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IMMUNOCHEMICAL STUDIES ON THE METABOLISM OF NITROSAMINES BY ETHANOL-INDUCIBLE CYTOCHROME P-450*

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SUMMARY: The ethanol-induced rabbit liver microsomal cytochrome P-450, P-450_{LM3a}, has been shown previously to efficiently catalyze the demethylation of N-nitrosodimethylamine (NDMA) with a K_m of 2.9 mM. Since the predominant K_m in hepatic microsomes from ethanol-treated rabbits is 0.07 mM, the role of P-450_{LM3a} in the activation of this carcinogen has been uncertain. In the present study, antibodies to P-450_{LM3a} were shown to almost completely inhibit NDMA demethylation by the purified P-450 in a reconstituted system as well as the low-K_m activity of liver microsomes from control or ethanol-treated rabbits. In contrast, the antibody did not inhibit the high-K_m NDMA demethylase activity in the microsomes. These results indicate that P-450_{LM3a} is the major P-450 responsible for the low-K_m NDMA demethylase activity. In addition, evidence is provided for the existence of a cytochrome immunochemically similar to P-450_{LM3a} in liver microsomes from rats, mice, and guinea pigs that effectively catalyzes the demethylation of NDMA.

Nitrosamines, a group of widely occurring carcinogens, are known to require metabolic activation for conversion to their carcinogenic and cytotoxic forms (1,2). The activation process, generally involving the oxygenation of the α -carbon, has been shown to be a cytochrome P-450-dependent reaction (2-6). Nevertheless, the enzymology of nitrosamine metabolism is not clearly understood because the metabolism of N-nitrosodimethylamine (NDMA), a commonly studied prototype nitrosamine, is different from many well characterized monooxygenase reactions (7-9). Previously, we have studied the induction of a high affinity

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Abbreviations used: NDMA, N-nitrosodimethylamine; NDMAd, N-nitrosodimethylamine demethylase; control and ethanol-induced microsomes, hepatic microsomes from untreated and ethanol-treated rabbits, respectively.

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microsomal NDMA demethylase (NDMAd), with a K_m of 0.07 mM, in rats by ethanol, acetone, isopropanol, and pyrazole, as well as by conditions such as fasting and diabetes (10-14). The results suggest that the enhanced microsomal NDMAd activity is due to the induction of a specific P-450 isozyme which is efficient in catalyzing the metabolism of NDMA. Direct evidence for this hypothesis has been obtained recently by a study of the metabolism of nitrosamines in reconstituted systems with purified rabbit liver P-450 isozymes (5). The ethanol-induced form, P-450_{LM3a}, is much more active (lower K_m and higher V_{max}) than five other purified isozymes in catalyzing the demethylation and denitrosation of NDMA. The other isozymes, however, may be more active with other nitrosamines; for example, the phenobarbital-induced P-450_{LM2} is more active than P-450_{LM3a} in catalyzing the metabolism of N-nitrosomethylaniline (5).

An intriguing problem in the enzymology of NDMA metabolism is the cause of the multiple K_m values found for liver microsomes (2,14). Similar to the situation in rats, at least 3 K_m values (0.07, 0.27, and 36.8 mM) have been observed in liver microsomes from control rabbits. The lowest K_m form is predominant in the microsomes of ethanol-treated rabbits (5). The purified P-450_{LM3a}, however, displays a single K_m of 2.9 mM which is higher than the K_m of 0.07 mM in microsomes. It is not known whether this low-K_m microsomal NDMAd is due to P-450_{LM3a} or other enzyme species. A similar discrepancy was observed in ethanol-induced rats between a partially purified P-450 isozyme and microsomes (Tu and Yang, submitted for publication). In order to resolve this problem and to elucidate further the role of P-450 isozymes in nitrosamine metabolism, an immunochemical study was undertaken using antibodies prepared against P-450_{LM3a}.

MATERIALS AND METHODS

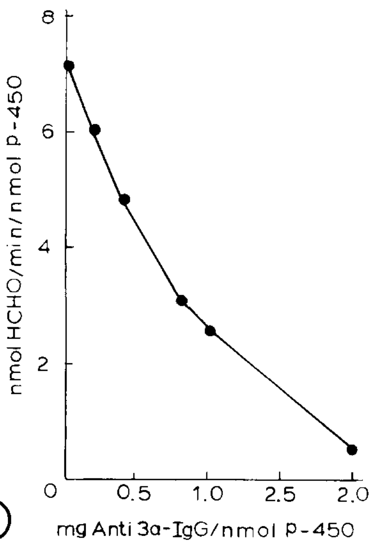
Microsomal Enzymes. Hepatic microsomes were prepared from control or ethanol-treated adult New Zealand male rabbits (2.0 to 2.5 kg) according to previous procedures (15,16). P-450_{LM3a} was purified from ethanol-induced microsomes as described previously (16). The P-450_{LM3a} preparation was electrophoretically homogeneous with a specific content of 19.0 nmol P-450 per mg protein. Electrophoretically pure NADPH-P-450 reductase was prepared from rabbit liver microsomes (17). The specific activity of the reductase was 54 units (μmol cytochrome c reduced/min/mg protein).

Antibody. The antibody to P-450_{LM3a} was produced by immunization of yearling female sheep; the IgG fractions from immune and pre-immune sera were isolated by ammonium sulfate precipitation and DEAE-cellulose column chromatography (18). The final IgG preparation (anti-3a IgG) did not exhibit significant cross-reactivity with rabbit P-450 isozymes LM_2, LM_{3b}, LM_{3c}, LM_4 or LM_6. A detailed characterization of this antibody preparation is published elsewhere (18).

Enzyme assays. NDMAd activity was assayed by HCHO formation as described previously (13). The reaction mixture contained microsomes (0.2 nmol P-450) or the reconstituted monooxygenase system (0.1 nmol P-450, 0.63 unit NADPH-P-450 reductase, 7.5 μg dilauroylphosphatidylcholine), anti-3a IgG, NDMA, and an NADPH-generating system in a final volume of 0.25 ml. Pre-immune IgG was added to the incubations as a control so that the total IgG content was the same in all the incubation tubes in the experiment. N-Nitrosomethylaniline and benzphetamine demethylase activities were also assayed by HCHO formation (13).

RESULTS AND DISCUSSION

As shown in Figure 1, anti-3a IgG effectively inhibited the NDMAd activity of P-450_{LM3a} in a reconstituted monooxygenase system. It is estimated that a 50% inhibition could be produced by 0.7 mg of anti-3a IgG per nmol P-450. At 2 mg/nmol P-450, the antibody caused a 93% inhibition. NDMA was used at a concentration of 5 mM, about 1.7 times the K_m of NDMAd for P-450_{LM3a}. The inhibitory



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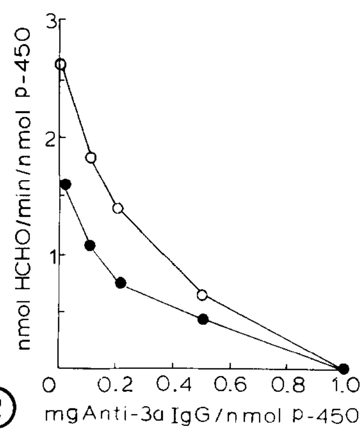

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Figure 1. Inhibition of reconstituted NDMA demethylase activity by anti-3a IgG. The reaction mixture contained 0.1 nmol P-450_{LM3a}, 0.63 unit NADPH-P-450 reductase, 7.5 μg dilauroylphosphatidylcholine, anti-3a IgG at the indicated concentration, an NADPH-generating system, and 5 mM NDMA in a final volume of 0.25 ml. The incubation time was 20 min.

Figure 2. Inhibition of rabbit microsomal NDMA demethylase activity by anti-3a IgG. The reaction mixture contained ethanol-induced rabbit liver microsomes (0.25 nmol P-450) and 0.2 mM NDMA (●—●) or 5 mM NDMA (○—○) in a final volume of 0.25 ml. Other conditions were similar to those for Figure 1.

TABLE I IMMUNOCHEMICAL INHIBITION OF RABBIT MICROSOMAL MONOOXYGENASE ACTIVITIES

	Control microsomes			Ethanol-induced microsomes		
Substrate	Without antibody	With antibody	% Inhibition	Without antibody	With antibody	% Inhibition
NDMA, 5 mM	0.45	0.13	71	2.39	0.24	90%
NDMA, 200 mM	1.56	1.21	22	2.59	1.18	54%
Nitrosomethyl-aniline, 1 mM	0.65	0.42	35	2.21	1.61	27%
Benzphetamine, 1 mM	2.29	1.79	22	4.28	2.24	47%

The reaction mixture contained ethanol-induced or control rabbit liver microsomes (0.25 nmol P-450) and different substrates. Anti-3a IgG was added at a concentration of 1 mg/nmol P-450. Other conditions are described in Materials and Methods. The monooxygenase activities (in nmol HCHO/min/nmol P-450) and percent inhibition are shown.

action of anti-3a IgG was also studied with ethanol-induced microsomes (microsomes from ethanol-treated rabbits) at NDMA concentrations of 0.2 and 5.0 mM (Figure 2). At 0.2 mg anti-3a IgG/nmol P-450, the inhibition was 55 and 47% for reactions with 0.2 and 5.0 mM NDMA, respectively. At 1.0 mg anti-3a IgG/nmol P-450, the antibody almost completely inhibited the NDMAd activity at both substrate concentrations. An NDMA concentration of 0.2 mM was selected because it should produce 74% of the V_{max} of the low-K_m NDMAd (K_m = 0.07 mM) and only 6% of the V_{max} of the demethylase activity with a K_m of 2.9 mM. The results indicate that even though purified $P\text{-}450_{LM3a}$ has a K_m of 2.9 mM, anti-3a IgG is very effective in inhibiting the microsomal low-K_m NDMAd activity.

The specificity of the inhibitions was studied with both ethanol-induced microsomes and control microsomes (microsomes from untreated rabbits) and with different substrates (Table 1). When assayed with 5 mM NDMA, 1 mg anti-3a IgG/nmol P-450 inhibited 71 and 90% of the NDMAd activity of the control and ethanol-induced microsomes, respectively. The inhibition was 22 and 54%, respectively, for these two microsomal preparations when 200 mM NDMA was used. The results are consistent with the hypothesis that $P\text{-}450_{LM3a}$ is responsible for a larger portion of the NDMAd activity in ethanol-induced microsomes than in control microsomes and that, at 200 mM, NDMA is metabolized by other P-450 isozymes in addition to $P\text{-}450_{LM3a}$. The other forms appeared to produce about

TABLE II. INHIBITION OF NDMAd ACTIVITY OF MICROSOMES FROM RATS, MICE, AND GUINEA PIGS BY ANTI-3a IgG

Source of Microsomes	Activity		% Inhibition
	Without antibody	With antibody	
Control rats	1.34	0.39	71
Ethanol-treated rats	6.73	0.55	92
Control mice	2.28	0.21	91
Control guinea pigs	2.23	0.65	71
Acetone-treated guinea pigs	8.45	2.01	76

The reaction mixture contained microsomes (0.25 nmol p-450) and 5 mM NDMA. Anti-3a IgG was added at a concentration of 1 mg/nmol P-450. Other conditions are described in Materials and Methods. NDMAd activities are shown in nmol HCHO/min/nmol P-450.

40% of the NDMAd activity in the ethanol-induced microsomes when assayed with 200 mM NDMA; yet this substrate concentration did not produce a much higher demethylase activity than did 5 mM NDMA. This suggests substrate inhibition of the low-K_m form of NDMAd, in agreement with previous postulations (5,10,13). Rabbit P-450_{LM2} (phenobarbital inducible), which has a very high NDMAd K_m value (5), was not inhibited by anti-3a IgG at 1 mg/nmol P-450 (data not shown). The metabolism of N-nitrosomethylaniline is known to be catalyzed more efficiently by P-450_{LM2} than by P-450_{LM3a} (5). The nitrosomethylaniline demethylase was inhibited 35 and 27% by the antibody in assays containing control and ethanol-induced microsomes, respectively (Table 1). The metabolism of benzphetamine was also inhibited by 22 and 47%, respectively, in control and ethanol-induced microsomes. Because P-450_{LM3a} also catalyzes the metabolism of benzphetamine and nitrosomethylaniline (5,15), it appears that the inhibitory action of anti-3a IgG on the metabolism of these two compounds is due to the inhibition of P-450_{LM3a}.

Anti-3a IgG was also effective in inhibiting liver microsomal NDMAd activity of other species (Table II). The extents of inhibition with microsomes from control and ethanol-treated rats were about the same as those seen with rabbit microsomes (Table 1). The antibody also effectively inhibited the activities of uninduced mouse microsomes as well as microsomes from control and acetone-treated guinea pigs. The results indicate that NDMA is metabolized in the liver of these species by P-450 species that are immunochemically similar to P-450_{LM3a}.

From a comparison of the results in Figures 1 and 2, it appears that the antibody exerted a more potent inhibitory action on the NDMAd activity in microsomes than in the reconstituted system. This is probably due to the fact that P-450_{LM3a} accounts for only a portion of the total microsomal P-450 (i.e., the anti-3a IgG to P-450_{LM3a} ratio was higher than the anti-3a IgG to P-450 ratio shown in Figure 2). The present results are also consistent with the previously observed specific inhibition of alcohol oxidation and aniline hydroxylation by anti-3a IgG (18). It was also established in this previous study that the inhibitory action of anti-3a IgG is due to its direct action on P-450_{LM3a} and does not involve reactive oxygen radicals, which have been suggested to mediate certain P-450-catalyzed reactions (19). It was also demonstrated previously that superoxide and hydroxyl radicals play at most a minor role in the P-450-catalyzed NDMAd activity (Tu and Yang, submitted for publication).

Although P-450_{LM3a} has a K_m of 2.9 mM for the NDMAd activity in the reconstituted system, anti-3a IgG effectively inhibits the low-K_m (0.07 mM) NDMAd in microsomes. These results suggest that P-450_{LM3a} is responsible for the low-K_m NDMAd activity in microsomes and the kinetic parameter of this activity is different when the cytochrome is in the microsomal membrane or in the reconstituted system. According to this concept, differences in the local environment in the membrane may also contribute to the multiplicity of the K_m values of NDMAd, even though the multiple K_m values are mainly due to the existence of different P-450 isozymes in microsomes. An alternative interpretation is that the low-K_m microsomal NDMAd activity is due to another P-450 species which crossreacts with anti-3a IgG. Although this interpretation is not favored because P-450_{LM3a} appears to be the major P-450 species responsible for the ethanol-induced NDMAd activity, it remains to be examined.

The present work demonstrates that anti-3a IgG effectively inhibits the low-K_m microsomal NDMAd activity of rabbit liver as well as the liver microsomal NDMAd activity of rats, mice, and guinea pigs. The results suggest the presence in these rodents of a P-450_{LM3a} type cytochrome which is inducible by ethanol, acetone, isopropanol, pyrazole, imidazole, fasting, and other treatments (10-14,16,20-22). This type of cytochrome is believed to be the major P-450 species responsible for the metabolism of toxicologically important compounds such as NDMA, ethanol, acetone, aniline, carbon tetrachloride, and enflurane (14,18,20-25).

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