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EFFECTS OF ANTI-NADPH-CYTOCHROME c REDUCTASE AND ANTI-CYTOCHROME b_5 ANTIBODIES ON THE HEPATIC AND PULMONARY MICROSOMAL METABOLISM AND COVALENT BINDING OF THE PULMONARY TOXIN 4-IPOMEANOL

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SUMMARY: An antibody prepared against purified rat liver NADPH-cytochrome c reductase inhibited both the pulmonary and hepatic microsomal covalent binding of 4-ipomeanol as well as the respective NADPH-cytochrome c reductase activities, findings which are consistent with previous studies which indicated the participation of cytochrome P450 in the metabolic activation of the toxin. An antibody prepared against purified rat liver cytochrome b_5, which strongly inhibited both the rat hepatic and pulmonary NADH-dependent cytochrome c reductases, and was inactive against the respective NADPH-dependent cytochrome c reductases, had little effect on metabolic activation of 4-ipomeanol by hepatic microsomes, but strongly inhibited both the NADH-supported and the NADPH-supported pulmonary microsomal metabolism and covalent binding of the compound. These results suggest that metabolic activation of 4-ipomeanol involves a two-electron transfer in which transfer of the second electron via cytochrome b_5 is rate-limiting in lung microsomes.

INTRODUCTION

In the current view of cytochrome P-450-dependent drug oxidations by liver microsomes, cytochrome P-450, after binding with a substrate, is initially reduced by accepting one electron from NADPH-cytochrome P-450 reductase. Upon binding with molecular oxygen and the receiving of a second electron from NADPH or NADH, an "active oxygen" is formed and one atom of the oxygen is inserted into the substrate. For some drug oxidations by hepatic microsomes, the transfer of the second electron has been proposed as a rate-limiting step, and several studies have suggested that cytochrome b_5

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participates in this process (1). The possible role of cytochrome b_5 in cytochrome P-450-dependent monooxygenase reactions in microsomes from extrahepatic tissues has received little attention. Sasame et al. (2) reported evidence for the involvement of cytochrome b_5 in the renal microsomal hydroxylation of an endogenous substrate, lauric acid, but no previous studies suggested a role for cytochrome b_5 in the oxidation of xenobiotic substrates by microsomal preparations from extrahepatic tissues. To explore this question we have compared the effects of antibodies against purified NADPH-cytochrome c reductase and cytochrome b_5 on the in vitro metabolism and covalent binding of the pulmonary toxin 4-ipomeanol (1-[3-furyl]-4-hydroxypentanone) (3-5) in hepatic and pulmonary microsomes of rats.

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 and preparation of microsomes - Microsomes were prepared as previously described (6) from lungs and livers of male, Sprague-Dawley rats (150-180 g) obtained from Taconic Farms (Germantown, N.Y.).

Antibodies - The preparation of antibodies against purified rat liver NADPH-cytochrome c reductase and cytochrome b5 from trypsin-solubilized rat liver microsomes have been described (2). The corresponding γ-globulin fractions obtained similarly from nonimmunized animals were used for control incubations.

Assays - Incubation mixtures contained 1-2 mg of microsomal protein in 1 ml of 0.05 M phosphate buffer, pH 7.4. The microsomal suspensions were pre-incubated 10 min at room temperature with the antibodies; control incubation mixtures were treated similarly with an equivalent amount of control γ-globulin. Protein concentrations were measured by the Lowry method (7) using bovine serum albumin as the standard. Reactions with 4-ipomeanol were started by addition of the radiolabeled substrate (1 μmole) 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 (6). 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/C_{18} 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. Representative samples were subjected to further analyses by thin-layer chromatography and gas chromatography/mass spectroscopy under conditions previously described (3); these analyses indicated that the radioactive fractions measured in this way contained only 4-ipomeanol. Cofactor-dependent covalent binding and/or disappearance of 4-ipomeanol was determined by comparison of amounts of the

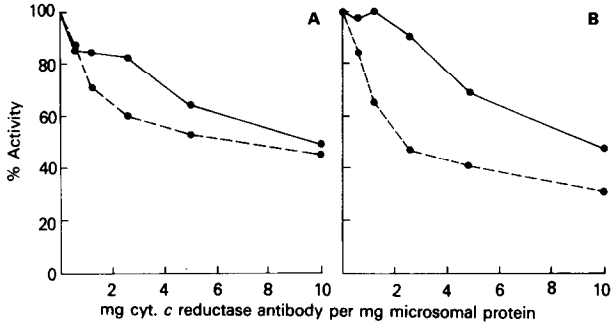

Fig. 1 Inhibition of NADPH-cytochrome c reductase (broken lines) and NADPH-dependent covalent binding of 4-ipomeanol (solid lines) in lung (A) and liver (B) microsomes, by an antibody prepared against purified NADPH-cytochrome c reductase.

compound covalently bound and/or remaining in the supernatants from incubations run with and without the presence of the appropriate cofactors (NADPH and/or NADH at 2 x 10^{-3}M concentrations). Recovery of unmetabolized 4-ipomeanol using this procedure was essentially quantitative.

Other aliquots of the γ-globulin-microsomal mixtures were assayed for NADH-dependent and NADPH-dependent cytochrome c reductase activities according to the method of Williams and Kamin (8) using horse heart cytochrome c as the substrate.

All assays were run in triplicate. In experiments where results have been expressed as % activities, the average control values for the liver and lung microsomal preparations respectively were: NADPH-cytochrome c reductase, 190 and 74 nmoles/mg/min; NADH-cytochrome c reductase, 1600 and 575 nmoles/mg/min; 4-ipomeanol covalent binding, 3.9 and 4.7 nmoles/mg/5 min. Standard errors for all assays averaged less than 10% of the respective mean values obtained.

RESULTS AND DISCUSSION

Inhibition of NADPH-cytochrome c reductase activities, by an antibody prepared against rat liver NADPH-cytochrome c reductase, was accompanied by inhibition of the activation of 4-ipomeanol both in lung (graph A) and liver (graph B) microsomal incubation mixtures (Figure 1).

Since NADH-cytochrome c reductase consists of a flavoprotein and cytochrome b_5, the anti-cytochrome b_5 antibody activity was assayed by its inhibitory effect on NADH-mediated reduction of cytochrome c (2). The anti-cytochrome b_5 antibody strikingly inhibited both the NADH-mediated reduction of cytochrome c, and the NADPH-mediated metabolic activation of 4-ipomeanol

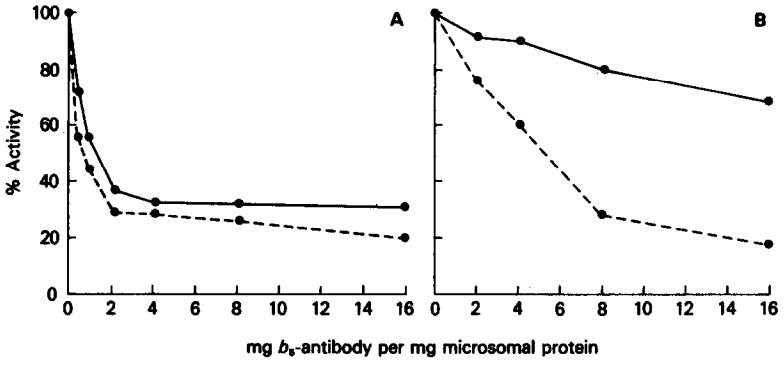

Fig. 2 Effect of an antibody to cytochrome b_5 on NADH-cytochrome c reductase (broken lines) and on NADPH-dependent covalent binding of 4-ipomeanol (solid lines) in lung (A) and liver (B) microsomes.

by lung microsmes (Figure 2A); indeed the two respective inhibition curves shown in figure 2A are practically superimposable. In contrast, the inhibition of NADH-mediated cytochrome c reduction in liver microsomes was accompanied by only very small decreases in the rate of 4-ipomeanol activation (Figure 2B). Pre-incubation of the anti-cytochrome b_5 antibody with cytochrome b_5 (purified from trypsin treated rat liver microsomes) abolished its inhibitory effect on NADH-mediated reduction of cytochrome c and on the activation of 4-ipomeanol (Figure 3).

Both the covalent binding and the disappearance of 4-ipomeanol were measured in incubation mixtures of lung microsomes in the presence of NADPH or NADH, or a mixture of the two cofactors (Table 1). The anti-cytochrome b_5 antibody markedly inhibited the NADH-cytochrome c reductase. The antibody also inhibited (60-70%) both the disappearance and the covalent binding of 4-ipomeanol, whether the cofactor added was NADPH or NADH, or a mixture of the two.

It is of interest that NADH gave nearly 30% of the activities, expressed either as covalent binding or as total metabolism of 4-ipomeanol, that were obtained with NADPH as cofactor (Table 1). Moreover, the stimulatory effects of NADPH and NADH were not synergistic; indeed they were not even additive

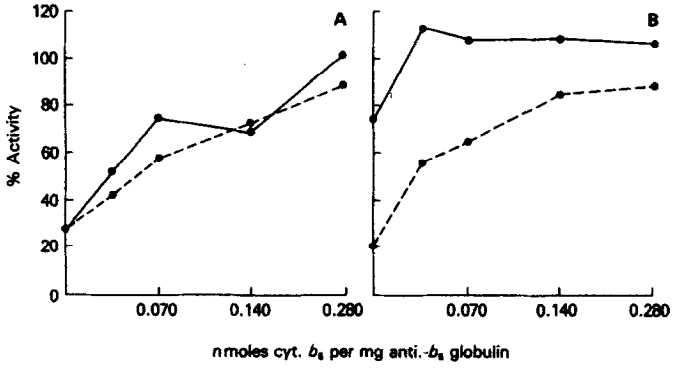

Fig. 3 Effect of preincubations (25 min) with purified cytochrome b_5 on the ability of cytochrome b_5 antibody to inhibit NADH-cytochrome c reductase (broken lines) and NADPH-dependent covalent binding of 4-ipomeanol (solid lines) in lung (A) and liver (B) microsomes.

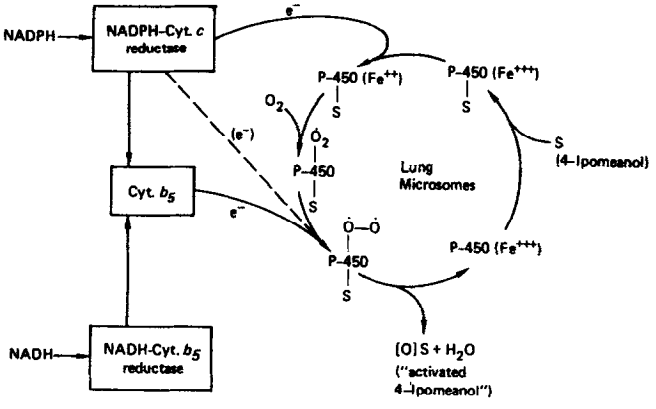

Fig. 4 Schematic representation showing potential roles of cytochromes P-450 and b_5 in the metabolic activation of 4-ipomeanol by pulmonary microsomes.

(Table 1). These findings were similar to those obtained previously for the renal microsomal hydroxylation of lauric acid (2).

Based on the results of these studies, in conjunction with past studies which showed that a $CO:O_2$ atmosphere inhibited the microsomal metabolism of 4-ipomeanol (6), the reactions are apparently catalyzed by a cytochrome P450. From the current view of the mechanism of these enzyme systems in liver

TABLE 1

Effect of an anti-cytochrome b_5 antibody on disappearance and covalent binding of 4-ipomeanol and on cytochrome c reductase activities in lung microsomal incubation mixtures containing NADPH, NADH, or a mixture of NADPH and NADH.

Assay	Cofactor(s)	-Ab	+Ab	% Inhibition
Covalent Binding	NADPH + NADH	4.79	1.97	60.4
of 4-Ipomeanol	NADPH	4.20	1.73	58.8
(nmoles/mg prot./6 min)	NADH	1.39	0.34	75.5
Disappearance of	NADPH + NADH	9.10	3.90	57.1
4-Ipomeanol	NADPH	8.58	2.75	70.0
(nmoles/mg prot./6 min)	NADH	3.72	0.85	77.2
Cyt. c reductase				
(nmoles reduced/mg	NADPH	77.8	72.0	7.4
prot./min)	NADH	510.0	171.4	66.4

microsomes (9), we propose a mechanism for the in vitro microsomal activation of 4-ipomeanol, schematically represented in figure 4, which involves a two-electron transfer, in which one or both electrons can be derived via NADPH-cytochrome c reductase, but in which transfer of the second electron via cytochrome b_5 is rate-limiting in lung microsomes. We suggest that future studies should address the possible toxicologic significance of cytochrome b_5 in the in vivo metabolic activation of xenobiotics, especially in extra-hepatic tissues, such as the lung and the kidney.

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