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PROTECTIVE ROLE OF ENDOGENOUS PULMONARY GLUTATHIONE AND OTHER SULFHYDRYL COMPOUNDS AGAINST LUNG DAMAGE BY ALKYLATING AGENTS

INVESTIGATIONS WITH 4-IPOMEANOL IN THE RAT

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Abstract—Because endogenous glutathione is known to participate in the detoxification of highly reactive, hepatotoxic drug metabolites, we studied the role of this substance in the pulmonary toxicity of 4-ipomeanol [1-(3-furyl)-4-hydroxypentanone] in rats. 4-Ipomeanol was an appropriate model for these studies since previous investigations have indicated that an alkylating metabolite, formed *in situ*, is responsible for selective lung damage by 4-ipomeanol. Toxic doses of 4-ipomeanol preferentially depleted rat lung glutathione. Pretreatment of animals with piperonyl butoxide, an inhibitor of the metabolic activation of 4-ipomeanol, prevented both the depletion of lung glutathione and the pulmonary toxicity of 4-ipomeanol. Prior depletion of lung glutathione by diethylmaleate increased both the pulmonary covalent binding and the toxicity of 4-ipomeanol, whereas administration of cysteine and cysteamine decreased both the covalent binding and the toxicity. These *in vivo* studies, in conjunction with previous *in vitro* studies which showed inhibitory effects of sulfhydryl compounds on the covalent binding of 4-ipomeanol, are consistent with the view that pulmonary glutathione plays a protective role against pulmonary alkylation and lung toxicity by 4-ipomeanol, probably by reacting with the toxic metabolite(s) to form nontoxic conjugate(s). Pulmonary glutathione may similarly provide protection against other electrophilic drugs or metabolites that can damage the lungs.

Chemically reactive, electrophilic chemicals or metabolites may be involved in drug-induced pulmonary injury (see Ref. 1 for review) as they are in hepatotoxicity (see Ref. 2 for review). An important question is whether glutathione (reduced form; GSH) may be involved in the *in vivo* detoxification of such substances in lung and thereby play a protective role against pulmonary damage, analogous to the protective role it plays in the liver [2]. However, there are several difficulties inherent in the design of *in vivo* studies to explore this hypothesis. Important among these are the problems of determining site(s) (e.g. liver vs lung) of formation and/or detoxification of the ultimate toxic products, as well as the possible occurrence of translocations of GSH between the various tissues. However, it can be argued that many of these experimental problems can be avoided through the study of appropriate model compounds. Accordingly, in this paper studies are presented concerning the role of GSH as a modulator of pulmonary alkylation and toxicity in rats by the furan derivative, 4-ipomeanol [1-(3-furyl)-4-hydroxypentanone].

4-Ipomeanol was an especially attractive model agent for these experiments. The compound is a highly selective pulmonary alkylating agent and cytotoxin in several species, including rats [3, 4]. This extraordinary pulmonary specificity results from the metabolic activation of 4-ipomeanol *in situ* in the lung. The highly reactive, toxic product binds immediately to pulmonary macromolecules or, alternatively, as suggested by the present studies, it may be detoxified by binding covalently with low molecular weight substances (e.g. primarily GSH) present in the cytosol to form less reactive, excretable conjugates.

The possibility that GSH might modulate the pulmonary covalent binding and damage by 4-ipomeanol was suggested by previous *in vitro* and *in vivo* studies. The addition of GSH to incubation mixtures containing radiolabeled 4-ipomeanol, NADPH, and rat pulmonary microsomes prevented the covalent binding of 4-ipomeanol metabolites, and resulted in the formation of GSH/4-ipomeanol conjugates [5]. The rates of conjugate formation were not enhanced by pulmonary or hepatic cytosol preparations, indicating that the conjugation reactions did not require catalysis by GSH-transferase enzymes present in the soluble fractions. Preliminary *in vivo* experiments [3] indicated that pretreatment with diethylmaleate, an agent known to deplete tissue GSH [6], caused a marked enhancement of pulmonary alkylation and toxicity by 4-ipomeanol in rats.

Thus, the specific goals of the present *in vivo* studies were: (1) to explore further the possible protective role of GSH against alkylation and damage of the lungs of rats by reactive 4-ipomeanol metabolites, and (2) to test whether exogenously

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administered sulfhydryl reagents could alter the pulmonary covalent binding and toxicity of 4-ipomeanol *in vivo*.

MATERIALS AND METHODS

Animals. Rats (Sprague–Dawley, male, 180–200 g) were obtained from Taconic Farms (Germantown, MD). Animals were housed in groups of five to eight animals each for at least 1 week after arrival from the supplier and were allowed free access to standard rat chow and water.

Procedures. The measurement of GSH [7], the synthesis of [5-^{14}C]-4-ipomeanol [8], the assay of covalently bound 4-ipomeanol, and the measurement of pulmonary toxicity and lethality all were accomplished as described previously [3]. LD_{50} values (± 95% confidence limits), determined using at least four to five dose levels with groups of five to ten animals each, were calculated by the method of Litchfield and Wilcoxon [9].

Drug treatments. 4-Ipomeanol (solution in 25% propylene glycol/water), cysteamine·HCl, and cysteine (solutions in saline) were administered i.p. at a constant dose volume of 1 ml/100 g animal weight. Diethylmaleate and piperonyl butoxide were administered subcutaneously as solutions in corn oil (dose volume, 1 ml/100 g animal weight).

RESULTS

Depletion of pulmonary GSH by 4-ipomeanol. Figure 1 shows that the i.p. administration of 4-ipomeanol caused dose-related decreases in pulmonary GSH concentrations. Within the wide range of doses studied, there was no apparent dose threshold either in the GSH depletion curve or in the covalent binding of 4-ipomeanol metabolites to pulmonary protein. Both the pulmonary covalent binding and the depletion of pulmonary GSH reached maximal levels very rapidly (30–40 min), and the two phenomena seemed clearly related temporally (Fig. 2). At none of the doses tested did 4-ipomeanol significantly deplete glutathione in the liver or kidneys (data not shown).

Pretreatment of rats with piperonyl butoxide

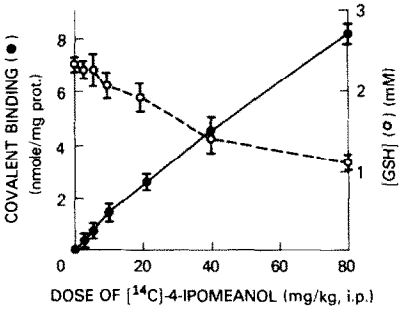

Fig. 1. Glutathione concentrations and covalently bound 4-ipomeanol metabolites in lungs of rats 1 hr after i.p. administration of various doses of [^{14}C]-4-ipomeanol. Values plotted are means (± S.E.) of determinations on groups of five animals each. All doses of greater than 20 mg of 4-ipomeanol per kg caused significant ($P < 0.01$) decreases in GSH.

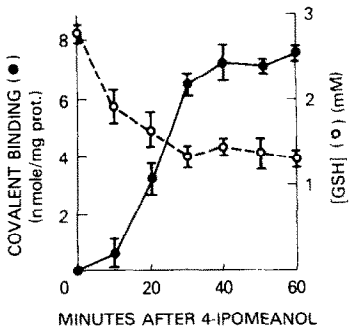

Fig. 2. Glutathione concentrations and covalently bound 4-ipomeanol metabolites in lungs of rats at various times after i.p. administration of 80 mg of [^{14}C]-4-ipomeanol per kg. Values plotted are means (± S.E.) of determinations on groups of five animals each.

almost completely prevented the depletion of pulmonary GSH by 4-ipomeanol (Table 1). This experiment confirmed that the depletion of lung GSH by 4-ipomeanol was due to reactive product(s) of 4-ipomeanol metabolism and not the parent compound itself; piperonyl butoxide has been shown to be a potent *in vivo* inhibitor of the pulmonary metabolic activation and toxicity of 4-ipomeanol [3]. Other studies have shown that this pretreatment compound clearly does not inhibit lung tissue uptake of the parent compound [10]; in fact, lung concentrations of unmetabolized 4-ipomeanol are markedly elevated by pretreatment with piperonyl butoxide [10].

Effects of diethylmaleate on tissue GSH and on the covalent binding of 4-ipomeanol metabolites. In a preliminary experiment, diethylmaleate was administered subcutaneously to rats, and the hepatic and pulmonary GSH concentrations were measured. The maximum GSH depletions after a 0.4 ml/kg dose were achieved within about 1 hr after the pretreatment (data not shown); therefore, in subsequent studies animals were used routinely 1 hr following the diethylmaleate pretreatment.

Figure 3 shows that diethylmaleate caused dose-related decreases in pulmonary and hepatic GSH and caused dose-related increases in the covalent binding of 4-ipomeanol metabolites in these tissues. Interestingly, the lung appeared to be more sensitive to the effects of diethylmaleate; the lower doses of diethylmaleate (e.g. 0.2 to 0.4 ml/kg) invariably caused more pronounced effects on the GSH and covalent binding values in the lung compared to the liver. Previous studies [3] showed that diethylmaleate treatment markedly enhanced the lethal pulmonary toxicity of 4-ipomeanol. This finding also was confirmed in the present study (data not shown); the LD_{50} value for i.p. administered 4-ipomeanol was decreased markedly (from 22 mg/kg to 6 mg/kg) by diethylmaleate treatment, and histological examinations indicated that the lungs were the major site of damage both in the control and in the pretreated rats.

Effects of sulfhydryl reagents on in vivo *covalent binding and toxicity of 4-ipomeanol metabolites.* Table 2 shows that pretreatments of animals with the sulfhydryl compounds cysteine or cysteamine

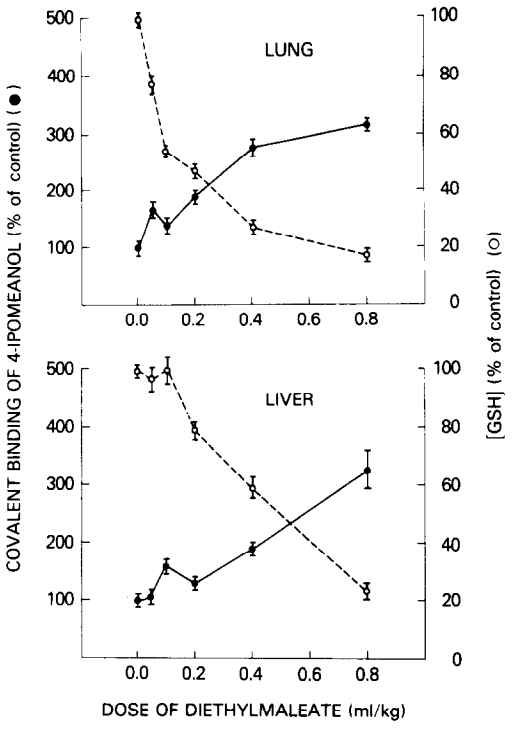

Fig. 3. Glutathione concentrations and covalently bound 4-ipomeanol metabolites in lungs and livers of rats after s.c. administration of various doses of diethylmaleate. Data plotted with broken lines show GSH concentrations in lungs and livers of rats 1 hr after pretreatment with various s.c. doses of diethylmaleate; control (100%) values were 2.5 and 7.7 mM GSH for lung and liver respectively. Data plotted with solid lines show amounts of 4-ipomeanol metabolites bound covalently in lungs and livers of diethylmaleate-pretreated (s.c. injection, 1 hr prior to 4-ipomeanol) rats 1 hr after i.p. injection of 20 mg of [^{14}C]-4-ipomeanol per kg; control (100%) values were 2.5 and 0.9 nmoles/mg protein for lung and liver respectively. All values plotted are means (± S.E.) of determinations on groups of five animals each. The GSH data and the 4-ipomeanol binding data were determined in separate experiments. All values obtained from the diethylmaleate-treated animals were significantly different ($P < 0.01$) from the corresponding control values (i.e. from animals not pretreated with diethylmaleate).

Table 2. Effects of pretreatment with cysteine or cysteamine on *in vivo* covalent binding of 4-ipomeanol in rats

Pretreatment	Dose of [^{14}C]-4-ipomeanol (mg/kg, i.p.)	Covalent binding of 4-ipomeanol* (nmoles/mg protein) Lung	Liver
Saline	15	1.3	0.3
	40	3.4	1.1
Cysteine†	15	0.8‡	0.2
	40	1.8‡	0.7‡
Cysteamine§	15	0.7‡	0.1‡
	40	1.5‡	0.4‡

* Values are means of determinations on five rats per group. Standard errors were all less than 14% of the respective means. Determinations were performed 2 hr after administration of the radiolabeled 4-ipomeanol.

† Cysteine dose: 300 mg/kg, i.p., 5 min prior and 20 min after 4-ipomeanol.

‡ Significantly different from control value ($P < 0.01$; Student's *t*-test).

§ Cysteamine dose: 350 mg/kg, i.p., 30 min prior to 4-ipomeanol.

markedly inhibited the *in vivo* covalent binding of 4-ipomeanol metabolites to macromolecules of lung and liver. Table 3 shows that these pretreatments likewise decreased the toxicity of 4-ipomeanol; the LD_{50} values were approximately doubled by the pretreatments, and histological examinations indicated that the lungs were the major site of tissue damage both in the control and in the pretreated animals. Preliminary measurements confirmed that these pretreatment regimens consistently produced 30–45% elevations of nonprotein sulfhydryl concentrations (NPSH), both in the lung and in the liver, within the time frame of the experiments (no attempt was made to differentiate GSH from total NPSH).

DISCUSSION

These studies support the view that pulmonary GSH serves as an endogenous protective factor against lung injury by 4-ipomeanol, probably by

Table 1. Effect of piperonyl butoxide pretreatment on the depletion of pulmonary glutathione by 4-ipomeanol in the rat

Pretreatment	4-Ipomeanol dose (mg/kg, i.p.)	Pulmonary glutathione* (mM)
Vehicle only	None	2.10 ± 0.04
	60	1.40 ± 0.03†
Piperonyl butoxide‡	None	2.01 ± 0.08
	60	2.00 ± 0.06

* Each value is the mean ± S.E. of determinations on five animals per group; measurements were performed 1 hr after the administration of 4-ipomeanol.

† Significantly different from control value ($P < 0.01$; Student's *t*-test).

‡ Piperonyl butoxide dose: 1200 mg/kg, s.c., 45 min prior to 4-ipomeanol. Piperonyl butoxide is a potent inhibitor of the pulmonary covalent binding and toxicity of 4-ipomeanol in rats [3].

Table 3. Effects of pretreatment with cysteine or cysteamine on lethal pulmonary injury by 4-ipomeanol in the rat

Pretreatment	LD_{50} (± 95% confidence limits) of 4-ipomeanol* (i.p., 36 hr) (mg/kg ± confidence limits)
Vehicle only	22 ± 4
Cysteine†	40 ± 6
Cysteamine‡	47 ± 10

* In both groups the lung was the primary site of damage by 4-ipomeanol (bronchiolar necrosis, edema, congestion, and hemorrhage). There was little or no apparent lung damage in sulfhydryl-pretreated rats receiving 15–20 mg/kg doses of 4-ipomeanol, whereas these doses of 4-ipomeanol caused severe lung injury in vehicle-pretreated controls. All animals surviving 36 hr usually survived indefinitely and lungs showed normal histology after 3–4 weeks.

† Cysteine dose: 300 mg/kg, i.p., 5 min prior to and 20 min after 4-ipomeanol.

‡ Cysteamine dose: 300 mg/kg, i.p., 30 min prior to 4-ipomeanol.

providing a detoxification pathway for conjugation and removal of toxic, alkylating metabolites of the furan derivative. Consistent with this hypothesis were the findings that the administration of 4-ipomeanol caused a rapid, dose-dependent depletion of GSH, selectively from the lungs, and that this depletion could be prevented by pretreatment with piperonyl butoxide, an inhibitor of the metabolic activation of 4-ipomeanol.

There was no apparent threshold for the covalent binding of 4-ipomeanol, and there was no evidence that total lung GSH had to be substantially depleted before the tissue covalent binding of 4-ipomeanol could occur. This was in contrast to the threshold phenomenon for hepatic GSH depletion and covalent binding which occurs with hepatotoxic agents such as acetaminophen [11] and bromobenzene [12]. It seems likely that pulmonary cellular GSH offers only an *alternative* site (i.e. versus tissue macromolecules) for reaction of electrophilic 4-ipomeanol metabolite, rather than a *preferred* site of reaction. It may also be of relevance that the cytosolic GSH-transferase enzymes markedly enhance the rates of conjugation of GSH with reactive metabolites of acetaminophen and bromobenzene, whereas these soluble-fraction enzymes appear to have little effect on the rates of GSH conjugate formation with microsomally activated 4-ipomeanol [5]. Any interpretations, however, should be considered cautiously, because of limitations in the present experimental procedures for measuring whole-organ levels of GSH. Depletion of GSH primarily in a specific lung cell type may be difficult to detect by conventional procedures if total pulmonary GSH is distributed widely throughout the organ and possibly in a variety of cell types. This question is of special relevance since 4-ipomeanol has been shown to covalently bind preferentially to pulmonary nonciliated bronchiolar (Clara) cells [10].

Also in support of a protective role for GSH against lung damage by 4-ipomeanol were the findings that the prior depletion of pulmonary GSH by diethylmaleate led to striking enhancements both in the amounts of 4-ipomeanol metabolite bound covalently to the lung and in the pulmonary damage by the compound. The diethylmaleate pretreatment also caused decreases in hepatic GSH and increases in the hepatic covalent binding of 4-ipomeanol metabolites; however, the amounts of 4-ipomeanol metabolite bound covalently in the livers of the control or the pretreated animals were very low in comparison to the amounts in the lung, and in neither group was there any histological evidence of liver damage by 4-ipomeanol (and 4-ipomeanol treatment alone did not decrease hepatic GSH). Another study, reported in detail in a separate paper [13], demonstrated that diethylmaleate pretreatment did not alter the tissue distribution or the blood levels of unmetabolized 4-ipomeanol, and it did not alter the excreted amounts of ipomeanol-4-glucuronide (a major urinary metabolite of 4-ipomeanol in rats [14]). Thus, the effects of diethylmaleate on 4-ipomeanol covalent binding and toxicity are best accountable by the removal of GSH available for conjugation with reactive 4-ipomeanol metabolites rather than by effects upon other routes of detoxification of 4-ipomeanol.

The inhibitory effects of cysteine and cysteamine on the pulmonary covalent binding and toxicity of 4-ipomeanol also suggest an important modulatory role for sulfhydryl compounds in 4-ipomeanol toxicity. The effects of these compounds may be explainable by their providing an increased pool of nucleophiles for conjugation with the toxic, electrophilic 4-ipomeanol metabolite. Previous *in vitro* studies (S. D. Nelson and M. R. Boyd, unpublished results) have shown that these compounds form conjugates with microsomally activated 4-ipomeanol and that they inhibit the covalent binding of alkylating metabolites of 4-ipomeanol to microsomal protein; however, at high concentrations ($>10^{-3}$ M) these agents also clearly inhibit the *formation* of the reactive 4-ipomeanol metabolites. Thus, the *in vivo* inhibitory effects of these agents on tissue alkylation and toxicity by 4-ipomeanol cannot be fully evaluated without further studies of the effects of these agents on the total metabolic profile of 4-ipomeanol *in vivo*; the protective effect of these compounds could, at least in part, be accountable by a direct inhibitory effect on the *in vivo* metabolic activation of 4-ipomeanol.

To conclude, because there is evidence that many instances of chemically induced lung injury may be due to the highly reactive, electrophilic nature of the agent involved, or the ultimate toxic products derived therefrom, it is likely that GSH may have an important general protective role against pulmonary injury by such materials. Therefore, any factors (e.g. other drugs or chemicals, nutritional status, circadian rhythms and irradiation) which conceivably could alter the availability of pulmonary GSH may likewise be important modulators or determinants of susceptibility to drug-induced lung injury.

Finally, recent studies with 4-ipomeanol in avian species [15] provide an additional perspective. These investigations are of particular interest since the lungs of birds contain little or no enzyme activity for

mediating the formation of an alkylating metabolite of 4-ipomeanol, but such activity is very high in the avian liver. 4-Ipomeanol was bound covalently, and depleted GSH preferentially, in the avian liver, and it was a potent hepatotoxin in birds. There was relatively very little pulmonary covalent binding of 4-ipomeanol metabolites, no significant depletion of pulmonary GSH, and no apparent pulmonary toxicity with 4-ipomeanol in birds.

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