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N-Demethylation of Cocaine to Norcocaine

Evidence for Participation by Cytochrome P-450 and FAD-Containing Monooxygenase

MICHELLE W. KLOSS,[1] GERALD M. ROSEN, AND ELMER J. RAUCKMAN

Departments of Pharmacology and Surgery, Duke University Medical Center, Durham, North Carolina 27710

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SUMMARY

Experiments were conducted to determine which microsomal enzymes are involved in the *in vitro* hepatic oxidative *N*-demethylation of cocaine to norcocaine, the first step in the biotransformation of cocaine to its ultimate hepatotoxic metabolite. Cocaine was found to undergo conversion to norcocaine by two alternate pathways, one involving only cytochrome P-450 and the other requiring both cytochrome P-450 and FAD-containing monooxygenase. In the first pathway, cocaine was directly *N*-demethylated to norcocaine by cytochrome P-450; this reaction was enhanced by phenobarbital induction and was inhibited by both *n*-octylamine and metyrapone. The second route was found to be a two-step reaction involving cocaine *N*-oxide as an intermediate. In this pathway, cocaine is first oxidized to cocaine *N*-oxide by FAD-containing monooxygenase, followed by a cytochrome P-450-catalyzed *N*-demethylation to norcocaine. This latter step was enhanced by phenobarbital treatment and inhibited by *n*-octylamine. Cocaine *N*-oxide also exhibited a Type I binding spectrum with mouse hepatic microsomes. In addition, a model system consisting of ferrous sulfate was found to catalyze the *N*-demethylation of cocaine *N*-oxide. On the basis of these experiments, it is concluded that cytochrome P-450 and FAD-containing monooxygenase participate in the initial oxidation of cocaine to norcocaine. We also propose a mechanism to account for the conversion of cocaine *N*-oxide to norcocaine.

INTRODUCTION

Cocaine, a central nervous system stimulant, has been found to be hepatotoxic in mice, causing fatty infiltration, necrosis, elevation of serum transaminase levels, and depression of a variety of hepatic enzyme systems (1–3). Evans and Harbison (4) and Thompson *et al.* (5) first suggested that a metabolite of cocaine, rather than cocaine itself, was responsible for the observed hepatotoxicity, based on data obtained when known cytochrome P-450-inducing agents were used. It is now known that the minor hepatic metabolic pathway, cocaine to norcocaine to *N*-hydroxynorcocaine to norcocaine nitroxide, is responsible for the observed cocaine-mediated hepatotoxicity (6).

In all studies to date, it has been assumed that cytochrome P-450 is solely responsible for all hepatic metabolic oxidations of cocaine to its hepatotoxic metabolite. Recently, however, we have demonstrated that hepatic FAD-containing monooxygenase, a major Phase I oxidative enzyme that functions independently of cytochrome P-450 (7), can catalyze the conversion of norcocaine to *N*-hydroxynorcocaine, both *in vitro* and *in vivo* (8).

This study was designed to examine the hepatic oxidative *N*-demethylation of cocaine to norcocaine *in vitro* and to determine the extent of involvement of both cytochrome P-450 and FAD-containing monooxygenase in this biotransformation. Our results indicate that both systems can participate in the *in vitro* conversion of cocaine to norcocaine. We further suggest a possible mechanism by which cocaine is oxidized to norcocaine *in vitro*, and presumably *in vivo*. This mechanism proposes two alternate pathways for norcocaine formation: one employing only cytochrome P-450, the other being catalyzed sequentially by FAD-containing monooxygenase and cytochrome P-450, respectively, with cocaine *N*-oxide as an intermediate metabolite.

MATERIALS AND METHODS

General comments. Unless otherwise indicated, chemicals were purchased from Sigma Chemical Company (St. Louis, Mo.). The following chemicals were obtained from various sources: cocaine HCl (Mallinckrodt, St.

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Louis, Mo.); NADPH (Boehringer Mannheim Biochemicals, Indianapolis, Ind.); metyrapone and *n*-octylamine (Aldrich Chemical Company, Milwaukee, Wisc.).

Animals. DBA/2Ha and C3H/St male mice, at least 11 weeks of age, were obtained from Health Research Laboratories (West Seneca, N. Y.) and housed on ground corncob bedding. Animals received food (Purina rodent chow No. 5001) and tap water ad libitum. Mice were maintained under these conditions, in an isolation room, for at least 14 days prior to use. C3H/St mice were used exclusively for gonadectomy experiments, while all other experiments employed DBA/2Ha mice.

Gonadectomy and treatments. For gonadectomy, mice were anesthetized with methoxyflurane (Metofane, Pitman-Moore) for approximately 3 min and were castrated 2 months prior to use. Phenobarbital-treated animals recieved 0.1% sodium phenobarbital in their drinking water for 5 days prior to sacrifice.

Cocaine *N*-oxide was synthesized from cocaine according to the method of Misra *et al.* (9).

Analytical measurements. Washed mouse hepatic microsomes were prepared according to the method of Schenkman and Cinti (10). The resulting microsomal suspension contained 6–9 mg of protein per milliliter. The cytochrome P-450 content was assessed by the method of Omura and Sato (11). FAD-containing monooxygenase (dimethylaniline monooxygenase, EC 1.14.13.8) activity was measured as methimazole-*S*-oxidase activity by following NADPH oxidation spectrophotometrically (12). For experiments involving K_m determinations, washed mouse hepatic microsomes were used, and substrate concentrations varied from 1 mM to 5 μM cocaine as described previously (8). Purified porcine FAD-containing monooxygenase was kindly supplied by Dr. Daniel M. Ziegler, University of Texas (Austin, Tex.). The difference spectrum of cocaine *N*-oxide with phenobarbital-induced mouse hepatic microsomes was obtained according to the method of Jefcoate (13). For the assay, the microsomal protein concentration was 1.2 mg/ml in 0.1 mM sodium phosphate buffer (pH 7.4), and cocaine *N*-oxide in dimethyl sulfoxide was added to a final concentration of 3.8 mM. Demethylation of cocaine and cocaine *N*-oxide, *in vitro*, was estimated by monitoring production of formaldehyde as described previously (3). The final concentration of cocaine or cocaine *N*-oxide in the incubation medium was 5 mM. Microsomal protein was estimated by the method of Bradford (14), using bovine γ-globulin as standard. Ferrous catalyzed dealkylation of cocaine *N*-oxide to norcocaine was carried out according to the method of Ferris *et al.* (15), using the Nash reaction (3) to estimate the amount of formaldehyde produced. The reaction mixture contained 2.3 mmoles of cocaine *N*-oxide and 3.3 mmoles of ferrous sulfate in 50 ml of 0.49 N sulfuric acid; the mixture was heated to reflux under a nitrogen atmosphere for 5 hr.

Statistics. Statistical evaluations between sample means were made by the two-tailed Student's *t*-test. The level of significance chosen was $p < 0.05$.

RESULTS

The data in Table 1 demonstrate the effect of cytochrome P-450 induction and inhibition on the *N*-demethylation of cocaine *in vitro*. In agreement with the data of Evans and Harbison (4), the use of phenobarbital-induced hepatic microsomes as compared with control microsomes resulted in significantly enhanced production of formaldehyde during cocaine oxidation. Alternatively, the addition of *n*-octylamine or metyrapone, both of which are potent inhibitors of cytochrome P-450 (16, 17), caused a significant reduction in the extent of cocaine demethylation *in vitro*. These data clearly implicate a role for cytochrome P-450 in the dealkylation of cocaine.

TABLE 1

N-Demethylation of cocaine by mouse hepatic microsomes: effect of cytochrome P-450 induction and inhibition

Results are means ± standard error of the mean ($n = 6$). Conditions were as stated under Materials and Methods. The cocaine concentration was 5 mM.

Sample	HCHO	Cytochrome P-450
	nmoles/mg protein	*nmoles/mg protein*
Control	126 ± 5	0.718 ± 0.053
Phenobarbital-induced	264 ± 14[a]	2.80 ± 0.14[a]
Control + 2 mM metyrapone	30 ± 1[a]	0.718 ± 0.053
Control + 3 mM *n*-octylamine	17 ± 1[a]	0.718 ± 0.053

[a] $p < 0.05$, significantly different from control.

To determine the extent of involvement of hepatic FAD-containing monooxygenase in the dealkylation of cocaine, hepatic microsomes from castrated male C3H/St mice were used in cocaine *N*-demethylation experiments. The results are shown in Table 2. We have previously demonstrated (8, 18) that castration of male C3H/St mice results in the selective enhancement of hepatic FAD-containing monooxygenase, with no resulting alteration in hepatic cytochrome P-450. It can be seen from the data presented in Table 2 that microsomes prepared from castrated mice produce greater amounts of formaldehyde from cocaine *N*-demethylation than do microsomes from noncastrated mice.

Since these data suggested a role for FAD-containing monooxygenase in cocaine *N*-demethylation, we examined the ability of purified FAD-containing monooxygenase to oxidase cocaine *in vitro*. It was found that 1 mM cocaine, added to purified porcine FAD-containing monooxygenase, in the presence of *n*-octylamine and tricine buffer at pH 8.3 stimulated NADPH oxidation by 2.6 nmoles of NADPH per minute, indicating that cocaine is indeed a substrate for FAD-containing monoox-

TABLE 2

N-Demethylation of cocaine by mouse hepatic microsomes: effect of castration

Results are means ± standard error of the mean ($n = 6$). Conditions were as stated under Materials and Methods.

Sample	HCHO	FADM[a]	Cytochrome P-450
	nmoles/mg protein		*nmoles/mg protein*
Sham-operated mice	147 ± 10	1.63 ± 0.22	0.274 ± 0.027
Castrated mice	180 ± 2[b]	3.11 ± 0.11[b]	0.303 ± 0.026

[a] FAD-containing monooxygenase activity, measured as nanomoles of NADPH oxidized per minute per milligram of protein.

[b] $p < 0.05$, significantly different from control.

ygenase. With hepatic microsomes, cocaine was determined to have an apparent K_m of 9 μM and a V_{max} of 0.54 nmole/mg·min for FAD-containing monooxygenase; under identical conditions, methimazole, one of the best substrates for this enzyme (19), gave values of 12 μM and 2.01 nmoles/mg·min. The reaction of cocaine to yield cocaine *N*-oxide *in vitro*, using either purified FAD-containing monooxygenase or mouse hepatic microsomes, failed to produce enough cocaine *N*-oxide to detect qualitatively using thin-layer chromatography. From spectrophotometric data, it was calculated that the reaction proceeded too slowly (0.81 μg of cocaine oxidized per minute) to produce thin-layer chromatography-detectable quantities of cocaine *N*-oxide over a reasonable period of time.

To evaluate the possible fate of cocaine *N*-oxide after its formation from cocaine via FAD-containing monooxygenase, we examined the extent of microsomal conversion of cocaine *N*-oxide to norcocaine as measured by the production of formaldehyde. These results are shown in Table 3. Our data suggest that cytochrome P-450 mediates this dealkylation. Microsomes from phenobarbital-treated mice demonstrated an enhanced production of formaldehyde from cocaine *N*-oxide dealkylation; in the presence of *n*-ocytlamine, formaldehyde generation was decreased.

To establish further that cocaine *N*-oxide is a substrate for cytochrome P-450, a difference spectrum using microsomes and cocaine *N*-oxide was obtained (Fig. 1). The spectrum is characteristic of a Type I binding spectrum, suggesting that cocaine *N*-oxide is a substrate for cytochrome P-450 (13).

Finally, in order to gain insight into the mechanism by which cocaine *N*-oxide is demethylated to norcocaine, we examined the extent of formaldehyde production during cocaine *N*-oxide dealkylation by a model cytochrome P-450 system. Ferris *et al.* (15) have previously reported the ferrous-catalyzed demethylation of a number of amine *N*-oxides to give formaldehyde and the corresponding secondary amines. Utilizing ferrous sulfate as the cytochrome P-450 model, we observed that, during the anaerobic incubation of cocaine *N*-oxide with the iron salt in an acidic medium, 8.5 μmoles of formaldehyde were produced during the 5-hr incubation period. Norcocaine could not be isolated from the reaction mixture because of its extensive hydrolysis under the acidic conditions.

TABLE 3

Demethylation of cocaine N-oxide by mouse hepatic microsomes: effect of cytochrome P-450 induction and inhibition

Results are means ± standard error of the mean ($n = 6$). Conditions were as stated under Materials and Methods. The cocaine *N*-oxide concentration was 5 mM.

Sample	HCHO	Cytochrome P-450
	nmoles/mg protein	*nmoles/mg protein*
Control	222 ± 12	0.718 ± 0.053
Phenobarbital-induced	477 ± 44[a]	2.80 ± 0.14[a]
Control + 3 mM *n*-octylamine	59 ± 06[a]	0.718 ± 0.053

[a] $p < 0.05$, significantly different from control.

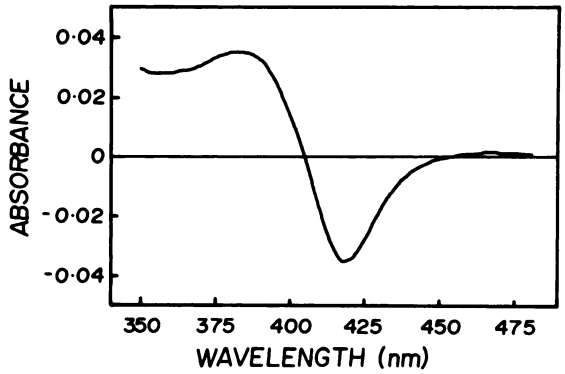

FIG. 1. *Difference spectrum of cocaine N-oxide with phenobarbital-induced DBA/2Ha hepatic microsomes*

Conditions were as stated under Materials and Methods.

DISCUSSION

Previous studies have indicated that the initial step in the hepatic biotransformation of cocaine to norcocaine is carried out by cytochrome P-450 (4). Our results, however, clearly demonstrate that both cytochrome P-450 and FAD-containing monooxygenase are involved in the oxidation of cocaine to norcocaine *in vitro*. Our data suggest that the two enzymes act in concert to oxidize cocaine via two alternate routes (Fig. 2). In one pathway, cocaine can be *N*-demethylated to norcocaine directly by cytochrome P-450. This conclusion can be substantiated by our results. The participation of the alternate mechanism in which cocaine is initially *N*-oxidized to cocaine *N*-oxide by FAD-containing monooxygenase and then *N*-demethylated by cytochrome P-450 is demonstrated by our findings that enhanced production of formaldehyde was observed in microsomes obtained from castrated C3H/St mice in which the activity of FAD-containing monooxygenase was increased while cytochrome P-450 was unchanged. This mechanism is also supported by our observations that both of the individual mechanisms occurred with control microsomes and that phenobarbital-induced hepatic microsomes metabolized co-

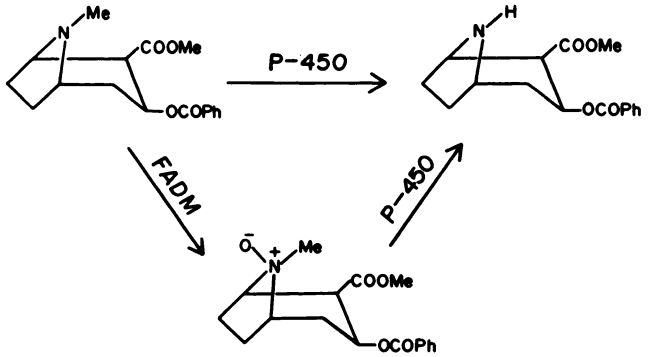

FIG. 2. *Metabolic scheme to account for the demethylation of cocaine to norcocaine*

The direct pathway envisions the production of norcocaine from cocaine via cytochrome P-450 demethylation. The alternate route suggests that cocaine is initially oxidized by FAD-containing monooxygenase to yield cocaine *N*-oxide. This metabolite is then demethylated by cytochrome P-450. *P-450*, Cytochrome P-450; *FADM*, FAD-containing monooxygenase.

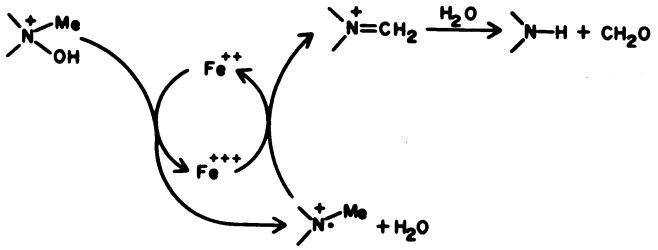

FIG. 3. *Proposed mechanism for cytochrome P-450 demethylation of cocaine N-oxide*

In this scheme, donation of an electron from ferrocytochrome P-450 gives the radical cation and ferrocytochrome P-450. Transfer of an electron to ferricytochrome P-450 leads to the formation of the imine and ferrocytochrome P-450. Hydrolysis gives norcocaine.

caine *N*-oxide to a greater extent than did control microsomes, whereas *n*-octylamine inhibited this reaction. Finally, cocaine *N*-oxide showed a Type I binding spectrum (Fig. 1). Compounds which exhibit a Type I binding spectrum are almost invariably substrates for cytochrome P-450 (13). The amount of formaldehyde generated by control microsomes at saturating concentrations of cocaine is 42 nmoles/mg in 15 min at 25°. It can also be calculated that, in the presence of *n*-octylamine to inhibit cytochrome P-450, only 10.5 nmoles/mg of cocaine *N*-oxide can be produced in 15 min by FAD-containing monooxygenase. Thus, the maximal possible contribution by the cocaine *N*-oxide pathway is only 10.5 nmoles/mg in 15 min or 25% of the formaldehyde production. It can therefore be concluded that the direct cytochrome P-450 *N*-demethylation of cocaine by control microsomes from male DBA/2Ha mice accounts for at least 75% of norcocaine production.

From our experiments with a model cytochrome P-450 system, we propose a mechanism to account for the demethylation of cocaine *N*-oxide (Fig. 3). We suggest that after cytochrome P-450 binds cocaine *N*-oxide, it accepts an electron from NADPH-cytochrome P-450 reductase to give ferrocytochrome P-450. Donation of an electron to cocaine *N*-oxide with loss of hydroxide gives ferricytochrome P-450 and the radical cation. Transfer of an electron to ferricytochrome P-450 with loss of a proton leads to the production of the imine and ferrocytochrome P-450. Hydrolysis of the imine gives norcocaine. This mechanism proposes a catalytic role for cytochrome P-450. Evidence to support this hypothesis has been obtained by Ferris *et al.* (15).

It is clear, then, that cocaine can be biotransformed to norcocaine by two different pathways. Both mechanisms appear to involve cytochrome P-450, and, in the route involving cocaine *N*-oxide, FAD-containing monooxygenase plays an important role in the bioactivation of cocaine to norcocaine. The relative extent to which each enzyme participates in the dealkylation of cocaine *in vivo* remains unclear. However, since the activities of both enzymes appear to be modulated by a wide variety of internal and external factors (e.g., hormonal, environmental, and chemical inducers), the fact that each enzyme is involved in cocaine bioactivation may be an important consideration in the study of cocaine-induced hepatotoxicity.

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Send reprint requests to: Dr. Gerald M. Rosen, Department of Pharmacology, Duke University Medical Center, Durham, N. C. 27710.