epoxide intermediates, which were trapped and measured as GSH conjugates. These metabolites were identified and quantified with the use of 1-[14C]nitronaphthalene (500 μM, 5000 dpm/nmol). The identity of the metabolites formed was confirmed by comigration of purified standards using reversed-phase HPLC. The identity and regiochemistry of each metabolite was established by mass spectrometry/mass spectrometry and NMR as reported by Watt et al. (1999). Figure 1, A and B, show the UV and radiochemical HPLC profiles, respectively, of an extract of an incubation for the metabolism of 1-nitronaphthalene with recombinant CYP2F2. Peaks were detected corresponding to standard 1-nitronaphthalene conjugates 2, 3, 4, 1, and 6. The coelution of all peaks was confirmed by spiking samples with known standards for each of the conjugates. A standard for conjugate 2 was not available; therefore, the peak comigrating with conjugate 2 was identified primarily by relative retention time. Recombinant CYP2F2 preferentially formed epoxides at the 7,8-position and to a lesser extent at the 5,6-position of 1-nitronaphthalene with a ratio of 5:1. Metabolism was not detected in incubations containing noninfected Tn5 insect cell lysates, cytochrome P450 reductase, NADPH regenerating system, GSH/GST, and 1-nitronaphthalene (data not shown). Metabolism was inhibited by bubbling CYP2F2 incubations with CO and conjugates were not observed in incubations that did not contain GSH and GST (data not shown). Before conducting kinetic studies, optimal incubation conditions for 1-nitronaphthalene metabolism by recombinant CYP2F2 were defined. Linearity with time (up to 30 min) and protein (up to 10 pmol of CYP2F2) was established as was the optimal ratio of CYP:reductase (1:14.7 nmol of P450/nmol of reductase) for use in all subsequent incubations (data not shown). In addition, the concentration of detergent (CHAPS) was optimized to obtain maximal activities.

Experiments were conducted to determine whether addition of cytochrome b5 altered the rate of 1-nitronaphthalene metabolism under the optimal incubation conditions established above. Incubations containing CYP2F2 and optimal amounts of reductase were supplemented with recombinant human cytochrome b5 at 1:1 and 3:1 ratios (nmol of b5/nmol of P450). Addition of cytochrome b5 did not have any effect on the metabolism or alter the pattern of metabolite formation of 1-nitronaphthalene (data not shown).

Michaelis constants were determined for the formation of total glutathione conjugates of 1-nitronaphthalene using substrate concentrations varying between 0.00375 and 0.5 mM. No alterations in the ratios of individual peaks were observed at the different substrate concentrations examined. Data were analyzed by linear regression of double reciprocal plots for the estimation of kinetic parameters. The Km for metabolism of 1-nitronaphthalene to glutathione conjugates was determined to be 21.5 μM with a kcat of 17.1 min−1 (Fig. 2). The specificity constant of CYP2F2 for 1-nitronaphtha-

![Fig. 1. HPLC profiles of an extract from an incubation containing 500 μM 1-[14C]nitronaphthalene (5000 dpm/nmol), 2.5 pmol of recombinant CYP2F2, 36.8 pmol of reductase, NADPH regeneration system, and GST/GSH (under Experimental Procedures) for 10 min at 37°C. A, HPLC UV profile showing the formation of 1-nitronaphthalene conjugates 2 and 4, 1 and 3, and 6 corresponding to the 8-, 7-, and 6-thioethers, respectively (see panel above A). B, HPLC radiochemical profile of 0.5-min fractions of the same sample as A confirming that the peaks detectable by UV were derived from the parent, 1-[14C]nitronaphthalene.

![Fig. 2. Double reciprocal plot for the determination of the apparent Km and Vmax for 1-nitronaphthalene metabolism by recombinant CYP2F2. Incubations contained 2.5 pmol of CYP2F2, 36.7 pmol of reductase, NADPH regenerating system, GST, GSH, and 1-nitronaphthalene (3.75, 5, 6.6, 10, 17.5, 25, 33, 50, 100, and 500 μM). Incubations were for 10 min at 37°C. Data is reported as the mean of two incubations per substrate concentration. The rate is reported as total 1-nitronaphthalene conjugates formed (nmol · nmol of P450−1 · min−1). No changes were noted in the ratios of conjugates generated at varying substrate concentrations.](https://i.imgur.com/0Z0Z0.png)
lene was calculated to be $1.33 \times 10^4$ M$^{-1}$ s$^{-1}$.

**Metabolism of 2-Methylnaphthalene by Mouse Liver Microsomes and Recombinant CYP2F2.** The metabolism of 2-methylnaphthalene may result in the ring oxidation at the 3,4-, 5,6-, or 7,8-positions and/or the oxidation of the methyl group at the 2 position (Fig. 3). Although only three epoxides are shown, ring oxidation may generate as many as six distinct enantiomeric epoxides which, in turn, could generate up to 12 glutathione conjugate diastereomers in the presence of GSH and GST (depending on the stereochemistry). The epoxides may rearrange to phenols or may also be metabolized to dihydrodiols by epoxide hydrolases. Oxidation of the methyl group could result in the formation of 2-naphthalene methanol, which may be metabolized to 2-naphthaldehyde and further to 2-naphthoic acid. Due to the instability of these products, it was extremely difficult to quantitatively measure their formation. For the purpose of these studies our analysis focused primarily on the ring oxidation of 2-methylnaphthalene since these metabolites could be quantified consistently and the formation of side chain metabolites were measured, at best, qualitatively.

Mouse liver microsomes incubated with NADPH regeneration system and GSH/GST generated at least nine of the possible twelve 2-methylnaphthalene GSH conjugates (Fig. 4A). These peaks were individually collected and prepared for mass spectral characterization. All spectra yielded major fragments at $m/z$ 488 (M + Na), 466 (M$^+$), 448 (M – H$_2$O), and 306 (GS$^-$), which are consistent with a hydroxy glutathionyl dihydro-2-methylnaphthalene derivative (Fig. 5).

Recombinant CYP2F2 was capable of generating five peaks, corresponding to conjugates 1, 2, 3, 6, and 7 of the 2-methylnaphthalene glutathione conjugates generated by mouse liver microsomes, at significant rates as shown in the UV and radiochemical profiles from an extract of a complete incubation containing 2-methylnaphthalene and CYP2F2 (Fig. 4, B and C). Metabolites were identified by cochromatography with metabolites generated in mouse liver microsomal incubations and were measured by using $^{14}$C-labeled substrate (500 $\mu$M, 5000 dpm/nmol). Metabolism was not detected in similar incubations containing uninfected Tn5 insect cell lysates in place of recombinant CYP2F2 (data not shown). Bubbling CYP2F2 incubations with CO or excluding GSH and GST from the incubations inhibited the formation of metabolites (data not shown).

No radioactive, UV-absorbing peaks, which coelute with either 2-naphthalene methanol or 2-naphthoic acid, were observed in complete incubations of 2-methylnaphthalene with CYP2F2. However, formation of 2-naphthaldehyde was easily measurable, suggesting that recombinant CYP2F2 is capable of hydroxylating the methyl group at the 2 position with the resulting formation of 2-naphthaldehyde. This peak was not present in the negative control incubations (see above for the 2-methylnaphthalene conjugates). Incubations lacking GSH and GST resulted in a marked increase in this peak. This peak appeared to be composed of multiple compounds poorly resolved from 2-naphthaldehyde using the methods described here. Subsequent chromatography of samples using an altered HPLC gradient allowed for more complete separation of nonpolar 2-methylnaphthalene metabolites (under Experimental Procedures). Under these conditions, two other unidentified peaks were observed in the chromatogram at retention times of 72.5 and 74.5 min, indi-

![Fig. 3. Schematic of the possible metabolites generated from the metabolism of 2-methylnaphthalene by cytochrome P450s. Metabolism of 2-methylnaphthalene may proceed to reactive epoxides, which subsequently may be trapped as glutathione conjugates in the presence of GSH/GST in a manner similar to metabolites of naphthalene and 1-nitronaphthalene. The methyl group at the 2 position may also be oxygenated leading to further metabolites.](image-url)
Incubations containing CYP2F2, reductase, GSH, GST, NADPH regeneration system, GST, GSH, and 2-methylnaphthalene (2.5, 3, 3.75, 5, 6.6, 10, 17.5, 25, 33, 50, 100, and 500 μM). Incubations were for 5 min at 37°C. Data are reported as the mean of two incubations per substrate concentration. The rates are reported as total 2-methylnaphthalene conjugates formed (nmol) nmol of P450. Alterations in the ratio of conjugates were not observed at any of the substrate concentrations examined.

Experiments were conducted to determine whether addition of cytochrome b5 altered the rate of 2-methylnaphthalene metabolism under the optimal incubation conditions established above. Incubations containing CYP2F2, reductase, GSH, and GST were supplemented with recombinant human cytochrome b5 at 1:1 and 3:1 ratios (nmol of b5:nmol of P450). Addition of cytochrome b5 did not have any effect on the rate of metabolism or on the pattern of metabolites generated from 2-methylnaphthalene (data not shown).

All GSH conjugates were totaled and these values were used for the determination of kinetic parameters for 2-methylnaphthalene at concentrations varying from 0.0025 to 0.5 mM. No alteration in the relative rates of metabolites generated was observed at any of the substrate concentrations examined. Data were analyzed by linear regression of the double reciprocal plot for the estimation of kinetic parameters. The $K_m$ of recombinant CYP2F2 with 2-methylnaphthalene was estimated to be 3.7 μM with a $k_{cat}$ of 67.6 min⁻¹ (Fig. 6). The specificity constant of CYP2F2 for 2-methylnaphthalene was calculated to be 3.05 × 10³ M⁻¹ s⁻¹ (Table 1).

**Metabolism of Anthracene by CYP2F2.** Incubations examining the metabolism of anthracene were not optimized for time, protein, and P450:reductase ratio; rather, the parameters established for 2-methylnaphthalene and 1-nitronaphthalene (which were also similar to those of naphthalene) were used under the assumption that there would not be dramatic differences with this substrate. The metabolism of anthracene by CYPs has been reported to yield two enantiomeric epoxides (1R,2S- and 1S,2R-oxides) (van Bladeren et al., 1984, 1985) which, in turn, yield four glutathione conjugates in the presence of GSH and GST. HPLC of an extract from mouse liver microsomal incubations with anthracene, GSH, and GST yielded a UV profile (Fig. 7A) with one large and several smaller peaks. Due to limited quantities, a mass spectrum of only the largest of the UV-absorbing peaks, eluting at 30.5 min, was obtained. Prominent ions at m/z 524 (M + Na), 502 (M⁺), 484 (M – H₂O), and 306 (GS⁻) were consistent with the formation of a hydroxy-glutathionyl-1,2-dihydroanthracene adduct (data not shown).

Incubations containing [¹⁴C]anthracene were used to assess whether recombinant CYP2F2 was capable of epoxidizing this compound. Incubations containing 50 pmol of CYP2F2, reductase, GSH, GST, NADPH regeneration system, and [¹⁴C]anthracene (100 μM; 10,000 dpm/nmol) were performed along with a negative control incubation lacking reductase. As demonstrated by the HPLC radiochemical profile (Fig. 7B), [¹⁴C]anthracene is metabolized by CYP2F2 to yield a variety of radiolabeled products. All radioactive metabolites corresponded with compounds detected at 247 nm and neither the radioactive nor UV peaks were present in the negative control (data not shown). A radiolabeled peak coelutes with the anthracene conjugate peak formed by mouse liver microsomal incubations at an estimated rate of 0.024 nmol/nmol/min. Total anthracene metabolites were formed by recombinant CYP2F2 at a rate of 0.140 ± 0.045 nmol/nmol/min (Table 1).

**Metabolism of Benzo[a]pyrene by CYP2F2.** Incubation conditions previously established as optimal for 2-methylnaphthalene and 1-nitronaphthalene were used in the studies to determine whether CYP2F2 is capable of metabolizing benzo[a]pyrene. The metabolism of benzo[a]pyrene by CYPs has been reported to yield a number of regioisomeric epoxides (Thakker et al., 1985) which, in turn, leads to a...
large number of diastereomeric glutathione conjugates in the presence of GSH and GST. Of specific interest is the epoxide formed at the 7,8-position since this is the precursor to the carcinogenic benzo[a]pyrene-7,8-dihydrodiol-9,10-dihydroepoxide. Figure 7C shows the HPLC UV profile for the glutathione conjugates synthesized from the benzo[a]pyrene-4,5- and -7,8-epoxides (peaks eluting at 24–29 and 32–39 min, respectively). An extract from mouse liver microsomal incubations of benzo[a]pyrene with [3H]GSH and GST yielded UV-absorbing peaks with retention times virtually identical to those derived from the 4,5- and 7,8-epoxides. Radiochemical profiles measuring [3H]GSH yielded peaks corresponding to the UV-absorbing peaks, thereby confirming them as GSH-related metabolites (data not shown). In addition, several other metabolites (which were not identified) were generated as would be expected from the combined metabolism by various CYPs.

Incubations containing [14C]benzo[a]pyrene were used to assess whether recombinant CYP2F2 was capable of metabolizing this compound. Incubations containing 50 pmol of CYP2F2, reductase, GSH, GST, NADPH regeneration system, and benzo[a]pyrene (100 μM; 50,000 dpm/nmol) were performed along with a negative control incubation lacking reductase. The HPLC radiochemical profile (Fig. 7D) demonstrated the formation of a variety of radiolabeled products, including metabolites at 24 to 29 and 32 to 39 min. These metabolites cochromatographed with GSH conjugates generated by synthesis from authentic epoxides. These peaks were not present in the negative control. Total benzo[a]pyrene metabolites were formed by recombinant CYP2F2 at a rate of 0.040 ± 0.001 nmol/nmol/min (Table 1); the formation of conjugates at the 4,5- and 7,8-positions account for 30 and 70% of this rate, respectively (Table 2).

**Relative Levels of Cytochrome P450 in Microsomes from Airway, Trachea, and Parenchyma.** Cytochrome P450 levels were determined in dissected mouse lung airway, parenchyma, and trachea. Figure 8A (left) shows sample difference spectra from microsomes of each compartment used to quantify the cytochrome P450s. Airway microsomes contained 0.13 ± 0.11 nmol of P450/mg of microsomal protein, significantly more than in either trachea (0.04 ± 0.03 nmol of P450/mg of microsomal protein) or parenchyma (0.03 ± 0.02 nmol of P450/mg of microsomal protein) (Fig. 8A). The level in airway was 3.5 to 4 times higher than those found in trachea or parenchyma. This level was significantly different from both trachea (p < 0.01) and parenchyma (p < 0.001), whereas the difference between trachea and parenchyma was not statistically significant.

**Naphthalene Metabolism in Microsomes from Airway, Trachea, and Parenchyma.** The data in Fig. 8B show the rates of metabolism of naphthalene to dihydrodiol and to enantiomeric epoxides (trapped as GSH adducts) in microsomal preparations prepared identically to those used to obtain difference spectra data above. In most cases, the microsomal samples used for metabolism experiments were fractions of the same samples used to obtain difference spectra. The data are expressed both on a per milligram of microsomal protein (left) and per total P450 (right) basis. The data show the formation of the 1R,2S- and 1S,2R-naphthalene oxide and

<table>
<thead>
<tr>
<th>Substrate</th>
<th>kcat</th>
<th>Km</th>
<th>Specificity Constant</th>
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<tr>
<td>Naphthalene&lt;sup&gt;a&lt;/sup&gt;</td>
<td>104</td>
<td>3</td>
<td>5.8 × 10&lt;sup&gt;5&lt;/sup&gt;</td>
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<tr>
<td>2-Methylnaphthalene</td>
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<td>3.7</td>
<td>3.05 × 10&lt;sup&gt;5&lt;/sup&gt;</td>
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<tr>
<td>1-Nitronaphthalene</td>
<td>17.1</td>
<td>21.5</td>
<td>1.33 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>Anthracene&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.14 ± 0.04</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Benzo[a]pyrene&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.04 ± 0.00</td>
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</table>

<sup>a</sup> Activity for recombinant mouse CYP2F2 (Shultz et al., 1999).
<sup>b</sup> Activity is based on total metabolites formed and is expressed as the mean ± S.D. of three incubations performed using 100 μM substrate.
TABLE 2

| Total Metabolites | Bis[|P-4,5-oxide | Bis[|P-7,8-oxide |
|------------------|----------------|----------------|
| Mouse CYP2F2<sup>a</sup> | 0.04 ± 0.00 | 0.01 ± 0.00 |
| Human CYP1A1<sup>b</sup> | 0.27 ± 0.05 | 0.03 ± 0.00 |
| Human CYP1A2<sup>a</sup> | 0.02 ± 0.01 | 0.02 ± 0.01 |
| Human CYP1B1<sup>b</sup> | 2.69 ± 0.22 | <0.01 |
| Human CYP2E1<sup>b</sup> | <0.01 | <0.01 |
| Human CYP3A4<sup>b</sup> | <0.01 | <0.01 |

<sup>a</sup>Activity is expressed as the mean ± S.D. of three incubations performed using 100 μM substrate.
<sup>b</sup>Activities for the recombinant human CYPs are the mean ± S.D. of three incubations containing 20 μM substrate (Shimada et al., 1999).

Fig. 8. A, cytochrome P450 levels in microsomes prepared from dissected mouse lung subcompartments (right) expressed per milligram of microsomal protein. The data are the mean ± S.D. (n = 16) with each n consisting of pooled lung compartments from five to seven mice. Asterisks indicate significant differences ("p < 0.001,"s < 0.01) using one-sample paired t tests based on the difference between subcompartment P450 levels. The left panel contains a representative spectrum from each lung compartment. B, metabolism of naphthalene to naphthalene dihydrodiol, 1,2R-naphthalene oxide, and 1R,2S-naphthalene oxide by microsomes prepared from dissected airways, trachea, and parenchyma. Data are expressed as (nmol of metabolite) · mg of microsomal protein<sup>-1</sup> · min<sup>-1</sup> (left) and as (nmol of metabolite) · nmol of P450<sup>-1</sup> · min<sup>-1</sup> (right). Data are the mean ± S.D. (n = 11) with each n consisting of pooled lung compartments from five to seven mice.

This work extends our previous studies to show that CYP2F2 metabolizes two other lung toxicants, 1-nitronaphthalene and 2-methylnaphthalene, with relatively high turnover (17.1 and 67.7 min<sup>-1</sup>, respectively) and low K<sub>m</sub> (21.5 and 3.7 μM, respectively). Additionally, CYP2F2 metabolizes anthracene and benzo[|g]pyrene albeit at low rates. The levels of total CYP in mouse airways are more than 3 times those in parenchyma and trachea, and this is consistent with the relative rates of naphthalene metabolism by microsomal preparations from these same subcompartments. The ability of CYP2F2 to metabolize a variety of lung toxicants requiring metabolic activation and the apparent content of this protein in mouse lung supports the view that CYP2F2 is important in the unique sensitivity of mouse lung to a number of toxicants.

Recombinant CYP2F2 metabolizes 1-naphthalenone with considerable regioselectivity; the ratio of 7,8- to 5,6-oxide is 5:1. The regioselectivity did not change at varying substrate concentrations. This ratio is similar to that observed in incubations using mouse lung microsomes where conjugates derived from 7,8-oxide versus the 5,6-oxide were produced at a ratio of 3:5:1 (Watt and Buckpitt, 2000). These data are consistent with the view that CYP2F2 plays a major but not necessarily exclusive role in the overall formation of 1-nitronaphthalene epoxides. Our kinetic data suggest that CYP2F2 will be especially important in substrate turnover at levels of 1-nitronaphthalene likely to be in the lung after administration of toxic doses. Recent work by Halladay et al. (1999) showed that levels of 1-nitronaphthalene in blood reached C<sub>max</sub> concentrations of approximately 4 μg/ml (22 μM) 1.5 h after i.p. administration of 100 mg/kg. The calculated V<sub>area</sub> of 3 l/kg suggests that the compound is well distributed to tissues and that the maximal concentrations in the lung might be higher than blood concentrations. Levels of parent compound in the lung are well within concentrations efficiently metabolized by CYP2F2 (K<sub>m</sub> = 21.5 μM).

Previous work on mouse lung metabolism of 2-methylnaphthalene has demonstrated the formation of 3,4-, 5,6-, and 7,8-dihydrodihydroxy metabolites as well as products derived from the oxidation of the methyl group (Breger et al., 1983; Griffin et al., 1983). Formation of the 7,8-dihydriodiol predominates in both lung and liver microsomal incubations from mice and rats. Previous work demonstrating the formation of three regioisomeric epoxides is consistent with the data presented here showing that at least nine glutathione conjugates, identified by mass spectrometry, are generated in mouse liver microsomal incubations. Only five of these were detected in extracts of complete incubations containing CYP2F2 and 2-methylnaphthalene. Although this is consistent with the possibility that the metabolism of 2-methylnaphthalene by CYP2F2 is highly regioselective, definitive structural assignments, which will require proton NMR, for each of the conjugates are not available. This work also shows that CYP2F2 is capable of oxidizing the side chain methyl, presumably to form an alcohol.

The kinetics of 2-methylnaphthalene metabolism by CYP2F2 further supports the importance of this protein in pulmonary metabolism of small aromatic hydrocarbons. Although data on the disposition of 2-methylnaphthalene are not currently available, the physiochemical characteristics of this compound are similar to naphthalene and 1-nitronaphthalene. As discussed above, peak levels of these compounds are on the order of 20 μM after i.p. administration. Thus,

Discussion

Previously, we demonstrated that recombinant CYP2F2 metabolizes naphthalene at high rates (k<sub>cat</sub> of ~104 min<sup>-1</sup>) with a biologically relevant K<sub>m</sub> (3 μM) (Shultz et al., 1999).
depending on the level of CYP2F2 present in mouse lung, the low $K_M$ (3.7 μM) and high $k_{cat}$ (66.7 min$^{-1}$) imply that CYP2F2 would be an important isozyme involved in the metabolism of 2-methylphenanthrene in vivo.

Although there is no evidence that anthracene is carcinogenic, the metabolism and tumorigenicity of benzo[a]pyrene has been documented extensively. The biological activity of benzo[a]pyrene is clearly related to the formation of epoxide and diol epoxide derivatives and the lung of the mouse is a target tissue for this polycyclic aromatic hydrocarbon and its metabolites (for review, see Thakker et al., 1985). Although CYP1A and 1B have been shown to be important in the metabolic activation of benzo[a]pyrene and anthracene, these proteins are present in very low levels in uninduced lung (Ryu and Hodgson, 1999). The fact that CYP2F2 metabolizes a number of naphthalene derivatives with high turnover numbers and that this protein appears to be a quantitatively important pulmonary P450 raised the possibility that this protein was involved in the metabolic activation of larger polycyclic aromatic hydrocarbons. Incubations of [14C]anthracene with CYP2F2 generated multiple radiolabeled metabolites not present in extracts of incubations lacking cytochrome P450 reductase. One of the metabolites was identified as a glutathione conjugate of hydroxydihydronaphthalene. Although other, unidentified, peaks were present, which are possibly phenols, these also would have been generated via intermediate epoxides. The total rates of anthracene metabolism by CYP2F2 (0.14 nmol/nmol/min) were between 100- and almost 1000-fold lower than those observed with naphthalene, 1-nitronaphthalene, or 2-methylnaphthalene. Previous work by van Bladeren et al. (1985) using purified rat liver cytochrome P450s showed much higher turnovers of anthracene with CYP1A1 (17.7 nmol/nmol/min) than by CYP2B1 (0.18 nmol/nmol/min) in incubations containing 50 μM substrate. CYP1A1 is present constitutively in mouse lung at very low levels (Chichester et al., 1991) and may not contribute significantly to anthracene metabolism in uninduced animals. CYP2B1 and CYP2F2 appear to have similar catalytic activities with anthracene; thus, the relative contributions of these two proteins to the overall metabolic disposition of anthracene will depend upon the quantities of protein present. Comparison of the turnover of benzo[a]pyrene by CYP2F2 with other P450 proteins yields a similar picture (Table 2). The rates of turnover to the 7,8-epoxide catalyzed by CYP1A1 and CYP1B1 are 10- and 100-fold higher than by CYP2F2 (Shimada et al., 1999), but the expression levels of CYP1A1 and B1 in the lung are very low (Chichester et al., 1991; Ryu and Hodgson, 1999). The 7,8-oxide is generated at nearly 3 times the rate of the 4,5-oxide, suggesting that the active site of CYP2F2 may be very rigid, thus orienting substrates so that bulky groups are facing away from the protoporphyrin heme. This is consistent with the preponderance of 7,8-epoxide intermediate generated from 1-nitronaphthalene and fits with the high degree of stereoselectivity associated with naphthalene epoxidation (66.1 ratio of 1R,2S- to 1S,2R-epoxide).

The finding that airways contained approximately 4-fold higher levels of total cytochrome P450 than the trachea or parenchyma is consistent with numerous studies showing that several of the pulmonary cytochrome P450s are highly localized in Clara cells of airways (for review, see Plopper, 1993). It is also consistent with the higher rates of metabolism of a variety of substrates in various species (Lee et al., 1998; Watt et al., 1998). The rates of naphthalene metabolism, when expressed on the basis of nanomoles of metabolite per nanomoles of P450 per minute are similar in airways and trachea but are nearly double those in parenchymal microsomes. These findings, in conjunction with the data on the relative ratios of 1R,2S-1S,2R-oxide generated by recombinant CYP2F2, and microsomes from airway, trachea, and parenchyma (66:1, 35:1, 21:1, and 5:1, respectively) support the view that CYP2F2 is the major P450 involved in the metabolism of this substrate. We note, however, that the apparent ratios of epoxides, which are based on the ratios of glutathione conjugates generated in microsomal incubations, could potentially be influenced by the enantioselectivity of the epoxide hydrolases present in these preparations. This factor should be small because the amounts of diol generated are low. Finally, the apparent turnover of naphthalene in microsomes from dissected airways is higher than by the recombinant enzyme (∼170 nmol/nmol of P450/min versus 100–150 nmol/nmol of CYP2F2/min), and this supports the argument that CYP2F2 generates the vast majority of naphthalene epoxides in mouse lung.

One way to address the question of relative roles of specific CYP proteins in the metabolism of a given substrate is to analyze the specific contribution of an isof orm like CYP2F2 to overall CYP levels in each of the subcompartments of the lung. This will require pure standards of CYP2F2 for Western blot analysis. Although recombinant CYP2F2 is highly expressed in Tn5 cells, comparison of CYP2F2 from Tn5 microsomes quantified spectrally versus by protein estimated from Coomassie-stained gels indicates that a significant portion of expressed CYP2F2 is nonfunctional (does not contain the heme protoporphyrin). In our hands, roughly 50% of the CYP present is spectrally active (R. M. Baldwin, unpublished data). Spectral determination alone is therefore not a good measure of total enzyme (functional and nonfunctional) present. Further purification and more direct quantitative methods are required for conducting such experiments.

These studies have provided evidence for the importance of CYP2F2 in the metabolic activation of a number of murine lung tox icants. In future work, it will be important to ascertain the role of CYP2F orthologs in the rat (CYP2F4) and primate (CYP2F1) to determine whether the catalytic activities of these proteins are similar to those of the mouse. Initial indications suggest that CYP2F4 has almost identical catalytic activities to the mouse protein with naphthalene (Baldwin et al., 1999) but that CYP2F1 metabolizes substrates such as naphthalene and 3-methylindole only slowly (Lanza et al., 1999). Again, the overall contribution of any P450 to the metabolic activation and subsequent toxicity of chemicals in the lung will depend not only on the catalytic activity of the protein but also on its abundance and cellular localization in the lung.

References


