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Relationship of Cytochrome P450 Activity to Clara Cell Cytotoxicity. IV. Metabolism of Naphthalene and Naphthalene Oxide in Microdissected Airways from Mice, Rats, and Hamsters

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SUMMARY

Parenteral administration of naphthalene produces a dose-dependent and tissue-, species-, and cell-selective lesion of murine Clara cells. The rate and stereoselectivity of naphthalene metabolism by microsomal preparations correlate with tissue and species differences in cytotoxicity. Because earlier studies used microsomes obtained from whole tissue, differences in susceptibility of proximal and distal airways could not be related to differences in the metabolic activation or detoxication of naphthalene. Specific subcompartments of the respiratory system, obtained by microdissection, have been used to study the cytochrome P450-dependent metabolism of naphthalene and the epoxide hydrolase/glutathione transferase-dependent metabolism of naphthalene oxide. The rates of naphthalene metabolism were substantially higher in mouse airways than in comparable airways of hamsters or rats. Rates of metabolism were higher in distal airways than in the trachea of all species studied. Metabolism in mouse airways was highly stereoselective, whereas that in hamster and rat tissues was not. Nonciliated cells at all airway levels in mice were heavily labeled with an antibody to cytochrome P450 2F2; little labeling was observed in any portion of rat and hamster lungs. Postmitochondrial supernatants prepared from mouse and hamster airways metabolized racemic naphthalene oxide to diol and glutathione adducts at substantially higher rates than did comparable preparations from rats. Although glutathione levels varied 2–4-fold at different airway levels in the three species studied, levels at the most susceptible site (mouse distal bronchioles) were as high as or higher than those at other, less susceptible, sites. These studies support the view that the rate and stereoselectivity of naphthalene metabolism to naphthalene 1*R*,2*S*-oxide catalyzed by cytochrome P450 2F2 are critical determinants in the species-specific and region-selective cytotoxicity of naphthalene in mice. The lack of major differences in the catalytic activity or enantioselectivity of putative detoxication enzymes (epoxide hydrolase or glutathione transferases) between mouse and hamster tissue, combined with data showing that the differences in the metabolic fate of naphthalene oxide in proximal versus distal airways are not dramatic, suggests that the initial epoxidation of naphthalene is an important factor in site-selective toxicity. These studies support the need to use tissue from defined airway levels for studies on the relationship of biochemical and metabolic factors important in cellular injury by lung toxicants, such as naphthalene, where there are dramatic regional differences in susceptibility to injury within the respiratory system.

The initial discovery that the Clara cell is the primary target cell for 4-ipomeanol toxicity in lungs of adult rodents (1) has been followed by a number of reports demonstrating the high sensitivity of this cell type to the cytotoxic effects of chemicals requiring cytochrome P450 monooxygenase-dependent metabolism. These chemicals include chlorinated hydrocarbons (CCl_4, dichloroethylene, and trichloroethylene), other furan derivatives (perilla ketone and 3-methylfuran), indoles (3-methylindole), phosphorothioates, and aromatic hydrocarbons such as naphthalene and 2-methylnaphthalene (for reviews, see Refs. 2 and 3). The sensitivity of the Clara cell to chemicals undergoing cytochrome P450-dependent metabolic activation is consistent with the finding that the Clara cell is an important locus of monooxygenase enzymes in the lung (4, 5). In addition, the pulmonary Clara cell is a potential target for carcinogens such as 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; the tumorigenic actions of this agent have been attributed to both the ability of monooxygenases within the Clara cell to activate

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ABBREVIATION: CDNB, 1-chloro-2,4-dinitrobenzene.

the compound and the inability to efficiently repair O^6-methylguanine once it has been formed (for review, see Ref. 6).

Chemical-induced Clara cell toxicity shows a high degree of species selectivity and regional selectivity within the airway. For example, naphthalene, 2-methylnaphthalene, and the chlorinated ethylenes produce Clara cell necrosis in mouse but not rat or hamster lung (7–10). Likewise, mouse distal airways are 3–5-fold more sensitive to naphthalene than are Clara cells present in the trachea and lobar bronchus. Administration of naphthalene at low doses (50–100 mg/kg) resulted in Clara cell necrosis in the most distal airway segments but had no effect on bronchial epithelium (11). Proximal airways were injured at 300–400 mg/kg. Rat airways were insensitive at all doses of naphthalene tested (including the LD_{50} of 1600 mg/kg). Hamster distal airway epithelium was unaffected at doses as high as the LD_{50} (800 mg/kg), but injury occurred in the bronchial epithelium at these doses (11).

The metabolic and biochemical factors that control the species, tissue, and regional sensitivity to naphthalene cytotoxicity have been studied in a number of different systems. Results of *in vivo* work have demonstrated the involvement of the cytochrome P450 monooxygenases and glutathione in the formation and deactivation, respectively, of reactive metabolites that become bound covalently to macromolecules in lung, liver, and kidney (12). Although the levels of bound metabolites in the lung correlate with the severity of lung injury, reactive metabolite binding in nontarget tissues (liver and kidney) of mice is as high as in the lung. Several factors are potentially involved in this apparent anomaly. There are numerous electrophilic metabolites generated during naphthalene metabolism, including the 1,2-epoxide (13), a diepoxide (14), and quinones (15, 16), but these metabolites appear to differ considerably in their ability to result in Clara cell injury in either isolated perfused lungs (17) or isolated murine Clara cells (18). In addition, at least some of these metabolites appear to be capable of circulating in the blood (19, 20) and, therefore, the level of covalent binding that occurs in a particular tissue is not necessarily a good indication of the rate of formation of such metabolites in that tissue.

Earlier work showing substantial differences in the rate and stereoselectivity of naphthalene epoxide formation in target and nontarget tissue microsomes from mice and from sensitive and nonsensitive species suggested that differences in the rate and stereochemistry of the initial metabolic step may be key factors in determining the susceptibility of cells to naphthalene cytotoxicity (21). However, because those studies utilized whole-tissue homogenates, the contributions of cytochromes P450 from Clara cells from terminal airways (highly sensitive), from proximal airways (less sensitive), and from parenchymal regions of the lung (insensitive) could not be delineated. In addition, no information could be obtained about the capabilities of these target and nontarget areas to detoxify naphthalene oxide. A major reason that mouse distal airways are highly sensitive to naphthalene could be related to the lack of epoxide hydrolase/glutathione transferases capable of rapidly converting the large amounts of naphthalene 1*R*,2*S*-oxide generated to less toxic derivatives. We have recently developed procedures to obtain carefully defined segments of airways that can be used to study both the metabolic activation and the detoxication of chemically unstable metabolites in species ranging from mice to nonhuman primates and, possibly, to humans. In this paper, we describe the application of these techniques to the investigation of the metabolism of naphthalene and naphthalene oxide in mouse, rat, and hamster airways. This work was conducted to identify critical metabolic differences that might be associated with the striking differences in sensitivity between rodent species and at different airway levels of mice.

Materials and Methods

Animals. All animals were males and were virus antibody free (Charles River Breeding Laboratories, Wilmington, MA). The following strains and weight ranges were used: CFW mice, 20–30 g; Sprague-Dawley rats, 150–225 g; Syrian Golden hamsters, 70–90 g. Animals were housed in cage racks supplied with High-efficiency particulate air-filtered air and had free access to food (Purina Rodent Chow) and water, with a 12-hr/12-hr light/dark cycle, for at least 5 days before use. Rodents were killed with an overdose of pentobarbital.

Chemicals. All chemicals were reagent grade or better. Glutathione was obtained from Sigma Chemical Co. (St. Louis, MO). Racemic naphthalene oxide was synthesized according to the method of Yagi and Jerina (22); the final product was stored in 99.5% ethanol/0.5% triethylamine under nitrogen at −80°. Concentrations were determined at 266 nm using an extinction coefficient of 8500 mM^{-1} cm^{-1}. Monobromobimane was obtained from Calbiochem (San Diego, CA). Diethylenetriaminepentaacetic acid was purchased from Aldrich Chemical Co. (Milwaukee, WI).

Dissection of airway segments. These methods have been described in detail in a preceding publication (23). Briefly, animals were killed and the tracheae were cannulated. Lungs were infused with low-melting point agarose and immediately cooled on ice in Waymouth's medium for 30 min (4°). Airways were blunt dissected under a dissecting microscope to obtain distal trachea, lobar bronchus, major and minor daughter pathways, terminal bronchioles, parenchyma, and blood vessels.

Tissue preparation for immunohistochemistry. Lungs of five adult male mice, rats, or hamsters were fixed with either ethanol/acetic acid (4:1) or 1% paraformaldehyde (in 0.2 M phosphate buffer, pH 7.2). The fixative was perfused at a pressure of 30 cm into a cannulated lobar bronchus after removal of the lungs by thoracotomy. The fixed lobes were sliced perpendicularly to the long axis of the major conducting airway. Slices of lung were embedded in paraffin. Representative sections (5–7-μm thick) were reacted with antibodies to selected cytochrome P450 isoforms and cytochrome P450 reductase. The antisera against the cytochrome P450 2B4 isozyme and NADPH-cytochrome P450 reductase were elicited in goats; characterization of these antibodies has been described previously (4). Antisera against the cytochrome P450 2F isozyme were raised in rabbits (24). Immunohistochemical localization of cytochrome P450 isozymes using these antibodies was done by standard procedures, using Vectastain kits purchased from Vector Laboratories (Burlingame, CA) (25). Sections from lungs of each species were used in the same immunohistochemical experiment with the same dilutions of primary antisera. Controls included substitution of primary antisera with phosphate-buffered saline or normal serum. The dilutions of primary antibody were varied to establish reaction product densities that ranged from nondetectable to heavily labeled for one of the species, if there was heterogeneity of expression among species.

Incubations with naphthalene or naphthalene epoxide. To determine the rates and stereoselectivity of naphthalene epoxidation, incubations were prepared on ice and contained the following, in a total volume of 500 μl of Waymouth's medium: microdissected airway, glutathione (5 mM), glutathione transferases (2.5 CDNB units from mouse liver, partially purified on glutathione-agarose affinity columns), and naphthalene (0.5 mM). Naphthalene was added last and incubations were capped and transferred to a shaking water bath at 37° for 45 min. At the end of the incubation, the reaction was quenched by addition of 500 μl of ice-cold methanol. The tissue was separated from

medium by centrifugation through a centrifuge filter and the tissue was recovered and dissolved in 1 N NaOH for protein determination. The filtrate was evaporated under reduced pressure for high pressure liquid chromatographic analysis of diol and glutathione conjugates.

To determine the rates of metabolism of naphthalene epoxide, lung subcompartments were homogenized in microglass homogenizers in 0.1 M phosphate buffer, pH 7.4, and samples were centrifuged at 9000 × *g* for 15 min. Incubations were prepared on ice and contained postmitochondrial supernatant (entire sample) and 2 mM glutathione, in a total volume of either 250 μl (mice) or 500 μl (rats and hamsters). Samples were preincubated for 2 min at 37°, racemic naphthalene oxide (0.1 mM final concentration) was added, and incubations were continued for 3 min. One volume of methanol was added to stop the reaction. Blanks, containing no enzyme, were included with every set of incubations.

Analysis of naphthalene metabolites. Naphthalene is metabolized by cytochrome P450 monooxygenases to 1*R*,2*S*- and 1*S*,2*R*-oxides. The 1*R*,2*S*-oxide, in the presence of glutathione and glutathione transferases, forms a single glutathione adduct at the allylic position on the naphthalene ring. Naphthalene 1*S*,2*R*-oxide is metabolized to two adducts (both benzylic and allylic thioethers). These epoxides could be trapped efficiently in the presence of saturating concentrations of glutathione and glutathione transferase, to yield adduct 2 (from the 1*R*,2*S*-oxide) and adducts 1 and 3 (from the 1*S*,2*R*-oxide), which were separated and quantified by high pressure liquid chromatography (21).

Measurement of reduced glutathione in lung subcompartments. Preliminary studies showed that the glutathione levels in microdissected airways do not change over the time needed to complete the dissection. All dissections were started between 9:00 and 11:00 a.m., to reduce the influence of diurnal fluctuation of glutathione concentrations. Reduced glutathione was measured, after derivatization with monobromobimane, by high pressure liquid chromatography with fluorescence detection (26). All samples were homogenized in 100 μl of ice-cold 800 mM methanesulfonic acid containing 20 mM diethylenetriaminepentaacetic acid, using glass/glass microhomogenizers. The homogenates were transferred to microcentrifuge tubes, and 100 μl of 4 M sodium methanesulfonate were added. Samples were stored at −80° for up to 1 week before derivatization and analysis. Glutathione standards were included with each sample set, and the response was linear over the range of sample values (20 pmol to 2 nmol).

Protein assay. Precipitated protein was dissolved in 1 N NaOH, and the amount of protein in the sample was determined by the method of Lowry *et al.* (27), with bovine serum albumin as a standard.

Results

Rates of Metabolism of Naphthalene in Mouse, Rat, and Hamster Tracheobronchial Airways

Tracheobronchial airways and parenchyma, obtained by microdissection, were incubated with naphthalene in the presence of glutathione and glutathione-*S*-transferases to trap naphthalene oxides generated in the incubations. Metabolism in mouse airways occurred at substantially higher rates than in comparable incubations of hamster or rat airways (Fig. 1). At most airway levels, the rates of substrate turnover in mice were at least twice as high as in hamsters and were 3–5 times greater than in rats. The overall rate of metabolism was significantly higher in more distal airways than in proximal airways. The formation of dihydrodiol and glutathione conjugates in incubations of mouse distal bronchioles was more than double the rate of metabolism in tracheal incubations. Likewise, metabolism in hamster minor daughter segments and distal bronchioles was more than double the rate of metabolism of naphthalene in the trachea. In rats, with the exception of the minor daughter pathways, the rates of substrate turnover were very

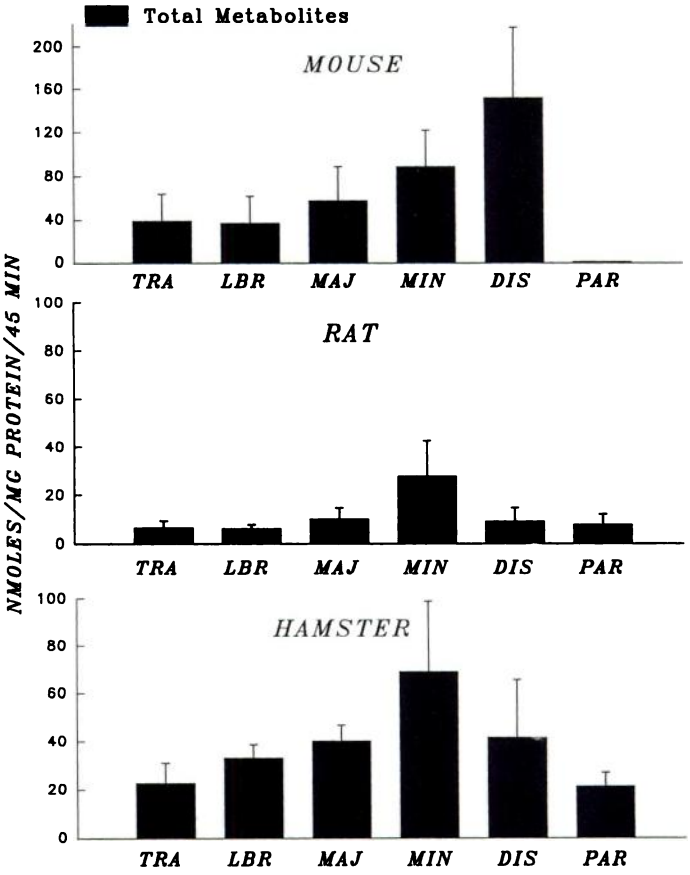

Fig. 1. Comparison of the rates of metabolism to dihydrodiol and glutathione conjugates (total) in incubations of dissected airways from mice, rats, and hamsters. Incubations contained airway segment and 0.5 mM naphthalene and were performed in the presence of glutathione (5 mM) and glutathione transferase (2.5 CDNB units). Values are the mean ± standard deviation for separate incubations using airway segments dissected from four to six animals. Data for mice are presented here for comparison (23). The *y*-axis scale presenting the rates for mice is double that for rats and hamsters. *TRA*, trachea; *LBR*, lobar bronchus; *MAJ*, major daughter segment; *MIN*, minor daughter segment; *DIS*, distal bronchiole; *PAR*, parenchyma (proximal to distal).

similar. Naphthalene metabolism could not be detected in mouse parenchymal tissue. In contrast, parenchyma from both rats and hamsters metabolized naphthalene at easily measurable rates.

Stereoselectivity of Naphthalene Epoxidation in Tracheobronchial Airway Incubations from Mice, Rats, and Hamsters

Naphthalene metabolism in mice occurred with an apparent high degree of stereoselectivity (Fig. 2). The only metabolites detected in any of the incubations were naphthalene dihydrodiol and glutathione conjugate 2, which is derived from naphthalene 1*R*,2*S*-oxide. The rates of diol formation were 30–50% of the rates of conjugate formation in mouse lung airway incubations. Conjugates 1 and 3, generated from naphthalene 1*S*,2*R*-oxide, could not be detected in any of the incubations. In contrast, all three glutathione adducts were observed in rat and hamster airway incubations. In rats, the ratios of adducts 1 and 3 to adduct 2 (which provides an indication of the stereoselectivity of epoxide formation) were approximately equal at all airway levels. In hamsters, the ratios of adducts 1 plus 3 to adduct 2 exceeded 2:1, indicating that metabolism

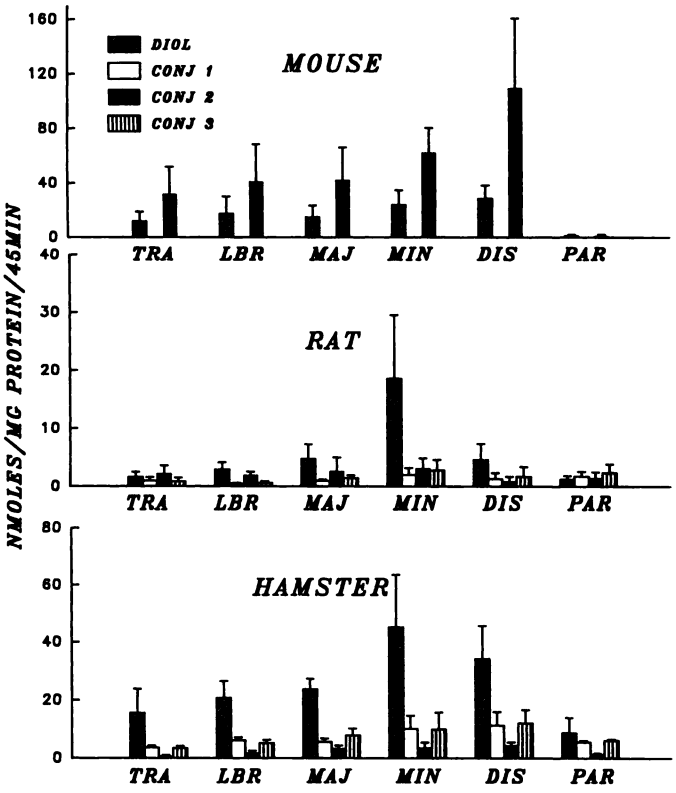

Fig. 2. Metabolism of naphthalene to dihydrodiol (*DIOL*) and glutathione conjugates (*CONJ*) in microdissected airways from mice, rats, and hamsters. Conditions were as described for Fig. 1. Conjugates 1 (allylic adduct) and 3 (benzylic adduct) are derived from the 1*S*,2*R*-oxide; conjugate 2 is from the 1*R*,2*S*-oxide. Note the differences in scales.

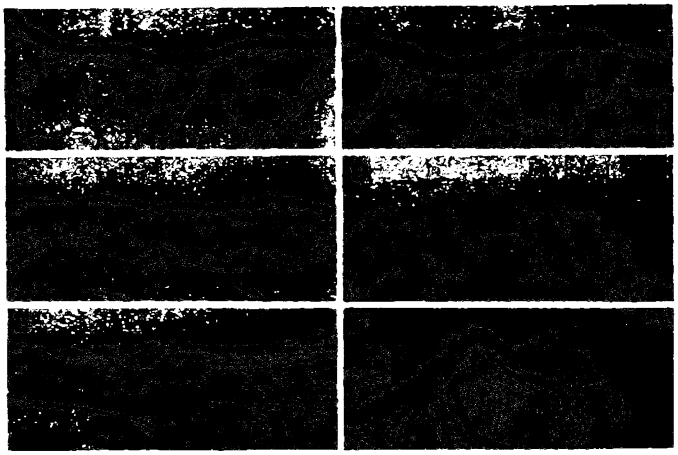

Fig. 3. Localization of cytochrome P450 2F in mouse (A and B), rat (C and D), and hamster (E and F) bronchial (A, C, and E) and bronchiolar (B, D, and F) airways. Antibody dilution, 1/2000.

occurred with a slight preference for the 1*S*,2*R*-oxide. In rats and hamsters, diol was a prominent metabolite and, at many airway levels, was generated at rates greater than or equal to that of conjugates.

Immunohistochemistry

Cytochrome P450 2F. Cytochrome P450 2F was detected by immunohistochemistry in the nonciliated cells in mouse proximal (lobar) bronchi (Fig. 3A). There appeared to be a small amount of reaction product in the apices of some cells in the proximal bronchi of rats and hamsters (Fig. 3, C and E). In terminal bronchioles, there was heavy labeling throughout mouse nonciliated cells (Fig. 3B). In comparison, there was very little labeling in the epithelial cells of either rat or hamster terminal bronchioles (Fig. 3, D and F). There did not appear to be any labeling in any other aspects of the lung in mice or other species with primary antiserum against cytochrome P450 2F.

Cytochrome P450 2B4. Cytochrome P450 2B4 was detected in nonciliated cells in mouse, rat, and hamster proximal (lobar) bronchi (Fig. 4, A, C, and E). When antigen was detectable, it appeared to be primarily in the apices of these cells. Not all nonciliated cells appeared to be equally labeled within the bronchi. In the alveoli, heaviest labeling was in mouse and rat tissues. At an antibody dilution of 1/10,000, cytochrome P450 2B4 was detected in nonciliated cells in mouse, rat, and hamster terminal bronchioles (Fig. 4, B, D, and F). The labeling was primarily in the apices of these cells. Although there were more labeled cells in mouse tissue than in rat or hamster tissues, the density of labeling was heaviest in rat samples, followed by mouse tissue, with less in hamster tissue. There was a small amount of labeling in the endothelial cells of some rat, hamster, and mouse vessels. Increased labeling was noted in type II pneumocytes in some areas of the lung.

NADPH-cytochrome P450 reductase. In proximal (lobar) bronchi, cytochrome P450 reductase could be detected in mouse, rat, and hamster nonciliated cells (Fig. 5, A, C, and E). The heaviest labeling appeared to be in mouse tissue; more cells were labeled in this species and the anti-reductase antibody appeared to label a larger portion of the cytoplasm. Fewer labeled cells and less dense labeling were observed in rat and hamster tissues. In the terminal bronchioles, epithelial cells were labeled for cytochrome P450 reductase in mice, rats, and hamsters (Fig. 5, B, D, and F). Labeling was heaviest and in more cells in mice than in hamsters or rats. The least intense labeling was observed with hamster alveolar cells. There was labeling of what appeared to be some type II cells in the lung parenchyma, especially in mice (Fig. 5, A and B).

Metabolism of Racemic Naphthalene Oxide in the 9000 × *g* Supernatant from Tracheobronchial Airways

The high degree of stereoselectivity in naphthalene metabolism in tracheobronchial airways of mice, compared with rats

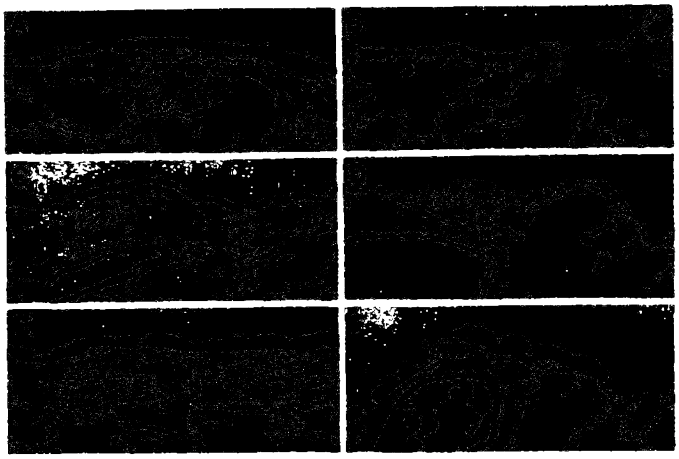

Fig. 4. Localization of cytochrome P450 2B4 in mouse (A and B), rat (C and D), and hamster (E and F) bronchial (A, C, and E) and bronchiolar (B, D, and F) airways. Antibody dilution, 1/10,000.

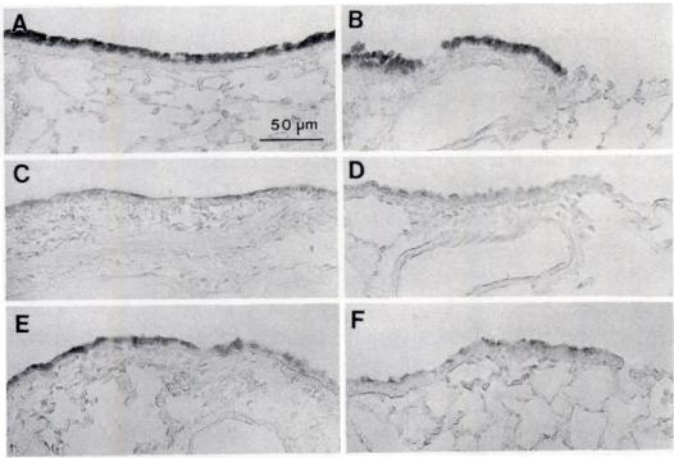

Fig. 5. Localization of NADPH-cytochrome P450 reductase in mouse (A and B), rat (C and D), and hamster (E and F) bronchial (A, C, and E) and bronchiolar (B, D, and F) airways. Antibody dilution, 1/10,000.

and hamsters, suggested that the rates and possibly the enantioselectivity of metabolism of the epoxide might be important determinants of the susceptibility of the cells to naphthalene toxicity. To determine the rates and enantioselectivity of metabolism by epoxide hydrolases or glutathione transferases, postmitochondrial supernatants prepared from airways obtained by microdissection were incubated with racemic naphthalene oxide in the presence of glutathione. The overall rate of naphthalene oxide metabolism was 8–10-fold higher in hamster and mouse tissues, compared with rat tissues, at all airway levels (Fig. 6; Table 1). In all three species, there was a 2–3-fold difference between total conjugate formation in the minor daughter airways and that in the trachea. Diol accounted for a small percentage of the total metabolites produced in both mouse and rat tissues; at many airway levels in hamsters, diol accounted for more than half of the metabolites isolated in these experiments. There were 2–3-fold differences in the amount of diol, as a percentage of total metabolites (diol plus conjugates), recovered from incubations of distal airways, compared with proximal airways, for all three species studied. There was little apparent enantioselectivity in the metabolism of naphthalene oxide to conjugates at any airway level in the three rodent species examined. The ratios of adduct 2 to adducts 1 plus 3 were approximately 2, 3, and 1.3 in mice, rats, and hamsters, respectively.

Glutathione Levels in Microdissected Airways

Glutathione levels varied about 2-fold at different airway levels in mice, rats, and hamsters (Table 2). The lowest glutathione levels were present in either the lobar bronchus (rats) or the major daughter airway segment (mice and hamsters). In general, the level of glutathione in the distal bronchioles and parenchyma was as high or higher than that in other airway segments.

Discussion

The design of experiments to discern the relationships between the formation and fate of chemically reactive metabolites and injury to pulmonary epithelium is difficult because of the cellular heterogeneity of the lung, the highly focal nature of the distribution of several xenobiotic-metabolizing enzymes, and the possible involvement of other, metabolically active tissues

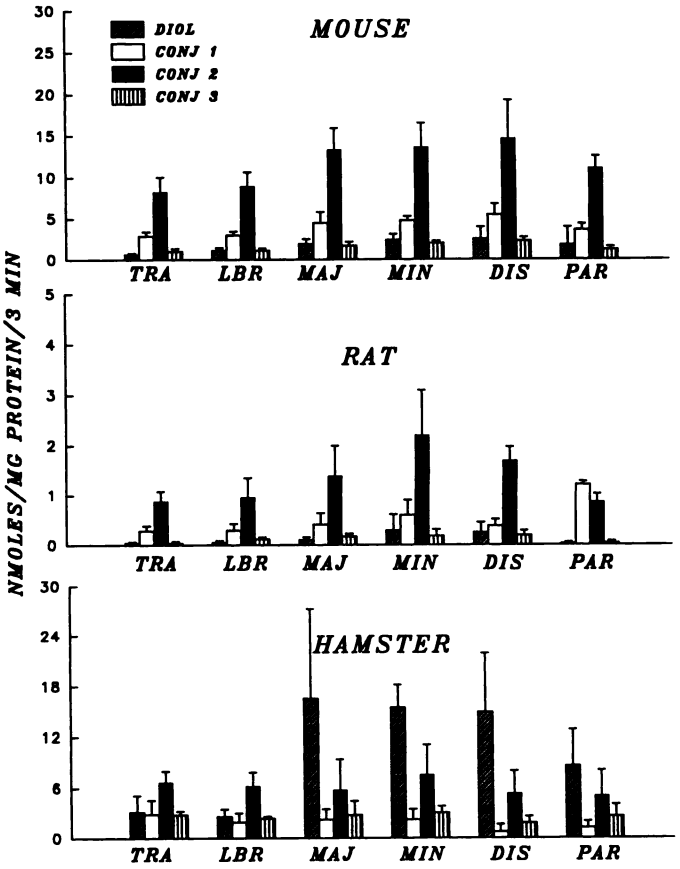

Fig. 6. Metabolism of racemic naphthalene 1,2-oxide to diol and glutathione conjugates (*CONJ*) in the 9000 × *g* supernatant prepared from airways obtained by microdissection. Data are from 3-min incubations of racemic epoxide (0.1 mM), reduced glutathione (2 mM), and postmitochondrial supernatant. In each case, nonenzymatic control values were subtracted. Values are the mean ± standard deviation for separate incubations using airway segments dissected from four to six animals. The scale used for mice and hamsters is 0–30 nmol/mg/3 min; that for rats is 0–5 nmol/mg/3 min. *TRA*, trachea; *LBR*, lobar bronchus; *MAJ*, major daughter segment; *MIN*, minor daughter segment; *DIS*, distal bronchiole; *PAR*, parenchyma

in the formation of circulating toxic metabolites. Although work in whole animals, in perfused lung, and in microsomes can provide valuable insight into the overall disposition and metabolic fate of specific chemicals, these experimental approaches are not capable of assessing the contribution of a particular cell type or cells in a specific region of the lung to the overall formation and fate of toxic metabolites. Likewise, *in vivo* approaches are unlikely to provide a useful means of testing the cytotoxic potential of unstable metabolites that have been prepared synthetically. Techniques are available for preparation of isolates enriched in Clara and alveolar type II cells, but the quality of these isolates is species dependent. Although isolates containing >80% Clara cells can be prepared from both mice and rabbits (18), similar enrichments have not been reported for rat tissue (28). The application of these procedures to larger species such as primates would entail considerable expense. In addition, enzymatic digestion of the tissue to release Clara cells from the lung can lead to extensive proteolysis of xenobiotic-metabolizing enzymes (29).

The work presented here describes the application of a technique capable of yielding defined segments of lung tissue that

TABLE 1

Rates of formation of dihydrodiol and glutathione conjugates in incubations of naphthalene oxide with postmitochondrial supernatants from mouse, rat, and hamster tracheobronchial airways segments

Airway subcompartment	Diol + conjugates			Diol			Ratio of conjugate 2/conjugates 1 + 3		
	Mouse	Rat	Hamster	Mouse	Rat	Hamster	Mouse	Rat	Hamster
	nmol/mg/3 min			*% of total*					
Trachea	12.8	1.3	16.6	5.0	3.0	25.4	2.1	2.5	1.2
Lobar bronchus	14.8	1.4	13.1	7.6	3.2	19.7	1.9	2.3	1.5
Major daughter segment	21.4	2.1	27.3	9.0	4.7	60.5	2.1	2.5	1.2
Minor daughter segment	27.8	3.3	28.2	8.7	8.9	54.6	1.1	2.8	1.4
Distal bronchiole	24.9	2.5	22.7	10.2	9.4	65.5	1.9	3.0	2.2
Parenchyma	17.7	1.4	17.5	10.0	17.6	49.1	2.3	3.3	1.3

TABLE 2

Glutathione levels in subcompartments of mouse, rat, and hamster lung

Values are the mean ± standard deviation for four to six animals.

Lung subcompartment	Glutathione		
	Mouse	Rat[a]	Hamster
	nmol/mg of protein		
Trachea	5.9 ± 1.4	5.6 ± 1.7	10.8 ± 1.3
Lobar bronchus	5.3 ± 1.8	3.5 ± 0.1	9.9 ± 1.0
Major daughter segment	2.8 ± 0.8	4.4 ± 0.9	5.3 ± 0.6
Minor daughter segment	7.7 ± 1.2	6.1 ± 2.3	6.7 ± 1.2
Distal bronchiole	8.8 ± 1.4	9.1 ± 2.5	10.6 ± 1.7
Parenchyma	8.6 ± 2.0	10.6 ± 3.0	11.1 ± 2.0

[a] Values are taken from Ref. 36, for comparison.

are suitable for investigating the relationships between metabolism and toxicity. The technique is generally applicable to a number of species, including primates (30), and can be used to examine metabolic processes in highly defined areas within the lung. The technique has been applied to studies of the rates of metabolism of both the parent hydrocarbon naphthalene and the putative toxic metabolite naphthalene oxide (17). The data on the metabolism of naphthalene (measured as diol and trapped glutathione adducts) provide a partial explanation for the species and regional differences in cytotoxicity that are observed after parenteral administration of naphthalene. The striking differences in the susceptibility of mice, in which Clara cell vacuolation occurs in terminal bronchioles at naphthalene doses as low as 50 mg/kg, in comparison with hamsters and rats, in which LD_{50} doses (800 mg/kg in hamsters and 1600 mg/kg in rats) produce slight or no pulmonary injury, respectively, are reflected in substantial differences in the rates of naphthalene metabolism in distal bronchioles prepared from mice, rats, and hamsters. Moreover, the gradation in sensitivity within a single species, i.e., mice, where terminal bronchioles are injured at 50 mg/kg but doses of 300 mg/kg or greater are necessary for cytotoxicity in proximal bronchi, correlates with higher rates of cytochrome P450-dependent metabolism in isolates from distal bronchioles, compared with more proximal airways. Species and airway level differences in the rates of formation of naphthalene oxide, measured as diol and trapped glutathione conjugates (Figs. 1 and 2), are similar to the variations observed in the rates of formation of reactive metabolites that become covalently bound to proteins in the explants (31). Although the differences in rates of metabolism between susceptible and nonsusceptible airway levels observed in the current studies are substantial, the data were generated using high substrate concentrations that are likely to be saturating. Previous work in microsome preparations from mouse, rat, and hamster lung indicated that the apparent K_m for naphthalene oxide formation is <0.05 mM. It is possible that, with the use of lower substrate concentrations, the differences in activity noted here would be even greater. It also should be noted that the substrate concentrations are much higher than observed in the lung after administration of naphthalene *in vivo*. Although these experiments suggest that the higher rates of metabolism in distal airways are associated with increased sensitivity to naphthalene cytotoxicity, the data are not capable of indicating whether the apparent differences in rates of metabolism are the result of inherent differences in Clara cells in proximal and distal airways or whether the differences are simply based on the volume fraction of Clara cells at the two airway levels (11). Previous work showing that the putative toxic metabolite naphthalene oxide is capable of diffusing across cell membranes (17) supports the view that the total amount of epoxide generated at a given airway level is important in determining the susceptibility of cells within that airway. A more definitive answer to this question may come from ongoing studies examining the sensitivity of explants of proximal and distal airways to carefully controlled concentrations of naphthalene oxide.

Earlier work has demonstrated that cytochrome P450 2F2, an enzyme isolated from mouse liver, catalyzes the stereoselective epoxidation of naphthalene (24). Immunolocalization of cytochrome P450 2F2 correlates well with the rates of formation of naphthalene 1*R*,2*S*-oxide in mouse, rat, and hamster airway explant incubations. High levels of protein antigen were detected in mouse terminal bronchioles; these same regions catalyzed the rapid and stereoselective conversion of naphthalene to its 1*R*,2*S*-oxide. At the same antibody dilutions protein was not detected in lung parenchyma, and activities could not be measured in this region. Likewise, little of the cytochrome P450 2F2 protein was detected by imunocytochemistry in rats or hamsters and the rates of naphthalene metabolism were correspondingly lower. In addition, the stereoselective epoxidation noted in mouse airways was not apparent in hamster or rat airways. However, these data should be interpreted cautiously, because diol was a quantitatively important metabolite in incubations of both rat and hamster airways and possible enantioselective metabolism by epoxide hydrolases could influence the ratio of glutathione adducts trapped during these experiments.

Cytochrome P450 2B4 is present in both Clara cells and type II alveolar cells of mice. However, based on the lack of detectable metabolism in lung parenchyma, cytochrome P450 2B4 does not appear to play an important role in naphthalene metabolism in the lung. These data are consistent with the results of recent studies showing that the cytochrome P450 2B

suicide substrate 2-ethynylnaphthalene (32) does not inhibit naphthalene metabolism by mouse lung microsomal enzymes.[1]

Differences in the rates of naphthalene metabolism probably do not wholly explain the susceptibility differences noted between species and at different airway levels, however. Although some level of cytochrome P450-dependent activation of the parent substrate is a necessary element in naphthalene cytotoxicity, the balance between the activating and detoxifying pathways is key to determining the susceptibility of a given region. Glutathione transferases and epoxide hydrolases are capable of metabolizing epoxide substrates with a high degree of enantioselectivity (33, 34). One of the possibilities that must be considered in attempting to explain the site-selective toxicity of naphthalene is that target areas of the lung lack either the glutathione transferases or the epoxide hydrolases that are capable of efficiently metabolizing the epoxide. This would explain earlier studies showing that Clara cells are selectively injured in mouse lungs perfused with naphthalene oxide (17). Mouse and hamster postmitochondrial supernatants metabolized naphthalene oxide to diol and glutathione conjugates at comparable rates, whereas metabolism in rat airways was considerably slower (Table 1). In fact, the rates of metabolism in rat airways were only twice those of control. There appeared to be a slight preference of the transferases toward the 1*R*,2*S*-oxide in all three species. As indicated earlier, however, the enantioselective metabolism of either naphthalene 1*R*,2*S*-oxide or naphthalene 1*S*,2*R*-oxide by epoxide hydrolases could account for the differences in the ratios of glutathione adducts noted here.

The apparent sensitivity of epithelial cells in the proximal intrapulmonary bronchi but not the distal bronchioles of hamsters does not appear to be related to strikingly high rates of naphthalene conversion to diol and conjugates in proximal bronchi or to excessively low rates of hydration/conjugation of the epoxide in proximal bronchi, compared with distal bronchioles. The only striking difference between these two airways is in the glutathione levels, which in proximal bronchi are approximately half of those in the distal bronchioles (Table 2).

In summary, the striking differences in the rates of conversion of naphthalene to the epoxide in tracheobronchial airways of susceptible, compared with nonsusceptible, species and the correlation of these rates with the presence, in target but not nontarget species, of a cytochrome P450 isoform that catalyzes the rapid and stereoselective metabolism of naphthalene support the view that the rates of metabolism of the parent hydrocarbon are a key factor in determining the species-, site-, and cell-selective toxicity of naphthalene. This view is further supported by the finding that glutathione levels in highly susceptible sites of mouse lung are as high as those in less susceptible regions of the tracheobronchial airway and in airways from nonsusceptible species. In addition, the rates of naphthalene oxide metabolism in mice are the same as or faster than those in either hamsters or rats and thus do not appear to account for the differences in sensitivity of the tracheobronchial airway epithelium to naphthalene. We are currently attempting to provide more direct evidence for the involvement of the epoxide in bronchiolar epithelial cell toxicity by examining the toxicity and glutathione depletion caused by naphthalene oxide and naphthoquinones in airways isolated from both sensitive and nonsensitive species. Presumably, if the rates of generation of the epoxide are a key element in determining site-selective toxicity, then the sensitivity of hamster airways incubated with naphthalene oxide should be similar to that of mouse tissue. Due to lower apparent transferase/epoxide hydrolase activities, rat airways should be more sensitive.

[1] A. Buckpitt, C. Plopper, A. Chang, and W. Alworth, unpublished observations.

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