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COMPARISON OF RATIOS OF COVALENT BINDING TO TOTAL METABOLISM OF THE PULMONARY TOXIN, 4-IPOMEANOL, IN VITRO IN PULMONARY AND HEPATIC MICROSOMES, AND THE EFFECTS OF PRETREATMENTS WITH PHENOBARBITAL OR 3-METHYLCHOLANTHRENE

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SUMMARY: Studies of the ratios of the amounts of 4-ipomeanol covalently bound to the total amounts metabolized support the view that the high rates of in vitro pulmonary microsomal alkylation by 4-ipomeanol reflect high rates of NADPH-mediated metabolic activation of the compound rather than a relative deficiency of a microsomal detoxication pathway. Moreover, the ability of 3-methylcholanthene pretreatment, but not phenobarbital pretreatment, to shift the in vivo target organ alkylation and toxicity of 4-ipomeanol from the lung to the liver in rats could not be explained by a major alteration in the balances between microsomal toxicatior and detoxication pathways measurable in the in vitro systems examined, nor upon a major change in the nature of the reactive 4-ipomeanol metabolites produced in the lungs or livers of the pretreated animals.

INTRODUCTION

4-Ipomeanol [1-(3-furyl)-4-hydroxypentanone] is a highly selective pulmonary alkylating agent and cytotoxin in several animal species, including the rat (1,2). The pulmonary covalent binding of 4-ipomeanol results from the metabolic activation of the compound in situ in the target tissue; the formation of the highly reactive product is mediated by a cytochrome P-450 monooxygenase enzyme system (1-3). The liver also contains cytochrome P-450 enzymes capable of mediating the covalent binding of 4-ipomeanol, but the amounts of hepatic alkylation by 4-ipomeanol in vivo generally are much lower, compared to the lung (2,3). In vitro studies indicated that the K_m for the covalent binding pathway in rat pulmonary microsomes was only about 1/15 that for the hepatic microsomes (3). Moreover, the corresponding V_{max} values were nearly equal, even though the total cytochrome P-450 content of the pulmonary microsomes was only about 1/10 that of hepatic microsomes (3). Thus it seemed likely that 4-ipomeanol was converted to a reactive product

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much more efficiently by the pulmonary system compared to the hepatic system. However, the net amount of material bound covalently could have reflected not only the rates of formation of reactive 4-ipomeanol metabolites, but also the rates of reactions (eg. - enzymatic hydration of an epoxide intermediate) preventing the covalent binding of the reactive products. Another interesting phenomenon is the 3-methylcholanthrene-induced shift in the specificty for the *in vivo* target organ alkylation and toxicity of 4-ipomeanol from the lung to the liver (1,4). It seemed likely that this alteration resulted from an enhanced rate of activation of 4-ipomeanol in the livers of the 3-methylcholanthrene-treated rats. However, *in vivo* pretreatments with either 3-methylcholanthrene or phenobarbital markedly enhanced the *in vitro* covalent binding of 4-ipomeanol in rat hepatic microsomes (3), although phenobarbital did not shift the target organ specificity for alkylation and toxicity in rats *in vivo* (1,4).

Therefore, it was of interest to compare the relative amounts of covalently bound 4-ipomeanol with the total amounts of 4-ipomeanol metabolized in pulmonary and hepatic microsome preparations from rats pretreated with phenobarbital, 3-methylcholanthrene, or vehicle only.

MATERIALS AND METHODS

Chemicals - The synthesis, and verification of chemical and radiochemical purity of 4-ipomeanol-5-^{14}C have been described (6). The specific activity of the compound used in these studies was 0.6 mCi/mmole. All other chemicals were purchased from Sigma Chemical Company (St. Louis, Mo.).

Animals, pretreatments, and preparation of microsomes - Male, Sprague-Dawley rats (150-200g) were obtained from Taconic Farms (Germantown, N.Y.). Phenobarbital pretreatment consisted of twice daily i.p. injections (solution in saline) of 50mg/kg for 5 days. 3-Methylcholanthrene (dissolved in corn oil) was given i.p. as a single dose of 45mg/kg. Control animals received injections of vehicle only. Animals were sacrificed by decapitation 24 hours after the final pretreatment and pulmonary and hepatic microsomes were prepared as described previously (3). Microsomal cytochrome P-450 levels were checked by the method of Omura and Sato (5).

Assays - Triplicate incubation mixtures contained 1-2 mg of microsomal protein and 1 µmol NADPH (NADPH was deleted from control incubations performed in duplicate) in 1.0 ml of 0.05 M phosphate buffer, pH 7.4. Protein concentrations were measured by the Lowry method (6) using bovine serum albumin as the standard. Reactions were started by addition of radiolabeled 4-ipomeanol (1 µmol) and terminated, after incubation at 37° for 6 min, by the addition of 2 volumes of cold methanol. The precipitates were assayed for covalently bound radioactivity

as previously described (3). 4-Ipomeanol remaining in the methanolic supernatants was assayed by high pressure liquid chromatography (Waters Model ALC 202). Aliquots (100 µl) of the supernatants were injected onto a 0.39 X 30 cm µBondapak/ C18 column (Waters) and eluted with 35% methanol/water at a flow rate of 1 ml/min. The 4-ipomeanol had a retention time of 8.5 min and was qualitatively detected by its UV absorbance at 254 nm, and quantitated by collection and liquid scintillation counting. Recovery of unmetabolized 4-ipomeanol using this precedure was essentially quantitative. Representative samples were subjected to further analyses by thin-laye chromatography and gas chromatography/mass spectroscopy under conditions previously described (3); these analyses confirmed that the radioactive 4-ipomeanol fraction contained only 4-ipomeanol.

RESULTS AND DISCUSSION

Table 1 summarizes the results of these experiments. In the control animals, as well as in the inducer-pretreated animals, both the NADPH-dependent total metabolism (disappearance) and the covalent binding values per nmol P-450 invairably were much higher in pulmonary microsomes compared to hepatic microsomes. Moreover, the ratios of covalent binding to total metabolism were similar in the pulmonary and hepatic microsomal preparations, and they were not substantially altered by pretreatments either with phenobarbital or with 3-methylcholanthrene. These results confirm that the previously-documented high rate of *in vitro* covalent binding of 4-ipomeanol in rat pulmonary microsomes compared to rat liver microsomes reflects a high rate of oxidative metabolic activation of the compound, rather than a relative deficiency of a microsomal detoxication pathway. The *in vivo* pretreatments with phenobarbital or 3-methylcholanthrene increased the *in vitro* covalent binding and the NADPH-dependent total metabolism of 4-ipomeanol in rat hepatic microsomes, when the data were expressed per mg microsomal protein; however, when expressed per nmol P-450 the total amounts metabolized or covalently bound were unchanged by phenobarbital, but were decreased significantly by 3-methylcholanthrene. Phenobarbital did not alter significantly the total metabolism or covalent binding of 4-ipomeanol in pulmonary microsomes; however, 3-methylcholanthrene decreased both the total metabolism and the covalent binding, when the values were expressed with reference either to the total microsomal protein or to the cytochrome P-450 content. These studies are consistent with the view that the 3-methylcholanthrene-induced *in vivo* enhancement of hepatic alkylation and toxicity

TABLE 1

In vitro covalent binding and total metabolism (disappearance) of 4-ipomeanol in pulmonary and hepatic microsomes from rats pretreated with phenobarbital, 3-methylcholanthrene, or vehicle only

Source of microsomes	In vivo pretreatment	Covalent binding[a] per mg microsomal protein	Covalent binding[a] per nmol P-450 [b]	Total metabolism[a] per mg microsoal protein	Total metabolism[a] per nmol P-450[b]	Ratio: covalent binding / total metabolism
Liver	Vehicle only	5.72	5.66	16.02	15.86	0.36
	Phenobarbital	13.12[c]	5.25	41.21[c]	16.48	0.32
	3-Methylcholanthrene	7.61[c]	3.81[c]	18.58[c]	9.29[c]	0.41
Lung	Vehicle only	3.18	39.75	6.80	85.00	0.47
	Phenobarbital	3.63	45.37	7.05	88.12	0.51
	3-Methylcholanthrene	2.71[c]	27.10[c]	6.34[c]	63.40[c]	0.40

[a] Values are means of triplicate determinations; standard errors (omitted for clarity) averaged less than 10% of the respective mean values.

[b] P-450 values were lung: 0.08, 0.08, and 0.10 nmol/mg protein and liver: 1.01, 2.50, and 2.00 nmol/mg protein, for rats pretreated with vehicle only, phenobarbital, or 3-methylcholanthrene, respectively.

[c] Significantly different from respective control value ($P < 0.05$).

by 4-ipomeanol results from enhanced rates of formation of the ultimate toxic metabolite in the liver, rather than a major change in the balance between microsomal pathways, detectable in the present in vitro systems, for the formation and detoxication of the active metabolite. The failure of phenobarbital to shift the in vivo target organ specificity for covalent binding and toxicity of 4-ipomeanol cannot be explained by a failure of the pretreatment to enhance the hepatic microsomal enzyme activity mediating the formation of the hepatotoxic 4-ipomeanol metabolite (the in vitro covalent binding activity actually was enhanced to a greater extent by phenobarbital than by 3-methylcholanthrene), nor to a major alteration in the balance between microsomal toxication and detoxication metabolic pathways which could be measured under the conditions studied. The most likely explanation for the difference in the in vivo effects of phenobarbital compared to 3-methylcholanthrene is that, in vivo, phenobarbital induces a detoxifying metabolic pathway to a greater extent than the toxic pathway (4). Some support for this hypothesis recently was obtained in studies showing a large enhancement by phenobarbital, but not by 3-methylcholanthrene, of the urinary excretion of ipomeanol-4-glucuronide in rats (7). Other studies have indicated also that the pattern of adducts formed in rat hepatic and pulmonary microsomal suspensions in the presence of 4-ipomeanol and glutathione were qualitatively similar in preparations from control animals and from animals pretreated with phenobarbarbital or 3-methylcholanthrene (8); these studies suggested the reactive 4-ipomeanol metabolites produced in the liver and the lungs of control rats, or of rats pretreated with either inducer, were qualitatively similar.

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