Nitric Oxide and the Neurotoxic Effects of Methamphetamine and 3,4-Methylenedioxymethamphetamine

TERRI TARASKA and KEVIN T. FINNEGAN
Departments of Psychiatry (T.T., K.T.F.), Pharmacology and Toxicology (K.T.F.) and Neuroscience (K.T.F.), University of Utah School of Medicine and the Veterans Administration Medical Center, Salt Lake City, Utah
Accepted for publication October 21, 1996

ABSTRACT
The role of nitric oxide (NO) in the long-term, amine-depleting effects of methamphetamine (METH) and 3,4-methylenedioxymethamphetamine (MDMA) was investigated in the rodent central nervous system. The NO synthase inhibitor N\textsubscript{G}-nitro-L-arginine methyl ester (L-NAME) antagonized the dopa-mine- and serotonin-depleting effects of both METH and MDMA. The protective actions of L-NAME in METH-treated mice were reversed by prior administration of the NO generator isosorbide dinitrate. However, pretreatment with N\textsubscript{G}-monomethyl-L-arginine or N\textsubscript{G}-nitro-L-arginine, two other NO synthase inhibitors, failed to block the neurotoxic effects of METH or MDMA. L-NAME was also the only NO synthase inhibitor that antagonized the hyperthermic effects of METH, reducing colonic temperatures in mice by a mean of 3°C, in comparison with control. Moreover, if the hyperthermic effects of L-NAME in METH-treated mice were prevented by raising the ambient room temperature, the dopamine-depleting actions of the stimulant were fully restored. The latter findings suggest that it is the hyperthermic actions of L-NAME, rather than its NO inhibitory properties, that are responsible for the prevention of neurotoxicity. Together with the results of the N\textsubscript{G}-monomethyl-L-arginine and N\textsubscript{G}-nitro-L-arginine experiments, the data suggest that NO plays little or no role in the toxic mechanism of action of METH or MDMA.

Amphetamine and several of its analogs (METH and MDMA) produce persistent decreases in the CNS concentrations of DA and serotonin (5-HT) in rodents and monkeys (for reviews, see Seiden and Ricaurte, 1987; Finnegan and Schuster, 1989; Gibb et al., 1990). The decline in neurotransmitter levels is accompanied by equally persistent decreases in the number of DA and 5-HT reuptake sites and in the synthetic capacities of the enzymes tyrosine and tryptophan hydroxylase. Coupled with morphological data showing the presence of degenerating axons and terminals in these same brain areas (Ellison et al., 1978; Ricaurte et al., 1982; Moliver et al., 1990), the data have been interpreted as indicating that the amphetamines damage DA and 5-HT neurons in experimental animals. Although previous studies have implicated changes in both dopaminergic and glutamatergic neurotransmission in the mechanism of the toxicity (Schmidt et al., 1985; Stone et al., 1988; Sonsalla et al., 1989; Finnegan et al., 1990), the neurochemical events responsible for the neuronal damage remain uncertain.

NO is a versatile substance implicated in the regulation of macrophage killing, vascular tone and neurotransmission (for reviews, see Moncada et al., 1991; Nathan, 1992; Iadecola et al., 1994). NO is a known mediator of tissue injury in the periphery; based on this observation, its involvement in the pathophysiology of cell death in the CNS has received considerable recent attention. As reviewed previously (Choi, 1988; Coyle and Puttfarcken, 1993), glutamate release and the overstimulation of glutamate receptors appear to underlie the neuron-damaging effects of several acute CNS insults, including ischemia/hypoxemia, hypoglycemia and traumatic injury. Glutamate rapidly stimulates NO production by a mechanism that involves NMDA receptor stimulation and the translocation of calcium (Garthwaite et al., 1988, 1989; Bredt and Snyder, 1989, 1990; Knowles et al., 1989). The resultant increase in intracellular calcium is thought to trigger a calmodulin-mediated phosphorylation event that in turn activates the enzyme responsible for the synthesis of NO (NOS). In vitro, the inhibition of NOS prevents the neuron-damaging effects of NMDA and glutamate in cortical cell culture (Dawson et al., 1991). In vivo, CNS NO concentrations become elevated shortly after the onset of cerebral ischemia (Kader et al., 1993; Malinski et al., 1993; Sato et al., 1993), whereas the inhibition of NO production is reported to decrease cellular damage, as indicated by reductions in infarction volume (Nowicki et al., 1991; Buisson et al., 1992; Nagafuji et al., 1992; Ashwal et al., 1993). Results such as...
these have lead to the proposal that the neuron-damaging effects of glutamate are mediated by NO. Reductions in cell damage (Garthwaite and Garthwaite, 1994) or infarction volume (Yamamoto et al., 1992; Kuluz et al., 1993) have not always been observed, however, suggesting that any role for NO in glutamate-induced neurotoxicity is complex.

Mechanisms by which NO could produce cell damage include the inhibition of iron-containing enzymes such as complexes I and II of the mitochondrial respiratory chain (Stadler et al., 1991), thiol inactivation and protein ribosylation (Dimmeler et al., 1992; Zhang et al., 1994), alterations in DNA synthesis (Wink et al., 1991) or the reaction of NO with superoxide to generate the potent oxidant peroxynitrite and other destructive oxygen radicals (Radi et al., 1991). Because several of the above mechanisms may also play a role in amphetamine-induced neuronal damage, it is possible that the modes of action of NO and the amphetamines may overlap. Indeed, the idea that NO might be directly involved in the toxic mechanism of action of the amphetamines is especially attractive because glutamate has been suggested to play a central role in both. METH is reported to increase synaptic concentrations of glutamate (Nash and Yamamoto, 1992, 1993; Abekawa et al., 1994), and the neurotoxic effects of the stimulants, like glutamate, are blocked by NMDA antagonists (Sonsalla et al., 1989, 1991; Finnegan et al., 1990, 1991). Although open to debate (Bowyer et al., 1993, 1994; Farfel and Seiden, 1995), one interpretation of these data is that the amphetamines damage neurons by stimulating glutamate release. If increases in NO mediate the injurious effects of excess glutamate, it might be predicted that NOS inhibitors would also block the toxic effects of the amphetamines. The aim of the present studies was to test this hypothesis by evaluating the abilities of several NOS inhibitors to block the long-term, amine-depleting effects of METH in mice and MDMA in rats.

Materials and Methods

Animals and drugs. Male CF-1 mice (30 g) or male Sprague-Dawley rats (200 g) were used (Sasco, Omaha, NE). Animals were housed in an American Association for the Accreditation of Laboratory Animal Care-approved facility, under conditions of constant room temperature (23°C) and humidity (45%). The rodents were fed ad libitum and water was available. All experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the local Animal Care Committee.

The NOS inhibitors l-NAME, N\textsuperscript{G}-monomethyl-l-arginine and N\textsuperscript{G}-nitro-l-arginine were purchased from the Sigma Chemical Co. (St. Louis, MO). Isosorbide dinitrate, a compound that spontaneously generates NO in solution, was also obtained from Sigma. METH and MDMA were generously donated by the National Institute on Drug Abuse (Washington, DC). Drug doses are expressed as the free base, and all drugs were dissolved in 0.9% saline immediately before injection. METH or MDMA was administered s.c. to rats four times, with injections separated by intervals of 2 hr. In general, multiple doses of the NOS inhibitors were given concurrently with the administration of the stimulants. Doses of the NOS inhibitors used were based on published literature values and on pilot studies. The exact dose, route of administration and schedule for each drug used in the experiments are detailed in the figure legends.

Assay of catecholamines and indoleamines. One week after drug administration, mice were sacrificed by cervical dislocation, whereas rats were sacrificed by decapitation. The striatum of both species was rapidly dissected free, and the tissue samples were frozen in liquid nitrogen (Finnegan et al., 1989). Samples of the hippocampus and frontal cortex were also obtained from rats. Levels of amines in the striatum (DA, homovanillic acid, dihydroxyphenylacetic acid, 5-HT and 5-hydroxyindoleacetic acid) and in the hippocampus and frontal cortex (5-HT and 5-hydroxyindoleacetic acid) were assayed using high-pressure liquid chromatography, coupled with electrochemical detection, as described previously (Finnegan et al., 1989). The concentrations of the various amines (in nanograms per milligram of tissue) were determined by comparison with standard curves.

Measurement of colonic temperature. Colonic temperatures of mice were obtained using an electronic thermometer attached to a rectal temperature probe (TRI-R instruments, Jamaica, NY). The probe was lubricated with surgical lubricant (Surgilube; Surgical Supply, Inc., Melville, NY) and inserted exactly 2.5 cm into the rectum for 30 sec. Colonic temperature was recorded immediately before drug administration (base line) and at 30-min intervals thereafter.

Statistics. A one-factor analysis of variance was used when the data involved measurements of catecholamines and indoleamines (Sigmastat; Jandel Scientific). When the overall analysis was statistically significant, differences between individual groups were compared post hoc by Student t test (corrected for multiple comparisons by the Bonferroni method). Colonic temperature data were analyzed using a two-factor analysis of variance (treatment × time) with repeated measures across time. Differences between individual groups were compared post hoc using the Student-Newman-Kuels method. The significance level was set at P < .05.

Results

As shown in figure 1, METH significantly decreased the concentrations of DA in the striatum of mice sacrificed 1 week after completion of the multiple-dose regimen (38% of control). Similar declines in the levels of striatal dihydroxyphenylacetic acid and homovanillic acid were also noted (data not shown). Pretreatment with the NOS inhibitor

![Fig. 1. Effects of l-NAME on METH-induced DA depletions in the mouse striatum.](image)
L-NAME blocked the DA-depleting effects of METH in a dose-related fashion. The dose-dependent nature of the antagonism was most apparent at the two lowest doses of L-NAME tested (37 and 75 mg/kg/injection) and appeared to plateau at higher doses (150 and 300 mg/kg/injection). The administration of L-NAME alone (150 mg/kg/injection) did not significantly affect the concentration of striatal DA. These observations are consistent with the notion that NO is involved in the toxic mode of action of METH.

The protection by NOS inhibitors against glutamate- or NMDA-induced neuronal cell damage is reversed by the administration of compounds that spontaneously liberate NO (Dawson et al., 1991). We therefore investigated whether the NO generator isosorbide dinitrate would restore the toxic effects of METH in the presence of L-NAME. Consistent with the findings in the previous experiment, METH alone profoundly lowered striatal DA concentrations to 23% of control (fig. 2), whereas pretreatment with L-NAME provided significant protection against the toxicity (79% of control). Administration of isosorbide dinitrate, however, restored the DA-depleting effects of the stimulant in mice concurrently given L-NAME and METH; that is, isosorbide dinitrate appeared to reverse the protective effects of L-NAME (fig. 2). The restoration of METH-induced toxicity was most dramatic in the mice receiving the larger dose of isosorbide dinitrate (500 mg/kg/injection); the mean DA depletion in this group was the same as that observed in mice given METH alone (23% of control). The administration of isosorbide dinitrate by itself or in combination with METH produced no significant effect on striatal DA levels, in comparison with the appropriate control. These data appear to provide additional support for the proposal that threshold concentrations of NO are required for the expression of METH-induced neurotoxicity.

Two other NOS inhibitors were investigated, to rule out the possibility that the neuroprotective effects of L-NAME might have resulted from some nonspecific action. In one set of experiments, mice were injected with 50 mg/kg N\textsuperscript{G}-nitro-L-arginine twice each day for 4 days and then given METH on day 5. This dosing regimen for N\textsuperscript{G}-nitro-L-arginine was reported to inhibit NOS activity in the mouse CNS by >95% (Dwyer et al., 1991). Surprisingly, no protection was observed (fig. 3). Higher multiple doses of N\textsuperscript{G}-nitro-L-arginine (75 mg/kg injection) likewise provided no protection. Similar results were found when a third NOS inhibitor, N\textsuperscript{G}-monomethyl-L-arginine, was studied; that is, pretreatment of mice with multiple doses of N\textsuperscript{G}-monomethyl-L-arginine did not alter the DA-depleting effects of METH (fig. 3).

The role of NO in the neurotoxic effects of MDMA was also investigated. MDMA is structurally related to METH but preferentially targets CNS 5-HT, rather than DA, neurons in rats (Stone et al., 1987). As shown in figure 4, pretreatment with L-NAME significantly attenuated the long-term, 5-HT-depleting effects of MDMA in both the hippocampus and frontal cortex of rats. As was found for METH, however, the neuroprotective effects of L-NAME were not shared by a second inhibitor of NOS, in this case N\textsuperscript{G}-nitro-L-arginine. The finding that L-NAME blocks the amine-depleting effects of METH and MDMA but other NOS inhibitors do not suggests

**Fig. 2.** Effects of isosorbide dinitrate on striatal DA depletions in mice treated concurrently with L-NAME and METH. Groups of mice (n = 10/group) were treated with multiple doses of either saline, L-NAME (75 mg/kg/injection i.p.), isosorbide dinitrate (ISO) (300 or 500 mg/kg/injection i.p.) or L-NAME given concurrently with isosorbide dinitrate. All groups received four doses of METH (10 mg/kg/injection s.c., with injections separated by intervals of 2 hr). L-NAME was administered twice, 30 min before the first and fourth injections of METH; isosorbide dinitrate was given four times, with each dose being administered 15 min before an injection of METH. All animals were sacrificed 1 week later for the assay of DA and its metabolites in the striatum. Results are presented as the mean ± S.E.M. of the concentration of striatal DA for each treated group. Differences between individual groups were assessed post hoc by Student t test (corrected for multiple comparisons by the method of Bonferroni), after a one-factor analysis of variance revealed a significant difference among groups (F = 45.74, P < .001). a, significantly different from saline control (P < .05); b, significantly different from METH alone (P < .05); c, significantly different from METH plus L-NAME (P < .05).

**Fig. 3.** Effects of N\textsuperscript{G}-monomethyl-L-arginine (MMA) or N\textsuperscript{G}-nitro-L-arginine (NA) on METH-induced DA depletions in the mouse striatum. Groups of mice (n = 10/group) were treated with either saline or N\textsuperscript{G}-nitro-L-arginine (50 or 75 mg/kg/injection i.p., two injections/day) for 4 days. On day 5, approximately 14 hr after the last dose of N\textsuperscript{G}-nitro-L-arginine, mice were given four injections of METH (10 mg/kg/injection, with injections separated by intervals of 2 hr). All animals were sacrificed 1 week later for the assay of DA and its metabolites in the striatum. In a second experiment, groups of mice (n = 10/group) were treated with either saline or four injections of N\textsuperscript{G}-monomethyl-L-arginine (30 mg/kg/injection i.p., with injections separated by intervals of 2 hr) concurrently with four doses of METH (10 mg/kg/injection s.c., with injections separated by intervals of 2 hr). Doses of N\textsuperscript{G}-monomethyl-L-arginine were given 30 min before each injection of METH. All animals were sacrificed 1 week later for the assay of DA and its metabolites in the striatum. Results are presented as the mean ± S.E.M. of the concentration of striatal DA for each treated group. Striatal DA concentrations from the control and METH-alone-treated mice from the two experiments were pooled for analysis and the results presented in the figure. Differences between individual groups were assessed post hoc by Student t test (corrected for multiple comparisons by the method of Bonferroni), after a one-factor analysis of variance revealed a significant difference among groups (F = 29.33, P < .001). a, significantly different from saline-treated control (P < .05).
that the protective actions of the drug arise as a consequence of some action other than its inhibition of NOS.

Recent studies have shown that temperature plays an important role in the neuron-damaging effects of METH. Placing the animals in a cold environment (e.g., a walk-in refrigerator) during the period of METH administration, for example, or administering drugs that lower body temperature blocks the toxicity (Bowyer et al., 1993, 1994). Based on these findings, we examined the effects of L-NAME (fig. 5, top) or N^3-nitro-L-arginine (fig. 5, bottom) on colonic temperature in METH-treated mice. As illustrated in figure 5, top, L-NAME (administered at doses that provide significant protection against the toxic effects of METH) induced a profound hypothermic response when given concurrently with the stimulant. Compared with saline- or METH-alone-treated mice, animals given the combination of L-NAME and METH displayed a mean decrease in colonic temperature of almost 3°C. In contrast, colonic temperatures in mice treated concurrently with N^3-nitro-L-arginine and METH (fig. 5, bottom) were not significantly different from those observed in saline- or METH-alone-treated mice; that is, N^3-nitro-L-arginine did not produce hypothermia when given with METH.

The data raise the possibility that the hypothermia produced by L-NAME may be responsible for its neuroprotective effects. If this is true, preventing the hypothermia might restore the toxicity of METH. This was accomplished by periodically moving the animals from the laboratory (23°C) to an incubator (33°C) for 10 min whenever their colonic temperatures were observed to fall below 37°C. In this fashion, mice given L-NAME and METH concurrently and mice given METH alone were made similar with respect to body temperature. As shown in figure 6, striatal DA levels in L-NAME/

Discussion

It is a commonly held maxim that drugs exert multiple pharmacological effects and that any given pharmacological action can be produced by many different drugs. The findings here highlight the fact that several commonly used inhibitors of NOS are no exception to this rule; they also emphasize the value of using multiple drugs in an effort to circumvent the problem of nonspecificity. We observed that the NOS inhibitor L-NAME protected against the long-term, DA-depleting
effects of METH, a finding that tends to support the notion that this diffusible gas plays a key role in the neurotoxic mechanism of action of the stimulant. In accordance with this idea, pretreatment with the NO donor isosorbide dinitrate restored the DA-depleting effects of METH in l-NAME-treated mice. l-NAME also partially prevented the long-term, 5-HT-depleting effects of the related neurotoxin MDMA. These findings are reminiscent of those provided by other investigators, showing that inhibitors of NOS prevented the neuron-damaging effects of glutamate and its analogs in cell culture (Dawson et al., 1991) and in vivo (Nowicki et al., 1991; Buisson et al., 1992; Nagafuji et al., 1992; Ashwal et al., 1993), a result that was often reversed by the addition of NO donors or the NOS substrate L-arginine. The conclusion that NO is involved in METH-induced toxicity is also attractive because much of the work on NO and neuronal cell death has centered on the action of glutamate, an excitatory amino acid that has also been implicated in the toxic mechanism of action of both METH and MDMA (Son-salla et al., 1989; Finnegam et al., 1990; Nash and Yamamoto, 1992).

Our subsequent studies, however, seem to dispute this initial conclusion because Nω-monomethyl-L-arginine and Nω-nitro-L-arginine, two other Nω-substituted L-arginine analogs commonly used to inhibit NOS activity (for reviews, see Moncada et al., 1991; Nathan, 1992; Iadecola et al., 1994), provided no protection against the amine-depleting effects of METH or MDMA. Although the negative findings might be explained by inadequate dosing or perhaps by reductions in the delivery of the inhibitors to the CNS, we view these explanations as unlikely. Reduced delivery might occur because the inhibition of NOS is known to produce substantial effects on cerebral vascular tone, blood flow and arterial blood pressure (Iadecola et al., 1994). However, METH- or MDMA-induced amine depletions in animals pretreated with Nω-monomethyl-L-arginine or Nω-nitro-L-arginine were very similar to those observed in animals given the neurotoxins alone, indicating that such hemodynamic effects did not affect the delivery of the neurotoxins, and by extension the NO inhibitors, to the brain. That l-NAME was neuroprotective, of course, is also consistent with this conclusion. Inadequate dosing of the two NOS inhibitors is another possibility. However, Dwyer et al. (1991) reported that the systemic administration of 50 mg/kg Nω-nitro-L-arginine twice daily for 4 days inhibited NOS activity in the CNS by 95%. We evaluated the effects of 50 and 75 mg/kg injection Nω-nitro-L-arginine using the same schedule of administration and found no protection. These doses may actually be in excess of those required, because other investigators have shown that considerably lower doses (e.g., 5–10 mg/kg Nω-nitro-L-arginine) are capable of reducing infarction volume and neurological deficits in a rodent model of stroke (Nowicki et al., 1991; Nagafuji et al., 1992). The systemic administration of a single 30 mg/kg dose of Nω-monomethyl-L-arginine profoundly reduces blood flow in the cerebral cortex and various deep structures of brain (Tanaka et al., 1991; Adachi et al., 1993), although this finding probably results more from the inhibition of endothelial NOS. Nonetheless, because animals in the present experiments were treated multiple times with equivalent or higher doses of the two inhibitors, adequate inhibition of the brain isozyme seems likely to have been achieved. Because only one of the three NOS inhibitors used blocked the amine-depleting effects of METH or MDMA, we conclude that NO is unlikely to be involved in the toxic mechanism of action of the stimulants. Other factors, evidently, must account for the neuroprotective effects of l-NAME.

One possibility in this regard is the effect of l-NAME on core body temperature. Recent studies have shown that reducing body temperature by placing rats in a cold environment prevents METH-induced neurotoxicity, whereas increasing body temperature potentiates the toxicity (Bowyer et al., 1993, 1994). Similar findings have been reported for MDMA (Schmidt et al., 1990). These data indicate that, whatever the mechanism of the toxicity, it is evidently strongly regulated by body temperature. When colonic temperatures in mice treated with l-NAME and METH were investigated, we found that the NO inhibitor induced profound hypothermia, reducing the mean core body temperature by almost 3°C, in comparison with saline- or METH-alone-treated mice. This reduction in colonic temperature is twice that previously noted to be effective in blocking the neuron-damaging actions of METH (Bowyer et al., 1993), indicating that the hypothermic actions of l-NAME could very well account for its neuroprotective effects. In contrast, Nω-nitro-L-arginine, one of the NO inhibitors that did not protect against METH- or MDMA-induced toxicity, produced no significant effect on body temperature in METH-treated mice. Moreover, periodic exposure to warmer ambient temperatures fully restored the DA-depleting effects of METH in l-NAME-treated mice. All of these data support the argument that it is the hypothermic actions of l-NAME, and not its NO inhibitory properties, that are responsible for its abil-
ity to antagonize the long-term, amine-depleting effects of METH and MDMA. The ability of isoribose dinitrate to restore the toxicity in L-NAME/METH-treated mice is puzzling in this regard but perhaps reflects an antagonism of the hypothermia produced by L-NAME. This was not investigated, however, and remains a topic for future research.

Our conclusions concerning L-NAME are consistent with a number of recent studies demonstrating that the neuroprotective effects of a variety of agents appear to result from their common ability to block the hyperthermic actions of the amphetamines and to lower body temperature. Glutamatergic antagonists, such as MK-801 and CGS19755, reduce METH-induced toxicity to a degree predicted by their inhibition of the hypothermia, whereas increasing the ambient temperature abolishes the neuroprotection (Bowyer et al., 1994; Albers and Sonsalla, 1995). NMDA antagonists are also known to block the 5-HT-depleting effects of MDMA (Finnegan et al., 1990), and in a analogous series of experiments Farfel and Seiden (1995) showed that MK-801 and CGS19755 do so by inducing hypothermia in MDMA-treated animals. Similarly, Albers and Sonsalla (1995) have reported that the mechanism by which several dopaminergic drugs (e.g., the catecholamine synthesis inhibitor α-methyl-p-tyrosine or the DA receptor antagonists haloperidol, sulpiride and SCH 23390) block METH-induced toxicity involves reductions in body temperature. In addition to dopaminergic and glutamatergic antagonists, hypothermia appears to explain the protective actions of a variety of other drugs (ethanol, pentobarbital, diethyldithiocarbamate, aminoxyacetic acid, propanolol and dilantin) (Albers and Sonsalla, 1995; Miller and O’Callaghan, 1995). All of these data suggest that hypothermia is a common factor in the protective effects of many different drugs, and they support the contention that reductions in body temperature may similarly underlie the ability of L-NAME to block the toxic effects of METH and MDMA. Given the structural diversity of these compounds, it is likely that multiple mechanisms are involved in their capacity to induce hypothermia and that, therefore, it is the hypothermia itself that is key. Although this is undoubtedly an important clue, the significance of the hypothermia with respect to the mechanism of toxicity remains to be determined.

Acknowledgements
The authors gratefully acknowledge the technical assistance of Jennifer Clikeman in the execution of these studies.

References
Nash, J. F. and Yamamoto, B. K.: Methamphetamine neurotoxicity and striatal


Send reprint requests to: Kevin T. Finnegan, M.D., Ph.D., Psychiatry Service (116A), Veterans Administration Medical Center, 500 Foothill Blvd., Salt Lake City, UT 84148.