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INHIBITION OF THE MICROSOMAL N-HYDROXYLATION OF 2-AMINO-6-NITROTOLUENE BY A METABOLITE OF METHIMAZOLE

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Summary: Inhibition studies were used to investigate the identity of the microsomal enzyme(s) responsible for the NADPH-dependent N-hydroxylation of 2-amino-6-nitrotoluene. The N-hydroxylation reaction was inhibited by several cytochrome P-450 inhibitors as well as by methimazole, a substrate for flavin-containing monooxygenase. Heat inactivation of flavin-containing monooxygenase had no effect on the rate of the reaction but abolished the inhibition by methimazole. These results indicate that the flavin-containing monooxygenase-mediated metabolism of methimazole produced an inhibitor of the cytochrome P-450-catalyzed N-hydroxylation reaction. When glutathione was included in the incubation the inhibition by methimazole was abolished, presumably due to the reduction of oxygenated metabolites of methimazole. These results show that methimazole inhibition does not necessarily implicate flavin-containing monooxygenase in microsomal N-hydroxylation reactions.

N-Hydroxylation is widely regarded as an essential step in the bioactivation of aromatic amines to carcinogenic species (1). Numerous studies on the microsomal oxidation of arylamines to arylhydroxylamines have implicated the involvement of two NADPH-dependent monooxygenase systems, cytochrome P-450 and flavin-containing monooxygenase (FMO[1]; EC 1.14.13.8) (2-9). Because of the species variation in the specific contents of microsomal FMO and cytochrome P-450 (10), the relative participation of these enzymes in microsomal oxidations is frequently evaluated by inhibition studies (4,6-9). Methimazole (N-methyl-2-mercaptoimidazole), a high affinity substrate for FMO (11), is often used as an inhibitor of FMO-catalyzed oxidations. The unusual heat sensitivity of FMO (12) can also be used to investigate its involvement in microsomal oxidation reactions.

Preliminary studies have shown that reduced metabolites of the hepatocarcinogen 2,6-dinitrotoluene can be N-hydroxylated by rat liver microsomes

[1] Abbreviations used: FMO, flavin-containing monooxygenase; ANT, 2-amino-6-nitrotoluene.

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to produce hydroxylamines (13) and that microsomal preincubation to inactivate FMO had no effect on the rate of N-hydroxylation of 2-amino-6-nitrotoluene (ANT). Herein we report that methimazole inhibits the microsomal N-hydroxylation of ANT. However, the data indicate that the inhibition is due to a product of the FMO-mediated metabolism of methimazole rather than to methimazole itself.

MATERIALS AND METHODS

ANT, octylamine, N-methylimidazole, nitrosobenzene, and sodium sulfite were obtained from Aldrich Chemical Co. NADPH, glutathione, bathophenanthroline, ferric nitrate, and methimazole were obtained from Sigma Chemical Co. Metyrapone was obtained from CIBA Pharmaceuticals Co. 2-Diethylaminoethyl-2,2-diphenylvalerate (SKF-525A) was a gift from Smith Kline and French. Phenylhydroxylamine was synthesized by ascorbate reduction of nitrosobenzene in aqueous solution. The product was extracted with ether and recrystallized. The melting point and mass spectrum were consistent with the expected product. All other chemicals were reagent grade and obtained from commercial sources.

Liver microsomes of 200-250 g male Fischer-344 rats (CDF®(F-344)/CrlBR, Charles River Breeding Laboratories, Kingston, NY) were prepared by differential centrifugation (14,15). Protein concentrations were determined by the biuret method. Cytochrome P-450 concentrations were determined by the method of Omura and Sato (16).

The reaction mixtures contained potassium phosphate buffer (0.1M), pH 7.7, ANT (0.1 mM), NADPH (1.0 mM), and 4.3 mg of microsomal protein in a final volume of 3 ml. The mixtures in 10 ml flasks were preincubated at 37°C for 3 min. The reactions were initiated by the addition of NADPH, incubated with shaking for 3 min, and terminated by the addition of 3 ml of ethyl acetate.

Hydroxylamine formation was assayed in the ethyl acetate extracts of the incubations by the formation of a ferrous-bathophenanthroline complex as described by Tsen (17). Appropriate controls were run with each incubation. Hydroxylamine concentrations were calculated using an extinction coefficient of 33.93 mM^{-1} cm^{-1} determined at 535 nm by taking known amounts of phenylhydroxylamine through the same procedure.

All experiments were carried out in duplicate or triplicate under conditions where the rate of the reaction was linear with respect to both time and protein concentration.

RESULTS

The NADPH-dependent oxidation of ANT by rat liver microsomes produced 2-amino-6-nitrobenzyl alcohol and 2-hydroxylamino-6-nitrotoluene (Fig. 1). Control experiments demonstrated that neither ANT nor 2-amino-6-nitrobenzyl alcohol interferred with the colorimetric assay for hydroxylamine formation. Furthermore, omission of ANT, NADPH, or microsomes resulted in background absorbance values.

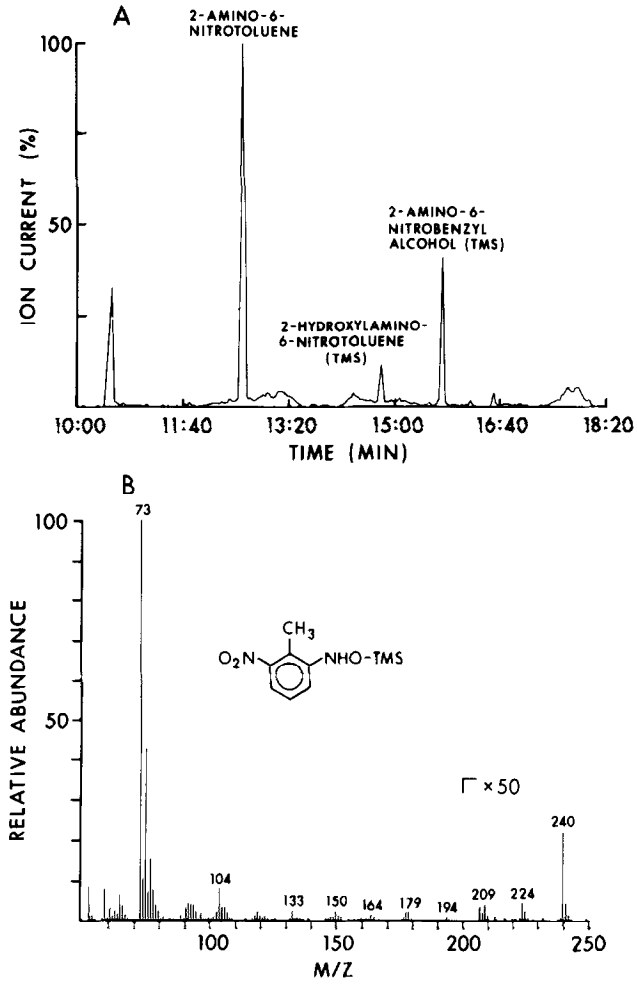

Figure 1. Gas chromatography-mass spectrometry of the microsomal metabolites of ANT. The reaction mixture was incubated for 30 min and extracted twice with 3 ml of ethyl acetate. The combined extracts were dried over magnesium sulfate and a 1 ml aliquot was derivatized with bis(trimethylsilyl)trifluoroacetamide and evaporated to dryness. The residue was dissolved in 30 µl of ethyl acetate and a 2 µl aliquot was injected onto a Finnegan 3223 gas chromatograph-mass spectrometer equipped with a 30 m capillary column coated with SE-54. Ionization was by electron impact with the filament operated at 70 eV.

A. Ion chromatogram of the ethyl acetate extract of the microsomal incubation.

B. Mass spectrum of the trimethylsilyl ether of 2-hydroxylamino-6-nitrotoluene. The retention time and mass spectrum were identical to that observed for the product of the zinc dust reduction of 2,6-dinitrotoluene. The fragmentation pattern is that expected for the hydroxylamine, with the characteristic loss of 16 (O) followed by the loss of the trimethylsilyl methyl groups.

The rate of ANT N-hydroxylation was decreased by the cytochrome P-450 inhibitors metyrapone, octylamine, and SKF-525A (Table I), suggesting the involvement of cytochrome P-450. Methimazole, a high affinity substrate for

Table I. Inhibition of the microsomal N-hydroxylation of ANT[a].

Additions	% Control
None	100
SKF-525A	87 (86-88)
Metyrapone	60 (58-62)
Octylamine	61 (53-69)
Methimazole	71 (68-74)
N-Methylimidazole	82 (80-84)
Sodium sulfite	97 (93-100)
Glutathione	103 (101-105)
Glutathione + Methimazole	102 (97-107)

[a]The incubation conditions and assay procedure were as described under "Materials and Methods". The inhibitor concentrations were 0.1 mM. The data are presented as % of control rate (range for 2-4 determinations). The control rate was 1.90 ± 0.12 nmol hydroxylamine formed/min/mg protein.

FMO (11), also inhibited the reaction (Table I). However, preincubation of the microsomes in the absence of NADPH at 37°C for 60 min to inactivate FMO (12) had no effect on the rate of the N-hydroxylation reaction (Table II). One possible explanation for these observations is that the FMO-mediated metabolism of methimazole produces an inhibitor of the N-hydroxylation reaction. This hypothesis was tested by heat inactivation of FMO. As shown in Table II, microsomal preincubation had no effect on the rate of the N-hydroxylation reaction but abolished the inhibition by methimazole. These

Table II. Effect of microsomal preincubation on the N-hydroxylation of ANT and its inhibition by methimazole[a].

Preincubation time (min)	Methimazole	nmol hydroxylamine formed/min/mg protein	% Control
0	-	2.19	100
0	+	1.62	74
60	-	2.14	98
60	+	2.08	95

[a]The incubation conditions and assay procedure were the same as in Table I. The data presented are average values of duplicate determinations which did not vary by > 5%.

results indicate that a product of the FMO-mediated metabolism of methimazole inhibits the cytochrome P-450-catalyzed N-hydroxylation of ANT.

Methimazole is sequentially monooxygenated by FMO to produce the sulfenic and sulfinic acids (11,18). The unstable sulfinic acid metabolite then reacts with water to produce N-methylimidazole and sulfite anion as the ultimate products (11). If either of these ultimate products of methimazole metabolism is responsible for the inhibition, then it should be a more potent inhibitor of the N-hydroxylation reaction than is methimazole under the same conditions. As shown in Table I, neither N-methylimidazole nor sulfite produced as much inhibition as methimazole, indicating that they are not responsible for the inhibition by methimazole. If the oxygenated products of methimazole metabolism are the inhibitors of the N-hydroxylation reaction, then inclusion of glutathione, which rapidly reduces the sulfenic acid metabolite (18), should prevent the inhibition. As shown in Table I, methimazole did not inhibit the reaction in the presence of glutathione.

DISCUSSION

The results of this study strongly suggest that cytochrome P-450 is responsible for the N-hydroxylation of ANT in rat liver microsomes. Although purified FMO may be able to catalyze this reaction, it does not appear to make a significant contribution in our system, since heat inactivation of microsomal FMO did not alter the rate of ANT N-hydroxylation.

The data also show that the FMO-mediated metabolism of methimazole produces an inhibitor of the cytochrome P-450-catalyzed N-hydroxylation of ANT. Sulfite and N-methylimidazole had little inhibitory effect on the reaction and there was no inhibition by methimazole in the presence of glutathione, suggesting that the true inhibitor is the sulfenic acid metabolite or some product derived from it. Although it is not presently known whether metabolites of methimazole can inhibit other cytochrome P-450-dependent oxidations, preincubation of rat liver microsomes with 1 mM methimazole and NADPH for 15 min resulted in a reduction of cytochrome P-450 content and a corresponding decrease in benzphetamine demethylation (19). These results

were ascribed to the formation of an activated metabolite of methimazole. While cytochrome P-450 can oxidize methimazole (20), this is a low affinity process (K_M = 18 mM) compared to the FMO-catalyzed reaction (K_M = 13 μM) (11). Thus, it is possible that the product of the FMO-catalyzed oxidation of methimazole was responsible for the reduction in microsomal cytochrome P-450 and benzphetamine demethylation previously reported (19).

These results lead us to conclude that methimazole inhibition should not be used as the sole criterion for establishing FMO involvement in a microsomal oxidation reaction. In the absence of specific antibodies to microsomal enzymes, other experiments such as heat inactivation of FMO should be done to confirm the results of inhibition studies.

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