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EFFECTS OF PHENOBARBITAL AND 3-METHYLCHOLANTHRENE ON THE *IN VIVO* DISTRIBUTION, METABOLISM AND COVALENT BINDING OF 4-IPOMEANOL IN THE RAT; IMPLICATIONS FOR TARGET ORGAN TOXICITY

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Abstract—The effects of phenobarbital (PB) and 3-methylcholanthrene (MC) on the distribution, metabolism and covalent binding of 4-ipomeanol were examined in the rat. An analysis of tissue extracts by high-pressure liquid chromatography (HPLC) showed that both treatments markedly decreased the concentrations of unmetabolized 4-ipomeanol at all times examined. PB treatment increased the urinary excretion of nonbound 4-ipomeanol metabolites, while MC treatment did not alter their excretion. Analysis of urine by HPLC indicated that the increased concentration of urinary metabolites found in the phenobarbital-treated rats was attributable primarily to an increased excretion of ipomeanol-4-glucuronide. These data indicate that the decreased pulmonary covalent binding and lethality of 4-ipomeanol in the rat after MC and PB were caused by alterations in the tissue distribution of the parent compound. Pulmonary concentrations of unmetabolized 4-ipomeanol were decreased by MC through an increased metabolism of 4-ipomeanol in the liver, primarily to toxic products that bind covalently in that tissue and lead to hepatotoxicity. PB produced a similar decrease in unmetabolized 4-ipomeanol concentrations in lung but by an enhanced *in vivo* metabolism and clearance of 4-ipomeanol, primarily through a "nontoxic" pathway, glucuronidation, and did not lead to hepatotoxicity.

The relationship of *in situ* metabolic activation and the target organ toxicity of 4-ipomeanol is well established [1]. Strong correlations between the site and degree of tissue damage and the *in vivo* levels of covalently bound 4-ipomeanol metabolites (hereafter referred to as "covalently bound 4-ipomeanol equivalents" to distinguish them from all other 4-ipomeanol metabolites) have been demonstrated [2]. Although the lung is the preferential target organ for 4-ipomeanol toxicity in the rat and several other species [3, 4], other animals occasionally demonstrate hepatic (hamster) or kidney (adult male mouse) necrosis in addition to the pulmonary damage [2].

In vivo studies in the rat have shown that treatment of rats with mixed-function oxidase inducers, phenobarbital (PB) and 3-methylcholanthrene (MC), decreased both the acute toxicity of 4-ipomeanol and the concentrations of covalently bound 4-ipomeanol equivalents in the lung [4]. MC treatment, however, markedly increased the amount of 4-ipomeanol equivalents bound covalently in the liver and produced a striking shift in the target tissue necrosis. Centrilobular hepatic necrosis accompanied by a marked diminution in the severity of pulmonary lesions were the major results of 4-ipomeanol administration to MC-treated rats [4]. No such alteration in the target organ specificity of 4-ipomeanol was observed with PB treatment.

In vitro studies have shown that both the PB treatment and the MC treatment increased the amount of covalently bound 4-ipomeanol equivalents with hepatic microsomes but produced little or no effect with the lung microsomal subcellular fractions [5]. The present investigations, therefore, were undertaken to elucidate the basis for the contrasting *in vitro* and *in vivo* effects of PB on hepatic binding, and to explore the effects of PB and MC treatments on the *in vivo* distribution and metabolism of 4-ipomeanol in the rat. Some of these studies have been presented previously in a preliminary form [6].

MATERIALS AND METHODS

Chemicals. Phenobarbital and 3-methylcholanthrene were obtained from the Sigma Chemical Co. (St. Louis, MO). All other chemicals and solvents were obtained from the Macalaster Bicknell Co. (Piscataway, NJ).

4-Ipomeanol. [^{3}H]4-Ipomeanol was prepared by catalytic tritiation of unlabeled 4-ipomeanol, prepared as previously described [7, 8], using T_2O or tritium gas as the tritium source and 5% Rh/Al_2O_3 as the catalyst [9]. Twenty-five percent propylene glycol/water was used as the vehicle for 4-ipomeanol dose solutions. Solutions were prepared such that administration of 1 ml/100 g body weight yielded the desired dose. The 4-ipomeanol was dissolved in the appropriate volume of propylene glycol prior to

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dilution with water to the required volume. The specific activity of the radiolabeled 4-ipomeanol was 0.96 mCi/mmole (purity > 99%).

Animals. Sprague–Dawley-derived male rats, weighing 130 ± 10 g, were obtained from Taconic Farms, Germantown, NY. All animals were held in a 12-hr light/dark cycle and were fed NIH open formula rat chow and water *ad lib.* for a period of 1 week prior to use.

Animal treatments. Phenobarbital treatment consisted of i.p. injection of a 50 mg/kg phenobarbital solution (in saline). Injections were given every 12 hr for ten repetitions. 4-Ipomeanol was administered 24 hr after the tenth phenobarbital treatment. 3-Methylcholanthrene was administered i.p. at a dose of 20 mg/kg (in sesame oil) 48 and 72 hr prior to 4-ipomeanol administration. All animals received the same number of injections of either drug or its appropriate vehicle. PB and MC dose solutions were prepared such that administration of a 1 ml/100 g body weight volume yielded the desired dose.

Tissue sampling and urine collection. Animals were injected i.p. with radiolabeled 4-ipomeanol and placed in glass metabolism cages. Vessels for urine collection were kept on ice throughout the experiment. Upon completion of the desired exposure period, a Dieffenbach serrafine artery clamp was placed on the exposed penis (to prevent urine loss), and the rat was removed from the metabolism cage. Rats were decapitated, and blood was collected in a 50-ml beaker containing 1.5 ml of ice-cold 5% sodium citrate. Blood samples were placed on ice for covalent binding and extraction procedures (described below). Animals were opened with a midline incision, and the bladder contents were removed. Bladders were rinsed twice with a 0.3-ml portion of cold water; the original contents and rinses were combined and placed in their respective collection vessels and kept on ice.

Tissues were removed, frozen immediately on dry ice, and then prepared for extraction and covalent binding assay by procedures listed below. Each metabolism cage was rinsed with 3 ml of ice-cold water followed by 2 × 3 ml washings with ice-cold methanol which contained 0.04 mg/ml of unlabeled carrier 4-ipomeanol. The water and methanol washings were combined with the original urine collections and the bladder contents, the volume was recorded, and the samples were kept at −20° for analysis by high-pressure liquid chormatography (HPLC).

Tissue preparation for determination of unmetabolized 4-ipomeanol. Tissues were homogenized in 1 weight/volume (wt/v) or volume/volume (v/v) (blood 2 ml) of ice-cold water with seven strokes of a Teflon homogenizer. Methanol, which contained 0.4 mg/ml of unlabeled carrier 4-ipomeanol, was added at a ratio of 4 wt/v or v/v, and the mixture was rehomogenized with an additional seven strokes of the pestle. The mixtures were decanted to 13-ml conical centrifuge tubes, capped, and centrifuged at 2000 *g* for 20 min on a Beckman J-6 refrigerated centrifuge (4°).

The supernatant fractions were transferred to their respective scintillation vials and placed on dry ice. The precipitate was washed twice with 4-ml aliquots of methanol which contained unlabeled 4-ipomeanol and, after centrifugation, the resulting supernatant fractions were decanted to their respective scintillation vials. Supernatant fractions were stored at −20° for HPLC analysis as described elsewhere. The remaining protein precipitate was used for the covalent binding assay described below.

Determination of radiolabeled metabolites covalently bound to tissue macromolecules. The precipitate which resulted from the above procedures was washed first with 10% TCA (tricholoroacetic acid) and then by exhaustive washing with methanol. The methanol washings were continued until no further radioactivity was detectable in the methanol supernatant fraction [4]. The pellet was dissolved in 1 N NaOH, and protein concentration was determined by the method of Lowry *et al.* [10]. Determination of radioactivity was accomplished by counting 0.8-ml aliquots of the NaOH digest in a solution consisting of 3 ml of 50% aqueous ethanol, 15 ml of ACS (Searle Analytic, Inc.) scintillation fluid, and 100 μl of glacial acetic acid. Covalently bound radioactivity was expressed as nanomoles bound per milligram of tissue protein.

HPLC determination of unmetabolized 4-ipomeanol and 4-ipomeanol metabolites in tissue extracts. All samples were chromatographed with a Waters Associates M6000A pump, U6K loop injector, and an M440 UV detector fitted with a 254 nm filter. A 200-μl aliquot of each sample was injected onto a C_{18} μBondapak semiprep column (Waters Associates; 30 × 0.78 cm) with a mobile phase of 40% methanol/water. An isocratic flow of 2 ml/min was used. The fractions containing radiolabeled 4-ipomeanol metabolites and unchanged 4-ipomeanol were collected directly into scintillation vials. Fifteen milliliters of ACS scintillation fluid was added, and the vials were counted in a Searle Mark III liquid scintillation counter. All samples were corrected for quench by automatic external standardization.

HPLC determination of ipomeanol-4-glucuronide. Instrumentation was identical to that of the previous method except that the mobile phase was changed to 20% methanol/water which contained 0.1 M EDTA. The isocratic flow rate was 2 ml/min. Urine samples were spun at 2000 *g* in a Beckman J6 refrigerated centrifuge for 20 min. Twenty-five microliters of the resulting supernatant fluid was injected onto the column, and 30-sec fractions were collected directly into scintillation vials for a period of 60 min [11]. All further procedures were identical to those described above.

RESULTS

A preliminary check was done to confirm the desired effects of the treatments on the liver microsomal enzymes. PB and MC produced 264 and 183% increases, respectively, in hepatic cytochrome P-450 content. PB treatment increased the yield of microsomal protein by 53%, while MC treatment produced no change. These results verified that the treated animals were well-induced when compared to the controls.

Figure 1 shows that both PB and MC decreased the amount of 4-ipomeanol equivalents covalently

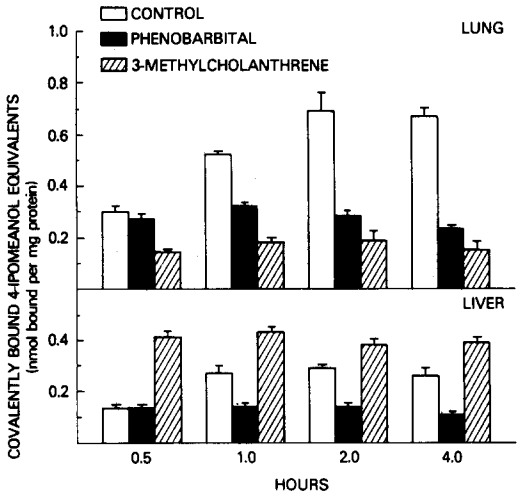

Fig. 1. Distribution of covalently bound 4-ipomeanol equivalents in lung and liver of control, PB-, and MC-treated rats at various times after i.p. administration of [^{3}H]4-ipomeanol (20 mg/kg). Each value shown is the mean ± S.E., determined on four animals.

bound to pulmonary macromolecules. PB treatment also decreased the amount of 4-ipomeanol equivalents covalently bound to liver macromolecules. However, MC treatment increased the amount of 4-ipomeanol equivalents bound to liver macromolecules. These results on covalent binding were consistent with observations from an earlier study [4].

Figure 2 shows that both PB and MC decreased the concentration of unmetabolized 4-ipomeanol in lung, liver and blood, especially at the early time periods when 4-ipomeanol concentrations in control rats were at their peak (0.5 and 1.0 hr). Figure 3 indicates that MC produced no significant effect on the urinary excretion of 4-ipomeanol metabolites, while PB increased the concentration of 4-ipomeanol metabolites at all time periods examined. HPLC analysis of the urine (Fig. 4) demonstrated that PB increased the excretion of ipomeanol-4-glucuronide (over control and MC groups) at all time periods examined. Indeed, the entire 15% increase in urinary radioactivity in PB-treated rats (Table 1) was due to an increase in ipomeanol-4-glucuronide excretion. The increased ipomeanol-4-glucuronide excretion results in a doubling in the total amount of the dose administered which is accountable as the glucuronide conjugate.

DISCUSSION

Results presented herein indicate that the decreased acute lethality and pulmonary covalent binding of 4-ipomeanol in rats after treatment with PB or MC are due to major changes in the tissue and blood distribution of the unmetabolized parent compound. While both treatments produced the same end result—decreased acute lethality and pulmonary covalent binding—through a reduction in tissue and blood concentrations of unmetabolized 4-ipomeanol, the principal *in vivo* metabolic changes underlying the effects of PB and MC are different.

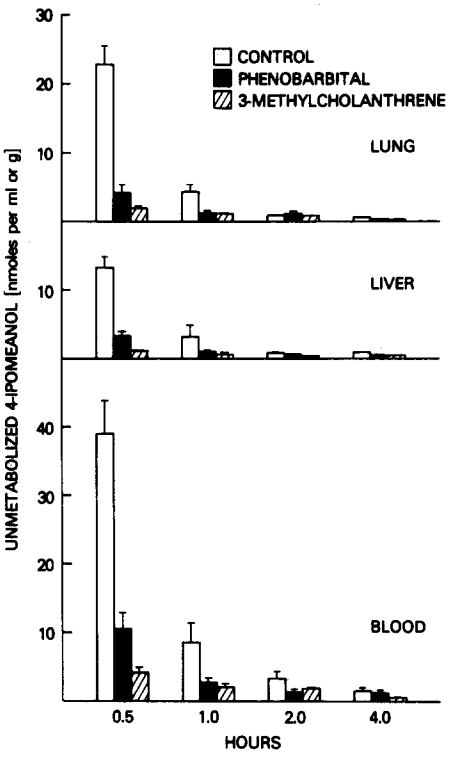

Fig. 2. Unmetabolized 4-ipomeanol in lung, liver, and blood of control, PB-, and MC-treated rats at various times after i.p. administration of 20 mg/kg of [^{3}H]4-ipomeanol. Values shown are the means ± S.E. of determinations on groups of four animals each.

MC treatment produced the greatest decreases in tissue concentrations of unmetabolized 4-ipomeanol (Fig. 2) and amounts of covalently bound 4-ipomeanol equivalents (Fig. 1), yet did not produce a significant effect on the urinary excretion of 4-ipomeanol metabolites or, specifically, of ipomeanol-4-glucuronide. A striking increase in hepatic covalent binding of reactive 4-ipomeanol intermediates was

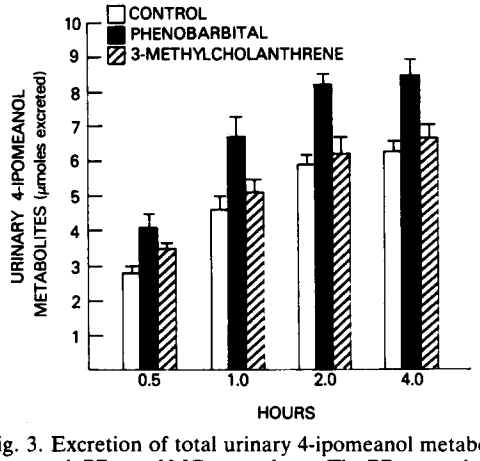

Fig. 3. Excretion of total urinary 4-ipomeanol metabolites in control, PB-, and MC-treated rats. The PB-treated group was significantly increased ($P < 0.05$, Student's t-test) over both the control and MC treatment groups at all time periods examined. Each value is the mean ± S.E of determinations on four animals.

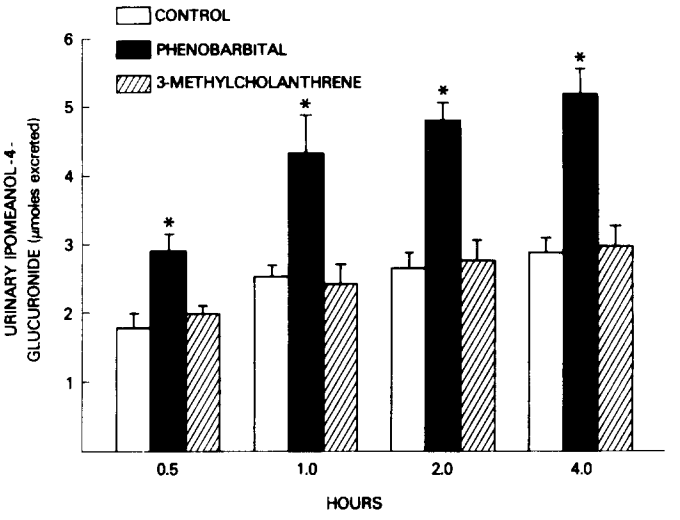

Fig. 4. Excretion of ipomeanol-4-glucuronide in control, PB-, and MC-treated rats. Each value is the mean ± S.E. of determinations on four rats receiving a 20 mg/kg dose of radiolabeled 4-ipomeanol. An asterisk (*) denotes a significant difference from control, $P < 0.05$.

confirmed in rats treated with MC; the increased hepatic covalent binding of the toxin has been shown to correlate with the production of centrilobular hepatic necrosis by 4-ipomeanol after MC treatment [4]. Also, *in vitro* studies have shown that MC produced a 200% increase in hepatic cytochrome P-450 concentrations and a 204% increase in the covalent binding of 4-ipomeanol intermediates in the liver microsomal subcellular fraction. This treatment had no effect on the *in vitro* covalent binding levels in the pulmonary microsomal subcellular fraction [5]. These data suggest that the decreased acute lethality and pulmonary covalent binding of 4-ipomeanol seen after MC treatment are associated with the increased clearance of unmetabolized 4-ipomeanol from the circulation via an increased metabolism of 4-ipomeanol to the reactive intermediate in the liver. The increased hepatic metabolism of 4-ipomeanol in the MC-treated rats is primarily through a toxification pathway which leads to increased hepatic covalent binding of 4-ipomeanol reactive intermediates and ultimately to centrilobular hepatic necrosis.

PB treatment also caused decreases both in the tissue concentrations of unmetabolized 4-ipomeanol and in the pulmonary covalent binding levels (Figs. 1 and 2). However, in contrast to MC, the PB decreased hepatic covalent binding of reactive 4-ipomeanol equivalents, even though *in vitro* studies [5] have shown that PB produced a 235% increase in hepatic cytochrome P-450 concentration and a 265% increase in the covalent binding of 4-ipomeanol-reactive metabolites to the liver microsomal subcellular fraction. This treatment also produced no effect on the *in vitro* covalent binding levels in the pulmonary microsomal subcellular fraction. PB did, however, produce a striking increase in the urinary excretion of 4-ipomeanol metabolites (Fig. 3). Moreover, the increase in urinary metabolites could be totally accounted for by an increase in a single urinary metabolite, ipomeanol-4-glucuronide, which is the major urinary metabolite of 4-ipomeanol in rats [11]. PB treatment also increased the metabolism of 4-ipomeanol as did MC treatment, but through a detoxification pathway, i.e. increased glucuronidation.

The two inducing agents used in these studies have provided a basis for classification of inducing agents into two distinct types: the "PB-type" and the "MC-type" [12]. The content of cytochrome P-450 is increased by PB and the content of cytochrome P-448 (P_1-450) by MC. *In vitro* studies [5] have shown that both of these inducing agents increase the hepatic microsomal metabolism of 4-ipomeanol to its reactive intermediate and this corresponds to the increase in the respective cytochrome P-450/P-448 as measured *in vitro*. Yet, this study clearly points

Table 1. 4-Hour urinary ipomeanol-4-glucuronide as a percent of administered dose of 4-ipomeanol and total urinary radioactivity*

	Percent of	
Pretreatment	Urinary radioactivity	Dose administered
Control	45	18
Phenobarbital	60	34
3-Methylcholanthrene	44	19

* Animals received a 20 mg/kg intraperitoneal injection of [^{3}H(G)]4-ipomeanol.

out the potential dangers of extrapolating *in vitro* data *a priori* to the possible *in vivo* effects of these two inducing agents. Both PB and MC induce a wide variety of enzymatic pathways in addition to the increased cytochrome content. Both inducing agents have been shown to induce glucuronyl transferase activities [13] but, as with cytochromes, either agent may induce different forms of glucuronyl transferase [14–17], with different substrate specificities. The present study clearly suggests that selective induction of a particular transferase can influence the toxicity of 4-ipomeanol.

The data also illustrate that, when dealing with experiments which involve inducing agents and compounds which are metabolized to highly toxic reactive intermediates in both hepatic and extrahepatic target tissues, it is the balance of the toxification and detoxification reactions or the overall net effect of the inducing agents on these pathways in each of the various tissues that may dictate the overall toxicity of a given compound. Moreover, inducer effects in one tissue clearly can influence those in another. As particularly illustrated by this study, the *in vivo* effects of inducers on the metabolism and toxicity of compounds activated *in situ* in extrahepatic tissues may be largely modulated by the effects of the inducers on the liver. Because of its large mass and high drug-metabolizing activity, the liver may predominantly regulate the amounts of unmetabolized compound reaching extrahepatic tissues where they could subsequently be metabolized to toxic products.

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