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PRODUCTION OF HYDROXYL RADICALS AND THEIR ROLE IN THE OXIDATION OF ETHANOL BY A RECONSTITUTED MICROSOMAL SYSTEM CONTAINING CYTOCHROME P-450 PURIFIED FROM PHENOBARBITAL-TREATED RATS

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SUMMARY

Ethanol oxidation by a reconstituted system composed of cytochrome P-450 purified from liver microsomes of phenobarbital-treated rats, NADPH-cytochrome c reductase, phospholipid and NADPH was inhibited by a series of hydroxyl radical scavenging agents. Inhibition was competitive with respect to ethanol and was specific in the sense that the metabolism of aminopyrine or benzphetamine by the reconstituted system was not affected by the scavengers. The generation of ethylene gas from 2-keto-4-thiomethylbutyric acid in an ethanol-sensitive manner provided chemical evidence consistent with the ability of the reconstituted system to generate hydroxyl radicals. These results suggest that the oxidation of ethanol by the reconstituted system reflects the interaction of ethanol with hydroxyl radicals generated during NADPH oxidation.

INTRODUCTION

The molecular mechanism underlying the oxidation of primary aliphatic alcohols by liver microsomes is not understood. Recent experiments indicated a role for hydroxyl radicals (•OH) in the microsomal alcohol-oxidizing system (1-4). A series of hydroxyl radical scavenging agents competitively inhibited the oxidation of ethanol and 1-butanol by microsomes (1-3). Neither catalase-dependent oxidation of ethanol nor microsomal metabolism of aminopyrine or aniline were affected by the scavengers (1,2). The transformation of a series of •OH scavengers to appropriate products provided chemical evidence consistent with the production of •OH during microsomal electron transfer (4). These observations

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suggested that the NADPH-dependent oxidation of alcohols by liver microsomes reflected the ability of the alcohols to interact with •OH generated by the microsomes. Since microsomes are contaminated with catalase which can also oxidize ethanol to acetaldehyde, the microsomal studies quoted above were carried out either in the presence of azide, an inhibitor of catalase, or with 1-butanol, an alcohol which is not an effective substrate for the peroxidatic activity of catalase.

In view of the complexities which may arise from the presence of catalase, it appeared important to evaluate the role of •OH in a catalase-free ethanol-oxidizing system. In recent years, highly purified preparations of microsomal cytochrome P-450 have been described (5-13) which are devoid of alcohol dehydrogenase and catalase activities (14-17). Reconstitution experiments employing cytochrome P-450, NADPH-cytochrome c reductase and phospholipid have demonstrated the NADPH-dependent oxidation of ethanol to acetaldehyde (14-17). The aim of the present study was to evaluate the role of •OH in ethanol oxidation by a reconstituted cytochrome P-450 system.

METHODS

Cytochrome P-450 from phenobarbital-treated rats and NADPH-cytochrome c reductase were prepared by methods described elsewhere (17). Ethanol oxidation was carried out in a system containing 0.1 nmol cytochrome P-450, 0.3 nmol NADPH-cytochrome c reductase, 20 ug dilauroylphosphatidylcholine, 100 mM potassium phosphate buffer, pH 7.4, 50 mM ethanol and 1 mM NADPH in a final volume of 1.0 ml. Reactions were initiated with ethanol, followed immediately with NADPH and were carried out for 30 min. at 37° in sealed 18 ml screw-capped tubes. Reactions were terminated by the injection of $HClO_4$ and thiourea (final concentrations of 0.1M and 0.01M respectively). Acetaldehyde production was determined by a head space method (18). Ethylene generation from 2-keto-4-thiomethylbutyric acid (KTBA) was determined in the enzyme system described above by a gas chromatographic method described elsewhere (4). Aminopyrine demethylase activity was determined using 10 mM aminopyrine in place of ethanol; formaldehyde was determined with the Nash reagent as previously described (2). Benzphetamine demethylase activity was determined by a radioactive procedure, using ^{14}C-benzphetamine (19). The data presented in the tables are from a typical experiment carried out in duplicate.

TABLE I

Effect of Hydroxyl Radical Scavenging Agents on Ethanol Oxidation, Benzphetamine Demethylation and Aminopyrine Demethylation

Scavenger	Concentration (mM)	Turnover Number (nmol/min/nmol cytochrome P-450)		
		Ethanol Oxidation	Benzphetamine Demethylase	Aminopyrine Demethylase
Control	—	27.7	22.1	11.7
Dimethylsulfoxide	5	21.7	20.1	-
	15	15.4	20.8	13.4
	30	10.9	20.8	15.1
	60	6.3	20.5	-
Mannitol	5	22.8	20.6	-
	15	20.0	20.6	10.3
	30	13.0	20.6	11.1
	60	10.5	20.4	10.5
KTBA	3	23.1	-	-
	5	18.9	21.8	9.2
	10	16.5	21.6	9.5
	20	11.2	21.2	-

RESULTS AND DISCUSSION

The addition of ethanol to the reconstituted system resulted in the production of acetaldehyde (Table I). Little or no acetaldehyde was produced when any of the following components were omitted: ethanol, NADPH, NADPH-cytochrome c reductase or phospholipid. Some ethanol was oxidized in the absence of cytochrome P-450 (about 25% of the rate of the complete reconstituted system). This has been observed previously (17) and may be related to recent studies in which production of •OH by NADPH-cytochrome c reductase in the presence of NADPH and phospholipid was observed by electron spin resonance (20). The effect of dimethylsulfoxide, mannitol and KTBA on ethanol oxidation by the reconstituted system was determined. These compounds are

effective •OH scavenging agents and were previously shown to decrease ethanol oxidation by intact microsomes (1, 2, 4). All three scavengers were effective inhibitors of ethanol oxidation in the reconstituted system (Table I).

The reconstituted system was also capable of metabolizing benzphetamine or aminopyrine (Table I). These drug metabolizing activities were not affected by concentrations of the scavengers which inhibited ethanol oxidation (Table I). Previous studies showed that the scavengers had no effect on aminopyrine demethylation or aniline hydroxylation by intact microsomes (1, 2). These results indicate that the action of the scavengers in the reconstituted system is relatively specific, and that alcohol oxidation can clearly be dissociated from the metabolism of other drugs.

To study the kinetics of inhibition in the reconstituted system, the concentration of ethanol was varied from 12.5 to 100 mM. The amount of acetaldehyde produced increased as the concentration of ethanol was raised (Table II). The Km for ethanol was about 25 mM while Vmax was about 40 nmol/min/nmol cytochrome P-450 (Lineweaver-Burk plot). The inhibition of ethanol oxidation by KTBA was dependent on the concentration of ethanol; KTBA was more inhibitory at the lower concentrations of ethanol (Table II). A Lineweaver-Burk plot of the data of Table II indicated that KTBA, at concentrations of 5, 10 and 20 mM increased the Km for ethanol to values of about 50, 55 and 75 mM, respectively, without significantly changing the Vmax (values of 37, 35, and 33 respectively), suggesting that KTBA was a competitive inhibitor of the reconstituted ethanol oxidizing system. Studies reported elsewhere also indicate that KTBA was a competitive inhibitor of ethanol oxidation by intact microsomes (21).

TABLE II

Effect of Ethanol Concentration on the Inhibition of Ethanol Oxidation by KTBA

KTBA Concentration (mM)	Acetaldehyde Generation[a] and (% Inhibition) Ethanol Concentration (mM) 12.5	25	50	100
0	14.4	22.4	30.1	31.9
5	8.5 (41)	14.4 (36)	20.7 (31)	26.3 (18)
10	7.4 (49)	11.6 (48)	17.9 (41)	21.0 (34)
20	4.9 (66)	8.1 (64)	13.0 (57)	18.2 (43)

[a] the rate of acetaldehyde generation is expressed as nmol acetaldehyde formed/min/nmol cytochrome P-450

Ethylene gas is generated when KTBA reacts with •OH (4). The formation of ethylene gas has been observed with model •OH-generating systems as well as during microsomal NADPH electron transfer (4, 21). When KTBA was added to the reconstituted system, ethylene production was also observed (Table III). Production of ethylene required the

TABLE III

Effect of Ethanol Concentration on the Rate of Ethylene Generation from KTBA

Ethanol Concentration (mM)	Ethylene Generation[a] and (% Inhibition) KTBA Concentration (mM) 3	10	20
0	3.95	5.62	6.02
12.5	2.51 (37)	4.29 (24)	4.88 (19)
25	1.65 (58)	3.50 (38)	4.00 (34)
50	0.91 (77)	2.42 (57)	3.13 (48)
100	0.54 (86)	1.50 (73)	2.32 (61)

[a] ethylene generation is expressed as nmol ethylene formed/min/nmol cytochrome P-450

presence of NADPH, KTBA and the components of the reconstituted system. In studies with intact microsomes, ethylene generation was suppressed by competing hydroxyl radical scavengers (4, 21). In the reconstituted system, ethylene generation was also suppressed by a competing scavenger, namely, ethanol (Table III). Moreover, the inhibition of ethylene generation from KTBA by ethanol was dependent on the concentration of KTBA; ethanol was more inhibitory at the lower concentrations of KTBA (Table III). These results are similar to those described in Table II, where the inhibition of ethanol oxidation by KTBA was dependent on the concentration of ethanol. A consistent interpretation of these data is that the two •OH scavenging agents are competing either for a common binding site or for a common intermediate, i.e. •OH.

In summary, these data indicate that the oxidation of ethanol by a reconstituted system containing cytochrome P-450 from phenobarbital-treated rats is inhibited by a series of •OH scavenging agents. The kinetics of inhibition by one scavenger, KTBA, was competitive with ethanol. The inhibition was specific in the sense that the the metabolism of two typical substrates (aminopyrine and benzphetamine) for mixed function oxidase activity was not affected by the scavengers. The generation of ethylene from KTBA, and its inhibition by ethanol are supportive evidence for the production of •OH by the reconstituted system. Recent physical studies, using electron spin resonance spectrometry, also provide evidence for the generation of •OH by a reconstituted microsomal system (20). A recent report (22) has confirmed that ethanol oxidation by a reconstituted system containing cytochrome P-450 from livers of chronic ethanol-fed rats is inhibited by dimethylsulfoxide, benzoate, mannitol and thiourea, all of which are •OH scavenging agents. Taken as a whole, these data suggest that 1) the oxidation of ethanol by

the reconstituted system may reflect the interaction of ethanol with •OH generated during NADPH oxidation by this system and 2) at least a portion of the ethanol oxidase activity of liver microsomes may be due to the production of •OH generated by the components of the cytochrome P-450 electron transport system.

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