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PRELIMINARY COMMUNICATIONS

FORMATION OF REACTIVE NAPHTHALENE METABOLITES BY TARGET VS NON-TARGET TISSUE MICROSOMES: METHODS FOR THE SEPARATION OF THREE GLUTATHIONE ADDUCTS*

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Intraperitoneal administration of naphthalene to mice results in dose-dependent necrosis of pulmonary bronchiolar epithelial cells but not in hepatic or renal damage [1-3]. Several factors have supported a relationship between the cytochrome P-450-mediated formation of reactive naphthalene metabolites and the bronchiolar damage. The primary target cell for naphthalene-induced cytotoxicity is the Clara cell, a pulmonary cell type with high cytochrome P-450 monooxygenase activity [4,5]. In addition, reactive metabolites from naphthalene become bound covalently to tissue macromolecules in the lung in a dose- and time-dependent manner [2]. Covalent binding and the severity of pulmonary bronchiolar necrosis are altered in a parallel fashion by pretreatment with the cytochrome P-450 monooxygenase inhibitor, piperonyl butoxide, or by pretreatment with the glutathione (GSH) depletor, diethyl maleate. However, the overall levels of reactive metabolite binding in liver, a non-target tissue, are higher than in lung at all doses and times studied. Moreover, pretreatment with inducers or inhibitors of the cytochrome P-450 monooxygenases or with agents that alter tissue GSH levels causes parallel alterations in pulmonary and hepatic covalent binding of reactive naphthalene metabolites. Thus, the observed target organ specificity for naphthalene-induced cytotoxicity is not reflected in the preferential covalent binding of reactive metabolites in the target tissue in vivo, nor is it reflected in substantially higher rates of covalent binding in microsomes from target vs non-target tissue [6]. One possible explanation for the lack of tissue specificity in covalent binding which is still consistent with a role of reactive metabolite formation in the bronchiolar necrosis is that the natures of the reactive metabolites produced by lung and liver differ. Since previous studies have shown that, in vivo, GSH depletion and the covalent binding of reactive naphthalene metabolites are interrelated events [2] and that GSH conjugates are formed from naphthalene [7,8], it seemed possible that the chemical nature of reactive metabolites formed microsomally from this aromatic hydrocarbon could be examined by trapping the electrophilic metabolites with GSH and analyzing the resulting adducts by high performance liquid chromatography (HPLC). This approach has been used successfully in studying reactive, potentially toxic or carcinogenic metabolites of a variety of chemicals [9]. Thus, the experiments reported here were done to establish an HPLC method for separating and quantifying the GSH conjugates of naphthalene and to explore the possibility that microsomes prepared from target tissue formed a particular reactive metabolite(s) at a different rate from non-target tissue.

Materials and Methods

Animals. Male Swiss-Webster mice (20-25 g) were purchased from the Charles River Breeding Laboratories, Wilmington, MA. Animals were housed on hardwood bedding, were allowed food and water ad lib. and were not used sooner than 5 days after receipt from the supplier. For induction of hepatic monooxygenases, phenobarbital was administered as a 0.1% solution in the drinking water for 5 days. Fresh water was supplied 24 hr before sacrifice.

Radiochemicals. 1-[^{14}C]Naphthalene (5 mCi/mmole) was purchased from Amersham Searle, Arlington Heights, IL, and shown to be >99.5% radiochemically pure by HPLC on a C_{18} column. L-Glutathione (glycine-2-[^{3}H]) (reduced form; 240 mCi/mmole) was purchased from the New England Nuclear Corp., Boston, MA, and was used without further purification.

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Chemicals. Components of the NADPH-generating system (NADP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase) were purchased from the Sigma Chemical Co., St. Louis, MO; GSH (reduced form) was from Calbiochem, La Jolla, CA. HPLC solvents were from Baker, Phillipsburg, NJ. All other chemicals were reagent grade or better.

Preparation of microsomal and cytosolic enzymes. Lung, liver and kidney microsomes were prepared by ultracentrifugation according to published procedures [10]. The 100,000 *g* supernatant fraction (containing the GSH transferases) from the first high speed centrifugation was chromatographed on a Sephadex G-25 column to remove endogenous GSH. Microsomal and cytosolic protein concentrations were determined by the method of Lowry et al. [11].

Incubations. Incubations were prepared on ice in a total volume of 2 ml, and contained microsomes (2-6 mg as specified), naphthalene (1.0 mM, added in 10 µl methanol), GSH (concentrations as specified), an NADPH-generating system (cofactor) [10], and cytosolic enzymes (1-2 mg). The incubation vessels were capped and transferred to a shaking incubator at 37° for the specified time. The vessels were again transferred to an ice bath, 4 ml of ice-cold methanol was added to stop the reaction, and the contents were transferred to centrifuge tubes.

Covalent binding. The covalent binding of radiolabel to precipitated microsomal protein was assayed as described previously [2].

Extraction and HPLC analysis. After removing the protein by centrifugation, the methanol/water supernatant fraction was extracted twice with 3 ml portions of trimethylpentane to remove unmetabolized naphthalene. An aliquot (1-4 ml) of the methanol/water phase was evaporated to dryness under vacuum and was reconstituted in mobile phase (200-500 µl) for analysis. HPLC was done on a Waters ALC 201 liquid chromatograph with a Beckman 5 µm ODS column (0.46 x 25 cm) eluted with 7-8% acetonitrile/1% glacial acetic acid/91-92% water at 1 ml/min. Radioactivity was collected into scintillation vials, 5 ml ACS was added, and the samples were counted for 20 min each in a Beckman 3150T liquid scintillation counter.

Results

Addition of GSH to incubations of phenobarbital-induced hepatic microsomes, [^{14}C]naphthalene, cytosolic enzymes and an NADPH-generating system resulted in a marked decrease in the covalent binding of reactive naphthalene metabolites compared to incubations done in the absence of GSH (Table 1). Addition of GSH to complete incubations also resulted in the formation of three U.V. absorbing and radioactive peaks (eluting at 47, 53 and 58 min, Fig. 1) which were not present in identical incubations without GSH (Fig. 1) or NADPH (data not shown). These same U.V. and radioactive peaks were present in extracts prepared from microsomal incubations containing naphthalene, cytosolic enzymes, NADPH-generating system and [^{3}H]GSH but were not present in incubations without cofactor (Fig. 1) or without naphthalene (data not shown). Formation of the compound eluting at 41 min was dependent upon the presence of NADPH in the incubation. In addition, this peak was not radioactive in extracts of complete incubations using [^{3}H]GSH, thus indicating that this compound is a non-GSH derived metabolite of naphthalene.

Table 1. Effect of addition of reduced glutathione on the covalent binding of reactive naphthalene metabolites to hepatic microsomal protein*

Incubation Conditions		Covalent Binding†
Cofactor	Glutathione	
+	-	21.1 ± 0.8
+	+	7.6 ± 1.6
-	-	0.1 ± 0.0

*Incubations were for 20 min at 37° and contained [^{14}C]naphthalene (1 mM, 832 dpm/nmole), phenobarbital-induced microsomes (6 mg), cytosolic enzymes (2 mg), and GSH (5 mM) or cofactor as specified.

†Values are the means ± S.E. of three incubations and are expressed as nmoles bound/mg protein/20 min.

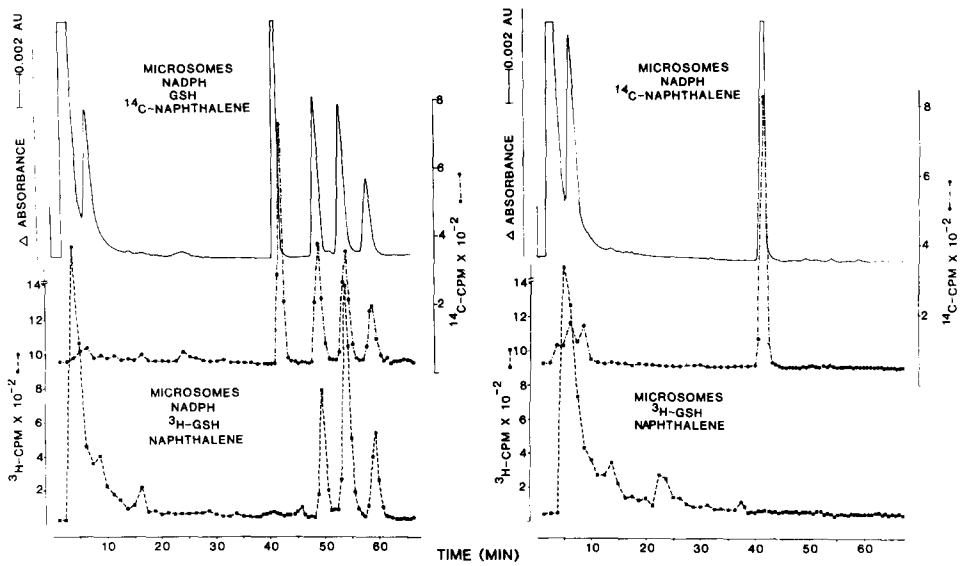

Fig. 1. Ultraviolet (254 nm) and radioactive profiles of extracts from 20-min incubations of phenobarbital-induced liver microsomes (6 mg) with naphthalene and/or GSH done under the following conditions: [^{14}C]naphthalene (832 dpm/nmole, 1 mM), cytosolic enzymes (2 mg) and cofactor done in the presence or absence of 5 mM GSH, or [^{3}H]GSH (1950 dpm/nmole, 0.1 mM), cytosolic enzymes and naphthalene (1 mM) done in the presence or absence of cofactor. Column eluant was collected at 1-min intervals for the first 15 min and at 30-sec intervals thereafter for scintillation counting.

To explore the possibility that reactive metabolites from naphthalene formed by target vs non-target tissue microsomes were chemically different or whether the relative rates of formation of the individual reactive metabolites differed, [^{14}C]naphthalene was incubated with GSH, cofactor and cytosolic enzymes in the presence of lung, liver, or kidney microsomes. Radioactive elution profiles of the aqueous extracts are shown in Fig. 2. Although the overall rates of formation of irreversibly bound metabolites by lung and liver microsomes were similar [6], both the total amount of GSH adduct and the relative proportions of the three adducts produced by pulmonary vs hepatic microsomes differed substantially. Lung microsomes produced the GSH adduct eluting at 47 min at rates which were nearly 10 fold higher than the rates of formation of the adducts eluting at 42 or 51 min. In contrast, similiar amounts of each of the naphthalene-GSH adducts were formed in liver microsomal incubations.

Discussion

Although previous NMR analysis of GSH adducts formed from naphthalene assigned the thioether bond at the C-2 position of the aromatic ring, this assignment was not entirely unambiguous [7]. The difficulty in determining the exact chemical nature of the GSH adduct may have been due, in part, to the presence of more than one conjugate in the final sample analyzed by NMR. As shown in the present study, at least three GSH adducts arise during the hepatic microsomal metabolism of naphthalene in the presence of GSH and cytosolic GSH transferases. The finding that multiple GSH adducts are formed under these conditions is consistent with previous studies showing that nucleophilic attack by GSH can occur on both electrophilic carbons of other epoxides such as styrene oxide [12] and benzo[a]pyrene-4,5-oxide [13] and with studies demonstrating that naphthalene is metabolized to not only the 1,2-oxide but to diepoxide and diol epoxide metabolites [14,15] as well. Such reactive metabolites are likely to conjugate with GSH.

In contrast to studies with the highly tissue-specific pulmonary toxicant and alkylating agent, 4-ipomeanol [16], where the relative rates of formation of the reactive metabolites formed by target (lung) and non-target (liver) tissue microsomes are similar [10], the studies with naphthalene indicate that there are substantial differences in the ratios of GSH adducts produced by pulmonary and hepatic microsomes.

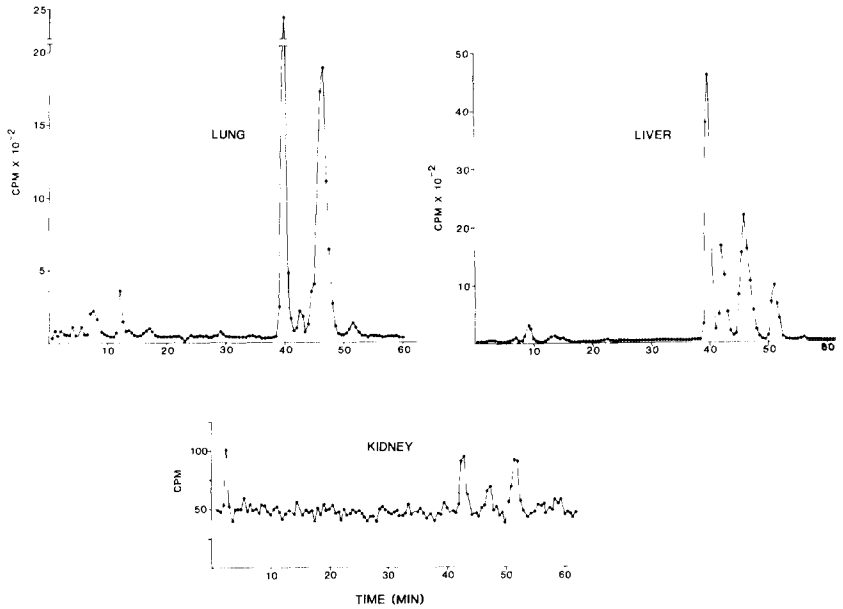

Fig. 2. Radioactive profiles of extracts from uninduced mouse liver, lung or kidney microsomal (2.5 mg) incubations with [^{14}C]naphthalene (1 mM, 1247 dpm/nmole), cofactor, cytosolic enzymes (1 mg) and GSH (5 mM). Incubations were for 15 min at 37°; extracts were prepared in an identical fashion, and radioactivity was collected from the column at 30-sec intervals.

Thus, it appears that the tissue selectivity for damage by 4-ipomeanol depends upon the kinetics of formation of reactive metabolites in target vs non-target tissue while for naphthalene, formation of a particular metabolite may be the primary determinant in the target tissue specificity of the compound. Consistent with earlier studies showing that the formation of covalently bound naphthalene metabolites by renal microsomes occurs at a very slow rate in comparison to liver or lung, very small amounts of conjugate were detected in extracts from kidney microsomal incubations.

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