Catalyzation of Cocaine *N*-Demethylation by Cytochromes P4502B, P4503A, and P4502D in Fish Liver

Emel Arinç and Azra Bozcaarmutlu

Biochemistry Graduate Program, Department of Biological Sciences, Middle East Technical University, 06531 Ankara, Turkey;
E-mail: *earinc@metu.edu.tr*

Received 14 January 2003; revised 11 April 2003; accepted 8 May 2003

ABSTRACT: Cocaine N-demethylation by microsomal cytochrome P450s is the principal pathway in cocaine bioactivation and hepatotoxicity. P450 isozymes involved in N-demethylation of cocaine have not been elucidated yet and they differ from species to species. In humans and mice, P4503A contributes to cocaine N-demethylase activity, whereas in rats, both P4503A and P4502B participate. In the present study, contribution of different P450 isozymes to cocaine N-demethylase activity was studied in vitro with fish liver microsomes. The specific cocaine N-demethylase activity was found to be 0.672 ± 0.22 nmol formaldehyde formed/min/mg protein (mean $\pm$ SD, $n = 6$). Cocaine N-demethylase exhibited biphasic kinetics, and from the Lineweaver–Burk plot, two K_m values were calculated as 0.085 and 0.205 mM for the high- and low-affinity enzyme. These results indicate that N-demethylation of cocaine in mullet liver microsomes is catalyzed by at least two cytochrome P450 isozymes. Inhibitory effects of cytochrome P450 isozyme-selective chemical inhibitors, ketoconazole, cimetidine, SKF-525A, and quinidine, on cocaine N-demethylase activity were studied at 50, 100, and 500 μM concentrations of these inhibitors. At 100 μM final concentrations, ketoconazole (P4503A inhibitor), SKF-525A (inhibitor of both P4502B and P4503A), and cimetidine (P4503A inhibitor) inhibited N-demethylation activity by 73, 69, and 63%, respectively. Quinidine, P4502D-specific inhibitor, at 100 μM final concentration, reduced N-demethylation activity down to 64%. Aniline, a model substrate for P4502E1, did not alter N-demethylase activity in the final concentration of 100 μM. IC_{50} values were calculated to be 20 μM for ketoconazole, 48 μM for cimetidine (both specific P4503A inhibitors), 164 μM for quinidine (P4502D inhibitor), and 59 μM for SKF-525A (inhibitor of both P4503A and P4502B). The contribution of P4502B to cocaine N-demethylase activity in mullet liver microsomes was further explored by the use of purified mullet cytochrome P4502B in the reconstituted system containing purified mullet P450 reductase and lipid. The turnover number was calculated as 4.2 nmol HCOH/(min nmol P450). Overall, these results show that P4503A and P4502B are the major P450s responsible for N-demethylation of cocaine, whereas contribution of P4502D is a minor one, and P4502E1 is not involved in the N-demethylation of cocaine in mullet liver microsomes. © 2003 Wiley Periodicals, Inc. J Biochem Mol Toxicol 17:169–176, 2003; Published online in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/jbt.10075

KEYWORDS: **Cocaine N-Demethylation; Kinetics; Leaping Mullet; Fish; Liver; Ketoconazole; Cimetidine; SKF-525A (Proadifen); Quinidine; Aniline**

INTRODUCTION

Cytochrome P450 (CYP) comprises a superfamily of hemoproteins that play a pivotal role in the activation and detoxification of xenobiotics such as drugs, pesticides, antioxidants, organic solvents, anesthetic agents, dyes, environmental pollutants, and food additives, and endogenous compounds including steroids, bile acids, vitamin D_3, fatty acids, prostaglandins, biogenic amines, and retinoids [1–8]. Among the cytochrome P450 gene families, *CYP1*, *CYP2*, and *CYP3* are currently thought to be responsible from the majority of hepatic xenobiotic metabolism [9].

Cocaine is a potent and dangerous central nervous system (CNS) stimulant obtained from the leaves of *Erythroxylon coca*. Cocaine is a highly addicting drug and can lead to many complications [10]. Cocaine also causes hepatotoxicity in humans and laboratory animals [11–15]. The cocaine-induced hepatotoxicity has been shown to be associated with the metabolites of cocaine, that is norcocaine and *N*-hydroxycocaine [12,16–18]. The first step in the bioactivation of cocaine is the N-demethylation of cocaine to norcocaine

Correspondence to: Emel Arinç.
Contract Grant Sponsor: Turkish Academy of Sciences (TÜBA).
Contract Grant Sponsor: Middle East Technical University, Ankara, Turkey.
Contract Grant Number: AFP-2001-01-08-02.

by cytochrome P450 isozymes [16,17,19–21]. The P450 isozymes involved in N-demethylation of cocaine have not been clarified yet, and they differ from species to species. In humans and mice, cytochrome P4503A catalyzes the N-demethylation of cocaine [17,20,22], whereas in rats, both P4503A and P4502B catalyze this reaction [14]. The toxicity of cocaine has been shown to be associated with only the P4502B-mediated metabolism in rats [14,19,20].

The balance between detoxification and bioactivation of a compound in a particular species or organ is highly dependent on the relative amounts and activities of the different forms of P450s that are expressed. Thus, identification of individual cytochrome P450 enzymes responsible for formation of specific metabolites of xenobiotics, including drugs, environmental toxins, and procarcinogens, is important from evolutionary, toxicological, and pharmacological standpoints. As in mammals, different cytochrome P450 isozymes have been identified in fish species [23–27]. Knowledge on the metabolism of xenobiotics by fish P450-dependent monooxygenases is important for environmental toxicology, chemical carcinogenesis, and comparative biochemistry [6,27]. There is no information on cocaine metabolism and the role of specific P450 isozymes catalyzing cocaine N-demethylation reaction in fish liver. Like in the case of smaller laboratory animals, cocaine has not been used as a veterinary drug in the fish. However, there exists extensive research on the involvement of P450 enzymes in cocaine metabolism, the objective of which is that the information gained from the smaller laboratory animal studies would be useful for the comparative drug metabolism studies including those for humans.

Leaping mullet (*Liza saliens*) is the most common fish in the Mediterranean. It has been used in biomonitoring studies in Izmir Bay, Turkey [28–30]. Biochemical and immunochemical properties of mullet liver cytochrome P450 reductase and P4501A1 have been characterized [26,31–33].

In the present study, we aimed to determine the specific isozymes of P450 participating in cocaine N-demethylation reaction in the liver of leaping mullet by using isoform-selective chemical inhibitors, isoform-specific substrate, and reconstitution studies using the purified components.

MATERIAL AND METHODS

Chemicals

Cocaine-HCl was purchased from Tarim ve Koy Isleri Bakanligi, Toprak Mahsulleri Ofisi, Ankara, Turkey. Acetic acid glacial, cupper sulfate, dimethyl sulfoxide (DMSO), ethylenediaminetetraacetic acid disodium salt (EDTA), formaldehyde (HCOH), glycerol, magnesium chloride, and methanol were purchased from E. Merck, Darmstadt, Germany. Ammonium acetate, ε-amino caproic acid (ε-ACA), bovine serum albumin, dilauroyl phosphatidycholine (DLPC), D-glucose-6-phosphate monosodium salt, D-glucose-6-phosphate dehydrogenase type XI, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), ketoconazole, β-nicotinamide adenine dinucleotide phosphate ($NADP^+$), phenylmethane sulfonyl fluoride (PMSF), proadifen (SKF-525A, *N*,*N*-diethylaminoethyl-2,2-diphenylvalerate hydrochloride), quinidine, and sodium potassium tartrate were purchased from Sigma Chemical Co., St. Louis, MI. All the other chemicals were of analytical grade and were obtained from commercial sources at the highest grade of purity available.

Fish Collection

The fish used in this study, leaping mullet (*Liza saliens*), were collected by fish net in Izmir Bay, Aegean coast of Turkey. The fish collected were of comparable size and weight (300–400 g).

Preparation of Leaping Mullet Liver Microsomes

The livers, weighing approximately 2–5 g, were removed immediately. The gall bladder was removed carefully with scissors so as to avoid the spillage of its contents that are known to be inhibitory to monooxygenase activities. The livers were placed in liquid nitrogen and transported from Izmir to the University laboratories in Ankara, about 650 km away. Liver microsomes were prepared with differential centrifugation as described by Arinç and Sen [34] except that the homogenization solution contained 10 mM EDTA, 0.25 mM ε-ACA, and 0.1 mM PMSF. In the last step, the washed microsomal pellet was resuspended in 10% glycerol containing 10 mM EDTA. For each gram of liver, 0.5 mL of suspension solution was used. Ten livers were used for the preparation of the first and second batch of microsomes, which were used for characterization of other mixed-function oxidase activities of mullet [35]. Other four batches were prepared from a single liver. The aliquots of microsomal suspensions containing approximately 25–35 mg protein/mL were gassed with nitrogen and stored in liquid nitrogen until use.

Protein Determination

Protein concentration in microsomes was determined according to the method of Lowry et al. [36] using bovine serum albumin as a standard.

Determination of Cytochrome P450 Content

Cytochrome P450 content was determined from the carbon monoxide difference spectra of dithionite reduced samples, using an extinction coefficient of 91 mM^{-1} cm^{-1} for the difference in the absorption between 450 and 490 nm, as described by Omura and Sato [37].

Determination of NADPH–Cytochrome P450 Reductase Activity

NADPH–cytochrome P450 reductase activity was assayed by measuring the rate of cytochrome *c* reduction at 550 nm spectrophotometrically, according to the method of Masters et al. [38], except that reaction was carried out at 25°C. The assay mixture contained 0.3 M potassium phosphate buffer (pH 7.7), containing 80 nmol cytochrome *c*, 130 nmol NADPH, and appropriate amounts of enzyme preparation in a final volume of 0.8 mL. The enzyme activity was calculated using the extinction coefficient of 19.6 mM^{-1} cm^{-1} for difference in absorbance between reduced and oxidized cytochrome *c* at 550 nm [39]. One unit of reductase was defined as the amount of enzyme catalyzing the reduction of 1 nmol cytochrome *c*/min under the described conditions.

Determination of Cocaine N-Demethylase Activity in Microsomes and Reconstituted Systems

The N-demethylation of cocaine was measured spectrophotometrically by measuring the amount of formaldehyde formed according to the method of Nash [40] as modified by Cochin and Alexrod [41]. A typical assay mixture contained 100 mM HEPES buffer (pH 7.8), 3.0 mM cocaine, 0.5 mM NADPH generating system, consisting of 2.5 mM magnesium chloride, 2.5 mM glucose-6-phosphate, 0.5 U glucose-6-phosphate dehydrogenase (EC 1.1.1.49; 1 U glucose-6-phosphate dehydrogenase reduces 1 nmol $NADP^+$/min at 25°C), 14.6 mM HEPES buffer (pH 7.8), 0.5 mM $NADP^+$, and 3 mg microsomal protein in a final volume of 0.5 mL. The reaction was initiated with the addition of NADPH generating system, and carried out for 10 min at 25°C with moderate shaking in a water bath.

Reconstitution of cocaine N-demethylase activity was performed by using electrophoretically pure mullet cytochrome P4502B [35] and cytochrome P450 reductase [26]. The reconstitution medium contained 0.2 nmol of purified mullet cytochrome P4502B, 90 U of cytochrome P450 reductase, and 0.04 mg of synthetic lipid, dilauroyl phosphatidycholine (DLPC). The mixture was mixed thoroughly and preincubated for 20 min at room temperature. The reaction proceeded for 10 min at 25°C after addition of 0.5 mM NADPH generating system in a final volume of 0.5 mL.

Kinetic Analysis of Cocaine N-Demethylation Reaction

Enzyme kinetics were carried out using substrate concentrations ranging from 0.1 to 4.0 mM (10 cocaine concentrations). Kinetic parameters (apparent K_m and V_{max} values) for the formation of norcocaine were analyzed by Lineweaver–Burk plot of reaction velocity ($1/V$) against 1/substrate concentration.

Inhibition of Cocaine N-Demethylation by Organic Solvents

Since the selected specific cytochrome P450 isozyme inhibitors, ketoconazole, SKF-525A (proadifen), cimetidine, and quinidine, used in this study are poorly soluble in water, organic solvents are used to aid solubilization of these chemical inhibitors. The effects of frequently used solvents, methanol, ethanol, and DMSO, on the enzyme activity were determined by adding these solvents in a final concentration of 1% of the reaction mixture containing cocaine in the absence of NADPH generating system. Control assay was also carried out. This mixture was preincubated for 15 min at 25°C with moderate shaking in water bath. The reaction was initiated by adding NADPH generating system and the reaction proceeded exactly 10 min at 25°C.

Inhibition of Cocaine N-Demethylation by Selective Inhibitors

Several chemicals known as selective inhibitors of P450 isozymes were preincubated in various concentrations with fish liver microsomes in the presence of cocaine to determine their potential inhibitory effect on cocaine N-demethylation reaction. Ketoconazole (P4503A inhibitor), SKF-525A (inhibitor of both P4502B and P4503A), cimetidine (P4503A inhibitor), and quinidine (P4502D inhibitor) were all dissolved in ethanol. The volume of ethanol added to the reaction mixture was 1% of the reaction volume. All four inhibitors were preincubated with 3 mg microsomal protein containing 3 mM cocaine and buffer for 15 min at 25°C before initiating the enzymatic reaction with NADPH generating system. In addition, the reaction mixture containing

SKF-525A was also preincubated with microsomes and NADPH generating system for 15 min at 25°C, and the reaction was initiated adding the substrate, cocaine.

Inhibition of Cocaine N-Demethylation by a Selective Substrate

Aniline, a P4502E1 substrate, was also preincubated with fish liver microsomes at 100 μM final aniline concentrations in the presence of cocaine for 15 min at 25°C prior to the addition of NADPH generating system to determine whether it could inhibit N-demethylation of cocaine.

RESULTS

Kinetics of Cocaine N-Demethylase Activity

Cocaine N-demethylase activity was measured in six different microsomal preparations. The average specific activities were found to be 0.672 ± 0.22 nmol formaldehyde formed/min/mg protein (mean ± SD, $n = 6$). Batch 1 and batch 2 microsomes were prepared from 10 fish livers, while in the preparation of remaining batches of microsomes, a single fish liver was used. Specific activity of mullet liver enzyme was found to be similar to that of rabbit liver [42], while it was less than that in either rat (3.8 nmol formaldehyde formed/min/mg protein) [14] or mice (1.59 formaldehyde formed/min/mg protein) [15] liver microsomes.

The kinetic parameters, apparent K_m and V_{max} of cocaine N-demethylation, were determined in mullet liver microsomes. Figure 1A illustrates the effect of cocaine concentration on cocaine N-demethylation activity. Ten different cocaine concentrations, starting from 0.1 mM to 4 mM, were used in the activity measurements. The activity appeared to be saturated at or above 1.5 mM cocaine. Lineweaver–Burk plot demonstrates biphasic kinetics (Figure 1B). The apparent K_m values were calculated as 0.085 and 0.205 mM, respectively. The respective V_{max} values for the high- and low-affinity fish liver enzyme activities were found to be 0.388 and 0.485 nmol formaldehyde formed/min/mg protein. These results indicate that N-demethylation of cocaine in mullet liver microsomes is catalyzed by at least two cytochrome P450 isozymes having different affinities for the substrate, cocaine. On the contrary, kinetics of human and rat liver microsomal enzymes exhibited a linear monophasic enzymatic reaction after transformation of Michaelis–Menten data to either Eadie–Hofstee or Lineweaver–Burk plot. The K_m values for rat [14] and adult human [21] liver microsomal enzymes were determined as 0.42 mM and 1.8 mM cocaine, respectively. Similar to what was observed with mullet enzyme in this study, Pellinen et al. [22] reported that murine liver cocaine N-demethylase enzyme also exhibited two K_m values as 0.040–0.060 mM and 2–3 mM. It is important to note that in the case of mullet, even the low-affinity enzyme with a K_m value of 0.205 mM exhibited about 10-fold higher affinity toward cocaine than did the human enzyme.

Effect of Solvents on Cocaine N-Demethylase Activity

To find out the most suitable organic solvent for the preparation of P450 selective inhibitors, the effects

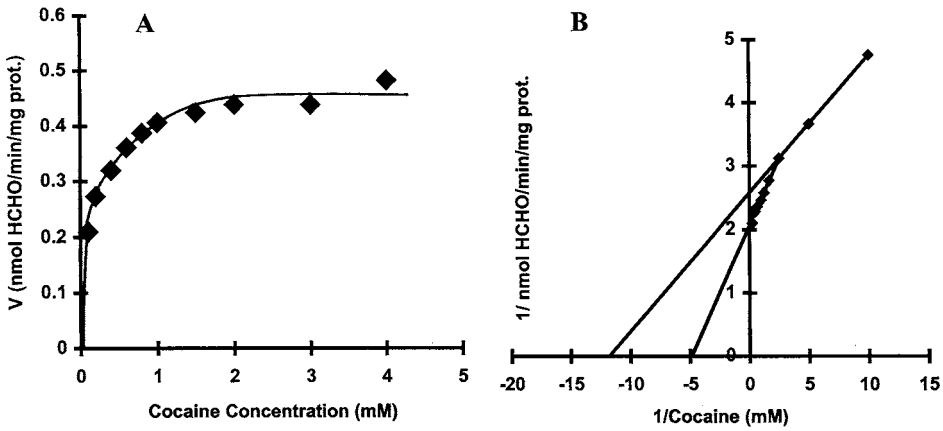

FIGURE 1. Kinetics of fish liver cocaine N-demethylase activity. (A) Substrate saturation curve of cocaine N-demethylase activity. Incubation mixture contained varying concentrations of cocaine ranging from 0.1 to 4.0 mM. The points are the average of duplicate determinations. (B) The correponding Lineweaver–Burk double-reciprocal plot. The apparent K_m values were calculated as 0.085 and 0.205 mM.

of three frequently used solvents, ethanol, methanol, and DMSO, on cocaine N-demethylase activity were determined by co-incubating these solvents in a final concentration of 1% with microsomes in the presence of 3 mM cocaine. Figure 2 illustrates the effects of these three different solvents on cocaine N-demethylase activity. The enzyme activity was inhibited approximately by 15% and 44% by ethanol and DMSO, respectively. On the other hand, cocaine N-demethylation rate was enhanced by about 230% with 1% methanol in the incubation medium. Since the organic solvent with the least effect on cocaine N-demethylation reaction was found to be ethanol, this solvent was used to dissolve ketoconazole, SKF-525A, quinidine, and cimetidine.

Inhibition of P450-dependent monooxygenation reactions by ethanol, methanol, acetone, and DMSO at 1% final concentration has been observed in several in vitro xenobiotic metabolism studies [14,20,21,43,44]. The reason for the enhancement of cocaine N-demethylase activity by about 230% with methanol is not clear. Hickman et al. [44] reported that acetone at 1% final concentration increased P4501A2-dependent caffeine N-demethylase activity by about 215% while methanol at the same concentration inhibited the same reaction by more than 95%. They did not attempt to explain the observed differences between the solvents but they recommended that inhibitory profile of each organic solvent used for each substrate in the presence of various P450s must be ascertained prior to use [44].

Participation of Cytochrome P4503A, P4502B, and P4502D in Cocaine N-Demethylation

Effects of various cytochrome P450 isozyme-specific inhibitors on the enzyme activity were determined in order to assess the participation of specific P450 isoforms that may contribute to N-demethylation of cocaine in mullet liver. Figure 3 shows the effects of various inhibitors, at a final concentration of 100 μM, on cocaine N-demethylation reaction.

The contribution of P4503A to cocaine N-demethylase activity was investigated using cimetidine and ketoconazole as P4503A-selective inhibitors [21,45–47].

As seen in Figure 3, ketoconazole and cimetidine at 100 μM final concentration inhibited cocaine N-demethylase activity markedly by 73 and 63%, respectively. Because both inhibitors were considered to be selective for P4503A, these data indicated that cocaine N-demethylation reaction was largely mediated by P4503A subfamily in mullet liver.

As seen in Figure 3, quinidine, a P4502D-specific inhibitor, at 100 μM final concentration reduced cocaine N-demethylase activity to 64%, suggesting the involvement of P4502D subfamily in cocaine N-demethylation in fish.

Aniline, a model substrate for P4502E1, at a final concentration of 100 μM, was incubated with cocaine to determine if it could inhibit the formation of norcocaine. As it can be seen in Figure 3,

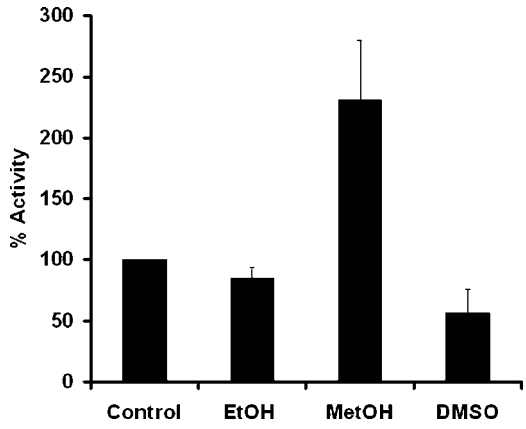

FIGURE 2. The effects of three different solvents (ethanol, methanol, and DMSO) on the rate of cocaine N-demethylation in leaping mullet liver microsomes. Solvents in a final concentration of 1% were preincubated with 3 mg microsomal protein containing 3 mM cocaine and buffer for 15 min at 25°C. Then the reaction was initiated with the addition of NADPH generating system. Data are means ± SD of four (for ethanol) and of two (for methanol and DMSO) independent experiments performed in duplicate.

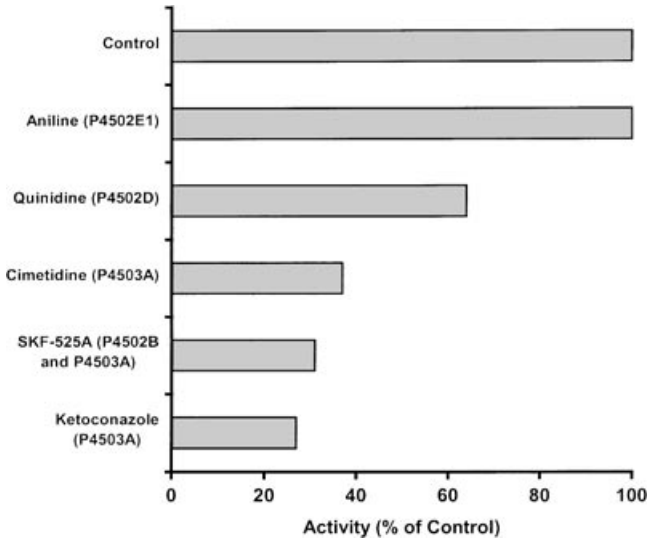

FIGURE 3. The effects of selective P450 inhibitors and a substrate probe on the rate of cocaine N-demethylation in leaping mullet liver microsomes. All chemicals were used at 100 μM final concentration. Each bar represents the mean of duplicate determinations.

aniline did not alter cocaine N-demethylation in mullet liver.

As illustrated in Figure 3, SKF-525A (proadifen), at a final concentration of 100 μM, substantially inhibited cocaine N-demethylase activity by 69%. Neither preincubation of SKF-525A with NADPH nor its preincubation with cocaine caused detectable differences in the degree of the inhibition. Since SKF-525A is an inhibitor of both P4502B and P4503A subfamily isoforms [48], the inhibition data by itself was not conclusive.

Participation of P4502B in fish liver cocaine N-demethylation activity was further investigated in a reconstituted system containing purified mullet liver P4502B [35], purified mullet P450 reductase [26], and artificial lipid DLPC. The results given in Table 1 demonstrated that purified fish P4502B substantially supported cocaine N-demethylase activity in the reconstituted system. For comparison, the results of reconstitution studies carried out by Poet and co-workers [14] with other purified P4502Bs are also given in Table 1. Purified mullet liver P4502B isozyme had similar ability in supporting N-demethylation rate to that of rat P4502B1 (Table 1). Poet et al. [14] showed that purified rat liver P4502B1 was three times more active in supporting cocaine N-demethylation reaction than rat P4502B2, while PB-inducible rabbit P4502B4 exhibited the lowest activity (Table 1).

Furthermore, the inhibitory effects of SKF-525A, ketoconazole, cimetidine, and quinidine on cocaine N-demethylase activity were studied at varying concentrations (50, 100, 500 μM) of these inhibitors so as to determine the IC_{50} values. As illustrated in Figure 4, all these chemicals inhibited cocaine N-demethylation reaction in a concentration-dependent manner. At 500 μM concentration, ketoconazole (P4503A inhibitor), SKF-525A (P4502B and P4503A inhibitor), cimetidine (P4503A inhibitor), and quinidine (P4502D inhibitor) inhibited cocaine N-demethylase activity by 96, 90, 78, and 73%, respectively. IC_{50} values were determined by linear regression (% control) against logarithmic inhibitor concentration and found to be 20 μM (for ketoconazole), 59 μM (for SKF-525A), 48 μM (for cimetidine), and 164 μM (for quinidine).

TABLE 1. Cocaine N-Demethylation Rates in a Reconstituted System Containing Purified P4502Bs

Purified P4502B	*Enzyme Activity (nmol HCOH/min/nmol P450)*
Fish liver P4502B[a]	4.20
Rat liver P4502B1[b]	5.05
Rat liver P4502B2[b]	1.70
Rabbit liver P4502B4[b]	0.70

[a] Reconstitution medium contained 0.2 nmol of purified fish liver P4502B, 90 U of purified fish liver P450 reductase, and 0.04 mg of DPLC in a final volume of 0.5 mL.
[b] Taken from Ref. [14].

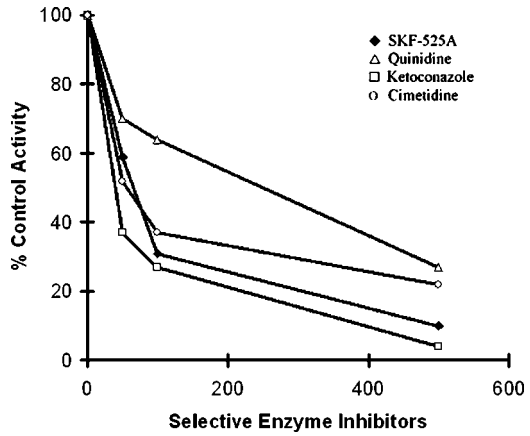

FIGURE 4. The effects of varying concentrations of SKF-525A, ketoconazole, quinidine, and cimetidine on cocaine N-demethylase activity. Values are the means of duplicate determinations.

DISCUSSION

Generally, more than one cytochrome P450 metabolizes the same compound. The P450 isoforms involved in the various steps of cocaine hepatic metabolism have not yet been completely elucidated and may differ from species to species. The N-demethylation of cocaine to norcocaine is suggested as the first step in the bioactivation of cocaine [14,16–21].

In the present investigation, we determined specific P450 isoforms participating in cocaine N-demethylation reaction in fish, leaping mullet, by using P450 isoform-selective inhibitors, isoform-specific substrate, and reconstitution studies using the purified components.

The present study demonstrated that P4503A subfamily participates in cocaine N-demethylation reaction in mullet liver microsomes. This conclusion was reached from the fact that both cimetidine and ketoconazole, diagnostic inhibitors of P4503A [21,45–47], potentially inhibited N-demethylase activity (Figures 3 and 4) with low IC_{50} values of 48 and 20 μM, respectively. The presence of members of P4503A family in liver was demonstrated in several fish species [6,27].

Results obtained with the inhibition studies carried out with quinidine, a P4502D-specific inhibitor [45,46], suggested the possible involvement of P4502D subfamily in cocaine N-demethylase activity in mullet. The expression of P4502D-like protein in fish was previously shown (http://drnelson.utmem.edu/cytochromeP450.html). Participation of P4502D subfamily in cocaine N-demethylase activity

has not been demonstrated in any species yet. An indirect evidence came from a recent work of Ramamoorthy et al. [49]. Like cocaine, ecstasy [(±)-3,4-methyenedioxymethamphetamine or MDMA] is a CNS stimulant. In vitro, MDMA is demethylated to (±)-3,4-dihydroxymethamphetamine by the polymorphic P4502D6. Cocaine was shown to be a potent inhibitor of MDMA metabolism in both human liver (P4502D6s) and P4502D6-expressing microsomes, with very low K_i values in micromolar (2.2 μM) and submicromolar (0.2 μM) range, respectively [49]. This indirect evidence suggests that cocaine, as in the case of "ecstasy," is most likely metabolized by P4502D6 in human liver.

Boyer and Peterson [50] observed that hepatotoxic effect of cocaine increased significantly following the treatment of mice with ethanol, but the major ethanol-inducible form of P450, P4502E1, was not involved in the metabolism of cocaine [19]. In agreement with the results of Boelsterli et al. [19], our results given in Figure 3 indicated no involvement of P4502E1 in cocaine N-demethylase activity in fish liver microsomes.

SKF-525A has been used in xenobiotic metabolism studies as a general inhibitor of the P450 activity since 1958. However, recent findings suggested that SKF-525A did not uniformly inhibit all P450 forms but the PB-induced forms, P4502Bs and P4503As, in rats [12,48]. In fish microsomes, SKF-525A inhibited cocaine N-demethylase activity (Figures 3 and 4) strongly with a low IC_{50} value of 59 μM, indicating possible involvement of P4502B in cocaine N-demethylation reaction. Reconstitution of fish cocaine N-demethylase activity in the presence of purified mullet P4502B and P450 reductase unequivocally demonstrated participation of P4502B in cocaine N-demethylation reaction in mullet.

In conclusion, the results of this investigation indicate that P4502E1 is not involved in N-demethylation of cocaine. P4503A and P4502B are the major P450s responsible for N-demethylation of cocaine whereas contribution of P4502D is minor in mullet liver.

REFERENCES

1. Lu AYH, Levin W. The resolution and reconstitution of the liver microsomal hydroxylation system. Biochim Biophys Acta 1974;344:205–240.
2. Arinç E, Philpot RM. Preparation and properties of partially purified pulmonary cytochrome P-450 from rabbits. J Biol Chem 1976;251:3213–3220.
3. Nebert DW, Gonzalez FJ. P-450 genes: Structure, evolution, and regulation. Ann Rev Biochem 1987;56:945–993.
4. Guengerich FP. Enzymatic oxidation of xenobiotic chemicals. Crit Rev Biochem Mol Biol 1990;25:97–153.
5. Schenkman JB, Jansson I. Introduction to cytochrome P450. In: Arinç E, Hodgson E, Schenkman JB, editors. Molecular Aspects of Oxidative Drug Metabolizing Enzymes: Their Significance in Environmental Toxicology, Chemical Carcinogenesis and Health. NATO-ASI Series. New York: Springer; 1995. pp 1–19.
6. Stegeman JJ. Diversity and regulation of cytochrome P450 in aquatic species. In: Arinç E, Hodgson E, Schenkman JB, editors. Molecular Aspects of Oxidative Drug Metabolizing Enzymes: Their Significance in Environmental Toxicology, Chemical Carcinogenesis and Health. NATO-ASI Series. New York: Springer; 1995. pp 135–158.
7. Nelson DR, Koymans L, Kamataki T, Stegeman JJ, Feyereisen R, Waxman DJ, Waterman MR, Gotoh O, Coon MJ, Estabrook RW, Gunsalus IC, Nebert DW. P450 superfamily: Update on new sequences, gene mapping, accession numbers, and nomenclature. Pharmacokinetics 1996;6:1–43.
8. Newcomb M, Hollenberg PF, Coon MJ. Multiple mechanisms and multiple oxidants in P450-catalyzed hydroxylations. Arch Biochem Biophys 2003;409:72–79.
9. Wringhton SA, Stevens JC. The human hepatic cytochrome P450 involved in drug metabolism. Crit Rev Toxicol 1992;22:1–22.
10. Cregler LL, Mark H. Medical complications of cocaine abuse. N Engl J Med 1986;315:1495–1500.
11. Evans MA, Harbison RD. Cocaine-induced hepatic necrosis in mice. Toxicol Appl Pharmacol 1978;45:739–754.
12. Thompson ML, Shuster L, Shaw K. Cocaine-induced hepatic necrosis in mice. Biochem Pharmacol 1979;28:2389–2395.
13. Perino LE, Warren GH, Levine JS. Cocaine-induced hepatotoxicity in humans. Gastroenterology 1987;93:176–180.
14. Poet TS, Mcqueen CA, Halpert JR. Participation of cytochrome P4502B and P4503A in cocaine toxicity in rat hepatocytes. Drug Metab Dispos 1996;24:74–80.
15. Pellinen P, Stenback F, Kojo A, Honkakoski P, Gelboin HV, Pasanen M. Regenerative changes in hepatic morphology and enhanced expression of CYP2B10 and CYP3A during daily administration of cocaine. Hepatology 1996;23:515–523.
16. Kloss MW, Rosen GM, Rauckman EJ. N-Demethylation of cocaine to norcocaine. Evidence for participation by cytochrome P450 and FAD-containing monooxygenase. Mol Pharmacol 1983;23:482–485.
17. LeDuc BW, Sinclair PR, Walton HS, Sinclair JF, Greenblatt DJ, Shuster L. Cocaine toxicity in cultured chicken hepatocytes: Role of cytochrome P450. Toxicol Appl Pharmacol 1994;125:322–332.
18. Pellinen P, Kulmala L, Konttila J, Auriola S, Pasanen M, Juvonen R. Kinetic characteristics of norcocaine N-hydroxylation in mouse and human liver microsomes: involvement of CYP enzymes. Arch Toxicol 2000;74:511–520.
19. Boelsterli UA, Lanzotti AC, Goldlin C, Oertle M. Identification of cytochrome P450IIBI as a cocaine-bioactivating isoform in rat hepatic microsomes and in cultured rat hepatocytes. Drug Metab Dispos 1992;20:96–101.
20. Poet TS, Brendel K, Halpert JR. Inactivation of cytochrome P450 2B protects against cocaine-mediated toxicity in rat liver slices. Toxicol Appl Pharmacol 1994;126:26–32.
21. Ladona MG, Gonzalez ML, Rane A, Peter RM, Torre RDL. Cocaine metabolism in human fetal and adult liver microsomes is related to cytochrome P4503A expression. Life Sci 2000;68:431–443.

22. Pellinen P, Stenbach F, Raunio H, Pelkonen O, Pasanen M. Modification of hepatic cytochrome P450 profile by cocaine-induced hepatotoxicity in DBA/2 mouse. Eur J Pharmacol Env Toxicol Pharmacol 1994;292:57–65.
23. Arinç E, Adalı O. Solubilization and partial purification of two forms of cytochrome P450 from trout liver microsomes. Comp Biochem Physiol 1983;76B:653–662.
24. Stegeman JJ, Kloepper-Sams PJ. Cytochrome P450 isozymes and monooxygenase activity in aquatic animals. Environ Health Perspect 1987;71:87–95.
25. Andersson T, Förlin L. Regulation of cytochrome P450 enzyme system in fish. Aquatic Toxicol 1992;24:1–20.
26. Sen A, Arinç E. Preparation of highly purified cytochrome P4501A1 from leaping mullet (*Liza saliens*) liver microsomes and biocatalytic, molecular and immunochemical properties. Comp Biochem Phys 1998;121C:249–265.
27. Buhler DR, Wang-Buhler J-L. Rainbow trout cytochrome P450s: Purification, molecular aspects, metabolic activity, induction and role in environmental monitoring. Comp Biochem Physiol 1998;121C:107–137.
28. Arinç E, Sen A. Hepatic cytochrome P4501A and 7-ethoxyresorufin O-deethylase induction in mullet and common sole as an indicator of toxic organic pollution in Izmir Bay, Turkey. Mar Environ Res 1999;48:147–160.
29. Arinç E, Sen A, Bozcaarmutlu A. Cytochrome P4501A and associated mixed-function oxidase induction in fish as a biomarker for toxic carcinogenic pollutants in the aquatic environment. Pure Appl Chem 2000;72:985–994.
30. Arinç E, Kocabiyik S, Su E. Induced CYP1A mRNA, protein and catalytic activity in the liver of feral fish, leaping mullet, *Liza saliens*. Comp Biochem Physiol 2001;128C:281–290.
31. Sen A, Arinç E. Purification and characterization of cytochrome P450 reductase from feral leaping mullet (*Liza saliens*) liver microsomes. J Biochem Mol Toxicol 1998;12:103–113.
32. Sen A, Arinç E. Further immunochemical and biocatalytic characterization of CYP1A1 from feral leaping mullet liver (*Liza saliens*) microsomes. Comp Biochem Physiol 2000;126C:235–244.
33. Sen A, Hu CH, Urbach E, Wang-Buhler JL, Yang YH, Arinç E, Buhler DR. Cloning, sequencing and characterization of CYP1A1 cDNA from leaping mullet (*Liza saliens*) liver and implications for the potential functions of its conserved amino acids. J Biochem Mol Toxicol 2001;15:243–255.
34. Arinç E, Sen A. Characterization of cytochrome P450 dependent mixed function oxidase system of gilthead seabream (*Sparus aurata*; Sparidae) liver. Comp Biochem Physiol 1993;104B:133–139.
35. Bozcaarmutlu A. Cytochrome P4502B-Type Protein from Feral Leaping mullet (*Liza saliens*) Liver Microsomes: Its Isolation and Biochemical and Immunological Characterization. PhD Thesis. Turkey: Middle East Technical University Ankara; 2002. pp 1–209.
36. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. J Biol Chem 1951;193:265–275.
37. Omura T, Sato R. The carbon monoxide-binding pigement of liver microsomes, II: Solubilization, purification and properties. J Biol Chem 1964;239:2379–2385.
38. Masters BSS, Williams CH, Kamin H. The preparation and properties of microsomal TPNH-cytochrome c reductase from pig liver. In: Coon MJ, Conney AH, Estabook RW, Gelboin HV, Gillette JR, O'Brien PJ, editors. Methods in Enzymology. New York: Academic Press; 1967. pp 709–720.
39. Yonetani T. Studies on cytochrome c peroxidase, II: Stoichiometry between enzyme, H_2O_2 and ferrocytochrome *c* and enzymic determination of extinction coefficients of cytochrome *c*. J Biol Chem 1965;210:4509–4514.
40. Nash T. The colorimetric estimation of formaldehyde by means of the Hantzsch reaction. Biochem J 1953;55:416–421.
41. Cochin J, Alexrod J. Biochemical and pharmacological changes in the rat following chronic administration of morphine, nalmorphine and normorphine. J Pharmacol Exp Ther 1959;125:105–110.
42. Ulusoy G, Boyuneğmez T, Bozcaarmutlu A. Kinetic and biochemical evidence suggest that at least two isoforms of P450 participates in N-demethylation of cocaine in rabbit. In: Abstract Book, XVII National Biochemistry Congress, Ankara, Turkey, June 24–27, 2002. pp 533–534.
43. Arinç E. Characterization of sheep liver and lung microsomal ethylmorphine N-demethylase. Comp Biochem Physiol 1985;80B:389–399.
44. Hickman D, Wang JP, Wang Y, Unadkat JD. Evaluation of the selectivity of in vitro probes and suitability of organic solvents for the measurement of human cytochrome P452 monooxygenase activities. Drug Metab Dispos 1998;26:207–215.
45. Halpert JR, Guengerich FP, Bend JR, Correia MA. Contemporary issues in toxicology: Selective inhibitors of cytochrome P450. Toxicol Appl Pharmacol 1994;125:163–175.
46. Bournie M, Meunier V, Berger Y, Fabre G. Cytochrome P450 isoform inhibitors as a tool for the investigation of metabolic reactions catalyzed by human liver microsomes. J Pharmacol Exp Ther 1996;277:321–332.
47. Kasahara M, Suzuki H, Komiya I. Studies on the cytochrome P450 (CYP)-mediated metabolic properties of miocamycin: Evalution of the possibility of a metabolic intermediate complex formation with CYP, and identification of human CYP isoforms. Drug Metab Dispos 2000;28:409–417.
48. Murray M. Complexation of cytochrome P-450 isozymes in hepatic microsomes from SKF 525-A-induced rats. Arch Biochem Biophys 1988;262:381–388.
49. Ramamoorthy Y, Yu A, Suh N, Haining RL, Tyndale RF, Sellers EM. Reduced (±)-3,4-methylenedioxymethamphetamine ("Ecstasy") metabolism with cytochrome P4502D6 inhibitors and pharmacogenetic variants in vitro. Biochem Pharmacol 2002;63:2111–2119.
50. Boyer CS, Peterson DR. Potentiation of cocaine-mediated hepatotoxicity by acute and chronic ethanol. Alcohol Clin Exp Res 1990;14:28–31.