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DISTRIBUTION AND METABOLISM OF THE PULMONARY ALKYLATING AGENT AND CYTOTOXIN, 4-IPOMEANOL, IN CONTROL AND DIETHYLMALEATE-TREATED RATS

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Abstract—Diethylmaleate (DEM), an agent which depletes tissue glutathione (GSH), increased the covalent binding and toxicity of 4-ipomeanol [1-(3-furyl)-4-hydroxypentanone] in rats. The distribution of unmetabolized 4-ipomeanol-[5-^{14}C] and its metabolites were studied in tissue extracts by high-pressure liquid chromatography (HPLC) in control and DEM-treated rats. At all time periods examined, DEM treatment produced no significant effect on the tissue distribution of unchanged 4-ipomeanol. In both groups, the relative tissue concentrations of unmetabolized 4-ipomeanol were in the order blood > lung > liver. In control rats, the relative tissue concentrations of nonbound, solvent-extractable 4-ipomeanol metabolites (hereafter referred to simply as "4-ipomeanol metabolites"), as well as the covalently bound 4-ipomeanol metabolites (hereafter referred to as "covalently bound 4-ipomeanol equivalents" to distinguish from all other metabolites) were in the order lung > liver > blood. The pulmonary levels of both the covalently bound 4-ipomeanol equivalents and the 4-ipomeanol metabolites were increased markedly by DEM treatment at all time periods examined. The total pool of urinary 4-ipomeanol metabolites was significantly decreased by DEM treatment, but the total amounts of excreted ipomeanol-4-glucuronide, the major metabolite of 4-ipomeanol in rats, were not significantly different in the control and DEM-treated rats. These data are consistent with the view that the increased pulmonary covalent binding and toxicity of 4-ipomeanol produced by diethylmaleate treatment in rats are due to the depletion of pulmonary GSH by the DEM and not a major DEM-induced alteration in the tissue distribution of the parent 4-ipomeanol.

4-Ipomeanol [1-(3-furyl)-4-hydroxypentanone] is a potent pulmonary toxin in rats and other experimental animals [1]. Previous reports have demonstrated that this compound is metabolically activated *in situ* by cytochrome P-450 mixed-function oxidase pathways in the lung to a highly reactive intermediate capable of interacting covalently with tissue macromolecular fractions [2–4]. After administration of radiolabeled 4-ipomeanol *in vivo*, the covalently bound 4-ipomeanol equivalents can be localized, using an autoradiographic technique, at its predominant site of formation in the lung, the non-ciliated bronchiolar lining cell (Clara cell) [2]. The Clara cells are a major site of necrosis by 4-ipomeanol in the lungs of several animal species. Reduced glutathione (GSH) modulates the pulmonary toxicity of 4-ipomeanol, and treatment of rats with diethylmaleate enhances the pulmonary toxicity and covalent binding of 4-ipomeanol [4, 5].

Because DEM depletes GSH in tissues [6, 7], there has been considerable use of this agent as an experimental modifier of GSH-dependent drug metabolism and drug toxicity, both *in vivo* and *in vitro*. However, there is little information concerning other effects of DEM beyond its action on GSH. An *in vitro* study by Anders [8] illustrated that DEM could produce either inhibition or stimulation of certain microsomal drug metabolism pathways. Since DEM might substantially alter GSH-dependent and/or GSH-independent drug metabolism *in vivo*, a detailed analysis of the influence of DEM on the *in vivo* distribution and metabolism of each specific parent compound and its metabolite(s) should be made. Such information will facilitate the interpretation of the mechanism by which DEM modifies the toxic effects of such chemicals, particularly those for which GSH serves as a protective factor.

In this paper we present a series of studies which support the view that the enhanced pulmonary toxicity and covalent binding of 4-ipomeanol in rats after diethylmaleate administration are due to a depletion of tissue GSH concentrations by diethylmaleate and not to an alteration in the tissue distribution of the parent compound. Some of these studies have been presented previously in preliminary form [9].

MATERIALS AND METHODS

Chemicals. Diethylmaleate was obtained from ICN Pharmaceuticals, Inc. (Plainview, NY), and all other required solvents and chemicals were obtained from the Macalaster Bicknell Co. (Millville, NJ).

4-Ipomeanol. 4-Ipomeanol-[5-^{14}C] was prepared as described elsewhere [3]. The specific activity of the radiolabeled 4-ipomeanol was 0.15 mCi/mmole. Unlabeled 4-ipomeanol was prepared similarly, using nonradiolabeled precursors.

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4-Ipomeanol dose solutions were prepared in 25% propylene glycol/water such that a volume of 1.0 ml/100 g body weight was administered to yield the desired dose. The compound was dissolved in the appropriate volume of propylene glycol prior to being diluted with water to the desired volume.

Animals. Male, Sprague–Dawley-derived rats, weighing 120 ± 10 g, were obtained from Taconic Farms, Germantown, NY. All animals were given NIH open formula rat chow and water *ad lib.* and kept in a 12-hr light/dark cycle for a period of 1 week prior to use. Animals were not fasted before use.

Pretreatment of animals. Diethylmaleate treatment consisted of a single s.c. injection (solution in sesame oil) 30 min prior to 4-ipomeanol injection i.p. The DEM dose solution was prepared such that administration of 1.0 ml/100 g body weight yielded a dose of 0.4 ml DEM/kg. The control groups were treated with sesame oil.

Collection of tissue and urine samples. After i.p. administration of radiolabeled 4-ipomeanol, animals were placed in glass metabolism cages for urine collection. Collection vessels were kept on ice throughout the experiment. Animals were removed from their cages after application of a Dieffenbach serrefine artery clamp to the exposed penis to prevent urine loss. Rats were decapitated and blood samples were collected in 50 ml beakers which contained 1.5 ml of cold 5% sodium citrate. Blood samples were kept on ice for extraction and covalent binding assay at a later time. Animals were opened with a midline incision and the bladder contents were removed with a 1 cc syringe equipped with a 25 gauge needle. The bladder was rinsed twice with 0.3 ml of cold water after removal of its initial contents. The rinses and original contents were added to their respective urine collection vessels.

Tissues were excised, trimmed of extraneous material, and immediately frozen by placement on dry ice for subsequent extraction and covalent binding assay as described below. To obtain excreted urine, each metabolism cage was rinsed with 3 ml of ice-cold water followed by 2 × 3 ml methanol (spiked with unlabeled 4-ipomeanol, 0.4 mg/ml) washings. The water and methanol washings were added to the combined urine collections, and the total volumes were recorded. Urine samples were kept at −20° for high-pressure liquid chromatography (HPLC) analysis as described below.

Preparation of tissues for determination of 4-ipomeanol and 4-ipomeanol metabolites. Tissue samples were homogenized in 1 weight/volume (w/v) or volume/volume (v/v) (blood 2 ml) of ice-cold water with seven strokes of a teflon homogenizer. Four w/v or v/v of cold methanol containing 0.4 mg/ml of unlabeled 4-ipomeanol were added and the mixture was homogenized with an additional seven strokes of the pestle. The resulting solutions were transferred to 13 ml conical centrifuge tubes, capped, and centrifuged at 2000 *g* for 20 min in a Beckman J-6 refrigerated centrifuge. The supernatant fractions were decanted to their respective scintillation vials and placed on dry ice. The resulting precipitates were washed twice with 4 ml aliquots of methanol solution containing carrier 4-ipomeanol and the supernatant fractions were decanted to their respective containers after centrifugation. The precipitates were used for the covalent binding assay described below. All samples were stored at −20° prior to analysis by HPLC as described below.

Assay for covalently bound 4-ipomeanol equivalents. The precipitates from above were first washed with 10% trichloracetic acid and then washed exhaustively with methanol until no further radioactivity was detectable in the washings [4]. After the final washing and centrifugation, the resulting pellet was dissolved in 1 N NaOH and protein was measured in an aliquot by the method of Lowry *et al.* [10]. Radioactivity was determined in 0.8 ml aliquots by counting in a mixture of 3 ml of 50% aqueous ethanol and 15 ml of "ACS" (Searle Analytic, Inc.) scintillation counting fluid and 100 μl of glacial acetic acid. Covalently bound radioactivity was expressed as nanomoles bound per milligram of tissue protein.

High-pressure liquid chromatography of tissue extracts for determination of unmetabolized 4-ipomeanol and 4-ipomeanol metabolites. All samples were chromatographed using a Waters Associates Inc. M600A pump, U6K loop injector, and a M440 UV detector fitted with a 254 nm filter. A 100 μl aliquot of each sample was injected onto a C_{18} μBondapak column (Waters Associates, Inc.) (30 × 0.38 cm) with a mobile phase of 30% methanol/water. An isocratic flow rate of 1 ml/min was used. Fractions containing the radiolabeled 4-ipomeanol metabolites and unchanged 4-ipomeanol were collected directly into scintillation vials. Fifteen milliliters of ACS counting mixture was added and the samples were counted for 10 min in a Searle Analytic Mark III liquid scintillation counter. All samples were corrected for quench by automatic external standardization.

High-pressure liquid chromatographic determination of ipomeanol-4-glucoronide. Instrumentation was similar to that described above except that a C_{18} μBondapak semiprep column (Waters Associates Inc.) (30 × 0.78 cm) was used. The mobile phase was 20% methanol/water which contained 0.01 M EDTA. The isocratic flow rate was 2 ml/min. A 100–200 μl aliquot of each sample was injected onto the column and 30-sec fractions were collected directly into scintillation vials for a period of 60 min. All further procedures were identical to those described above.

Isolation and identification of ipomeanol-4-glucuronide. Ipomeanol-4-glucuronide was isolated from urine by semipreparative HPLC followed by desalting on XAD-2 resin. The structure was confirmed by: (1) HPLC retention time; (2) liberation of unchanged 4-ipomeanol (as confirmed by HPLC retention time and mass spectrometry) after β-glucuronidase hydrolysis; (3) inhibition of β-glucuronidase hydrolysis by 1×10^{-3} M saccharo-1,4-lactone, a specific β-glucuronidase inhibitor; and (4) chemical ionization mass spectral analysis of the methylated/trimethylsyliated ipomeanol-4-glucuronide. These methods allowed for assignment of the structure of the major urinary metabolite of 4-ipomeanol in rats as ipomeanol-4-glucuronide. Complete details of the isolation, purification, and identification of ipomeanol-4-glucuronide will be published in detail elsewhere [11].

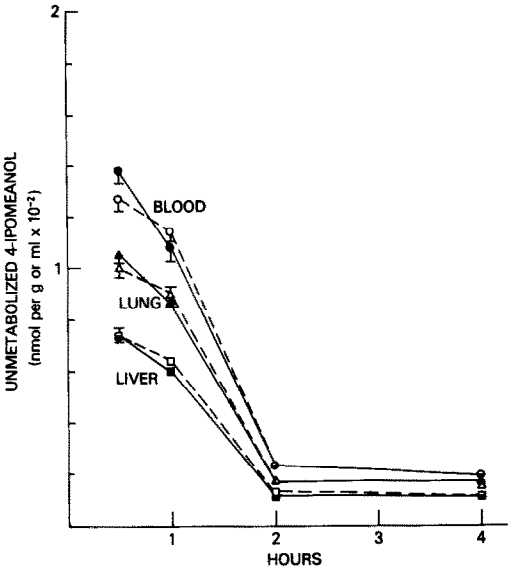

Fig. 1. Unmetabolized 4-ipomeanol-[5-^{14}C] concentration time courses in tissues of sesame oil (solid lines)- and diethylmaleate (broken lines, 0.4 ml/kg)-treated rats. Treatments were administered 30 min prior to the 4-ipomeanol-[5-^{14}C] (20 mg/kg, i.p.). Each value is the mean ± S.E. of five animals.

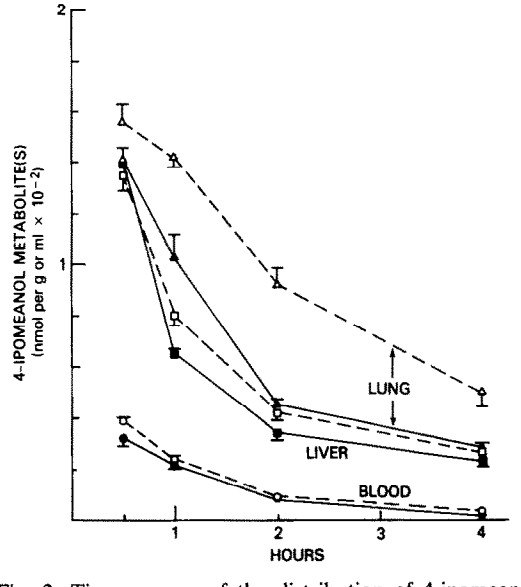

Fig. 2. Time courses of the distribution of 4-ipomeanol metabolites after the i.p. administration of a 20 mg/kg dose of 4-ipomeanol-[5-^{14}C] in sesame oil (broken lines)- and DEM (0.4 ml/kg, s.c., solid lines)-treated rats. 4-Ipomeanol-[5-^{14}C] was administered 30 min following the DEM treatment. The values listed are the means ± S.E. determined on five animals. The 1-, 2-, and 4-hr time point concentrations of 4-ipomeanol metabolites were significantly ($P < 0.05$, Student's t-test) elevated in DEM-treated rat lungs (△) over the sesame oil-treated group (▲).

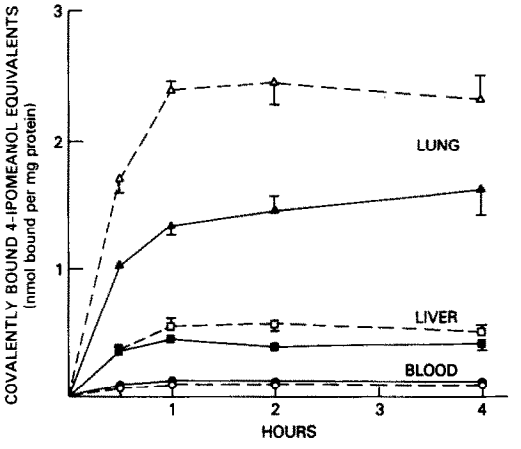

Fig. 3. Time courses of the covalent binding of radioactivity in rat tissues after i.p. administration of 4-ipomeanol-[5-^{14}C] (20 mg/kg) 30 min after a s.c. dose of sesame oil (solid lines) or diethylmaleate (0.4 ml/kg, broken lines). Values are the means ± S.E. of five animals.

RESULTS

Effects of DEM treatment on the tissue levels of unmetabolized 4-ipomeanol. The data illustrated in Fig. 1 show that DEM treatment produced no significant effect on the tissue and blood levels of unmetabolized 4-ipomeanol, at all time periods examined. In both groups of animals, the relative tissue concentrations of 4-ipomeanol were in the order blood > lung > liver. Thus, the increases in covalent binding and toxicity of 4-ipomeanol in the rat that are associated with DEM treatment are not due to alterations in the availability of parent 4-ipomeanol in the tissue for activation to reactive intermediate.

Effects of DEM treatment on the tissue levels of 4-ipomeanol metabolites. The tissue concentrations of nonbound, solvent-extractable 4-ipomeanol metabolites (hereafter referred to simply as "4-ipomeanol metabolites") at various times after administration of radiolabeled 4-ipomeanol are shown in Fig. 2. DEM treatment significantly enhanced the 1-, 2-, and 4-hr pulmonary levels of 4-ipomeanol metabolites. There was no consistent effect of DEM treatment on the liver or blood content of the 4-ipomeanol metabolites.

Tissue binding of radioactivity. The data presented in Fig. 3 show that, after rats were treated with DEM and given radiolabeled 4-ipomeanol (20 mg/kg) 30 min later, significantly more 4-ipomeanol metabolite(s) (hereafter referred to as "covalently bound 4-ipomeanol equivalents" to distinguish from all other metabolites) was covalently bound to the lung macromolecules. This preferential increase in only the pulmonary covalent binding of 4-ipomeanol equivalents after DEM treatment results, in part, from the dose of DEM administered. A dose of 0.4 ml/kg produces a greater depletion of GSH in the lung (74%) than in the liver (40%) [5]. These data are in agreement with previous observations suggesting that GSH participates in the detoxication of the 4-ipomeanol reactive intermediates [4, 5].

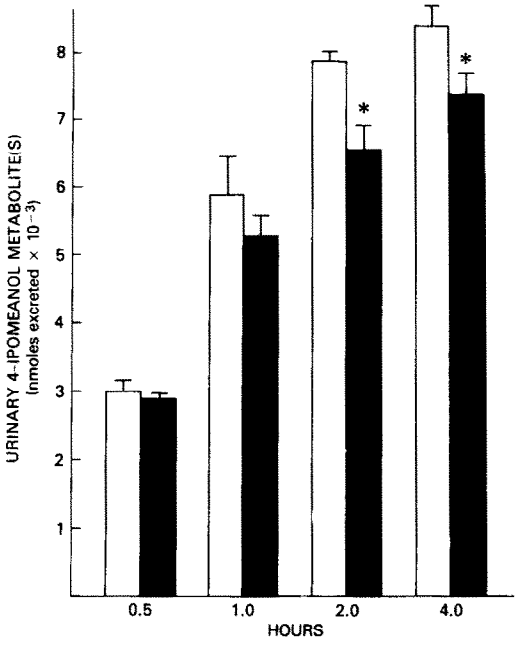

Fig. 4. Urinary excretion of 4-ipomeanol-[5-^{14}C] metabolites in sesame oil (open bars)- and DEM (0.4 ml/kg, s.c., solid bars)-treated rats. DEM and sesame oil were administered 30 min prior to i.p. injection of a 20 mg/kg dose of 4-ipomeanol-[5-^{14}C]. Each value listed is the mean ± S.E. of determinations on five animals. An asterisk (*) denotes significant difference from the sesame oil-treated group ($P < 0.05$).

Urinary excretion of 4-ipomeanol metabolites after DEM treatment. Radioactivity which appeared in the urine in both the control and DEM treatment groups consisted primarily of 4-ipomeanol metabolites. Unchanged 4-ipomeanol represented less than 0.1% of all radioactivity eliminated in the urine. DEM treatment did produce a small but significant decrease in the total amount of urinary metabolites at 2 and 4 hr, as shown in Fig. 4. The DEM-associated decrease in the amount of metabolites excreted in the urine was not due to a decrease in the excretion of ipomeanol-4-glucuronide, as shown in Fig. 5. The glucuronide conjugate is the major urinary metabolite of 4-ipomeanol in rats [11] and accounts for 51% of the total urinary radioactivity and 23% of the dose of administered 4-ipomeanol at 4 hr. Although there was a trend suggesting less ipomeanol-4-glucuronide excretion in the urine, there was not a significant decrease in the total amount of glucuronide conjugate eliminated via the urine.

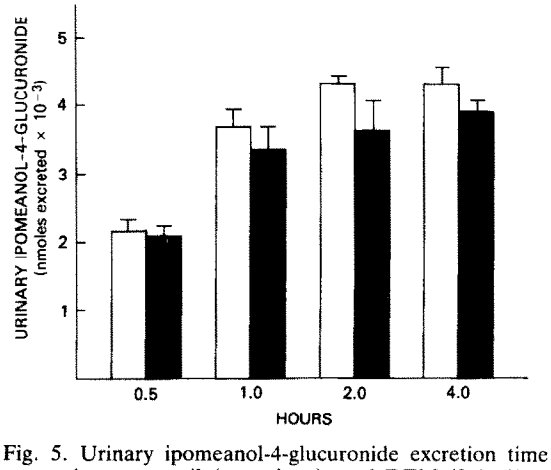

Fig. 5. Urinary ipomeanol-4-glucuronide excretion time course in sesame oil (open bars)- and DEM (0.4 ml/kg, s.c., solid bars)-treated rats. 4-Ipomeanol-[5-^{14}C] was administered i.p. 30 min after the pretreatments at a dose of 20 mg/kg. Five animals were used to determine the listed mean ± S.E.

DISCUSSION

The present studies illustrate that the increase in the covalent binding and enhanced toxicity of 4-ipomeanol produced by DEM treatment is not due to a major change in the tissue or blood distribution of the parent compound, 4-ipomeanol. Also, DEM treatment did not alter the production and excretion of the major urinary metabolite, ipomeanol-4-glucuronide. These observations clarify the mechanism of the DEM-enhanced covalent binding and toxicity. If DEM inhibited glucuronidation or another major detoxication pathway, it could thereby effectively increase the tissue concentration of 4-ipomeanol available for metabolism to the active intermediate. Therefore, DEM conceivably could, in this manner, increase the tissue covalent binding and toxicity of 4-ipomeanol by a mechanism independent of its effect on GSH. However, the present studies clearly argue against this possibility. These results are therefore consistent with the view that the enhanced pulmonary toxicity and covalent binding of 4-ipomeanol in rats after DEM administration are due to the depletion of the pulmonary GSH concentrations by DEM.

Figure 6 presents the current conceptualization of the important toxication and detoxication pathways for 4-ipomeanol in the rat. Previous studies [2–4] have demonstrated that 4-ipomeanol is metabolized to a highly reactive intermediate via the cytochrome P-450 mixed-function oxidase systems. This extremely labile electrophilic moiety is then capable of reacting with nucleophiles in the immediate vicinity of its formation. Reaction of the intermediate with tissue macromolecules is associated with the initiation of tissue necrosis (toxication pathway).

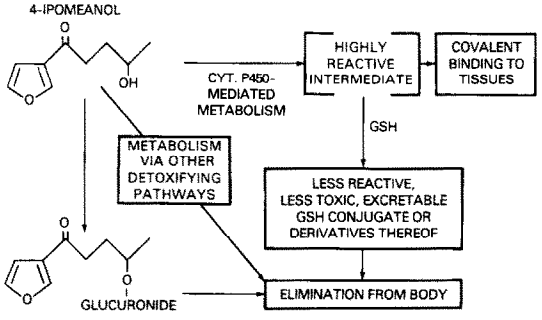

Fig. 6. Schematic representation of some important toxication/detoxication pathways for 4-ipomeanol in the rat.

There is a strong correlation between the amount of covalently bound 4-ipomeanol equivalents to pulmonary protein and the degree of lung damage [1]. The amount of radiolabeled material bound covalently to protein served as an index of the amount of reactive 4-ipomeanol intermediate formed. The reactive 4-ipomeanol derivative that is formed via the mixed-function oxidase system also reacts with the tissue glutathione. This reaction has been shown to be an important protective mechanism against the pulmonary damage and toxicity caused by the formation of the reactive intermediate (detoxication pathway) [4, 5]. The reaction of the reactive intermediate with GSH produces less reactive, less toxic, excretable conjugate(s) which may then be eliminated from the body. The data presented here support the view that DEM enhances the toxicity and covalent binding of 4-ipomeanol by depleting the pulmonary concentration of reduced glutathione, thereby making less GSH available for reaction with the reactive intermediate. This pathway appears to be an important detoxication step for the reactive intermediate of 4-ipomeanol in rat lung.

Although the conjugation of reactive 4-ipomeanol intermediate with GSH *in situ* in the lung is one important detoxication pathway, a major detoxication pathway for 4-ipomeanol in the whole animals *in vivo* is the elimination of 4-ipomeanol as the glucuronide conjugate [11]. Of the total dose of 4-ipomeanol administered, 23% is excreted in the urine as ipomeanol-4-glucuronide after 4 hr. The conjugation of 4-ipomeanol with glucuronic acid has been shown to be an important protective mechanism against the toxic effects of 4-ipomeanol. Changes in the amount of glucuronide conjugate produced correlate with the alterations in the toxicity of 4-ipomeanol *in vivo* in rats treated with various drug metabolism-inducing agents [12].

Diethylmaleate treatment did not alter the urinary excretion of ipomeanol-4-glucuronide but did decrease the total amount of 4-ipomeanol metabolites excreted, suggesting that DEM produced a decrease in the excretion of metabolites normally derived from GSH conjugation.

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