Pharmacology Biochemistry & Behavior, Vol 38 pp 345–351 © Pergamon Press plc, 1991 Printed in the U S A   0091-3057/91 $3 00 + 00

Assessment of the Role of α-Methylepinine in the Neurotoxicity of MDMA

THOMAS D. STEELE,[1] WILLIAM K. BREWSTER,* MICHAEL P. JOHNSON, DAVID E. NICHOLS* AND GEORGE K. W. YIM[2]

*Department of Pharmacology and Toxicology and *Department of Medicinal Chemistry and Pharmacognosy School of Pharmacy and Pharmacal Sciences, Purdue University, West Lafayette, IN 47907*

Received 13 April 1990

STEELE, T D, W K BREWSTER, M P JOHNSON, D E NICHOLS AND G K W YIM *Assessment of the role of α-methylepinine in the neurotoxicity of MDMA* PHARMACOL BIOCHEM BEHAV **38**(2) 345–351 1991 —To assess the potential involvement of metabolism of 3,4-methylenedioxymethamphetamine (MDMA) to the catechol α-methylepinine in producing serotonergic neurotoxicity, we attempted to correlate aspects of this reaction with the neurotoxicity profile of MDMA. In contrast to the stereoselectivity of *S*-(+)-MDMA in causing persistent declines in rat brain 5-hydroxyindole levels, no stereochemical component to the metabolic reaction was apparent Rat liver microsomes generated a significantly greater amount of α-methylepinine than did mouse microsomes, but similar amounts of metabolite were produced by brain microsomes from the two species Formation of α-methylepinine by hepatic, but not brain, microsomes was inhibited by SKF 525A and induced by phenobarbital, possibly indicating a tissue specificity in cytochrome P-450-dependent metabolism of MDMA To directly assess whether α-methylepinine is a likely mediator of MDMA neurotoxicity, the compound was administered intracerebroventricularly No persistent declines in biogenic amines or their metabolites were observed one week following treatment These data suggest that α-methylepinine alone is not responsible for the neurotoxic effects of MDMA

MDMA   α-Methylepinine   Neurotoxicity   Metabolism   Catecholamines

EVIDENCE documenting the persistent alterations of various neurochemical parameters by 3,4-methylenedioxymethamphetamine (MDMA) in several mammalian species has recently accumulated. The long-lasting declines in brain levels of 5-hydroxytryptamine (5-HT) and its acidic metabolite 5-hydroxyindole-3-acetic acid (5-HIAA) (2, 8, 30, 33), tryptophan hydroxylase activity (33,41), and 5-HT uptake sites (1,30), as well as histological (8) and immunocytochemical changes (24), suggest that high doses of MDMA produce neurodegeneration. Similar alterations are produced by other amphetamine derivatives such as *p*-chloroamphetamine (PCA) and methamphetamine (METH). MDMA more closely resembles PCA in that serotonergic markers are specifically affected (12, 15, 29), whereas METH alters both serotonergic and dopaminergic parameters (27). Despite extensive investigation, the mechanism by which various substituted derivatives of amphetamine produce neurotoxicity has not been clearly elucidated.

The effectiveness of specific inhibitors of 5-HT uptake in preventing drug-induced release of neurotransmitter (32) and persistent neurochemical changes (30) implies that an interaction of MDMA with an active transport mechanism for 5-HT is an essential requirement for these effects. Subsequent events in the neurotoxic pathway are less clearly defined Endogenous formation of the neurotoxins 6-hydroxydopamine (6-OHDA) from dopamine (DA) (35) and 5,6-dihydroxytryptamine (5,6-DHT) from 5-HT (7) has been suggested as a mechanism by which related amphetamine analogs METH and PCA produce neurotoxicity. Protection against METH-induced deficits in dopaminergic (28) and serotonergic (31) parameters by the tyrosine hydroxylase inhibitor α-methyl-*p*-tyrosine is consistent with a role for endogenous DA in the neurotoxicity of METH. However, depletion of endogenous stores of 5-HT with *p*-chlorophenylalanine does not prevent the persistent neurotoxic effects of PCA (12). Like METH, MDMA releases DA in vitro (18,32) and induces a transient release of DA in vivo (43). These findings have led to speculation that DA, which itself is cytotoxic (13), may mediate MDMA neurotoxicity. Indeed, depletion of central DA stores partially protects against MDMA-induced deficits of central serotonergic parameters (42).

Alternative speculations on the mechanism of MDMA-induced neurotoxicity have focused on the role of drug metabolism. Sup-

[1]Present address Department of Physiology, School of Medicine, University of Maryland at Baltimore, 655 West Baltimore Street, Baltimore, MD 21201

[2]Requests for reprints should be addressed to G K W Yim, Ph D, Department of Pharmacology and Toxicology, School of Pharmacy and Pharmacal Sciences, Purdue University, West Lafayette, IN 47907

port for the notion that a metabolite of MDMA may mediate neurotoxicity has stemmed from studies in which 5-HT uptake inhibitors blocked persistent serotonergic deficits produced by MDMA if given up to six hours following MDMA treatment (30). If brain levels of MDMA decline rapidly following peripheral treatment, as has been reported to occur with the primary amine, 3,4-methylenedioxyamphetamine (MDA) (22), some other compound might be responsible for the neurochemical changes that are produced long after initial drug administration. Furthermore, it has been reported that a bolus dose of MDMA administered centrally does not produce long-term changes in serotonergic markers, which suggests that a peripheral metabolite may be responsible for these alterations (34)

The propensity of catechols such as DA and α-methyl-DOPA to oxidize to reactive quinones and free radicals may account for their cytotoxic effects (10,13). This property of catechols is of interest with regard to MDMA because demethylenation of methylenedioxy-substituted aromatic amines, which results in the generation of a catechol, is mediated by a classical cytochrome P-450 metabolic pathway (6,16). Thus a hypothetical scheme for the neurodegenerative effects of MDMA is metabolism to α-methylepinine which is then oxidized to reactive quinoidal species, with coincident generation of toxic free radicals. To assess whether this metabolic route is critically involved in MDMA neurotoxicity, we have attempted to correlate characteristics of this conversion with certain aspects of the neurotoxic profile of MDMA. Since persistent declines in brain 5-HT are stereoselective for the *S*-(+)-enantiomer (30), we anticipated that similar stereoselectivity might be observed in the metabolic reaction if α-methylepinine were involved in the neurotoxic pathway. Since mice are less susceptible to MDMA neurotoxicity (19, 37, 40), we assessed whether differences in metabolism between rats and mice might contribute to differences in species sensitivity We also sought to determine if the in vitro conversion of MDMA to α-methylepinine by brain and hepatic microsomes occurred in similar cytochrome P-450-dependent manners, as classical modulators of drug metabolism had not helped to elucidate the role of metabolism in MDMA neurotoxicity (unpublished observations). Finally, to directly test the neurotoxic potential of the metabolic pathway, the effects of α-methylepinine on rat brain biogenic amines and metabolites following intracerebroventricular administration was assessed.

METHOD

Animals and Housing

Male Sprague-Dawley rats and male Swiss-Webster mice were obtained from Harlan Industries (Indianapolis, IN). Animals used in the drug metabolism experiments were group housed. Rats used in the intracerebroventricular cannulation studies were housed in individual stainless steel cages (25 × 21 × 20 cm) with a wire mesh floor. Food and water were available ad lib Room temperature was maintained at 22–24°C and lighting was controlled on a 12/12-h cycle (light onset at 0700).

Drugs and Chemicals

Previously described methods were employed for the synthesis of the stereoisomers of MDMA (23) and racemic MDMA (5). α-Methylepinine hydrobromide was prepared by chemical cleavage of the methylenedioxy ring of MDMA with BBr_3 (Fig. 1). Purity of these compounds was verified by elemental analysis, and standard chromatographic and spectroscopic methods. Phenobarbital sodium was from J. T. Baker, SKF 525A (2-diethylaminoethyl-2,2-diphenylvalerate hydrochloride) was from Smith, Kline and French Labs, and HPLC standards, β-nicotinamide adenine dinucleotide phosphate monosodium salt (B-NADP), glucose-6-phosphate disodium salt, and glucose-6-phosphate dehydrogenase (Type XII from Torula yeast) were from Sigma Chemical Co.

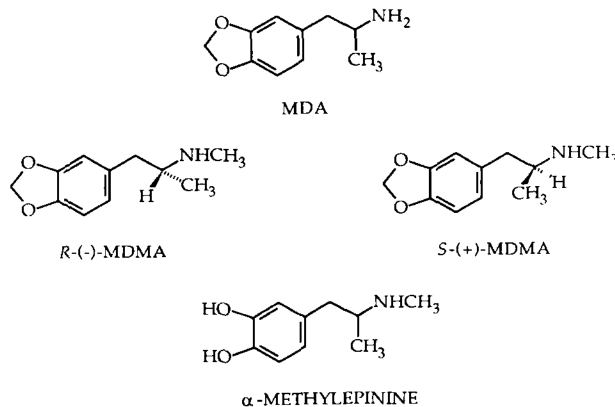

FIG 1 Chemical structures of the stereoisomers of MDMA and of α-methylepinine

In Vitro Metabolism Experiments

The in vitro demethylenation of MDMA to α-methylepinine was studied using liver and brain microsome preparations. For preparation of microsomes, rats (200–250 g) were sacrificed by decapitation and mice (25–30 g) by cervical dislocation, and brains and livers were removed and placed on ice. A 2.0 g portion of liver was homogenized in 6 volumes of 100 mM HEPES (pH 7.4) containing 1.15% KCl Brains were handled similarly with the exception that the homogenizing solution also contained 0.32 M sucrose. Homogenates were centrifuged at 9000 × g for 20 min at 4°C The resulting supernatants were centrifuged at 105,000 × g for 60 min at 4° Liver microsomal pellets were resuspended in 10 volumes and brain pellets in 3 volumes of 0.12 M phosphate buffer (pH 7.4). The protein concentration of the microsome preparations was approximately 1.0 mg/ml as determined by the method of Bradford using bovine serum albumin as the standard (3).

For liver microsome metabolism experiments, a 0.5 ml aliquot of the microsome preparation was preincubated for five minutes with 0.45 ml of a prepared solution of an NADPH generating system [final concentrations of components· 0.25 mM β-nicotinamide adenine dinucleotide phosphate (β-NADP), 3.0 mM glucose-6-phosphate, 1.2 units glucose-6-phosphate dehydrogenase, and 1.2 mM $MgCl_2$ in 0.12 M phosphate buffer, pH 7.4]. To blanks, 0.45 ml of phosphate buffer was added instead of the generating system. For brain microsome samples, the volumes of all components were reduced by one-half. The reaction was initiated by addition of MDMA (final concentration of 5 mM in 0.12 M phosphate buffer), and proceeded for the designated period of time. The reaction was terminated by placing the samples on ice and quenching with ice-cold 1 N perchloric acid. The samples were centrifuged at 3000 rpm for 15 min in a table-top centrifuge at 4–8°C to remove precipitated protein. The resulting clear supernatant was transferred to a glass vial and frozen at −70°C until time of assay

The effects of inhibition of cytochrome P-450-mediated metabolism were assessed by inclusion of SKF 525A in reaction

mixtures incubated with the stereoisomers of MDMA. The effect of induction of P-450 metabolism was assessed by treating rats for four days with 75 mg/kg phenobarbital per day and sacrificing the animals 24 h following the last phenobarbital dose. Incubation times of 10 min with liver microsomes and 5 min with brain microsomes were used. The times were selected as maximal points on the linear portion of the reaction time curve.

Intracerebroventricular Administration of α-Methylepinine

For the intracerebroventricular administration of α-methylepinine, male Sprague-Dawley rats weighing 250–300 g were implanted with a unilateral stainless steel cannula (length = 15 mm; outside diameter = 0.028") in the right side of the brain. The surgery was conducted under ketamine (90 mg/kg)/xylazine (10 mg/kg) anesthesia. A sagittal incision through the skin was made and the skull exposed The animal was placed in a stereotaxic apparatus to set the coordinates which were AP· −0.5 mm, L 1.75 mm with reference to bregma, and H. 2.8 mm below dura (25) A small hole was drilled with a dental drill to the dura. Two additional holes were drilled for the placement of anchor screws. The cannula was set in place with dental acrylic, plugged with a small wire, and the wound was closed with wound clips. The animals were allowed a recovery period of 4–7 days prior to testing for cannula placement and patency The wound was sprayed daily with furazolidone (Topazone) to prevent bacterial infection. To test for cannula placement and patency, the rats were administered 10 μg of *l*-norepinephrine bitartrate and their one-hour food intake was measured Only rats eating greater than 1 g of chow were used.

Animals were randomly assigned to one of three treatment groups which received either 0, 300, or 600 μg of α-methylepinine prepared in saline to deliver in a volume of 10 μl. The drugs were administered through a second cannula that fit inside the implanted cannula (o d. = .016") and set to protrude 0.5 mm below the tip of the implanted cannula. The delivery cannula was connected via polyethylene tubing to a Hamilton syringe with which the compound was infused over a 20–30-s time period. Animals were sacrificed by decapitation one week following treatment with α-methylepinine, brains were removed and dissected, wrapped in parafilm and foil, and frozen at −70° until the time of assay.

HPLC-EC Analysis of Biogenic Amines and Metabolites and Drug Metabolites

Preparation of brain tissue and subsequent analysis by high performance liquid chromatography with electrochemical detection (HPLC-EC) were essentially as described previously (37). The mobile phase consisted of 75% 0.05 M NaH_2PO_4/0.03 M citric acid/1.53 mM octyl sodium sulfate/0.1 mM EDTA (pH 2.75) and 25% methanol. The flow rate was 1.0 ml/min. The applied potential to the glassy carbon electrode was 800 mV. Data are reported as ng/mg tissue.

The amount of α-methylepinine in the in vitro metabolism samples was also quantitated by HPLC-EC. Preliminary experiments were conducted to identify α-methylepinine in the microsomal incubation extracts on the basis of the compound's chromatographic and electrochemical properties. This was accomplished by constructing hydrodynamic voltammograms (HDV) for authentic α-methylepinine and the coeluting chromatographic peak in the microsomal extracts (see the Results section). Sample aliquots of 40–50 μl were loaded into the HPLC-EC analyzer. The actual injection volume was produced using a 20 μl injection loop The mobile phase for the analysis was 92% 0.075 M NaH_2PO_4/0 1

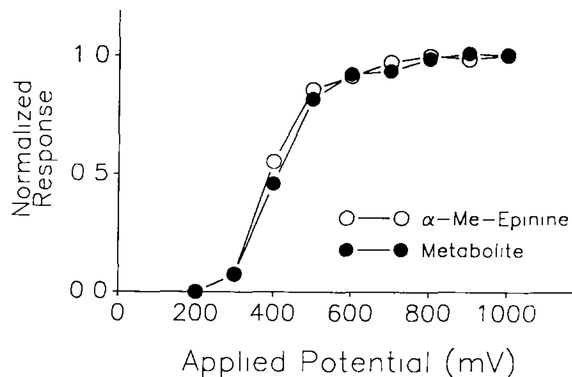

FIG. 2 Hydrodynamic voltammograms of α-methylepinine and liver microsomal metabolite of MDMA Aliquots of liver microsome incubation extract and authentic α-methylepinine were injected into the chromatograph and the current response monitored at applied potentials ranging from 200–1000 mV in 100 mV steps The data were normalized to the maximal response obtained at 1000 mV Each point represents the mean of two determinations

mM EDTA (pH 2 5) and 8% acetonitrile The flow rate was 1 6 ml/min and the applied potential was 800 mV. Peak heights were measured to the nearest millimeter, and the amount of α-methylepinine in the 20 μl injection sample was determined by interpolation from a standard curve. For sample quantitation, the final sample volumes were considered to be 1 2 ml for liver microsome samples, and 0.6 ml for brain microsome samples. The amount in the final sample volume is expressed as ng α-methylepinine/mg protein.

Statistical Analysis

For time course analysis of the conversion of MDMA to α-methylepinine, the line of best fit was determined by the least squares method and slopes of the regression lines compared. Differences among treatment groups and between species in the in vitro metabolism studies and among dosages in the intracerebroventricular study were compared with a one-way analysis of variance. The data obtained at the 10-min time point for the liver microsome studies, and the 5-min time point for the brain microsome experiments were used for these comparisons. Significant differences among group means were analyzed at the 0.05 probability level with a post hoc Newman-Keuls range test.

RESULTS

In all HPLC-EC analyses of extracts from microsomal incubations with MDMA a single electroactive predominant peak was detected. This peak consistently coeluted with authentic α-methylepinine under a variety of chromatographic conditions. To obtain a more rigorous identification of the metabolite, hydrodynamic voltammograms were constructed by determining the electrochemical response to 20 μl injections of standard α-methylepinine and microsomal extracts at electrode potentials ranging from +200 to +1000 mV. As shown in Fig 2, the voltammograms for the standard and metabolite are virtually identical, suggesting that the coeluting peaks represent the same compound (14).

As our initial approach to study the role of α-methylepinine in MDMA neurotoxicity, we sought to correlate aspects of the drug's metabolism to α-methylepinine with its neurotoxic profile. To

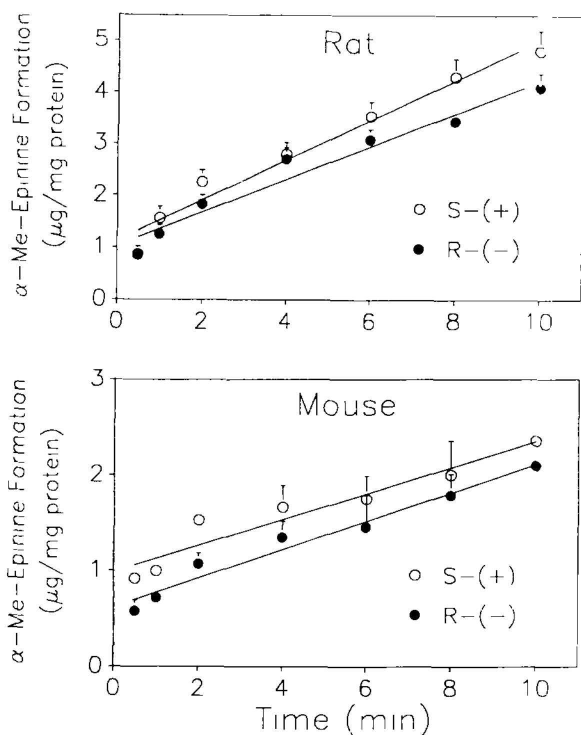

FIG 3 Time course of the formation of α-methylepinine from the stereoisomers of MDMA by rat (top panel) and mouse (bottom panel) hepatic microsomes Hepatic microsomes were incubated with 5 mM *S*-(+)-MDMA (open circles) or *R*-(−)-MDMA (closed circles) Shown are the means ± S E M of seven (rat) or three (mouse) independent trials expressed in ng/mg protein The line of best fit was determined by regression analysis Slopes were not significantly different (p>0 05)

assess the stereochemistry of the conversion, we compared the rates of formation of α-methylepinine from the two stereoisomers of MDMA. During the course of a 10-min incubation of rat hepatic microsomes with MDMA under the conditions imposed, the appearance of the metabolite from either stereoisomer occurred in a linear manner [Fig. 3a, correlation coefficients. *S*-(+) = .98, *R*-(−) = .98]. The regression line slopes for the stereoisomers were not significantly different, suggesting a similar rate of formation. The amounts of metabolite formed from the two stereoisomers after 10 minutes were also not significantly different (Fig. 4a).

As we have recently reported that Swiss-Webster mice are insensitive to MDMA neurotoxicity (37), we sought to determine if microsomes from this strain of mice did not form the metabolite, which would be consistent with a role of the metabolite in mediating neurotoxicity in rats. Incubation of mouse hepatic microsomes with either stereoisomer of MDMA resulted in α-methylepinine appearing as the primary electroactive metabolite, which was formed linearly with time [Fig. 3b; correlation coefficient: *S*-(+) = .98, *R*-(−) = .94]. As with rat hepatic microsomes, no significant stereochemical effect on α-methylepinine formation was observed (Fig. 4a).

The amount of α-methylepinine formed by rat hepatic microsomes from either stereoisomer of MDMA was significantly greater than that from the corresponding isomer by mouse hepatic microsomes after a 10-min incubation period (Fig. 4a). However, no significant differences in the amount of α-methylepinine formed by brain microsomes from the two species were observed (Fig. 4b)

TABLE 1

RAT BRAIN BIOGENIC AMINE AND METABOLITE LEVELS ONE WEEK AFTER INTRACEREBROVENTRICULAR ADMINISTRATION OF α-METHYLEPININE

Region	Dose (μg) of α-Methylepinine: 0	300	600
Hypothalamus			
5-HT	1 03 ± 0 11	1 22 ± 0 18	1 05 ± 0 04
5-HIAA	0 39 ± 0 04	0 42 ± 0 09	0 39 ± 0 06
NE	5 29 ± 0 48	5 77 ± 0 81	5 46 ± 0 34
DA	0 40 ± 0 08	0 32 ± 0 06	0 31 ± 0 04
Cortex			
5-HT	0 72 ± 0 08	0 68 ± 0 04	0 77 ± 0 09
5-HIAA	0 21 ± 0 02	0 24 ± 0 01	0 22 ± 0 02
NE	0 49 ± 0 05	0 51 ± 0 02	0 52 ± 0 04
DA	0 39 ± 0 08	0 33 ± 0 09	0 43 ± 0 08
DOPAC	0 017 ± 0 003	0 015 ± 0 006	0 016 ± 0 012
HVA	0 026 ± 0 003	0 021 ± 0 002	0 027 ± 0 003
Hippocampus			
5-HT	0.62 ± 0 05	0 68 ± 0 04	0 51 ± 0 02
5-HIAA	0 38 ± 0 03	0 49 ± 0 02*	0 51 ± 0 04*
NE	0 76 ± 0 09	0 74 ± 0 06	0 66 ± 0 03
Striatum			
5-HT	1 05 ± 0 14	1 04 ± 0 08	1 00 ± 0 06
5-HIAA	0 70 ± 0 06	0 62 ± 0 04	0 74 ± 0 06
NE	0 36 ± 0 06	0 24 ± 0 03	0 28 ± 0 02
DA	11 45 ± 0 92	9 16 ± 0 63	9 39 ± 1 15
DOPAC	0 56 ± 0 03	0 39 ± 0 04	0 56 ± 0 07
HVA	0 46 ± 0 02	0 37 ± 0 04	0 43 ± 0 04

Shown are the means ± S E M expressed in ng/mg tissue of five rats per group

*Significantly different (p<0 05) from saline-treated controls by one-way ANOVA with a post hoc Newman-Keuls test

Preliminary data in our lab had suggested that classical modulators of cytochrome P-450 metabolism did not alter the neurotoxicity of MDMA in the expected manner (i.e., SKF 525A did not inhibit and phenobarbital did not potentiate neurotoxicity) These results could be due to a tissue difference in cytochrome P-450 activity. A difference between central and peripheral P-450 pathways could be significant because of the likely inability of polar hepatic metabolites to cross the blood-brain barrier. To test this possibility, we determined the effect of phenobarbital and SKF 525A on rat liver and brain microsomal formation of α-methylepinine. Hepatic microsomes prepared from phenobarbital-treated rats formed more than twice as much α-methylepinine from either stereoisomer of MDMA compared to untreated microsomes, after a 10-min incubation period (p<0.001; Fig 5a) Incubation of rat hepatic microsomes with SKF 525A significantly reduced the formation of α-methylepinine from *S*-(+)- and *R*-(−)-MDMA by 66% and 61%, respectively (p<0.001; Fig. 5a). By contrast, brain microsome preparations do not appear to be similarly affected by these treatments, as no significant differences were observed between control and drug-treated rat brain microsomes with regard to their capacity to form α-methylepinine from either stereoisomer of MDMA (Fig 5b). These results suggest a difference in the responsiveness of the rat hepatic and brain microsomal systems to these classic metabolic tools.

To directly determine if α-methylepinine produces signs of

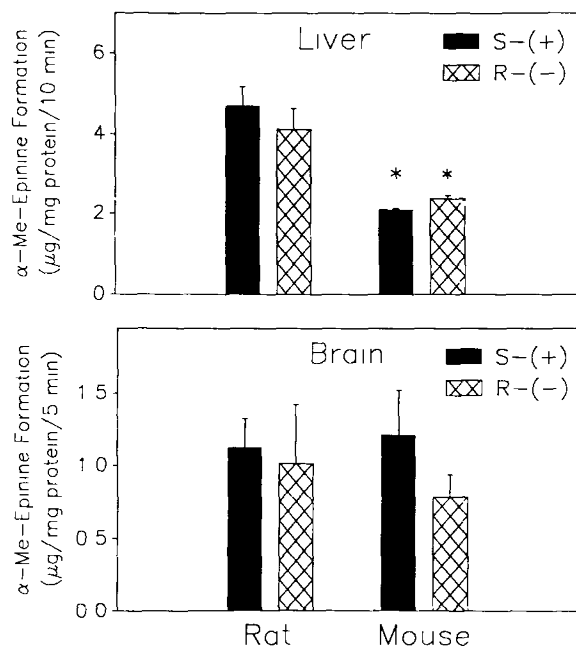

FIG 4 Comparison of the formation of α-methylepinine by liver and brain microsomes from rat and mouse Liver (top panel) and brain (bottom panel) microsomes were incubated for 10 and 5 min, respectively, with either 5 mM S-(+)-MDMA (filled bars) or R-(−)-MDMA (cross-hatched bars) Shown are the means ± S E M of three independent trials expressed in ng/mg protein/incubation time *Amount formed from either stereoisomer in mouse hepatic microsomes is significantly different ($p<0.05$) from that formed from the same stereoisomer in rat hepatic microsomes

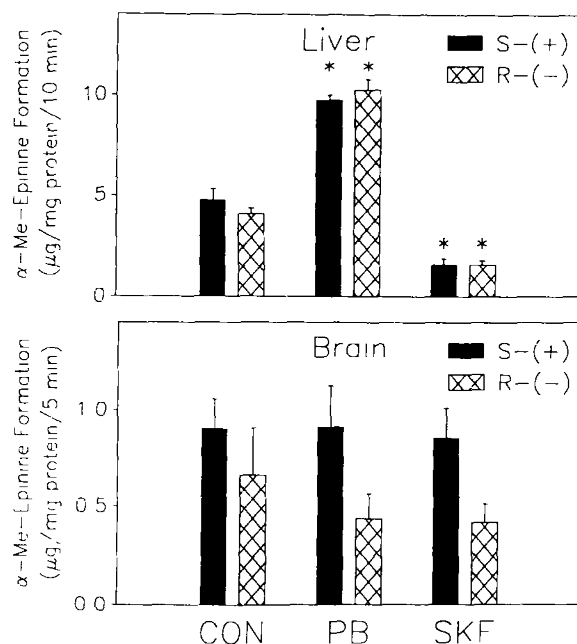

FIG. 5 Effect of phenobarbital and SKF 525A on the formation of α-methylepinine by rat hepatic and brain microsomes Liver (upper panel) and brain (lower panel) microsomes were prepared from control (CON) or phenobarbital-treated (PB) rats The effect of 1 mM SKF 525A (SKF) was assessed by including it in the reaction mixture The microsomes were incubated for the indicated time with either 5 mM S-(+)-MDMA (filled bars) or R-(−)-MDMA (cross-hatched bars) Shown are the means ± S E M of four independent trials expressed as ng/mg protein/incubation time *Significantly different ($p<0.05$) from control microsomes

neurotoxicity similar to those of MDMA, the compound was administered centrally to rats. Doses of 300 and 600 μg of α-methylepinine were injected through unilateral intracerebroventricular cannulae. In approximately two-thirds of the animal that received the metabolite, overt behavioral responses similar to the "serotonin syndrome" (forepaw treading, splayed hindlimbs, and backward movement) (17) were observed Two of the animals experienced convulsions All animals appeared normal 12 hours after drug administration The rats were sacrificed one week after treatment for analysis of brain biogenic amines and metabolites. As shown in Table 1, the only significant effect observed in animals receiving the α-methylepinine was an elevation of 5-HIAA in the hippocampus. The levels of biogenic amines and metabolites in the other brain regions did not significantly differ among treatment groups. These data are not consistent with the hypothesis that α-methylepinine is the mediator of the persistent neurotoxic effects of MDMA.

DISCUSSION

Because naturally occurring and synthetic methylenedioxyphenyl (MDP) compounds display biphasic effects of inhibition followed by induction of cytochrome P-450 and monooxygenase activity (26), determining the role of metabolism in the neurotoxicity of MDMA requires a multifaceted approach The metabolism of MDMA is further complicated because a major metabolite, MDA, does not form Type III difference spectra which is reflective of the formation of an inhibitory carbene-Fe(III) complex with cytochrome P-450 (4). Because of the known cytotoxicity of catechols such as DA (13,21) and α-methyl-DOPA (10), we suspected that α-methylepinine, the catechol metabolite of MDMA generated by demethylenation of the methylenedioxy ring, might be neurotoxic. However, the results of both the studies on the stereochemistry and species variation of this metabolic route and the intracerebroventricular study strongly suggest that α-methylepinine does not mediate MDMA neurotoxicity.

In contrast to the induction of persistent depletion of brain 5-HT which is stereospecific for S-(+)-MDMA (30), no significant degree of stereoselective formation of α-methylepinine by rat hepatic or brain microsomes was observed If neurotoxicity and other neurochemical effects which have a stereochemical component (18, 33, 36) are primarily mediated in vivo by S-(+)-α-methylepinine, some stereoselectivity for its formation from MDMA might be expected, although it is possible that only S-(+)-α-methylepinine is taken up into the nerve terminal Since the studies described here have focused on the appearance of a specific in vitro metabolite, other metabolic mechanisms not detected by our assay may confound our results. For instance, one stereoisomer might be more rapidly converted to α-methylepinine but then quickly converted to a second product. Although an extensive kinetic analysis was not conducted, the formation of metabolite from the MDMA enantiomers appeared to occur at relatively similar rates. In an in vivo study, a greater amount of S-(+)-MDA, the N-demethylated metabolite of MDMA, was present in plasma 4 hours following treatment of rats with racemic MDMA (11). Since MDMA and MDA differ in their interactions with cytochrome P-450 (4), preferential formation of MDA from one stereoisomer may alter the conversion of MDMA to α-methylepinine. As we

did not detect any α-methyldopamine, the catechol metabolite of MDA (22), in microsomal extracts we assume our conditions were favorable for studying the stereochemistry of α-methylepinine formation without interference from MDA. Therefore, at least in vitro there does not appear to be a stereochemical preference for one enantiomer in this metabolic pathway.

Rat hepatic microsomes converted a greater amount of MDMA to α-methylepinine than did mouse hepatic microsomes. A lower rate of formation of the catechol by the mouse could contribute to the lessened neurotoxic effects of MDMA in this species. On the other hand, a greater rate of hepatic metabolism in vivo should cause a more rapid elimination of the parent compound, as well as α-methylepinine, and might result in decreased neurotoxicity in the rat, if either of the two compounds were involved. A more rapid rate of drug metabolism in mice has been suggested as a mechanism by which the vulnerability of this species to MDMA (40) and PCA neurotoxicity (38) is diminished. Since there are well established species differences in metabolism of amphetamine and its analogs (9), it is quite possible that other metabolic pathways are responsible for the observed species variations Our findings that mouse liver microsomes do form the catechol, albeit more slowly, and the lack of species differences in α-methylepinine formation by brain microsomes argues strongly against this metabolite contributing to the differences in species susceptibility to MDMA neurotoxicity.

The metabolic probes phenobarbital and SKF 525A altered the hepatic microsomal metabolism of the stereoisomers of MDMA in the predicted manner (Fig. 5). However, the lack of effect of these compounds on brain microsomal formation of α-methylepinine suggests that in vivo treatment with phenobarbital would diminish and SKF 525A would increase the amount of unmetabolized drug available to the brain, where its rate of metabolism to the catechol would be unaffected. This potential tissue divergence, as well as the complicated metabolic profiles of MDMA and MDA, may represent significant obstacles in defining the contribution of cytochrome P-450-generated metabolites to MDMA neurotoxicity. Indeed, we (unpublished observations) and others [(2) and see in (34)] have been unable to show that SKF 525A and phenobarbital altered MDMA neurotoxicity in vivo in the anticipated manner.

In the direct evaluation of the neurotoxicity of α-methylepinine, the only significant neurochemical change present one week following treatment was an elevation in hippocampal 5-HIAA (Table 1). Although it is possible that insufficient doses were used, the highest dose used represents approximately 15% of a 20 mg/kg systemic dose of MDMA which effectively decreases brain 5-HT parameters for one week (30,32).

Furthermore, in a pilot study, 400 μg of *S*-(+)-α-methyldopamine, the catechol metabolite of MDA, was administered ICV and appeared to be without neurotoxic effect at one week. This dose of *S*-(+)-α-methyldopamine resulted in the appearance of a large chromatographic peak that coeluted with α-methyldopamine in brain extracts of rats that were sacrificed three hours posttreatment Thus it appears that the intraventricular treatment produced far greater levels of metabolite in the brain than would be present following systemic administration of the parent compound

Finally, the overt behavior observed in rats immediately following ICV α-methylepinine treatment was similar to the "serotonin syndrome" (i.e , forepaw treading, splayed hindlimbs, walking backwards) and was not observed after administration of the parent compound. Thus it seems likely that sufficient doses of the metabolite were administered.

It is possible that other metabolites are involved in MDMA neurotoxicity. The greater rate of N-demethylation of *S*-(+)-MDMA to MDA (11) is consistent with that expected for a metabolite which mediates MDMA neurotoxicity MDA is also sufficiently hydrophobic to reach the CNS if formed peripherally, and may also be more neurotoxic than MDMA (39) However, the major metabolites of MDA, α-methyldopamine and 3-O-methyldopamine, apparently do not produce signs of neurotoxicity similar to the parent (20). Thus it seems unlikely that these potentially reactive catechol products of drug metabolism are responsible for the neurodegenerative changes induced by MDMA and MDA.

REFERENCES

1 Battaglia, G , Yeh, S Y , O'Hearn, E , Molliver, M E , Kuhar, M J , DeSouza, E. B 3,4-Methylenedioxymethamphetamine and 3,4-methylenedioxyamphetamine destroy serotonin nerve terminals in rat brain Quantification of neurodegeneration by measurement of [^{3}H]-paroxetine-labelled uptake sites J Pharmacol Exp Ther 242 911–916, 1987
2 Battaglia, G., Yeh, S. Y , DeSouza, E B MDMA-induced neurotoxicity Parameters of degeneration and recovery of brain serotonin neurons Pharmacol Biochem Behav. 29 269–274, 1988
3 Bradford, M A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72 248–254, 1976
4 Brady, J F , DiStefano, E W , Cho, A K. Spectral and inhibitory interactions of (±)-3,4-methylenedioxyamphetamine (MDA) and (±)-methylenedioxymethamphetamine (MDMA) with rat hepatic microsomes Life Sci 39·1457–1464, 1986
5 Braun, U., Shulgin, A T ; Braun, G Centrally active N-substituted analogs of 3,4-methylenedioxyphenylisopropylamine (3,4-methylenedioxyamphetamine) J Pharmacol Sci 69 192–195, 1980
6 Casida, J , Engel, J , Essac, E , Kamienski, F , Kuwatsuka, S Methylene-^{14}C-dioxyphenyl compounds Metabolism in relation to their synergistic action Science 153 1130–1134, 1966
7 Commins, D L., Axt, K J , Vosmer, G , Seiden, L S Endogenously produced 5,6-dihydroxytryptamine may mediate the neurotoxic effects of para-chloroamphetamine Brain Res 419 253–261, 1987
8 Commins, D L , Vosmer, G , Virus, R M , Woolverton, W L , Schuster, C R , Seiden, L S Biochemical and histological evidence that methylenedioxymethamphetamine (MDMA) is toxic to neurons in the rat brain. J Pharmacol Exp Ther 241·338–345, 1987
9 Dring, L G , Smith, R L , Williams, R T The metabolic fate of amphetamine in man and other species Biochem J 116.425–435, 1970
10 Dybing, E , Nelson, S D , Mitchell, J R , Sasame, H A , Gillette, J R Oxidation of α-methyldopa and other catechols by cytochrome P-450-generated superoxide anion Possible mechanism of methyldopa hepatitis Mol Pharmacol 12 911–920, 1976
11 Fitzgerald, R L , Blanke, R. V , Rosecrans, J A , Glennon, R A Stereochemistry of the metabolism of MDMA to MDA Life Sci 45 295–301, 1989
12 Fuller, R W , Perry, K W , Molloy, B B Reversible and irreversible phases of serotonin depletion by 4-chloroamphetamine Eur J Pharmacol 33 119–124, 1975
13. Graham, D G , Tiffany, S. M , Bell, W R , Jr , Gutknecht, W F Autoxidation versus covalent binding of quinones as the mechanism of toxicity of dopamine, 6-hydroxydopamine, and related compounds toward c1300 neuroblastoma cells in vitro Mol Pharmacol 14 644–653, 1978.
14 Hamilton, M , Kissinger, P T The metabolism of 2- and 3-hydroxyacetanilide Determination of metabolic products by liquid chromatography/electrochemistry Drug Metab Dispos 14 5–12, 1986
15 Harvey, J A , McMaster, S. E , Yunger, L H *p*-Chloroamphetamine Selective neurotoxic action in brain Science 187 841–843, 1975
16 Hodgson, E , Philpot, R. M Interaction of methylenedioxyphenyl (1,3-benzodioxole) compounds with enzymes and their effects on

mammals Drug Metab Rev 3 231–301, 1974

17 Jacobs, B L An animal behavior model for studying central serotonergic synapses Life Sci 19 777–786, 1976

18 Johnson, M P, Hoffman, A H, Nichols, D E Effects of the enantiomers of MDA, MDMA and related analogues on [^{3}H]-serotonin and [^{3}H]-dopamine release from superfused rat brain slices Eur J Pharmacol 132 269–276, 1986

19 Logan, B J, Laverty, R, Sanderson, W. D, Yee, Y B Differences between rats and mice in MDMA (methylenedioxymethamphetamine) neurotoxicity Eur J Pharmacol 152 227–234, 1988

20 McCann, U D, Ricaurte, G A Major metabolites of MDA do not mediate its toxic effect on serotonin neurons in the rat brain Soc Neurosci Abstr 15 417, 1989

21 Maker, H S, Weiss, C, Brannan, T S Amine-mediated toxicity The effects of dopamine, norepinephrine, 5-hydroxytryptamine, 6-hydroxydopamine, ascorbate, glutathione and peroxide on the *in vivo* activities of creatinine and adenylate kinases in the brain of the rat Neuropharmacology 25 25–32, 1986

22 Marquardt, G M, DiStefano, V, Ling, L Metabolism of β-3,4-methylenedioxyamphetamine in the rat Biochem Pharmacol 27 1503–1505, 1978

23 Nichols, D E, Hoffman, A J, Oberlender, R A, Jacob, P, III, Shulgin, A T Derivatives of 1-(1,3-benzodioxole-5-yl)-2-butanamine Representatives of a novel therapeutic class J Med Chem 29 2009–2015, 1986

24 O'Hearn, E, Battaglia, G, DeSouza, E B, Kuhar, M J, Molliver, M E Methylenedioxyamphetamine (MDA) and methylenedioxymethamphetamine (MDMA) cause selective ablation of serotonergic axon terminals in forebrain Immunocytochemical evidence for neurotoxicity J Neurosci 8 2788–2803, 1988

25 Pellegrino, L J, Pellegrino, A S, Cushman, A J A stereotaxic atlas of the rat brain. New York Plenum Press, 1979

26 Philpot, R M, Hodgson, E The production and modification of cytochrome P-450 difference spectra by *in vivo* administration of methylenedioxyphenyl compounds Chem Biol Interact 4 185–194, 1971

27 Ricaurte, G, Schuster, C, Seiden, L S Long-term effects of repeated methylamphetamine administration on dopamine and serotonin neurons in the rat brain a regional study Brain Res 193 153–163, 1980

28 Ricaurte, G, Guillery, R W, Seiden, L S, Schuster, C, Moore, R Y Dopamine nerve terminal degeneration produced by high doses of methylamphetamine in the rat brain Brain Res 235 93–103, 1984

29 Sanders-Bush, E, Steranka, L R Immediate and long-term effects of *p*-chloroamphetamine on brain amines Ann NY Acad Sci 305 208–221, 1978

30 Schmidt, C J Neurotoxicity of the psychedelic amphetamine, methylenedioxymethamphetamine J Pharmacol Exp Ther 240 1–7, 1987

31 Schmidt, C J, Ritter, J K, Sonsalla, P K, Hanson, G R, Gibb, J W Role of dopamine in the neurotoxic effects of methamphetamine J Pharmacol Exp Ther 233 539–544, 1985

32 Schmidt, C J, Levin, J A, Lovenberg, W *In vitro* and *in vivo* neurochemical effects of methylenedioxymethamphetamine on striatal monoaminergic systems in the rat brain Biochem Pharmacol 36 747–755, 1987

33 Schmidt, C J, Taylor, V L Depression of rat brain tryptophan hydroxylase activity following the acute administration of methylenedioxymethamphetamine Biochem Pharmacol 36 4095–4102, 1987

34 Schmidt, C J, Taylor, V L Direct central effects of acute methylenedioxymethamphetamine on serotonergic neurons Eur J Pharmacol 156 121–131, 1988

35 Seiden, L S, Vosmer, G Formation of 6-hydroxydopamine in caudate nucleus of the rat brain after a single large dose of methylamphetamine Pharmacol Biochem Behav 21 29–31, 1984

36 Steele, T D, Nichols, D E, Yim, G K W Stereochemical effects of 3,4-methylenedioxymethamphetamine (MDMA) and related amphetamine derivatives on inhibition of uptake of [^{3}H]monoamines into synaptosomes from different regions of rat brain Biochem Pharmacol 36 2297–2303, 1987

37 Steele, T D, Nichols, D E, Yim, G K W MDMA transiently alters biogenic amines and metabolites in mouse brain and heart Pharmacol Biochem Behav 34 223–227, 1989

38 Steranka, L R, Sanders-Bush, E Long-term effects of continuous exposure to *p*-chloroamphetamine on central serotonergic mechanisms in mice Biochem Pharmacol 27 2033–2037, 1978

39 Stone, D M, Stahl, D C, Hanson, G R, Gibb, J W The effects of 3,4-methylenedioxymethamphetamine (MDMA) and 3,4-methylenedioxyamphetamine (MDA) on monoaminergic systems in the rat brain Eur J Pharmacol 128 41–48, 1986

40 Stone, D M, Hanson, G R, Gibb, J W Differences in the central serotonergic effects of methylenedioxymethamphetamine (MDMA) in mice and rats Neuropharmacology 26 1657–1661, 1987

41 Stone, D M, Merchant, K M, Hanson, G R, Gibb, J W Immediate and long-term effects of 3,4-methylenedioxymethamphetamine on serotonin pathways in brain of rat Neuropharmacology 26 1677–1683, 1987

42 Stone, D M, Johnson, M, Hanson, G R, Gibb, J W Role of endogenous dopamine in the central serotonergic deficits induced by 3,4-methylenedioxymethamphetamine J Pharmacol Exp Ther 247 79–87, 1988

43 Yamamoto, B K, Spanos, L J The acute effects of methylenedioxymethamphetamine on dopamine release in the awake-behaving rat Eur J Pharmacol 148 195–203, 1988