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THE DEMETHYLENATION OF METHYLENEDIOXYMETHAMPHETAMINE ("ECSTASY") BY DEBRISOQUINE HYDROXYLASE (CYP2D6)

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Abstract—The metabolism of methylenedioxymethamphetamine (MDMA, "ecstasy") was examined in a microsomal preparation of the yeast *Saccharomyces cerevisiae* expressing human debrisoquine hydroxylase, CYP2D6. Only one product, dihydroxymethylamphetamine (DHMA), was detected in the incubation mixture, and this product accounted for all of the substrate consumption at low concentration (10 μM). Mean ± SD values of apparent K_m(μM) and V_{max} (nmol/min per nmol P450) for the demethylenation of (+) and (−)-MDMA at low concentrations (1–1000 μM) were 1.72, 0.12 and 6.45, 0.10 and 2.90, 0.10 and 7.61, 0.06, respectively. At high concentrations (>1000 μM) substrate inhibition was noted, with K_i values of 14.2 and 28.2 mM, respectively, for the (+) and (−) enantiomers. Incubation of MDMA isomers with human liver microsomes indicated that their demethylenation is deficient in the poor metabolizer phenotype. Thus, MDMA is converted to the catecholamine DHMA by CYP2D6, and this may give rise to genetically-determined differences in toxicity.

Key words: ecstasy, methylenedioxymethamphetamine; debrisoquine; extensive metabolizer; poor metabolizer; brain P450; neurotoxicity

Reports of exaggerated responses and deaths [1, 2] after abuse of MDMA§ "ecstasy" have prompted concern as to whether some individuals may be at increased risk of acute toxicity. Longer term toxicity, characterized by persistent psychosis [3], is consistent with the ability of the drug to destroy serotoninergic nerve terminals [4], and there is evidence that some of the neurotoxic effects of MDMA may be mediated by a reactive catechol metabolite, DHMA, which is readily oxidized to the *o*-quinone and thence combines with nucleophilic thiols [5] (Fig. 1). In addition, aromatic hydroxylation and demethylenation of MDMA gives rise to a 6-hydroxydopamine analogue which is also believed to be neurotoxic [6] (Fig. 1). Thus, opening of the methylenedioxyphenyl ring of MDMA may be a key determinant of both acute toxicity, if this is mediated by parent drug, and of chronic toxicity mediated by active metabolites.

The demethylenation of MDMA to the catechol is a major metabolic pathway and is catalysed by cytochrome P450 in both liver and brain [5, 7, 8]. Previous work has established that this reaction is deficient in the female Dark Agouti strain of rat [9], a model of the PM phenotype for human CYP2D6 (debrisoquine 4-hydroxylase). CYP2D6 is a polymorphic member of the cytochrome P450 superfamily of enzymes and is absent in 5–9% of caucasians (PM) as a result of autosomal recessive inheritance of gene mutations [10]. We now provide further evidence for the role of CYP2D6 in the metabolism of MDMA using human liver microsomes from PM and EM subjects and microsomes from yeast expressing the human enzyme.

MATERIALS AND METHODS

Chemicals

(+)- and (−)-MDMA were obtained as their hydrochloride salts from the Research Technology Branch of the National Institute on Drug Abuse (Rockville, MD, U.S.A.). DHMA, the product of MDMA demethylenation, was prepared by a modification of the procedure described by Smissman and Borchardt [11]. Glucose-6-phosphate, glucose-6-phosphate dehydrogenase and superoxide dismutase were obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). All other chemicals were of the highest grade available.

Yeast microsomes

The yeast expression system for human CYP2D6 developed and validated by Ellis *et al.* [12] was used. Microsomes were prepared as described by Ching *et al.* [13].

Human microsomes

Samples of three human livers were used with the approval of the Ethics Committee of the Royal Hallamshire Hospital. Two of the livers (EM1 and

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§ Abbreviations: MDMA, methylenedioxymethamphetamine; DHMA, 3,4-dihydroxymethylamphetamine; MDA, methylenedioxyamphetamine; CYP2D6, cytochrome P4502D6; PM, poor metabolizer; EM, extensive metaboliser.

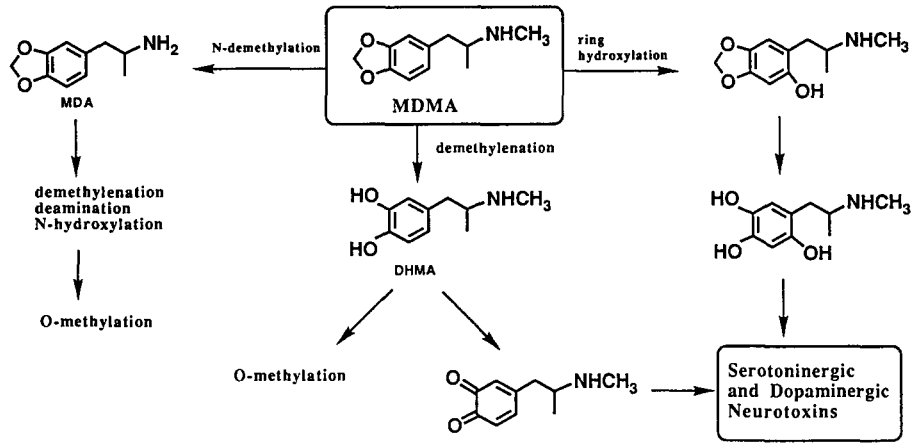

Fig. 1. Major metabolic pathways of MDA [5, 6]

EM2) were genotyped as extensive metabolizers being either homozygous wild-type or heterozygous for the G to A transition (C.R. Wolf, personal communication). EM1 was from a caucasian male road traffic accident victim of unknown age and the sample was obtained within 1 hr of the death of the patient, who was a non-drinker and non-smoker. The following drugs were given prior to or during organ removal: atracurium, midazolam, morphine, propofol, dobutamine, dopamine, alfentanil, verapamil, frusemide, ranitidine, cefuroxime and metronidazole. The sample was histologically normal. EM2 was from a 52-year old caucasian male non-smoker/occasional drinker, who had undergone a partial hepatectomy to remove a liver metastasis resulting from a primary rectal carcinoma. The patient was drug-free prior to admission to hospital and received the following drugs immediately before or during surgery: temazepam, midazolam, fentanyl, etomidate and atracurium. Although histological analysis was not performed, tissue which appeared tumour-free on visual inspection, was used for these studies. The third liver (PM) was homozygous for the G to A transition mutation and was from a 59-year old caucasian male renal transplant donor, whose smoking and drinking habits were not known. The following drugs were used prior to the death of the patient or during organ removal: dexamethasone, hydralazine, dopamine, dobutamine, chlorpromazine, frusemide, phenoxybenzamine, phentolamine and heparin. The sample was histologically normal. All three livers were active with respect to a range of other cytochrome P450-catalysed reactions. Microsomes were prepared as described previously [14].

MDMA metabolism

The incubation mixture consisted of 100 mM HEPES buffer (pH 7.6), superoxide dismutase (100 U), an NAPDH generating system (0.5 mM NADP +, 8 mM glucose-6-phosphate, 5 mM $MgCl_2$, and 1 U of glucose-6-phosphate dehydrogenase), the microsome preparation (from yeast containing 33–65 pmol cytochrome P450; from human liver containing 90–180 pmol of cytochrome P450), and substrate in a final volume of 1.0 mL [15]. The reactions were initiated by the addition of the microsomal preparation; they were carried out at 37°, and terminated after 5 min by the addition of 0.5 mL of 7.5% (v/v) perchloric acid. All of the metabolites assayed were stable in perchloric acid.

Analysis of products

GC/MS analyses. The supernatant from the quenched reaction mixture containing yeast microsomes was made alkaline with 1 mL of 1.5 M sodium carbonate buffer (pH 9.5) and 10 nmol of $[^2H_2]$MDMA, $[^2H_2]$MDA and $[^2H_2]$-6-OHMDMA were added as internal standards. Following extraction with dichloromethane (5 mL) and evaporation of the organic phase to 100 μL, the neutral and basic compounds were derivatized with acetic anhydride prior to GC/MS analysis. A Hewlett-Packard 5971A GC/MS system was employed in the selected ion mode using the m/z fragments 154 for MDMA, 164 for $[^2H_2]$MDMA, 162 for MDA and 164 for $[^2H_2]$MDA. The m/z = 154 fragment is the trifluoroacetylaminoethyl moiety, and 162 and 164 are the styrene fragments from the normal and deterium enriched compounds, respectively. The analysis of 6-OHMDMA utilized 274 and 276 for the analyte and internal standard, respectively. Standard curves were run with each batch of samples and the slopes of these curves differed by less than 5%. The lowest quantifiable levels of all the analytes were 25 pmol/sample.

HPLC analyses. Concentrations of DHMA were measured by HPLC with electrochemical detection as described by Kumagai *et al.* [15].

Other analyses

NADPH consumption was measured in a separate experiment with yeast microsomes in which the reaction was initiated by addition of NADPH (2 mM

Table 1. Material balance after incubation of MDMA with microsomes from yeast expressing CYP2D6

Substrate	MDMA consumed (nmol/min per nmol P450)	Catechol formed (nmol/min per nmol P450)
(+)-MDMA	4.58 ± 0.20	4.85 ± 0.05
(−)-MDMA	2.46, 3.12*	3.28 ± 0.15

Substrate (10 μM) was incubated with NADPH (1 mM) for 5 min and quenched.
The values are means ± SD of at least three determinations.
* Duplicate values since the third sample was lost.

final concentration) instead of the generating system. NADPH oxidation was measured at 15 sec intervals over 5 min by the change in UV absorption at 340 nm (UVikon 810 spectrophotometer), using an extinction coefficient of 6.22 mM/cm. After quenching, the reaction mixture was assayed for MDMA [15].

The formation of hydrogen peroxide was measured in a separate experiment with yeast microsomes in which sodium azide (0.2 mM) was added to the incubation mixture. The hydrogen peroxide was determined using the ferrithiocyanate assay described by Hildebrandt *et al.* [17]. MDMA and DHMA concentrations were also measured at the end of the incubation.

Data analysis

Kinetic data obtained over high (5–10,000 μM) and low (1–1000 μM) concentrations of MDMA were fitted by expressions for substrate inhibition and single enzyme kinetics, respectively [18], using a nonlinear regression program (BMDP Statistical Software). The expression for substrate inhibition was:

$$v_{obs} = V_{max}/(1 + K_s/[s] + [s]/K_s')$$

where K_s' is the equilibrium constant for formation of the inhibitory complex [19].

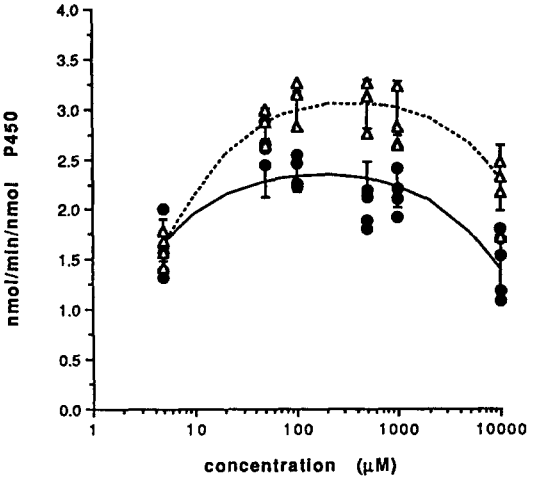

Fig. 2. Formation of DHMA from MDMA added to yeast microsomes expressing CYP2D6. Open triangles, data from incubations with (−)-MDMA; closed circles, data from incubations with (+)-MDMA. The lines are predicted values obtained by fitting a substrate inhibition model to the observed data.

RESULTS

Microsomes from control yeast transfected with plasmid without the *CYP2D6* cDNA did not demethylenate MDMA. The rate of MDMA demethylenation by microsomes from yeast expressing CYP2D6 was linear over about 7 min. The GC/MS procedure assayed the primary metabolites MDA and 6-hydroxy MDMA, but under the conditions of the incubation, neither compound was detected. The relationship between substrate consumed and DHMA formed was stoichiometric within experimental error (Table 1).

Initial kinetic studies with yeast microsomes revealed substrate inhibition (Fig. 2), with K_i values of 14.2, 3.4 and 28.2, 8.2 mM for the (+) and (−) isomers of MDMA, respectively. Subsequently, a detailed kinetic analysis of the yeast CYP2D6 was made using a substrate concentration range of 1–1000 μM (Fig. 3). The kinetic parameters obtained from this study are shown in Table 2.

The relationship between the consumption of NADPH and product formed is a measure of the efficiency of the reaction, and to assess this the quantities of hydrogen peroxide and product formed were compared with NADPH consumption. The results of this analysis, conducted at two concentrations, are shown in Table 3. At a substrate concentration of 10 μM approximately 75% of the consumed NADPH was accounted for by formation of hydrogen peroxide and DHMA, whereas at 10 mM only about 50% was accountable in this way. The total NADPH consumed at 10 mM was lower than that at 10 μM. The proportion of catecholamine formed only accounted for about 5–8% of the NADPH consumed at both concentrations.

Microsomes from the livers of the two EM subjects produced appreciably more DHMA from the isomers of MDMA than microsomes from the PM subject (Table 4). No products of N-demethylation or

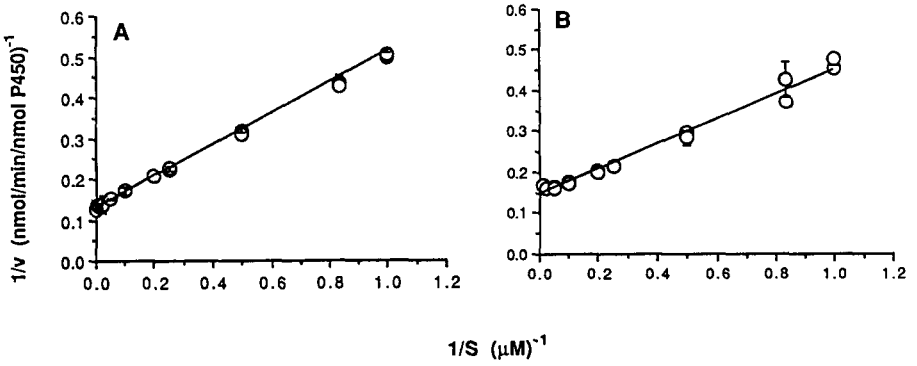

Fig. 3. Lineweaver–Burk plots of the demethylenation of MDMA by yeast microsomes expressing CYP2D6. Substrate concentration ranged from 1 to 1000 μM. Panel A, data for (−)-MDMA; panel B, data for (+)-MDMA. The lines are calculated from kinetic constants obtained by fitting the data with a single enzyme site model.

Table 2. Kinetic parameters for the demethylenation of MDMA by microsomes from yeast expressing CYP2D6

Substrate	(+)-MDMA	(−)-MDMA
K_m (μM)	1.72 ± 0.12	2.90 ± 0.10
V_{max} (nmol/min per nmol P450)	6.45 ± 0.10	7.61 ± 0.06

The substrate concentration range was 1–1000 μM and the cytochrome P450 concentration was 0.033 nmol/mL. The values are means ± SD of three determinations.

Table 4. MDMA metabolism to DHMA by human liver microsomes

	(+)-MDMA	(−)-MDMA
Genotype	(nmol/min per nmol P450)	
EM1	0.523	0.583
EM2	1.200	1.287
PM	0.114	0.009

Substrate concentration was 10 μM. No products of N-demethylation or aromatic hydroxylation were detected after the 5 min incubation.

aromatic hydroxylation were detected after the 5 min incubation using microsomes from any of the livers.

DISCUSSION

Oxidation of the methylenedioxyphenyl group is the predominant metabolic pathway for MDMA in man [8]. Our results using heterologously expressed CYP2D6 show that the reaction is catalysed by this enzyme, and the findings with microsomes from the livers of EM and PM subjects indicate that CYP2D6 makes a major contribution to the hepatic demethylenation of MDMA.

Members of the CYP2D enzyme family have also been found in mammalian brain [20]. Therefore, detection of the highly polar metabolite DHMA in the brain after administration of MDMA [7, 21] would be consistent with its formation from MDMA by CYP2D6 within that organ. Furthermore, the low K_m values for demethylenation cover the range

Table 3. NADPH consumption and product formation after incubating MDMA with microsomes from yeast expressing CYP2D6

Substrate	NADPH consumed	Peroxide formed	DHMA formed	Total products
(+)-MDMA (10 μM)	4.50 ± 0.01	3.33 ± 0.09	0.319 ± 0.002	3.65 ± 0.09
(+)-MDMA (10 mM)	3.54 ± 0.15	1.87 ± 0.03	0.249 ± 0.002	2.12 ± 0.03
(−)-MDMA (10 μM)	4.50 ± 0.01	3.39 ± 0.04	0.220 ± 0.006	3.61 ± 0.04
(−)-MDMA (10 mM)	3.86 ± 0.30	1.93 ± 0.03	0.310 ± 0.007	2.24 ± 0.03

The data are expressed as nmol/min and are the means ± SE of three determinations.

of brain concentrations of MDMA [22]. As DHMA is a dopamine derivative, its formation in the brain is of pharmacological and toxicological interest. The des-methyl analogue, α-methyldopamine, is an indirect dopamine agonist [23, 24], and when administered directly into the CNS causes dopamine-related hyperactivity. α-Methyldopamine has also been implicated in the hepatotoxic actions of α-methylDOPA [25]. Products formed following further metabolism of DHMA are considered to be potent neurotoxins [5, 6].

Schmidt [26] has shown that the long-term toxicity to serotonin neurones produced by MDMA in the rat is selective for the *S*(+)-isomer, and others [27] indicate that the enantiomers of MDA and MDMA may have different behavioural effects. However, there appears to be minimal stereoselectivity in the demethylenation reaction as catalysed by either rat [9], human or yeast preparations. The demethylenation of MDMA by CYP2D6 is consistent with the known regioselectivity of the enzyme in that it oxidizes basic aromatic compounds at sites distal to their basic centres [28]. Thus, although CYP2B4 oxidizes MDMA at both the nitrogen and aromatic ring centres [15, 16], only the latter is attacked by CYP2D6.

Substrate inhibition, noted in the kinetic studies with MDMA, is uncommon in reactions mediated by cytochromes P450. The present results are consistent with a mechanism in which the substrate forms a catalytically inactive complex at higher concentrations, a phenomenon observed previously with chemically related substances. Thus, amphetamine [29, 30], phentermine [30] and phenylethylamine [31] have been shown to exhibit substrate inhibition at millimolar concentrations in rat liver preparations. These reactions were not inducible by phenobarbitone or 3-methylcholanthrene, indicating catalysis by a constitutive enzyme. More recent studies of the 4-hydroxylation of methylamphetamine suggest that this reaction is mediated by the CYP2D system and also exhibits substrate inhibition (A. Hiratsuka, unpublished observations). Thus, the substrate inhibition that these alkylamines show may reflect a common interaction with CYP2D isoenzymes. Although the K_i values are higher than realistic *in vivo* concentrations, the structural basis of this effect contributes to an understanding of the regioselectivity of this family of enzymes.

The efficiency of demethylenation of MDMA by CYP2D6 appears to be very low as only a small proportion of the NADPH consumed is reflected in formation of the catecholamine product (Table 3). At the higher substrate concentration, where some substrate inhibition occurs, only 60% of the consumed NADPH was accountable as measureable product, and the proportion of catecholamine formed remained low. The possibility of alternate metabolic pathways was considered. The GC/MS procedure assayed the primary products of N-demethylation and ring hydroxylation but none was detected under the experimental conditions used. Since both catecholamine and hydrogen peroxide formation were decreased at the higher substrate concentration, hydrogen peroxide may result from an action of cytochrome P450 rather than from reduction of oxygen by the flavoprotein, cytochrome P450 reductase. Thus, when the haem protein is inhibited by substrate, formation of both catecholamine and hydrogen peroxide is inhibited.

In conclusion, we have shown that an important pathway of MDMA metabolism is catalysed by CYP2D6. Therefore, since this reaction is expected to be under monogenic control, the consequences of decreased demethylenation of MDMA in abusers who are PMs need to be established. While these individuals may be less susceptible to any chronic neurological effects of the drug, since they should produce less of the catechol metabolite, they may be at increased risk of acute toxicity because of impaired metabolism of the parent drug. Thus, genetically deficient metabolism of MDMA may help to explain why some users of "ecstasy" seem particularly sensitive to its effects. There are also implications with regard to interactions between MDMA and other substrates of CYP2D6.

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