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GLUTATHIONE DEPLETION BY NAPHTHALENE IN ISOLATED HEPATOCYTES AND BY NAPHTHALENE OXIDE *IN VIVO**,†

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Abstract—Previous studies have shown that naphthalene oxide and reactive naphthalene metabolites diffuse from intact, isolated hepatocytes. The amount of naphthalene oxide diffusing from the cells as a percentage of the total formed remained constant over a wide range of substrate concentrations, thus suggesting that depletion of glutathione might not be required prior to significant naphthalene oxide efflux. However, the relative intracellular versus extracellular covalent binding of reactive metabolites increased with increasing naphthalene concentrations, thereby suggesting that glutathione might be involved in modulating the extent of intracellular covalent binding. To examine this question in detail, intracellular glutathione levels were monitored in mouse hepatocytes incubated in the presence of various concentrations of naphthalene. Naphthalene produced a concentration- and time-dependent decrease in intracellular glutathione levels and, at higher concentrations, a marked decrease in the rate of glutathione efflux from hepatocytes. This decrease in hepatocellular glutathione levels correlated well with the shift in binding from predominantly extracellular to intracellular. Inclusion of glutathione and glutathione transferases in the cell incubation medium partially blocked the depletion of intracellular glutathione by naphthalene, thus suggesting that naphthalene oxide diffusing into the cell medium was partially responsible for intracellular glutathione depletion. Finally, *in vivo* administration of naphthalene oxide to mice produced a dose-dependent depletion of pulmonary but not hepatic or renal glutathione but only at doses that were greater than 75 mg/kg. These studies support the view that there is not a glutathione threshold for the efflux of naphthalene oxide from intact hepatocytes and suggest that naphthalene oxide is capable of diffusing into as well as out of isolated hepatocytes.

The tripeptide, glutathione, has been shown to play a central role in the metabolic detoxication of a variety of electrophilic carcinogenic and cytotoxic agents [1, 2]. Early studies by Mitchell, Jollow and others [3, 4] demonstrated a glutathione threshold for the covalent binding of reactive metabolites and for the centrilobular hepatic necrosis resulting from acetaminophen and bromobenzene. Similar results have been obtained in studies with naphthalene. Hepatic and pulmonary non-protein sulfhydryl levels are decreased in a dose-dependent manner at doses above 50 mg/kg [5]. In comparison, renal tissue is relatively resistant to naphthalene-induced depletion of non-protein sulfhydryls; doses of naphthalene above 300 mg/kg are required to produce significant decreases in renal non-protein sulfhydryls. Above this 300 mg/kg dose there is a marked threshold; covalent binding levels increase from approximately 0.8 nmol/mg protein at 300 mg/kg to 1.8 nmol/mg at 400 mg/kg, while non-protein sulfhydryl levels fall from 89% of control to 30% of control [5]. These data on the *in vivo* covalent binding and glutathione depletion, in conjunction with the finding that detectable rates of naphthalene metabolism by either NADPH or arachidonic acid dependent pathways are not observed in renal microsomal incubations [6, 7], suggested that the depletion of renal non-protein sulfhydryl levels was due primarily to metabolites circulating from other tissues such as liver and lung [8].

Based on the dose threshold observed in the depletion of non-protein sulfhydryls from the kidneys, we hypothesized that the release of naphthalene oxide and other reactive naphthalene metabolites from hepatocytes also would show a dose threshold. However, studies in isolated hepatocyte preparations indicated that, when expressed as a percentage of the total formed, naphthalene oxide release did not change substantially in hepatocytes incubated with naphthalene over a concentration range of 0.015 to 1.5 mM [9]. In contrast, when covalent binding was used to measure the formation and fate of reactive metabolites, there was a shift from predominantly extracellular to intracellular

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covalent binding with increasing substrate concentrations. Accordingly, the studies reported here were to examine the time course and effect of concentration of naphthalene on hepatocyte glutathione levels and determine whether the times and substrate concentrations used in earlier studies resulted in significant glutathione depletion. In addition, because the precise role of circulating reactive metabolites in naphthalene-induced bronchiolar injury has not been defined, we have measured intracellular non-protein sulfhydryl depletion in animals treated with naphthalene oxide intravenously as a means of evaluating the ability of circulating epoxide to enter tissues. Relatively high levels of naphthalene 1,2-oxide administered as an intravenous bolus dose were required to cause significant non-protein sulfhydryl depletion.

MATERIALS AND METHODS

Animals. Male Swiss Webster mice (20–25 g) were purchased from Charles River Breeding Laboratories, Wilmington, MA. Mice were given food and water *ad lib.* and were not used sooner than 5 days after receipt from the supplier.

Chemicals. Chemicals for the synthesis of (±)-naphthalene 1,2-oxide were purchased from the Aldrich Chemical Co., Milwaukee, WI. Components of the NADPH-generating system (NADP, glucose-6-phosphate dehydrogenase and glucose-6-phosphate) and glutathione reductase (Type III, yeast, Cat. No. G-4751) were purchased from the Sigma Chemical Co., St. Louis, MO. Collagenase (from *Clostridium histolyticum*) was obtained from ICN, Cleveland, OH. Fischer's medium was from Irvine Scientific, Irvine, CA. All other reagents were obtained from commercial suppliers and were reagent grade or better.

Preparation of isolated hepatocytes from mouse liver. These procedures have been described in detail in a previous publication [9]. Briefly, hepatocytes were prepared by retrograde perfusion of the mouse liver with Hanks' buffer containing EGTA* (0.5 mM) followed by a 12-min perfusion with collagenase (10 units/ml). After the perfusion, the liver was removed and submerged in additional quantities of Hanks' buffer, and the liver cells were dispersed gently with a pair of forceps. The cells were allowed to settle for 5 min on ice, the supernatant fraction was discarded, and the sedimented cells were resuspended in Fischer's medium containing 10% heat-inactivated fetal calf serum, 15 mM HEPES, and gentamicin (10 μg/ml) at pH 7.4. Viable hepatocytes were separated from nonviable cells by centrifugation on a Percoll density gradient [10]. Viable cells were washed and resuspended in Fischer's medium. Initial cellular viabilities, as assessed by trypan blue dye exclusion, were > 96%.

Hepatocyte incubations. All incubations were prepared on ice in screw-capped, silanized Erlenmeyer flasks. Hepatocytes were incubated at 10^6 cells/ml in Fischer's medium containing 10% heat-inactivated fetal calf serum, 15 mM HEPES buffer, and other reagents as described in the legends to the figures. Naphthalene was added at the concentrations specified in the figure legends in a volume of 4 μl methanol/ml incubation mixture. The flasks were gassed with 95% O_2/5% CO_2 and were sealed with a teflon-lined screw cap fitted with a three-way stopcock and narrow bore teflon tubing to permit sampling. The flasks were transferred to a shaking incubator at 37° (60 oscillations/min).

Synthesis of naphthalene 1,2-oxide. Racemic naphthalene 1,2-oxide was prepared according to the methods reported by Yagi and Jerina [11]. The final product was recrystallized from ether/petroleum ether and was stored at −80° in ethanol/0.1% triethylamine. The material yielded a single spot by thin-layer chromatography [the origin was prespotted with triethylamine and compounds were chromatographed on silica gel GF plates eluted with ethyl acetate/benzene/chloroform/triethyl amine (1:1:1:0.05)] [12]. In addition, the UV (maxima at 268 and 304 nm) and NMR spectra ($CDCl_3$) were consistent with those previously published by Vogel and Klarner [13]. Addition of acetic acid and methanol to the epoxide resulted in the formation of 1-naphthol.

Measurement of tissue non-protein sulfhydryl levels in naphthalene oxide treated mice. Naphthalene 1,2-oxide, synthesized as described above, was dissolved in absolute ethanol/0.1% triethylamine such that 1 μl was administered per gram body weight. The total infusion time was approximately 30 sec. Controls received vehicle only. The purity of the administered product was assessed by thin-layer chromatography immediately before and after administration to the animals, and detectable decomposition to 1-naphthol was not observed. Non-protein sulfhydryl levels were measured in mouse tissues 90 min after the intravenous administration of naphthalene oxide. Mice were killed by decapitation, lungs were thoroughly perfused with isotonic heparinized saline, and tissues were frozen at −80°. Absorbance was measured at 412 nm after coupling the non-protein sulfhydryls with dithiobisnitrobenzoic acid according to Ellman [14].

Measurement of thiol status in isolated hepatocytes. Glutathione, glutathione disulfide, cysteine and cysteine-glutathione mixed disulfide were measured separately in cells and cell medium by the HPLC technique of Reed *et al.* [15, 16]. Briefly, at the termination of the incubations, an aliquot of the incubation mixture was pipetted into a microcentrifuge tube containing 0.35 ml dibutyl phthalate (middle layer) and 0.4 ml of 10% perchloric acid with 2 mM EDTA (bottom layer). Centrifugation at 12,000 *g* for 1 min results in the transfer of cells into the perchloric acid layer, leaving the cell medium in the top layer. Aliquots of these two layers were removed and 5 nmol internal standard (δ-glutamylglutamic acid) was added. Perchloric acid was added to the cell medium to bring the total concentration to 10%. Samples were derivatized by the addition of iodoacetic acid and 2,4-dinitrofluorobenzene. Standards were prepared with glutathione, glutathione disulfide, cysteine and cysteine-glutathione mixed disulfide (a gift of Drs. John Livesey and Donald

* Abbreviations: EGTA, ethyleneglycolbis (aminoethylether)tetra-acetate; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; and CDNB, 1-chloro-2,4-dinitrobenzene.

Reed, Department of Biochemistry, Oregon State University, Corvalis, OR). Quantitation of the thiols in the sample was based on peak heights of the derivatized thiols eluted from a 25 cm, 5 μm amino-propyl column purchased from Custom LC, Houston, TX. Column eluate was monitored at 365 nm with a Waters Associates M440 fixed wavelength detector. Derivatized thiols were eluted from the column using the following solvent program: 75% solvent A/25% solvent B for 5 min followed by linear programming to 1% solvent A/99% solvent B over 20 min. Solvent A was 80% methanol/20% distilled water; solvent B was prepared by mixing 272 g sodium acetate trihydrate, 122 ml distilled water and 378 ml glacial acetic acid and diluting 200 ml of this mixture with 800 ml of solvent A. The derivatized thiols eluted with the following retention times: cysteine-glutathione mixed disulfide, 12 min; internal standard (δ-glutamylglutamic acid), 22 min; glutathione, 25 min; and glutathione disulfide, 28 min. All of the peaks were completely separated from each other and from interfering derivatized amino acids. Standard curves were linear over the range of sample values.

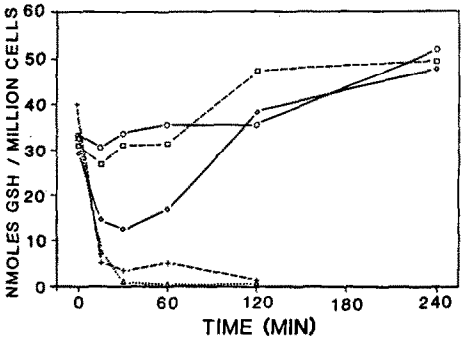

Fig. 2. Intracellular glutathione levels in hepatocytes incubated with menadione or various concentrations of naphthalene. Data are for methanol control (--□--), 0.005 mM naphthalene (—○—), 0.05 mM naphthalene (—◇—), 0.5 mM naphthalene (---△---), or 0.5 mM menadione (--+--). Values are the means of three separate hepatocyte incubations. Standard errors of the mean were less than 15% of the mean with the exception of the data for the 15-min control, 120-min 0.005 mM naphthalene, 15- and 30-min 0.05 mM naphthalene and 15-min 0.5 mM naphthalene which were less than 30% of the mean.

RESULTS

Glutathione depletion in isolated hepatocytes incubated with naphthalene: Effect of time and concentration. To determine whether the concentrations of naphthalene used in earlier studies [9] were sufficient to cause a marked decrease in intracellular glutathione levels, hepatocytes were incubated in the presence of naphthalene at concentrations of 0.005, 0.05 and 0.5 mM. Menadione (2-methyl-1,4-naphthoquinone, 0.5 mM) was included as a positive control, since Di Monte and his colleagues [17] have shown substantial depletion of glutathione by this compound in isolated hepatocyte incubations. The initial viability of all hepatocyte preparations was > 96% and remained > 90% during a 2-hr incubation with solvent, 0.005 or 0.05 mM naphthalene (Fig. 1). At 4 hr the percentage of cells excluding trypan blue in these three groups remained above 85%. In contrast, hepatocyte viability in incubations with 0.5 mM naphthalene decreased to 75% at 2 hr and was 10% at 4 hr. Likewise, cell viability was lost rapidly in incubations with 0.5 mM menadione (< 20% at 2 hr).

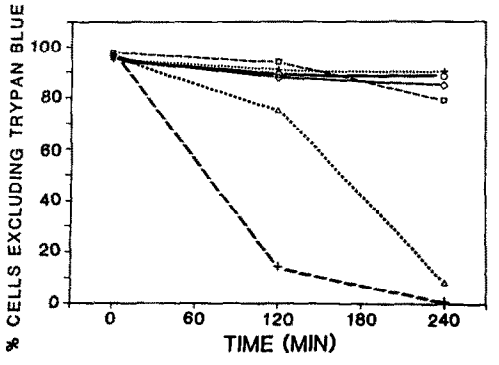

Fig. 1. Viabilities of isolated hepatocytes (as assessed by trypan blue dye exclusion) incubated under the following conditions: no additions, control (--□--), methanol (vehicle ---+---), 0.005 mM naphthalene (—○—), 0.05 mM naphthalene (—◇—), 0.5 mM naphthalene (---△---), and 0.5 mM menadione (--+--). Values are the means from three incubations.

Glutathione levels in hepatocytes incubated with vehicle (methanol) remained relatively constant over the first hour of incubation and thereafter gradually increased to approximately 150% of the starting levels at 4 hr (Fig. 2). Incubation with 0.005 mM naphthalene had little effect on glutathione levels in the hepatocytes, whereas 0.05 mM naphthalene produced a transient decrease to less than 50% of the levels in solvent control. Glutathione levels in hepatocytes incubated with 0.05 mM naphthalene were indistinguishable from solvent control at both the 2- and the 4-hr time point. Within 15 min of the start of the incubation with either naphthalene or menadione at a concentration of 0.5 mM, glutathione levels decreased to less than 20% of the levels in hepatocytes incubated with vehicle only. Glutathione levels remained very low over the first hour of incubation.

Effects of naphthalene and menadione on the efflux of glutathione from hepatocytes. To determine whether substantial depletion of intracellular glutathione resulted in a decreased rate of efflux of glutathione from hepatocytes, hepatocytes were incubated in Fischer's medium containing 0.2 mM cystine, and glutathione efflux was measured by monitoring the levels of cysteine-glutathione disulfide in the incubation medium [16]. The data in Fig. 3 indicate that glutathione efflux occurred at a rate of approximately 10 nmol/hr/million hepatocytes in incubations containing vehicle alone. Addition of 0.005 mM naphthalene to the incubations did not affect the rate of glutathione efflux, whereas incubation with 0.05 mM produced a transient decrease

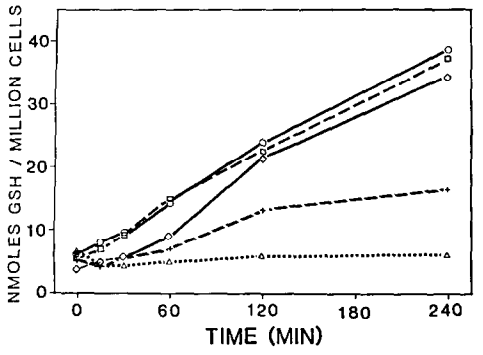

Fig. 3. Glutathione efflux from isolated mouse hepatocytes incubated in the presence of solvent (control --□--), menadione (--+--), or various concentrations of naphthalene (—○— 0.005 mM; —◇—) 0.05 mM; and --△-- 0.5 mM). All incubations were done in the presence of cystine (0.2 mM) to measure glutathione efflux. Values are the means of three incubations. Standard errors did not exceed 15% of their respective means.

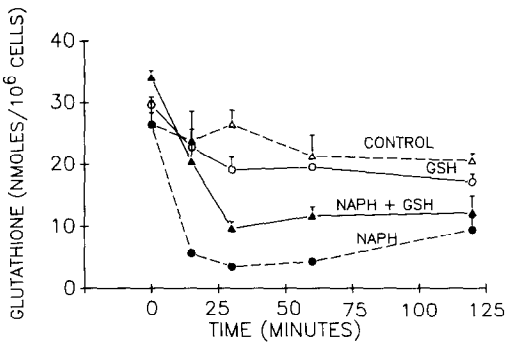

Fig. 4. Intracellular glutathione levels in hepatocytes incubated in the presence of naphthalene (0.15 mM) with or without glutathione. Glutathione transferases (5 CDNB units/ml) and glutathione reductase (0.5 units/ml) were added to all incubations containing glutathione. Values are the means ± SE for three separate incubations.

in the rate of efflux followed by a rebound. At 2 hr the total amount of glutathione trapped as disulfide with cystine was identical in hepatocytes incubated with vehicle or 0.05 mM naphthalene. Glutathione efflux was inhibited almost totally during the first hour of incubation with either 0.5 mM naphthalene or 0.5 mM menadione, a result which is consistent with the substantial decreases in intracellular glutathione observed.

Intracellular glutathione levels in hepatocytes incubated in the presence of glutathione and glutathione transferases. Earlier studies indicated that approximately a third of the total naphthalene oxide generated intracellularly could be trapped with extracellular labeled glutathione [9]. Thus, it seemed possible that glutathione depletion in a particular cell was due, in part, to reactive metabolites generated within that cell and, in part, to naphthalene oxide generated by other cells in the incubation. To determine whether the extent of intracellular glutathione depletion could be modulated by trapping naphthalene oxide diffusing from one cell to another, intracellular glutathione levels were compared in hepatocytes incubated with naphthalene (0.15 mM) in the presence or absence of glutathione and glutathione transferases. Hepatocytes incubated with vehicle only or with vehicle plus glutathione and glutathione transferases were utilized as controls. Hepatocyte viabilities in incubations containing naphthalene were slightly lower than in the control groups but remained above 75% for the 2-hr incubations. No differences in hepatocyte viabilities were noted in the naphthalene incubations done in the presence or absence of glutathione. The data in Fig. 4 show rapid depletion of intracellular glutathione levels in hepatocyte incubations containing naphthalene but no glutathione. Intracellular glutathione levels in these incubations remained less than 50% of the corresponding controls throughout the 2-hr incubation. In comparison, although significant depletion of intracellular glutathione occurred in hepatocytes incubated with naphthalene plus glutathione, glutathione levels in these cells remained consistently above those in cells incubated with naphthalene alone. These data are consistent with the concept that the presence of glutathione in the cell medium moderates the depletion of intracellular glutathione by trapping a portion of the naphthalene oxide effluxing from the cells and preventing the rediffusion and depletion of intracellular thiol levels. Inclusion of glutathione in the incubation medium did not alter the intracellular glutathione levels in comparison to control incubations containing vehicle alone. This compares favorably with the data in the isolated perfused liver and in isolated hepatocytes showing that glutathione is not taken up by intact liver cells [18, 19]. Thus, under the conditions used in these and previous studies [9], glutathione does not appear to be slowly leaking into the intracellular space.

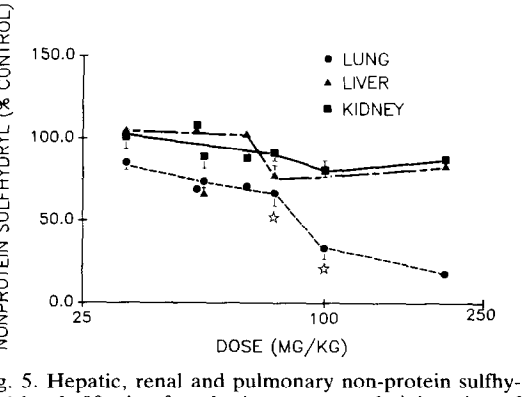

Fig. 5. Hepatic, renal and pulmonary non-protein sulfhydryl levels 90 min after the intravenous administration of (±)-naphthalene oxide. The data are from two separate experiments; the first was done using doses of 32, 48 and 64 mg/kg, while the second study used doses of 50, 75, 100 and 200 mg/kg. Control values were obtained from animals treated with vehicle only and were 401 ± 100 μg/g in lung, 2640 ± 150 μg/g in liver and 1400 ± 100 μg/g in kidney. Data are the mean or the mean ± SE for one to five animals per group. Stars indicate significant differences from control values ($P < 0.05$, two-tailed t-test).

Effect of intravenous administration of naphthalene 1,2-oxide on pulmonary, hepatic and renal non-protein sulfhydryl levels. Although previous studies have shown that as much as 35% of the total naphthalene oxide formed intracellularly can diffuse to the extracellular space in isolated hepatocyte incubations [9], there was no information concerning the relevance of this diffusion to the *in vivo* toxicity of naphthalene. If naphthalene oxide, released into the bloodstream by the liver, is involved in modulating the toxicity of naphthalene in the lung, it must be capable of reaching pulmonary cells. As a measure of this, tissue reduced sulfhydryl levels were assayed 90 min after the intravenous administration of naphthalene oxide. Intravenous administration of naphthalene oxide had no significant effect on hepatic or renal non-protein sulfhydryl levels and resulted in depletion of pulmonary non-protein sulfhydryl only at the three highest doses tested (75, 100 and 200 mg/kg) (Fig. 5). The decrease in pulmonary non-protein sulfhydryl levels was dose dependent; at 32 mg/kg the levels were 80% of control, while at 200 mg/kg lung non-protein sulfhydryl levels were less than 20% of the vehicle-treated controls.

DISCUSSION

Studies indicating that the pulmonary Clara cell is the principal target for naphthalene-induced injury [20, 21] are consistent with the apparent high degree of localization of cytochrome P-450 monooxygenases in this cell type [22–24] and the observed involvement of the P-450 system in the bronchiolar toxicity and covalent binding of reactive naphthalene metabolites in the lung [5]. Although a close interrelationship has been observed between the formation and fate of reactive metabolites in the lung with the extent and severity of naphthalene-induced bronchiolar injury, covalent binding of reactive metabolites in nontarget tissues is higher than in the lung. These data, in conjunction with the finding that reactive metabolites of naphthalene are semi-stable and appear to be capable of circulating, support the view that covalent binding/glutathione depletion measurements in a particular tissue are probably not good indicators of reactive metabolite formation *in situ* [8]. Marked differences in the rate and stereoselectivity of naphthalene epoxidation have been observed in microsomal preparations from target and nontarget tissues of the mouse and from lungs of sensitive versus nonsensitive species [25]. These data suggest that the tissue/cell selective cytotoxicity of naphthalene may be dependent upon the nature of reactive metabolites formed rather than the total levels of bound metabolite in a particular tissue. However, even if the generation of reactive metabolites *in situ* is critical to the bronchiolar necrosis in mice, circulating reactive metabolites may be important in altering the ability of pulmonary cells to effectively detoxify metabolites formed *in situ*. This view is consistent with the findings reported here since the efflux of naphthalene oxide, the major glutathione-depleting metabolite of naphthalene, was not dependendent upon substantial depletion of glutathione from the liver cell. Thus, even at low doses of naphthalene where hepatic glutathione levels would remain relatively high, the liver may be capable of contributing to glutathione depletion in the lung. The observation that substantial glutathione depletion did not appear to be requisite for the diffusion of naphthalene oxide suggests that, once formed on the endoplasmic reticulum, the epoxide is protected from both the cytosolic and microsomal detoxication enzymes. The apparent lack of a glutathione threshold for the efflux of naphthalene oxide is consistent with the data generated by Stowers and Anderson [26] on DNA adducts of benzo[*a*]pyrene diol epoxide. These workers demonstrated a poor correlation between the ability of tissues to catalyze the formation of the diol epoxide with the levels of DNA bound adduct *in vivo*, thus suggesting that adduct in tissues with little or no detectable P-450/prostaglandin synthetase may be derived from circulating diol epoxide formed elsewhere. In their studies, doses of benzo[*a*]pyrene were below those required to compromise the hepatic glutathione system.

Glutathione levels in freshly isolated control mouse hepatocytes were similar to those reported in rat hepatocytes [27]. In the 1- to 4-hr time period after isolation, intracellular glutathione levels rose moderately and this may reflect recovery of cellular glutathione lost during isolation. Moderate increases in intracellular glutathione levels also have been noted in rat hepatocytes [27]. Glutathione efflux in control mouse hepatocytes occurred at a rate of approximately 7.5 nmol/10^6 cells/hr and this is 2- to 3-fold the rate reported by Fariss and Reed [28] for rat hepatocytes (Fig. 3). The apparent sensitivity of glutathione efflux to depletion of intracellular glutathione levels (Fig. 2) is similar to that noted in the perfused liver (see review by Kaplowitz *et al.* [29]). Dawson *et al.* [30] and Hagen *et al.* [31] have reported that glutathione is taken up by lung when studied with either the isolated perfused lung system or isolated lung cells. Thus, it is possible that regulation of circulating glutathione levels in the blood may be important in determining the steady-state levels of glutathione in the lung. As a consequence, depletion of hepatic glutathione by compounds like naphthalene may influence the susceptibility of the lung to toxic insult from chemicals requiring glutathione for detoxication.

The data in Fig. 4 showing that addition of glutathione and glutathione transferases to the incubation medium partially blocked naphthalene-induced depletion of intracellular glutathione suggest that naphthalene oxide can diffuse across membranes in either direction. In comparison, very high doses of intravenously administered naphthalene oxide were required to produce non-protein sulfhydryl depletion in the lung *in vivo* (Fig. 5). There are several possible explanations for the apparent permeability of the hepatocyte to naphthalene oxide versus the relative insensitivity of the lung to glutathione depletion by intravenously administered epoxide. The diffusional barriers in the isolated hepatocyte system are substantially different from those encountered by naphthalene oxide infused intravenously, and this factor alone could account for the differences in the quantities of epoxide required to produce significant depletion of intracellular glutathione. Moreover, the stability of the epoxide is likely to be far greater in

hepatocyte medium than in blood. The reported half-life of naphthalene oxide in pH 7.4 phosphate buffer is 2.8 min [32]; the half-life in blood is likely to be far less due to the presence of gluthatione and the glutathione transferases [1, 33]. Thus, unless there is substantial clearance of the epoxide in a single pass through the lung, only a small portion of the injected metabolite is likely to be available for diffusion to pulmonary cells. Furthermore, in the experimental protocol outlined in these studies, hepatocytes are externally exposed to naphthalene oxide at a relatively slow rate. In comparison, the residence time of an intravenous bolus dose in the lungs is likely to be very short, and the apparent differences between the ability of the epoxide to penetrate the hepatocyte membrane versus membranes of the lung may simply reflect differences in exposure time. Current studies in which hepatocytes and the isolated perfused mouse lung are being exposed to naphthalene oxide by slow infusion should help clarify these points.

The results presented here and in a previous paper [9] show that the efflux of naphthalene oxide was not dependent upon the substantial depletion of intracellular glutathione. Conversely, the relative amounts of reactive metabolite bound intra- versus extracellularly appear to be affected markedly by the depletion of glutathione. Additional studies will be needed to attempt to determine the mechanism by which reactive metabolites, including naphthalene oxide, diffuse from hepatocytes and to determine to what extent such metabolites are present in the circulation *in vivo*. Knowledge of the exact nature of the product secreted from the liver cell *in vitro* and *in vivo* will be necessary before the significance of reactive metabolite circulation can be assessed completely.

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