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MOLECULAR PHARMACOLOGY, 45:664-672

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Metabolism and Cytotoxicity of Naphthalene and Its Metabolites in Isolated Murine Clara Cells

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Received June 25, 1993; Accepted December 3, 1993

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SUMMARY

Nonciliated bronchiolar epithelial (Clara) cells of mice are highly susceptible to toxicants that undergo metabolic activation, presumably because this cell type expresses high levels of cytochrome P450 monooxygenases. To establish the capability of these cells to metabolize an agent that causes Clara cell-selective toxicity *in vivo*, we evaluated the metabolism of naphthalene in isolated cells under two distinct conditions, i.e., in homogenized cell preparations supplemented with glutathione and glutathione *S*-transferases and in intact cells. In homogenized cell preparations naphthalene was metabolized to dihydrodiol (minor) and a single glutathione adduct (major) derived from the 1*R*,2*S*-epoxide. In intact cells the rate of formation of glutathione adduct was much lower and dihydrodiol predominated. Approximately 3–10% of racemic naphthalene oxide added to isolated homogenized cells was converted to glutathione adducts and dihydrodiol in 3-min incubations. At high concentrations of naphthalene oxide (0.25 and 0.5 mM), formation of the adduct derived from the 1*R*,2*S*-epoxide was favored. The intracellular glutathione concentration, measured by high performance liquid chromatography as the fluorescence of the monobromobimane-glutathione derivative, was 1.14 ± 0.13 nmol/10^6 cells. To determine whether Clara cell injury results from cytotoxic metabolites of naphthalene, we assessed viability of intact cells in response to different concentrations of naphthalene and naphthalene metabolites. At high naphthalene concentrations (0.5 and 1.0 mM) cell viability decreased to 63% or less of control, whereas lower concentrations (0.1 or 0.05 mM) did not alter viability significantly. Naphthalene-induced decreases in cell viability were blocked by preincubation of Clara cells with the cytochrome P450 monooxygenase inhibitor piperonyl butoxide. The cytotoxicity of naphthalene metabolites varied. Incubation of cells with 0.5 mM dihydrodiol, 1-naphthol, or 1,2-naphthoquinone decreased cell viability to an extent similar to that produced by 0.5 mM naphthalene. In contrast, 0.5 mM naphthalene oxide and 1,4-naphthoquinone significantly decreased viability more than the parent compound. Preincubation of Clara cells with piperonyl butoxide did not affect the loss in cell viability associated with naphthalene oxide. We conclude that isolated Clara cells 1) are capable of metabolizing naphthalene, a Clara cell-specific cytotoxicant, to two major metabolites, 2) have a detectable intracellular glutathione pool, and 3) are more susceptible to specific naphthalene metabolites than to the parent compound naphthalene.

Metabolic activation of biologically inert xenobiotics to electrophilic intermediates by cytochrome P450 monooxygenases is recognized as an obligate step in the cytotoxicity of many chemicals. The susceptibility of cells to these bioactivated chemicals depends upon the balance between activation and the abilities of the cells to detoxify the resulting electrophilic metabolites. In general, metabolites generated by cytochrome P450 monooxygenases cause cytotoxicity by a combination of processes that can involve 1) direct interaction with critical macromolecules in target cells and/or 2) depletion of cofactors important in detoxifying metabolites, such as glutathione, which may indirectly result in cell injury. Studies exemplifying these processes have demonstrated that depletion of tissue glutathione by administration of diethylmaleate enhances cytotoxicity and increases covalent binding in response to bioactivated pulmonary and hepatotoxic chemicals (1, 2). For many hepatotoxicants, the pathways of activation have been fully delineated. However, the subsequent interactions of electrophiles with macromolecules in the cells and the importance of these interactions in cellular injury remain to be fully characterized. For example, the hepatotoxicity of acetaminophen appears to depend upon the formation of *N*-acetyl-*p*-benzoquinonimine, but the relative importance of redox cycling and covalent interactions of the quinonimine with specific cellular proteins in cellular injury has not been established (see Refs. 3

This manuscript was made possible by Grants 04311 and 04699 from the National Institute for Enviromental Health Sciences, National Institutes of Health. The University of California, Davis, is a National Institute for Enviromental Health Sciences-sponsored Health Science Center (Grant P30-ES05707) and the authors would like to acknowledge support by the Center for the core facilities that have been used in this work.

ABBREVIATIONS: HPLC, high performance liquid chromatography; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N'*,*N'*-tetraacetic acid; CDNB, 1-chloro-2,4-dinitrobenzene; DME, Dulbecco's modified Eagle's.

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and 4 for reviews). The cytotoxic effects of acetaminophen in isolated hepatocytes demonstrate that the potencies of quinone metabolites are of the same order of magnitude as those of the primary oxidation product (5). Studies with benzene on the relationship of specific metabolites to cytotoxic insult have attributed toxicity to the phenol and catechol rather than the benzene oxide (6).

In contrast to the liver, a relatively homogeneous organ composed of only two or three cell types in the adult, the lung is composed of >40 cell types, each of which is a potential target for both inhaled and blood-borne toxicants. The nonciliated bronchiolar epithelial (Clara) cell is a locus for cytochrome P450 monooxygenases (7, 8) and the principal target for cytotoxicity in response to cytochrome P450-activated pulmonary toxicants, such as 4-ipomeanol (9–12). Previous studies have demonstrated the ability of Clara cells isolated from rabbits to bioactivate the Clara cell cytotoxicants 4-ipomeanol (13) and 3-methylindole (14). Dose- and time-dependent losses in Clara cell viability were noted in response to 3-methylindole (14).

Administration of naphthalene by either parenteral or inhalational routes produces a highly selective, dose-dependent lesion in murine bronchiolar Clara cells (15–18). An intraperitoneal dose of 50 mg/kg naphthalene causes vacuolation in Clara cells of the terminal bronchioles, whereas doses of 100 and 200 mg/kg cause necrosis and exfoliation (18). Naphthalene is metabolized by the cytochrome P450 monooxygenases to several reactive intermediates including naphthoquinones and epoxides (19–21), which can deplete glutathione, can become bound covalently, and may be involved in the cytotoxicity of the parent compound (22). Indeed, recent studies have suggested that reactive metabolites other than the primary oxidation product may play a role in the cytotoxic response (23, 24). In isolated hepatocytes, secondary metabolites of naphthalene have been implicated in cytotoxicity through the conversion of the parent compound to 1-naphthol, which then undergoes subsequent conversion to naphthoquinone metabolites that cause cell death by mechanisms involving glutathione depletion and redox cycling (20). Similar *in vitro* studies examining the ability of putative toxic naphthalene metabolites to directly produce losses in Clara cell viability have not been pursued. Moreover, their chemical instability, combined with rapid metabolism, precludes evaluation of the toxicity of these metabolites *in vivo*.

One key to understanding the susceptibility of Clara cells to toxic compounds in general, and to naphthalene in particular, lies in characterizing the intrinsic metabolic capabilities of this cell type. Determining whether the unique sensitivity of Clara cells to toxic insult is due to an abundance of activating enzymes or a lack of detoxifying enzymes will be essential for comprehending the metabolic response of these cells. For this reason, we have undertaken the present study with the following goals in mind: 1) to characterize the metabolism of naphthalene, a cytochrome P450-activated cytotoxicant, by its target cell population, Clara cells, and 2) to examine the contributions of naphthalene metabolites to Clara cell cytotoxicity.

Materials and Methods

Animals. Male Swiss-Webster mice (age, 4–5 weeks), weighing 20–25 g and virus antibody-free, were purchased from Charles River Breeding Laboratories (Wilmington, MA). Animals were allowed free access to food and water and were housed over autoclaved bedding, in American Association for Accreditation of Laboratory Animal Care-accredited facilities at the University of California, Davis, for at least 5 days before use.

Chemicals. All chemicals used were reagent grade or better. HPLC solvents were obtained from Fisher Scientific (Pittsburgh, PA). Most naphthalene metabolites were obtained from Aldrich Chemical Co. (Milwaukee, WI), although the naphthalene oxide was synthesized by previously published methods (25). Concentrations of the final product and possible contamination by 1-naphthol were determined by UV spectroscopy (26). Lyophilized porcine elastase for the cell isolation was purchased from Worthington Biochemical (Malvern, PA). F-12/DME medium was obtained from GIBCO (Grand Island, NY). Monobromobimane was purchased from Calbiochem (San Diego, CA).

Radiochemicals. [^{3}H]Naphthalene was obtained by reductive dehalogenation of 2,3-dibromonaphthalene. The radiochemical purity of the final product (after sublimation) was >98.5% by reverse phase HPLC. Final specific activities are provided in the figure legends.

Clara cell isolation. Clara cells were isolated as described previously (8). Briefly, animals were killed with an intraperitoneal injection of pentobarbital and the lungs were cleared of blood by perfusion with a balanced salt solution via the pulmonary artery. Lungs were removed from the chest, lavaged with a balanced salt solution containing 2 mM EGTA, and instilled with an elastase solution (4.0 units/ml). After a 20-min incubation with elastase, the lungs were minced, filtered through gauze, and centrifuged through a layer of calf serum. The cell pellet was resuspended in F12/DME medium, cells were plated onto dishes that had been previously coated with mouse IgG, and the cells were incubated at 37° for 1 hr in a 5% CO_2/95% air incubator. Cells were then collected and counted and the viabilty was measured by vital dye exclusion using erythrosine B (27). Ultrastructural characterization indicated that these preparations contained an average of 72 ± 5% Clara cells, 9 ± 8% ciliated cells, and 11 ± 5% type II cells. Erythrocyte contamination varied from 1 to 4%. Proteolysis, a potential problem with other preparations (13), was minimal (8).

Glutathione determination. Glutathione was determined according to the method of Fahey and Newton (28). Briefly, 10^6 cells were isolated and homogenized on ice with a glass homogenizer, in 100 µl of 200 mM methanesulfonic acid containing 5 mM diethylenetriaminepentaacetic acid. An equal volume of 4 M sodium methanesulfonate was added and samples were stored at −80° until derivatization. Samples were derivatized by the addition of 2 mM monobromobimane, 200 mM *N*-2-hydroxyethylpiperazine-*N'*-3-propanesulfonic acid, and 5 mM diethylenetriaminepentaacetic acid. After a 10-min incubation in dim light at room temperature, methanesulfonic acid was added to stop the reaction. The bimane derivative of glutathione was separated by HPLC on a Novapak C_{18} column (5 × 100 mm), using a mobile phase of 7.5% acetonitrile, 15 mM tetrabutylammonium phosphate, and 0.25% acetic acid, pH 3.4. Fluorescent derivative was measured at excitation and emission wavelenghths of 360 and 460 nm, respectively. Standard curves were linear up to 50 pmol/sample.

Glutathione transferase activity. After isolation, 10^6 Clara cells were sonicated in 0.1 M phosphate buffer, pH 6.5. Gluthathione *S*-transferase activity was determined spectrophotometrically using CDNB as the substrate (29).

Naphthalene and napthalene oxide metabolism. Clara cells (10^6 cells/ml) suspended in F-12/DME medium were incubated with 0.5 or 0.1 mM [^{3}H]naphthalene in 5 µl of methanol, with or without the addition of 5 mM exogenous glutathione, 5 CDNB units of glutathione transferase (affinity purified from mouse liver cytosol) (30), and a NADPH-regenerating system (1 µmol of NADP, 30 µmol of glucose 6-phosphate, and 1 unit of glucose-6-phosphate dehydrogenase). Incubations were prepared on ice in a total volume of 1 ml, substrate was added last, and incubations were transferred to a water bath at 37° for times indicated in the figure legends. Reactions were terminated by the addition of 2 volumes of ice-cold methanol.

The activity and enantioselectivity of the glutathione transferase

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were determined by incubation of the 9000 × g supernatant from 10^6 Clara cells with naphthalene oxide and 5 mM glutathione in a 37° water bath for 3 min. The reaction was stopped by the addition of 2 volumes of methanol.

Naphthalene glutathione conjugates generated in incubations with naphthalene or naphthalene oxide were determined by HPLC. In incubations with [^{3}H]naphthalene, unmetabolized labeled naphthalene was extracted with hexane and discarded. Aliquots of the methanol/water supernatant were evaporated to dryness under vacuum. The sample was reconstituted in water and injected onto a Novapak C_{18} column (8 × 100 mm), using a mobile phase of 3.8% acetonitrile, 1% acetic acid, and water to separate diastereomeric glutathione conjugates. As demonstrated in previous studies (31), the 1*R*,2*S*-naphthalene oxide forms a single glutathione adduct at the allylic position to produce conjugate 2, whereas the 1*S*,2*R*-naphthalene oxide forms conjugates 1 and 3 by addition of glutathione to either the benzylic or allylic position of the dihydronaphthalene ring to form the hydroxyglutathionyl adduct.

Cytotoxicity of naphthalene and naphthalene metabolites in isolated Clara cells. Naphthalene, 1,4-naphthoquinone, 1,2-naphthoquinone, 1-naphthol, and 1,2-dihydroxy-1,2 dihydronaphthalene were dissolved in methanol (5 μl/ml) and added to isolated Clara cells suspended in F-12/DME medium, to reach final concentrations of 1.0, 0.5, and 0.1 mM. Controls received methanol only. In experiments to determine whether cytochrome P450 monooxygenase-dependent metabolism was associated with the loss of cell viability from naphthalene and naphthalene oxide, isolated Clara cells were incubated with 0.05 mM piperonyl butoxide (dissolved in methanol and added at 5 μl/ml of incubation) for 30 min at 37° before the addition of naphthalene or the start of the infusion of naphthalene oxide. Control incubations contained piperonyl butoxide plus solvent. Incubations were carried out in silanized glass vials in a shaking water bath at 37° under a 95% air/5% CO_2 atmosphere. At the time points indicated in the figures, aliquots of the cell suspension were removed and cell viability was measured by vital dye exclusion using erythrosine B.

Naphthalene oxide, dissolved in 99.5% ethanol/0.5% triethylamine, was added to Clara cell incubations over 45 min with the aid of a syringe infusion pump (total volume delivered was 10 μl in a 1-ml incubation), to reach final concentrations of 0.5, 0.1, and 0.01 mM. Preliminary studies showed that the addition of the epoxide as a bolus caused virtually no cytotoxicity. Viability was determined at varying times after the start of the infusion. Ethanol/triethylamine was added in a similar manner to control incubations.

Results

Metabolism of naphthalene. The functional capability of the cytochrome P450 system in isolated Clara cells was evaluated by incubating naphthalene with homogenized cells in the presence of glutathione and glutathione transferases to trap naphthalene oxides. Naphthalene metabolism was stereoselective and the metabolism of 0.5 mM naphthalene occurred at easily measurable rates (4.07 ± 1.12 nmol/10^6 cells/45 min) (mean ± standard deviation) (Fig. 1a). The ratio of conjugate 2 to conjugates 1 and 3 (5.7:1) indicated that the formation of the 1*R*,2*S*-oxide predominated (2.76 ± 0.22 nmol/10^6 cells/45 min). Under these conditions dihydrodiol accounted for 20% of the total metabolites formed (0.83 ± 0.95 nmol/10^6 cells/45 min).

Isolated intact cells incubated with naphthalene without the addition of exogenous glutathione or glutathione transferases produced a metabolic profile strikingly different from that of the cell homogenates (Fig. 1b). The predominant metabolite produced was dihydrodiol, ranging from 59 to 64% of the total metabolites. Total conjugate formation in 10-min incubations was 0.30 ± 0.11 nmol/10^6 cells at 0.1 mM, 0.25 ± 0.05 nmol/10^6 cells at 0.25 mM, and 0.26 ± 0.14 nmol/10^6 cells at 0.5 mM. The differences in formation of relative amounts of glutathione conjugates versus dihydrodiol in incubations supplemented with glutathione and glutathione transferases (Fig. 1a) versus those without additional glutathione or the transferases (Fig. 1b) demonstrate that there are marked differences in the ability of these target cells to generate the epoxide, compared with their ability to catalyze the formation of dihydrodiol or glutathione conjugates. The inability of the cells to generate dihydrodiol or glutathione conjugates at rates commensurate with their ability to generate the epoxide may be due either to the relatively low concentration of glutathione in the cells (the glutathione level in isolated murine Clara cells was 1.14 nmol/10^6 cells) or to the presence of the transferases in supplemented incubations (Fig. 1a). The rate of metabolism of naphthalene was not dependent upon substrate concentration at concentra-

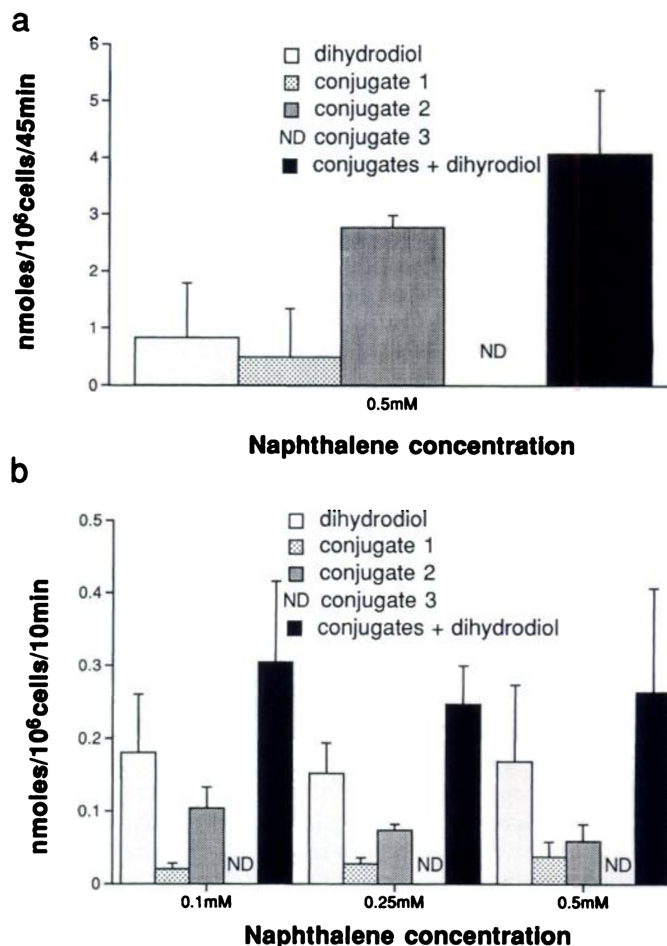

Fig. 1. Concentration response for the metabolism of naphthalene by isolated Clara cells. a, Metabolism of [^{3}H]naphthalene in the presence of glutathione and liver glutathione *S*-transferases. Homogenized Clara cells (10^6 cells) were incubated with [^{3}H]naphthalene (0.5 mM), glutathione (5 mM), and glutathione *S*-transferases (10 CDNB units) for 45 min. The supernatant was analyzed for metabolites by HPLC. Values are the mean ± standard deviation of three isolations with duplicate incubations. b, Metabolism of [^{3}H]naphthalene to dihydrodiol and glutathione conjugates by 10^6 intact Clara cells. [^{3}H]Naphthalene (0.1, 0.25, and 0.5 mM; specific activity, 10,000 dpm/nmol) was added as a bolus to Clara cells in a 37° water bath and incubated for 10 min. Metabolites were isolated and analyzed by HPLC as described in Materials and Methods. Two large unidentified metabolite peaks were consistently present in radioprofiles of all incubations. Values are the mean ± standard deviation of three isolations with duplicate incubations.

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tions between 0.1 mM and 0.5 mM. The metabolism of naphthalene (0.1 mM) was most rapid during the first 10 min and peaked by 30 min (Fig. 2); from 30 to 280 min there was no increase in the amount of metabolites produced (data not shown).

Glutathione *S*-transferase activity. In isolated sonicated cells the biotransformation activity of glutathione *S*-transferase with CDNB was 30.1 ± 8.9 nmol/10^6 cells/min (three experiments). To determine whether the glutathione *S*-transferase and/or epoxide hydrolase metabolized naphthalene oxide enantioselectively, the 9000 × *g* supernatant from homogenized cells was incubated with glutathione and racemic naphthalene oxide (0.05, 0.25, and 0.5 mM) for 3 min. The total rate of formation of dihydrodiol and conjugates varied from 0.92 ± 0.16 nmol/10^6 cells/3 min for 0.05 mM (7.32% conversion of the substrate) to 2.90 ± 1.20 for 0.25 mM (4.64% conversion) (Fig. 3). At all concentrations of naphthalene oxide, the major metabolite produced was the glutathione conjugate from the 1*R*,2*S*-oxide. The enantioselectivity of the glutathione *S*-transferase was more pronounced at high substrate concentrations (0.25 and 0.5 mM), as indicated by a ratio of 2.7:1 (conjugate 2:conjugate 1 plus conjugate 3), than it was at low substrate concentration (0.05 mM), with a ratio of 1.4:1.

Clara cell viability. Fig. 4a shows the percentages of isolated Clara cells still viable after timed exposures to different concentrations of naphthalene. Control cells, incubated with vehicle only, maintained approximately 80% viability throughout the 240-min incubation. Viability of cells incubated with either 0.05 or 0.1 mM naphthalene remained >90% of control values at both 120 and 240 min. At 240 min, higher concentrations of naphthalene (0.5 and 1.0 mM) resulted in significant decreases in Clara cell viability (61% and 12%, respectively, of control).

The effects of different concentrations of naphthalene oxide on Clara cell viability are presented in Fig. 4b. The addition of naphthalene oxide as a bolus to isolated cell incubations at concentrations as high as 0.5 mM caused no loss in cell viability (data not shown). In contrast, when cells were exposed to

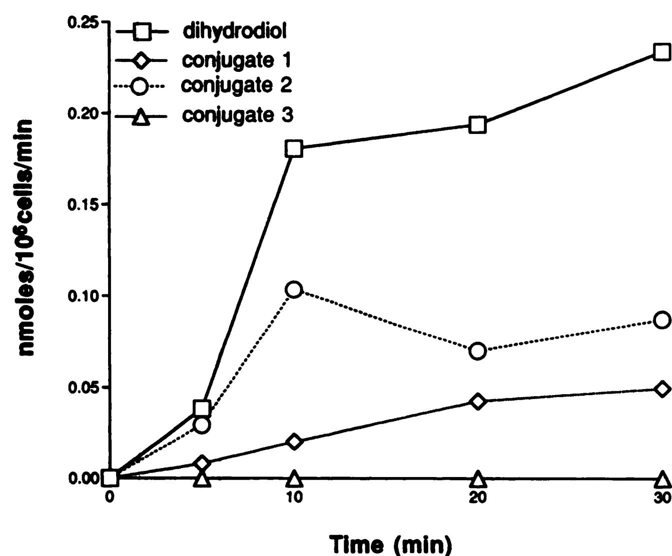

Fig. 2. Time course of metabolism of naphthalene in 10^6 isolated cells. The time course of formation of dihydrodiol and glutathione conjugates by 10^6 isolated Clara cells in incubations containing [^{3}H]naphthalene (0.1 mM) is shown. Metabolites were quantified by HPLC.

identical concentrations of naphthalene oxide delivered at a rate of 11.1 pmol/min/ml of incubation, Clara cell viability decreased significantly.

Preincubation of Clara cells with the cytochrome P450 monooxygenase inhibitor piperonyl butoxide (0.05 mM) did not have any effect on the viability of control Clara cells but markedly decreased the loss in cell viability caused by incubation with naphthalene (1.0 mM) (Fig. 5). In cells without inhibitor, 1.0 mM naphthalene decreased viability to <15% by 2 hr (Fig. 4). In contrast, viability was not significantly different from control in cells preincubated with piperonyl butoxide and then subjected to a 2- or 4-hr incubation with 1.0 mM naphthalene. Piperonyl butoxide had no effect on the loss in cell viability associated with naphthalene oxide infusion (Fig. 5). Four hours after the start of infusion of 0.5 mM naphthalene oxide, viability was not significantly different in nonpretreated cells, compared with piperonyl butoxide-treated cells (data not shown).

Viability decreased in all incubations by 120 min after the addition of 0.5 mM naphthalene metabolites (Fig. 6a). Racemic naphthalene oxide was the most cytotoxic; at 240 min (Fig. 6b) viability had decreased to 6 ± 3% of control. At 120 min, the cytotoxicity of naphthalene oxide was comparable to the decrease in viability caused by 1,4-naphthoquinone at 240 min (18 ± 6% of control). As a percentage of control, the decreases in viability caused by 1,2-naphthoquinone (53 ± 4%), 1-naphthol (70 ± 16%), and dihydrodiol (60 ± 8%) were similar to the decrease in viability caused by naphthalene (57 ± 20%) at 240 min.

Discussion

Naphthalene is metabolized by cytochromes P450 1A1, 2B, and 2F (32–34) to enantiomeric epoxides that are substrates for glutathione *S*-transferases and epoxide hydrolases (35) (Fig. 7). The present study has utilized preparations containing glutathione and glutathione *S*-transferase to trap unstable naphthalene oxides and to assess the stereoselectivity of naphthalene oxide formation through measurements of glutathione adducts. Under these conditions the metabolism of naphthalene by homogenates of isolated Clara cells was stereoselective, forming predominately 1*R*,2*S*-naphthalene oxide at easily measurable rates. This finding parallels studies using microsomes prepared from whole-lung homogenates (36), where the ratio of the 1*R*,2*S*- to 1*S*,2*R*-naphthalene oxide was 20:1, and from dissected airways (37), where only the 1*R*,2*S*-naphthalene oxide was detected. The ratio of glutathione adducts produced in the homogenates of isolated cells indicates that the cytochrome P450 isozymes present in Clara cells are capable of producing the naphthalene oxides intrinsically, suggesting that this reaction is the first step in the cytotoxicity pathway.

In contrast to the data showing that dihydrodiol is a minor metabolite in incubations of homogenized Clara cells supplemented with glutathione and glutathione *S*-transferase, data from incubations of intact Clara cells not supplemented with the glutathione-trapping system show that dihydrodiol predominates and the ratio of 1*R*,2*S*- to 1*S*,2*R*-naphthalene oxide is lower. Naphthalene oxide is a substrate for several different enzymes and, once formed, can potentially interact covalently with proteins in the cell. Therefore, the relative ratios of various metabolites arising from the epoxide are dependent upon the amounts of oxide generated in the cell, the availability of

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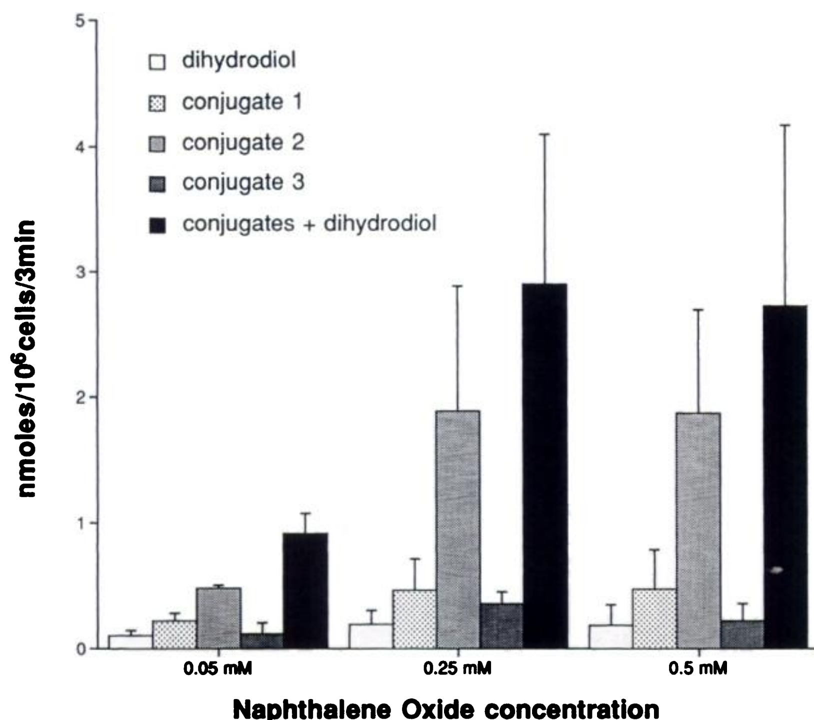

Fig. 3. Metabolism of naphthalene oxide in the presence of excess glutathione. Naphthalene oxide (0.05, 0.25, and 0.5 mM) was incubated with the 9000 × *g* supernatant from 10^6 homogenized Clara cells and glutathione (5 mM) for 3 min in a 37° water bath. The supernatant was analyzed for metabolites by HPLC.

cosubstrates such as glutathione, and the relative K_m values for epoxide hydrolases and glutathione transferases. The data in Fig. 1 indicate that the capacity of Clara cells to generate naphthalene oxide exceeds the ability of intact cells to conjugate this epoxide to glutathione or to form the dihydrodiol. Although the concentrations of naphthalene oxide generated within Clara cells cannot be determined reliably, the total epoxide generated (calculated from Fig. 1a) may well be above the reported K_m of epoxide hydrolase (1 μM) (33). The apparent mismatch in the ability of Clara cells to conjugate/hydrate naphthalene oxide is consistent with the relatively large amounts of reactive metabolite bound covalently to protein in incubations of naphthalene with isolated Clara cells[1] and with the apparent lack of dependence of metabolism on substrate concentration (Fig. 1b).

There are several possibilities to explain the lack of substrate dependence at the concentrations used in these studies. Our finding that there were no concentration-related differences in the amount of glutathione conjugates produced (Fig. 1b) suggests that glutathione concentrations in unsupplemented Clara cells may be limiting. However, cells were found to contain 1.1 nmol of reduced glutathione/10^6 cells, and this is substantially higher than the amount of glutathione adduct found (0.12 nmol/10^6 cells) at 0.5 mM substrate concentration (Fig. 1b). It is possible that a significant portion of the cellular glutathione is compartmentalized and is therefore unavailable for conjugation with naphthalene oxide. An additional possiblity is that the amount of glutathione remaining in the cytosol is well below the K_m for the transferase-mediated conjugation with naphthalene oxide. The overall profile of the metabolites produced is consistent with that obtained in the isolated perfused lung system, where conjugation with glutathione and conversion to dihydrodiol are the major pathways of naphthalene oxide metabolism (23). Further understanding of the metabolic disposition of metabolites from naphthalene oxide will require careful evaluation of epoxide hydrolase and glutathione *S*-transferase, as well as the cofactors for these enzymes.

The specific activity and enantioselectivity of glutathione *S*-transferases in Clara cells were investigated by evaluating the activity of the enzymes with CDNB and racemic naphthalene oxide as substrates. The activity of glutathione *S*-transferases with CDNB in this study was lower than 95 ± 35 nmol/10^6 cells/min, as reported by Forkert *et al.* (38). Differences in mouse strains and variations in the purity of the preparations utilized in their studies may account for the differences in activity. Myles *et al.* (39) have compared the distribution of biotransformation activities of Clara cells and type II cells isolated from hamsters and found that glutathione *S*-transferase activity toward CDNB is highest in type II cells, whereas 7-ethoxycoumarin *O*-deethylation is highest in Clara cells. This observation corresponds to studies by Forkert *et al.* (38) and suggests that monooxygenase-related activities are more concentrated in Clara cells than are glutathione *S*-transferase activities. Activity toward CDNB is only one assessment of the detoxification potential of the glutathione *S*-transferases within Clara cells, and it may not be totally representive because isozymes of glutathione *S*-transferase differ significantly in their binding properties and substrate specificities (40). The activity and enantioselectivity of Clara cell glutathione *S*-transferases with naphthalene oxides may be important determinants in the selective toxicity of naphthalene and may explain data showing that infusion of naphthalene oxide into isolated perfused lungs results in selective injury to Clara cells. Numerous studies have demonstrated dramatic differences in activity and enantioselectivity of purified glutathione *S*-transferases (41–43), as well as cytosolic fractions from a variety of tissues, for the metabolism of various aromatic hydrocarbon

[1] Cho M., C. Chichester, D. Merin, C. Plopper, and A. Buckpitt, unpublished manuscript.

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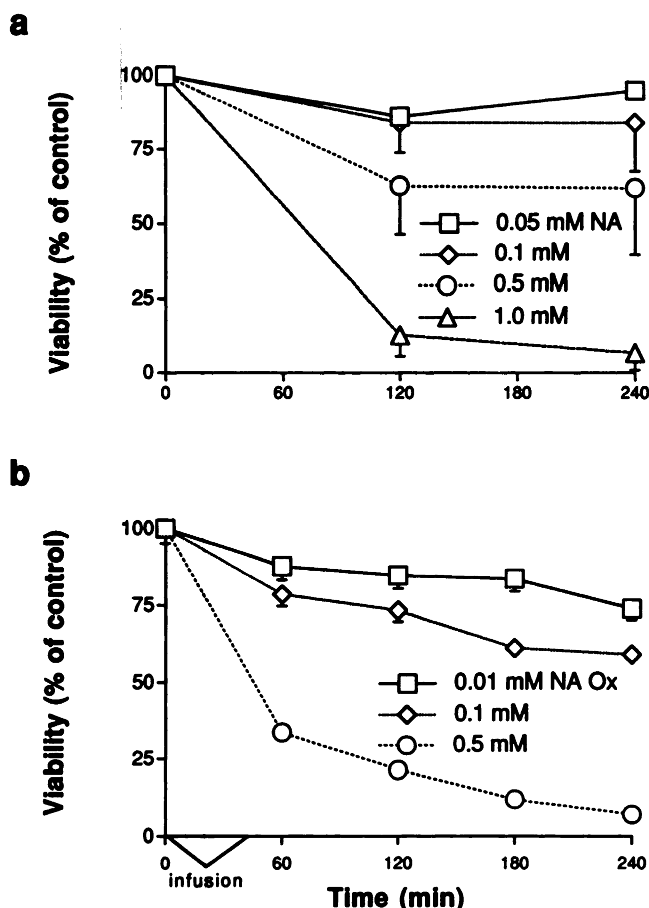

Fig. 4. Concentration-response curves for naphthalene- and naphthalene oxide-induced cytotoxicity in isolated Clara cells. Viability of isolated mouse Clara cells incubated in the presence of napthalene (*NA*) (0.05, 0.1, 0.5, or 1.0 mM) (a) or naphthalene oxide (*NA Ox*) (0.01, 0.1, or 0.5 mM) (b) was determined. Viabilities are expressed as the percentage of cells in the vehicle control that exclude erythrosine B. Values are the mean ± standard deviation of three isolations with duplicate incubations. Standard deviations not shown are obscured by the symbol.

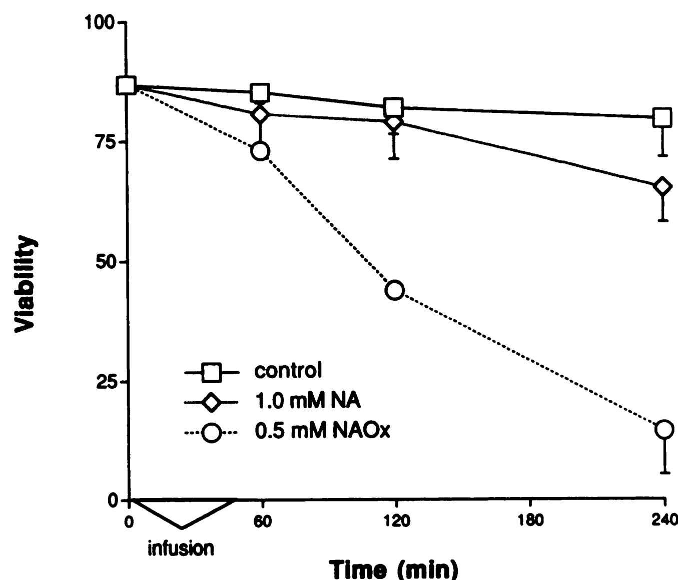

Fig. 5. Effect of preincubation with piperonyl butoxide on the cytotoxicity of naphthalene and naphthalene oxide. Clara cells were preincubated with piperonyl butoxide (0.05 mM) at 37° for 30 min before the addition of vehicle control (*control*) or naphthalene (*1.0 mM NA*) or the start of infusion of naphthalene oxide (*0.5 mM NA Ox*). Viabilities are expressed as the percentage of cells that exclude erythrosine B. Values are the mean ± standard deviation of three isolations. Standard deviations not shown are obscured by the symbol.

epoxides. For example, cytosol from both Clara and type II cells of the lung demonstrated a 3–4:1 preference for 4*R*,5*S*-benzo(*a*)pyrene oxide over the enantiomeric 4*S*,5*R*-epoxide. In the present work, the ratio of glutathione adducts generated from the 1*R*,2*S*-oxide to those generated from the 1*S*,2*R*-oxide is nearly 1:1 at low substrate concentrations. Only at the highest concentration used in these studies is there apparent enantio-selectivity by glutathione *S*-transferases for the 1*R*,2*S*-oxide. In addition to the glutathione *S*-transferases, epoxide hydrolase also shows enantioselective metabolism of the arene oxides (44). Under the experimental conditions reported in Fig. 3, the dihydrodiol is a minor product of the naphthalene oxides and does not appear to influence the ratio of glutathione adducts found. However, in the experiments where naphthalene was incubated with unsupplemented Clara cells, dihydrodiol constitutes approximately 62% of the products generated from the arene oxide. Under these circumstances, which are more likely to reflect concentrations of the naphthalene oxides generated in Clara cells *in vivo*, preferential metabolism of naphthalene oxide enantiomers by epoxide hydrolase may play a substantial role in determining the rates of detoxification. Additional work utilizing chemically prepared naphthalene oxide enantiomers will be needed to determine the precise rate constants for the metabolism of these agents.

Although isolated hepatocytes have been used extensively to evaluate the cytotoxicity of numerous hepatotoxicants, this appoach has not been explored thoroughly with Clara cell toxicants. Earlier work with several Clara cell toxicants has demonstrated loss of plating efficiency in short term Clara cell cultures (45); however, the abilities of these preparations to conduct biotransformations have not been elucidated. Other work with the cytochrome P450-activated Clara cell-selective cytotoxicant 3-methylindole showed concentration-related losses of Clara cell viability in short term incubations. Type II cells and macrophages were less sensitive to 3-methylindole concentrations (14). Although the imine methide derivative was implicated as the proximate toxic metabolite by the finding that deuterium-labeled 3-methylindole was less toxic than 3-methylindole, neither the toxicity of specific metabolites nor the specific detoxification pathways were examined. The present study shows that isolated Clara cells can bioactivate naphthalene stereoselectively and that the phase II systems are slightly enantioselective for the production of the glutathione conjugate of the 1*R*,2*S*-epoxide.

In addition to glutathione *S*-transferase activity, inadequate reduced thiol levels or the inability to generate additional quantities of thiol, either through synthesis or by uptake, may be an important factor in Clara cell-selective toxicity. The glutathione status of the Clara cells may also account for the metabolic profile observed when intact cells were exposed to naphthalene without additional glutathione and glutathione *S*-transferases. Devereux *et al.* (13) demonstrated that lower levels of glutathione correlated with increases in covalent binding of 4-ipomeanol in Clara cells isolated from rabbits. *In vivo* studies showing markedly enhanced cytotoxicity and levels of reactive metabolite binding in glutathione-depleted animals support the

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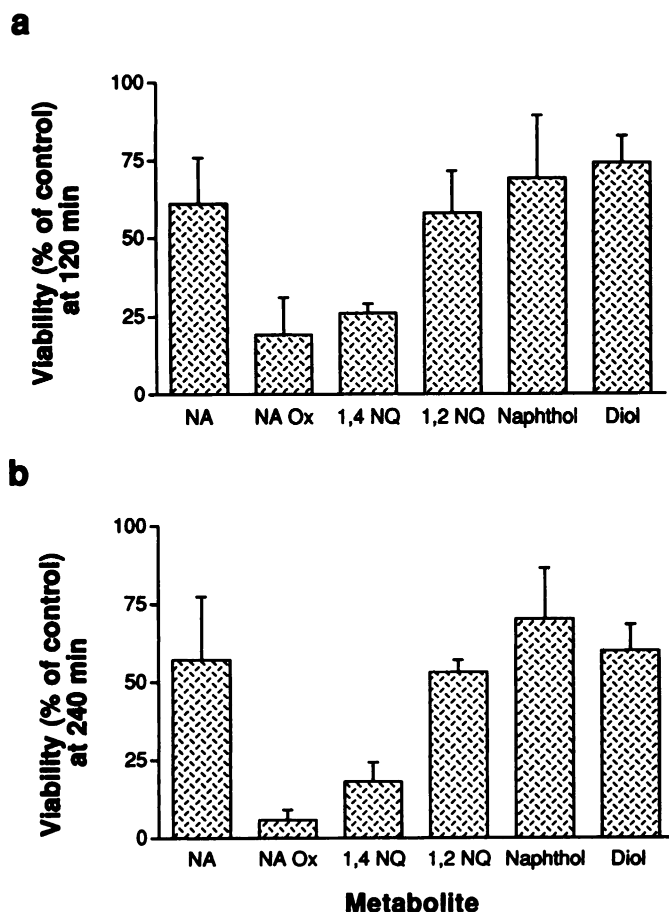

Fig. 6. Cytotoxic response of isolated Clara cells to naphthalene metabolites (0.5 mM). The viability of isolated Clara cells incubated with 0.5 mM naphthalene (*NA*), naphthalene oxide (*NA Ox*), 1,4-naphthoquinone (*1,4 NQ*), 1,2-naphthoquinone (*1,2 NQ*), 1-naphthol (*Naphthol*), or dihydrodiol (*Diol*) for 120 min (a) or 240 min (b) was determined. Viabilities are expressed as the percentage of cells in the vehicle control that exclude erythrosine B. Values are the mean ± standard deviation of three isolations with duplicate incubations.

view that cellular glutathione levels are an important determinant in naphthalene toxicity (1, 46). Our work showing that glutathione levels in Clara cells isolated from mice are <1.5 nmol/10^6 cells is consistent with earlier reports that Clara cell glutathione levels are <15% of the levels found in lung homogenates (13). These observations support the hypothesis that low glutathione levels may be a factor in the inherent Clara cell-selective toxicity of xenobiotics. These findings are also consistent with the very high levels of covalently bound metabolites observed in incubations of isolated Clara cells with [^{3}H] naphthalene (25–30 nmol/mg of protein),[2] compared with 0.5–2 nmol/mg of protein *in vivo* (1). Additional work is needed to assess cellular glutathione levels *in situ*, because Clara cells may lose glutathione during the isolation process and thus exhibit artifically low glutathione levels in the isolated cells.

Determining which metabolites are key factors in eliciting toxicity is an important factor in understanding the metabolic pathways that lead to tissue injury. The importance of cytochrome P450 monooxygenases in the formation of cytotoxic naphthalene metabolites is supported by the data showing that cells preincubated with piperonyl butoxide, at concentrations similar to those shown to markedly decrease the formation of reactive naphthalene metabolites in Clara cells,[1] were remarkably resistant to naphthalene-induced cytotoxicity. In this study three naphthalene metabolites, dihydrodiol, 1-naphthol, and 1,2-naphthoquinone, were not significantly more toxic than the parent compound, whereas 1,4-naphthoquinone and naphthalene oxide were. In comparison, in the isolated perfused lung system 1,2-naphthoquinone, but not 1,4-naphthoquinone, caused increases in the vacuolated cell mass (23). In our study naphthalene oxide was the most potent cytotoxicant tested; concentrations 10-fold lower than those of the parent compound caused cytotoxicity similar to that of naphthalene. The observation that naphthalene oxide produces a more dramatic loss in cell viability than does the rearrangement product 1-naphthol argues in favor of a role for naphthalene oxide in cellular toxicity. Although 1,4-naphthoquinone produces losses in cell viability similar to those produced by naphthalene oxide, it is probably not the primary species responsible for the cytotoxicity. If it were and naphthalene oxide had little influence on the process, then 1-naphthol would be expected to be a more potent cytotoxicant. Moreover, the finding that piperonyl butoxide blocked naphthalene but not naphthalene oxide toxicity supports the contention that the epoxide is a key participant in the process leading to the loss of cell viability in isolated Clara cells. This notion is supported by other studies showing that the administration of 1-naphthol *in vivo* (47) or the perfusion of 1-naphthol into isolated mouse lungs (23) does not elicit any substantial bronchiolar injury, as assessed by morphometric analysis. Although these data argue against a significant role for naphthoquinones in naphthalene-induced Clara cell necrosis, they do not exclude it as a possibility. Naphthoquinones appear to be involved in naphthalene-induced cataract formation (48, 49), and the quinones and their close structural analogs are potent cytotoxic agents in isolated hepatocytes (20, 50) [see review by Monks *et al.* (51)]. 1,4- and 1,2-Naphthoquinones react rapidly with both sulfhydryl and amine groups (50, 52), and it is possible that the relatively low cytotoxic potency of the naphthoquinones, compared with naphthalene oxide, is due to inactivation of naphthoquinones in the incubation medium. We are currently examining the nature of the protein-bound reactive metabolites and have evidence for both quinone and epoxide binding (53). These studies should provide important information regarding the significance of quinones and epoxides in the formation of covalent adducts with protein and the roles of these metabolites in cytotoxicity.

The reason for the lack of cytotoxicity when naphthalene oxide was added as a bolus to isolated Clara cells is not clear from our current data. We speculate that slow loading of the cells with naphthalene oxide resulted in less rearrangement of the epoxide to 1-naphthol and a more dramatic depletion of cellular glutathione, in comparison with the experiments where epoxide was added as a bolus to the cells. In future studies, differences in cytotoxicity and depletion of intracellular glutathione will be examined in detail after addition of both the epoxide and quinones to target cells.

A number of factors may contribute to cell-selective toxicity. This paper clarifies how a model Clara cell toxicant, naphthalene, can be metabolized by its target cell population. These data show that 1) the cytochrome P450-mediatated metabolism

[2] M. Cho, personal communication.

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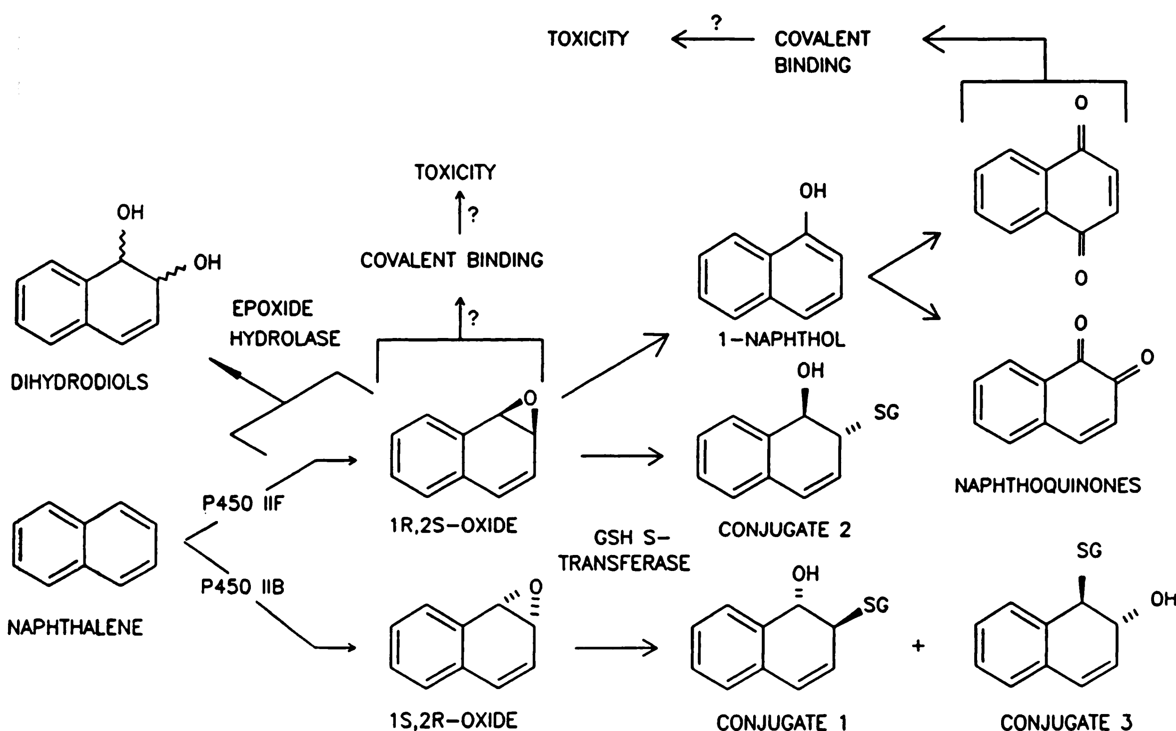

Fig. 7. Summary of the potential pathways of naphthalene metabolism. The rate of formation and the ratio of naphthalene oxides (1*R*,2*S*-oxide and 1*S*,2*R*-oxide) generated can be determined by adding excess glutathione and glutathione (*GSH*) *S*-transferases and capturing the enantiomers as conjugates 1, 2, and 3. In the absence of glutathione transferases and glutathione, the potential of murine Clara cells to deactivate naphthalene oxide can be evaluated.

of naphthalene by Clara cells is stereoselective for the formation of 1*R*,2*S*-oxide, 2) there appears to be a slight enantioselectivity of the glutathione *S*-transferases for the conjugation of naphthalene oxide, and 3) naphthalene oxide is the most toxic metabolite. We are currently examining the activity and enantioselectivity of epoxide hydrolase, the specificity of the covalent interaction of reactive naphthalene metabolites with cellular proteins, and the turnover of intracellular glutathione pools, in an effort to further define the biochemical/metabolic processes critical to naphthalene-induced Clara cell necrosis.

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