Evidence for the Involvement of Nitric Oxide in 3,4-Methylenedioxymethamphetamine-Induced Serotonin Depletion in the Rat Brain

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ABSTRACT

Production of reactive oxygen and/or nitrogen species has been thought to contribute to the long-term depletion of brain dopamine and serotonin (5-HT) produced by amphetamine derivatives, i.e., methamphetamine and 3,4-methylenedioxymethamphetamine (MDMA). In the present study, the effects of nitric-oxide synthase (NOS) inhibitors were examined on the long-term depletion of striatal dopamine and/or 5-HT produced by the local perfusion of malonate and MDMA or the systemic administration of MDMA. The effect of MDMA on nitric oxide formation and nitrotyrosine concentration also was determined. Perfusion with MDMA and malonate resulted in a 34% reduction of 5-HT and 49% reduction of dopamine concentrations in the striatum. The systemic administration of NOS inhibitors, $N^\omega$-nitro-$L$-arginine methyl ester hydrochloride and $S$-$methyl-L$-thiocitrulline (S-MTC), and the peroxynitrite decomposition catalyst Fe(III) tetrakis (1-methyl-4-pyridyl) porphyrin pentachloride attenuated the MDMA- and malonate-induced depletion of striatal dopamine and 5-HT. S-MTC also attenuated the depletion of 5-HT in the striatum produced by the systemic administration of MDMA without attenuating MDMA-induced hyperthermia. Additionally, the systemic administration of MDMA significantly increased the formation of nitric oxide and the nitrotyrosine concentration in the striatum. These results support the conclusion that MDMA produces reactive nitrogen species in the rat that contribute to the neurotoxicity of this amphetamine analog.

3,4-Methylenedioxymethamphetamine (MDMA), a ring-substituted amphetamine analog, is widely abused as a recreational drug, and there is concern that the drug produces damage to serotonergic nerve terminals (Green et al., 1995). MDMA selectively targets serotonergic neurons and produces long-term depletion of rat brain serotonin (5-HT) and its major metabolite 5-hydroxyindoleacetic acid (Stone et al., 1986). MDMA also produces a decrease in tryptophan hydroxylase activity (Schmidt and Taylor, 1987), reduction in the [$^{3}$H]paroxetine-labeled 5-HT reuptake sites (Battaglia et al., 1987), and reduced immunostaining of 5-HT terminals (O’Hearn et al., 1988). However, the exact mechanisms that mediate the toxic effects of MDMA on 5-HT terminals remain to be elucidated.

There is evidence to suggest that oxidative stress plays an important role in this process (Gudelsky, 1996; Colado et al., 1997; Shankaran et al., 1999a,b). MDMA increases the formation of hydroxyl radicals (Colado et al., 1997; Shankaran et al., 1999a,b), increases lipid peroxidation (Sprague and Nichols, 1995), and decreases the concentration of the endogenous antioxidants vitamin E and ascorbic acid (Shankaran et al., 2001). The administration of antioxidants attenuates the MDMA-induced long-term brain 5-HT depletion (Colado and Green, 1995; Gudelsky, 1996; Shankaran et al., 2001).

In addition to the potential role of reactive oxygen species in MDMA neurotoxicity, there is evidence for a role of reactive nitrogen species in the neurotoxicity produced by amphetamine analogs. Nitric oxide has been implicated as a mediator of neurotoxicity (Cerruti et al., 1995), and its involvement in the pathophysiology of neuronal cell death has received considerable attention. Several studies have demonstrated that nitric-oxide synthase (NOS) inhibitors provide protection against methamphetamine and MDMA-induced dopaminergic and serotonergic neurotoxicity in rodents. The relatively selective neuronal NOS inhibitor 7-nitroindazole attenuates methamphetamine-induced dopaminergic and serotonergic neurotoxicity in mice (Ali and Itzhak, 1998). The

ABBREVIATIONS: MDMA, 3,4-methylenedioxymethamphetamine; 5-HT, 5-hydroxytryptamine (serotonin); NOS, nitric-oxide synthase; $L$-NAME, $N^\omega$-nitro-$L$-arginine methyl ester hydrochloride; S-MTC, $S$-$methyl-L$-thiocitrulline dihydrochloride; FeTMPyP, Fe(III) tetakis (1-methyl-4-pyridyl) porphyrin pentachloride; DA, dopamine; ANOVA, analysis of variance.
administration of nonspecific NOS inhibitors, e.g., N\(^{-}\)-nitro-L-arginine or N\(^{-}\)-nitro-L-arginine methyl ester hydrochloride (L-NAME), has been shown to provide protection against MDMA-induced long-term 5-HT depletion in the rat hippocampus and cortex (Taraska and Finnegan, 1997; Zheng and Laverty, 1998). However, the neuroprotection afforded by NOS inhibitors in the aforementioned studies is accompanied by attenuation of MDMA-induced increase in body temperature.

It has been postulated that the hyperthermia produced by the systemic administration of MDMA plays an important role in the long-term degeneration of 5-HT axon terminals (Huether et al., 1997). Malberg and Seiden (1998) have reported that maintenance of rats at a cool ambient temperature resulted in attenuation of MDMA-induced hyperthermia and also prevented the MDMA-induced 5-HT depletion. Drugs that prevent the MDMA-induced rise in body temperature also provide neuroprotection against MDMA-induced long-term 5-HT depletion (Farfel and Seiden, 1995; Malberg et al., 1996). However, Colado et al. (2001) have reported recently that the neuronal NOS inhibitor S-methyl-L-thiocitrulline (S-MTC) provides significant neuroprotection against MDMA-induced long-term dopamine depletion in mice without attenuating the MDMA-induced hyperthermia.

In the present study, we have examined the effect of S-MTC on MDMA-induced hyperthermia and 5-HT depletion in the striatum of the rat. In addition, the effects of NOS inhibitors or a peroxynitrite decomposition catalyst on the depletion of dopamine and 5-HT elicited by the intrastriatal administration of malonate, a mitochondrial inhibitor, together with MDMA were examined. The combination of malonate and MDMA has been shown to deplete striatal dopamine and 5-HT without producing hyperthermia (Nixdorf et al., 2001), thereby obviating concern for the interaction of NOS inhibitors and MDMA on body temperature. Finally, the effect of MDMA on the generation of nitric oxide and the formation of nitrotyrosine in the striatum also was determined.

### Materials and Methods

#### Animal Procedures

Adult male rats (200–275 g) of the Sprague-Dawley strain (Charles River Breeding Laboratories, Portage, MI) were used in the studies. The animals were housed three per cage in a temperature- and humidity-controlled room with a 12-h light/dark cycle and allowed food and water ad libitum. Animals undergoing surgery were housed one per cage postoperatively. All procedures were in strict adherence to the National Institutes of Health guidelines and approved by the institutional animal care and use committee.

#### Chemicals, Drugs, and Drug Treatment

MDMA was provided by the National Institute on Drug Abuse (Bethesda, MD). Malonic acid disodium was obtained from Acros (Cincinnati, OH). L-NAME was obtained from Sigma-Aldrich (St. Louis, MO). S-MTC and Fe(III) tetrakis (1-methyl-4-pyridyl) porphyrin pentachloride (FeTMPyP) were obtained from Cayman Chemical (Ann Arbor, MI). All the drugs were dissolved in 0.15 M NaCl. MDMA (100 μM) and/or malonate (100 mM) was perfused locally in the striatum for 8 h. A similar dose regimen was used by Nixdorf et al. (2001). In other experiments, rats received MDMA (10 mg/kg i.p.) at 2-h intervals for a total of four injections. S-MTC (10 mg/kg i.p.) was administered 30 min before and 2.5 and 5.5 h after beginning treatment with MDMA (injection or perfusion). L-NAME (60 mg/kg i.p.) was given 1 h before and 4 and 8 h after beginning the perfusion in the striatum. Rats received FeTMPyP (20 mg/kg i.p.) 1 h before and 2 and 12 h after the perfusion of MDMA and malonate. The dosing regimens for S-MTC, L-NAME, and FeTMPyP were similar to those used previously (Taraska and Finnegan, 1997; Salvemini et al., 1998; Colado et al., 2001; Imam et al., 2001).

#### Biochemical Measurements

**Assay of Tissue Dopamine (DA) and 5-HT.** In the first experiment, rats received MDMA, malonate, MDMA and malonate, or no drug reversed dialyzed in the striatum. Rats were killed by decapitation, and the brains were removed and frozen with dry ice. The brains were sliced in 60-μm-thick sections until the entire probe track was visible. Then, a 400-μm section was taken, and tissue directly adjacent to the probe track was excised out from the 400-μm frozen section and was stored at ~80°C until further analysis. The dimensions of the excised tissue were 4.5 mm (length) × 1.0 mm (width) × 0.4 mm (depth). It was determined in a separate experiment that physical trauma due to probe insertion per se did not alter striatal DA or 5-HT concentrations (data not shown). In subsequent experiments in which rats were perfused with MDMA and malonate and injected with S-MTC, L-NAME, FeTMPyP, or vehicle, tissue surrounding the probe track was dissected together with a similarly sized sample of striatum from the contralateral side. This tissue from the contralateral side served as control, i.e., tissue exposed only to the NOS inhibitors alone and not MDMA and malonate. Finally, in experiments in which MDMA and/or S-MTC were injected systematically, rats were killed by decapitation, and samples of striatum (approx. 10 mg) were dissected from 1-mm coronal sections. All tissue samples were homogenized in ice-cold 0.2 N perchloric acid and homogenates were centrifuged at 14,000 rpm for 5 min. Concentrations of dopamine and 5-HT were determined in 20-μl aliquots of the supernatants that were injected onto a C18 reverse phase column connected to a LC-4B amperometric detector (BAS Bioanalytical Systems, West Lafayette, IN) equipped with a glassy carbon target electrode. The mobile phase for the separation of dopamine and 5-HT consisted of 35 mM citric acid, 54 mM sodium acetate, 50 mg/l disodium ethylenediamine tetraacetate, 50 mg/l octane sulfonic acid sodium salt, 3% methanol, and 3% acetonitrile, pH 4.1, pumped at a flow rate of 0.3 ml/min. Tissue dopamine and 5-HT concentrations are reported as nanograms per milligram of tissue or nanograms per milligram of protein for samples excised from the area of the probe tracks. Protein content was determined using a Lowry protein assay.

**Assay of Tissue Nitrotyrosine.** Rats were killed by decapitation and the brains were removed; the striatum was dissected out and stored at ~80°C. The tissue sample was acid hydrolyzed in 6 N HCl, and the hydrolyzed striatum protein sample was dissolved in 200 μl of mobile phase, which consisted of 50 mM K₂HPO₄, 50 mg/ml Na EDTA, and 5% methanol, pH 6.3. The sample was injected onto a Prodigy ODS column connected to a glassy carbon electrode maintained at 1 V versus an Ag/AgCl reference electrode (BAS Bioanalytical Systems). Nitrotyrosine/tyrosine values are reported as picograms per nanogram of tissue.

#### In Vivo Microdialysis Procedures

Rats were implanted with a stainless steel guide cannula under ketamine/xylazine (70/6 mg/kg i.m.) induced anesthesia 48 to 72 h before the insertion of the microdialysis probe. On the day of the dialysis experiment, a concentric style dialysis probe was inserted through the guide cannula into the striatum. The coordinates for the tip of the probe were A, 1.2 mm; L, 3.1 mm; and V, −7 mm from bregma, according to the stereotaxic atlas of Paxinos and Watson (1986). The microdialysis probes were constructed as described previously (Yamamoto and Pehek, 1990). The dialysis surface of the membrane (Spectra Por, 6000 mol. wt. cut-off, 210 μm outside diameter) for the
striatum was 4.5 mm in length. The microdialysis probe was connected to an infusion pump set to deliver Dulbecco’s phosphate-buffered saline containing 1.2 mM CaCl₂ at a rate of 1.8 μl/min. Perfusion with MDMA and malonate was started after a 2-h equilibration period.

**Assay of Dialysate Nitrite and Nitrate.** In experiments in which dialysis samples were collected for the analysis of nitrite/nitrate, three baseline samples were obtained at 1-h intervals beginning 1.5 h after insertion of the probe. Additional dialysis samples were obtained at 1-h intervals for 9 h after the first injection of MDMA or vehicle. The concentration of nitric oxide in dialysis samples was determined by quantifying nitrite/nitrate with the use of a kit obtained from Cayman Chemical. Nitric oxide is readily converted to nitrite and nitrate in the presence of oxygen. The initial step of the assay allows for the conversion of nitrate to nitrite by the enzyme nitrate reductase. Analysis of total nitrite is accomplished with the use of Griess reagents, and the end product is determined spectrophotometrically at 550 nm.

**Body Temperature Measurements**

On the day of the experiment, the rats were allowed to acclimate in their cages at 24°C for 2 h before body temperatures were measured. Measurements of rectal temperature were made using a telethermometer and a thermistor probe. The probe was lubricated with a small amount of petroleum jelly and inserted 5 cm into the rectum of each rat where it remained until a stable temperature was obtained. Measurements were taken every 30 min for a 1.5-h period before administration of MDMA (10 mg/kg i.p.) and every 60 min for a 7-h period after the first injection of MDMA.

**Statistical Analysis**

The effect of MDMA and malonate perfusions on dopamine and 5-HT concentrations in the striatum were analyzed with a one-way analysis of variance (ANOVA). The effects of NOS inhibitors L-NAME and S-MTC and the peroxynitrite decomposition catalyst FeTMPyP on MDMA- and malonate-induced DA and 5-HT depletion were analyzed with a two-way ANOVA. The effect of S-MTC on MDMA-induced 5-HT depletion was analyzed by two-way ANOVA. Drug-induced hyperthermia and nitrite/nitrate concentration in dialysis samples were analyzed by two-way repeated measures ANOVA. The effect of MDMA on nitrotyrosine formation in the striatum was analyzed by one-way ANOVA. Multiple pairwise comparisons were performed using the Student-Newman-Keuls test. Treatment differences for all the data were considered statistically significant at $p < 0.05$.

**Results**

Significant reductions of striatal dopamine and 5-HT concentrations were evident after the local coperfusion of MDMA (100 μM) and malonate (100 mM) for 8 h in the striatum (Fig. 1). MDMA and malonate perfusion in the striatum produced a significant $F(3,31) = 10.552; p < 0.001$ 49% reduction of dopamine and a 34% $F(3,31) = 8.085; p < 0.001$ reduction of 5-HT concentrations. Striatal dopamine concentrations were not significantly altered by the perfusion of MDMA alone ($p = 0.95$) or malonate alone ($p = 0.16$) (Fig. 1B). Striatal concentrations of 5-HT were similarly unaffected by the perfusion of MDMA ($p = 0.83$) or malonate alone ($p = 0.75$) (Fig. 1A). Reverse dialysis of MDMA and malonate into the striatum did not significantly alter body temperature (data not shown).

The effects of NOS inhibitors on the long-term depletion of striatal dopamine and 5-HT are shown in Fig. 2. Coperfusion of MDMA and malonate in rats treated with the nonspecific NOS inhibitor L-NAME (60 mg/kg i.p. × 3) did not result in long-term DA $F(1,28) = 12.616; p = 0.392$ and 5-HT $F(1,28) = 10.128; p = 0.235$ depletion in the striatum. Similar results were obtained in rats treated with the neu-
ronal NOS inhibitor S-MTC (10 mg/kg i.p. × 3) before and during co-perfusion with MDMA and malonate. Local perfusion of MDMA and malonate did not result in a long-term DA [\(F(1,33) = 12.578; p = 0.589\)] and 5-HT [\(F(1,32) = 4.111; p = 0.772\)] depletion in the striatum of rats treated with S-MTC. Treatment with L-NAME or S-MTC alone did not alter striatal dopamine and 5-HT concentrations.

In addition to the evaluation of the effects of NOS inhibitors on MDMA + malonate-induced dopamine and 5-HT depletion, the effect of a peroxynitrite decomposition catalyst FeTMPyP also was evaluated. In rats treated with FeTMPyP before and during the co-perfusion of MDMA and malonate, striatal 5-HT concentrations were not significantly depleted compared with control tissue [\(F(1,32) = 14.404; p = 0.083\)] (Fig. 3B). Although MDMA + malonate perfusion produced a significant (\(p < 0.001\)) depletion in striatal dopamine content in FeTMPyP-treated rats, the magnitude of the reduction was significantly (\(p < 0.001\)) less than that produced in the vehicle-treated rats perfused with MDMA and malonate (Fig. 3A). Treatment with FeTMPyP alone did not significantly alter the striatal dopamine and 5-HT concentrations.

The effect of S-MTC (10 mg/kg i.p. × 3) on the hyperthermia and long-term 5-HT depletion produced by the systemic dosing regimen of MDMA (10 mg/kg i.p. × 4) is depicted in Figs. 4 and 5. S-MTC administration did not attenuate the MDMA-induced increase in body temperature [\(F(1,12) = \]
The systemic administration of MDMA resulted in a 60% depletion of striatal 5-HT concentration in control animals. MDMA treatment did not significantly alter 5-HT concentrations in rats treated with $S$-MTC ($F(1,19) = 41.706; p = 0.05$) (Fig. 5). Administration of S-MTC alone did not alter striatal 5-HT concentrations.

The effect of systemic administration of MDMA (10 mg/kg i.p. x 4) on nitrotyrosine formation in the striatum is depicted in Fig. 6. The concentration of nitrotyrosine in the striatum of MDMA-treated rats was significantly ($p < 0.05$) greater than in vehicle-treated rats 24 h after the first drug injection.

To more directly evaluate the hypothesis that a neurotoxic regimen of MDMA produces an increase in the formation of nitric oxide, nitrite/nitrate concentrations in microdialysis samples were determined. The administration of MDMA (10 mg/kg i.p.) every 2 h for a total of four injections resulted in a delayed increase in nitric oxide formation. Extracellular concentrations of nitrite/nitrate increased significantly ($F(1,14) = 46.997; p < 0.001$) to approximately 175% of baseline values between the third and fourth injection of MDMA (Fig. 7). Nitrite/nitrate concentrations remained elevated for at least 3 h after the last injection of MDMA. A single injection of MDMA (10 mg/kg i.p.) did not significantly
increase extracellular concentrations of nitrite/nitrate (Fig. 7).

Discussion

The significant findings in this study are as follows. 1) Administration of NOS inhibitors, as well as a peroxynitrite decomposition catalyst, attenuated the long-term depletion of striatal 5-HT and dopamine depletion produced by the local perfusion of MDMA and malonate. 2) Pretreatment with the neuronal NOS inhibitor S-MTC afforded neuroprotection against MDMA-induced 5-HT depletion without attenuating MDMA induced hyperthermia. 3) A multiple-dose regimen of MDMA produced a significant increase in levels of nitrotyrosine and nitric oxide in the striatum. The results of the present study provide further evidence for the involvement of nitric oxide in MDMA-induced dopaminergic and serotonergic neurotoxicity.

In previous studies, administration of NOS inhibitors has been shown to provide protection against methamphetamine and MDMA-induced dopaminergic and serotonergic neurotoxicity in rats and mice (Taraska and Finnegan, 1997; Ali and Itzhak, 1998; Zheng and Laverty, 1998; Colado et al., 2001). However, the neuroprotection achieved by NOS inhibitors has been shown to be accompanied by a decrease in the hyperthermic effect of methamphetamine and MDMA, and suppression of psychostimulant-induced hyperthermia per se may contribute to the neuroprotective effects of drugs of several pharmacological classes (Farfel and Seiden, 1995; Malberg et al., 1996). Taraska and Finnegan (1997) also were unable to distinguish the neuroprotective action of the NOS-inhibitor L-NAME from its hypothermic action. Nevertheless, Ali and Itzhak (1998) have reported that 7-nitroindazole, a neuronal NOS inhibitor, provided neuroprotection without altering the methamphetamine-induced hyperthermia.

In the present study, the neuronal-preferring NOS inhibitor S-MTC attenuated the long-term 5-HT depletion in the striatum produced by the systemic administration of MDMA without suppressing the MDMA-induced hyperthermia. It has also been shown that S-MTC provides neuroprotection against MDMA-induced dopamine depletion in mice without suppressing the MDMA-induced increase in body temperature (Colado et al., 2001). This finding strongly suggests a role of neuronal NOS and nitric oxide in the 5-HT depletion produced by MDMA.

Alternatively, the neuroprotective effects of NOS inhibitors may have been due to suppressing MDMA-induced dopamine or 5-HT release. Shankaran et al. (1999a,b) have demonstrated that inhibition of dopamine or 5-HT transporters not only diminishes MDMA-induced dopamine or 5-HT release but also attenuates MDMA toxicity. The effect of NOS inhibitors on MDMA-induced dopamine release was not determined in the present study. However, neuroprotection against MDMA toxicity also was afforded by FeTMPyP in the present study. FeTMPyP is a peroxynitrite decomposition catalyst that acts by reducing the concentrations of peroxynitrite and not nitric oxide. The neuroprotective effects of FeTMPyP support the view that neuroprotective effects of NOS inhibitors are due to prevention of nitric oxide-dependent peroxynitrite formation rather than the inhibition of nitric oxide-mediated dopamine release.

The central administration of MDMA has been shown not to produce 5-HT neurotoxicity (Paris and Cunningham, 1992; Esteban et al., 2001), and this was confirmed in the present study. It has been postulated that a neurotoxic metabolite of MDMA, formed peripherally, mediates the toxicity of this amphetamine analog (Jones et al., 2004). In view of the present finding that co-perfusion of malonate and MDMA does deplete brain 5-HT, in accordance with Nixdorf et al. (2001), it can be speculated that such a neurotoxic metabolite of MDMA may have detrimental effects on mitochondrial function that promote neurotoxicity.

Moreover, whereas the systemic administration of MDMA is selectively neurotoxic to 5-HT in the rat, impairment of mitochondrial function with malonate results in MDMA-induced depletion of dopamine, as well as 5-HT (Nixdorf et al., 2001; present results). Dopaminergic neurons have been shown to be vulnerable to the toxic effects of mitochondrial inhibition, and it has been proposed that the malonate-induced inhibition of energy production and enhancement of glutamate release may render dopaminergic neurons vulnerable to the toxic effects of MDMA (Zeevalk et al., 1997; Nixdorf et al., 2001).

The depletion of striatal dopamine and 5-HT resulting from perfusion with MDMA and malonate was antagonized by the nonspecific NOS inhibitor L-NAME, as well as by the neuronal preferring inhibitor S-MTC. Treatment with L-NAME alone did not alter body temperature (data not shown). Thus, it seems reasonable to conclude that the dopamine and 5-HT neurotoxicity produced by MDMA and malonate also results from the production of nitric oxide.

Dysregulation of energy metabolism and energy depletion has been proposed to lead to an increase in intracellular Ca$^{2+}$ in the mitochondria (Huether et al., 1997; Ransom and Fern, 1997). Binding of Ca$^{2+}$ ions to calmodulin activates neuronal NOS and leads to a subsequent increased production of nitric oxide. The reaction of nitric oxide with superoxide forms the highly toxic peroxynitrite ion that is capable of oxidizing lipid membranes and sulfhydryl moieties, as well as hydroxylating and nitrating aromatics (Radi et al., 1991a,b; Van der Vliet et al., 1994). Water-soluble iron(III) porphyrins have been reported to be highly active peroxynitrite decomposition catalysts. These catalysts have been shown to increase the rate of peroxynitrite isomerization, preempting the formation of oxidizing radical species and generating the harmless nitrate anion (Salvemini et al., 1998). Further evidence for the specific involvement of peroxynitrite in the monoamine deamination produced by MDMA and malonate in the present study is the neuroprotection afforded by the peroxynitrite decomposition catalyst FeTMPyP. Treatment of mice with peroxynitrite decomposition catalysts has been shown previously to prevent methamphetamine-induced dopamine depletion (Imam et al., 2000, 2001). However, in these earlier studies, the attenuation of methamphetamine-induced dopamine depletion by peroxynitrite decomposition catalyst was accompanied by attenuation of methamphetamine-induced hyperthermia. In the present study, MDMA and malonate perfusion was not accompanied by hyperthermia and concerns related to alterations in body temperature were obviated.

Peroxynitrite ion is capable of hydroxylating and nitrating aromatics (Van der Vliet et al., 1994), and formation of the peroxynitrite ion results in nitrotyrosine formation, which is a stable biomarker for peroxynitrite formation. In further
support of the view that MDMA increases the formation of nitric oxide and peroxynitrite, administration of a neurotoxic regimen of MDMA resulted in an increased concentration of nitrotyrosine in the striatum. An increase in nitrotyrosine has also been shown to accompany the monoamine depletion produced by methamphetamine in mice (Imam et al., 2000, 2001).

Peroxynitrite formation gives rise to nitration of tyrosine residues in proteins. Kuhn et al. (2002) have shown that peroxynitrite produces nitration of tyrosine hydroxylase, and this has been implicated in dopamine neuronal degeneration. It is conceivable that MDMA and malonate promote peroxynitrite-induced nitration of tyrosine residues of the rate-limiting enzymes tyrosine or tryptophan hydroxylase. Other proteins, e.g., plasmalemmal transporters, critical for the functioning of dopamine or 5-HT neurons are also potential targets for the peroxynitrite-mediated nitration of tyrosine residues.

Direct evidence that supports the conclusion that MDMA increases nitric oxide formation is the present finding that extracellular concentrations of nitrite/nitrate were increased during and after the administration of a 5-HT-depleting regimen of MDMA. Nitric oxide readily reacts with oxygen to form nitrite/nitrate. Extracellular concentrations of nitrite/nitrate have been shown to be increased in ischemia and by the administration of lipopolysaccharide and cytokines (Liaw et al., 2002; Suzuki et al., 2002) and have been viewed as direct evidence of NOS-mediated nitric oxide formation. Indeed, nitrite/nitrate concentrations in dialysis samples are markedly reduced after treatment with an NOS inhibitor (Ohta et al., 1994).

It is well documented that a multiple dose regimen of MDMA produces long-term depletion; a single injection of MDMA (10 mg/kg) does not produce 5-HT depletion (Gudeksey et al., 1994). In the present study, nitric oxide formation was increased only in rats treated with the multiple dose regimen of MDMA and not a single administration of MDMA. Although this finding is only correlational in nature, it is further supportive of the role of nitric oxide formation in MDMA toxicity.

In summary, NOS inhibitors, including a neuronal inhibitor S-MTC, suppressed the depletion of dopamine and 5-HT produced by the central administration of MDMA and the mitochondrial inhibitor malonate, and the selective depletion of 5-HT resulting from the systemic administration of MDMA. Neuroprotection afforded by NOS inhibition seemed to be independent of alterations in body temperature. The systemic administration of MDMA also increased nitric oxide formation and nitrotyrosine concentration in the striatum. Collectively, the present data support the hypothesis that MDMA-induced neurotoxicity involves nitric oxide-dependent mechanisms.

Delineation of the role of nitric oxide in psychostimulant toxicity serves to illustrate the similarities in the neurochemical substrates in psychostimulant-induced monoamine depletion and in the toxicity associated with other neurodegenerative disorders. Further elucidation of the role of nitric oxide in these processes ultimately may result in the development of improved therapeutic strategies for neuroprotection.

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