Pure Appl. Chem., Vol. 72, No. 6, pp. 1001–1006, 2000.

Role of neuronal nitric oxide in methamphetamine neurotoxicity and protection by nNOS inhibitor*

D. Desaiah[1], S. L. N. Reddy[1], S. Z. Imam[2], and S. F. Ali[2]

[1]Department of Neurology, University of Mississippi Medical Center, Jackson, MS 39216, USA and [2]Division of Neurotoxicology, National Center for Toxicological Research, Jefferson, AR 72079, USA

Abstract: Methamphetamine (METH) is a potent psychostimulant known to produce neurotoxicity. The dopaminergic pathway is particularly sensitive to METH. Recent studies showed that 7-nitroindazole (7-NI), a selective inhibitor of neuronal nitric oxide synthase (nNOS), provided protection against METH neurotoxicity both *in vitro* and *in vivo*. The present studies were conducted to determine the nNOS activity in various regions of the brain of young adult male Sprague-Dawley rats treated with different doses of METH. Rats were injected ip with 5, 10, 20, and 40 mg/kg and 24 h after the rats were sacrificed and the brain regions (hippocampus, frontal cortex, and cerebellum) were quickly dissected. The cytosolic fractions were prepared, and the nNOS activity was determined using the ^{3}H-citrulline assay. The results showed that nNOS activity was significantly increased in all three brain regions of rats treated with METH. The increase was dose dependent reaching a maximum of 40–100% over the control values. Rats treated with 7-NI 30 min prior to METH injection provided protection against the toxicity and also showed a reduction of nNOS activity. The activation of nNOS is known to increase the synthesis of NO which is involved in the regulation of several neurotransmitter pathways including catecholaminergic system. Reducing the METH-induced production of NO by pretreatment with selective inhibitor of nNOS, 7-NI, provided protection against METH neurotoxicity.

INTRODUCTION

Methamphetamine (METH) is a common drug of abuse that causes a massive release of newly synthesized dopamine (DA) from the presynaptic vesicles and an increase in the extracellular DA concentration in the nigrostriatal system. High dose of METH caused long-lasting neurotoxicity associated with a marked decrease in dopamine level in the striatum [1–4]. Recent studies demonstrated a similar depletion in striatal dopamine nerve terminal markers in post-mortem, chronic METH users [5]. The role of nitric oxide in the manifestations of METH-induced neurotoxicity has been widely studied. Nitric oxide was found to be mediating METH-induced neurotoxicity in primary cultures of mesencephalic cells [6]. It has been reported that a selective neuronal nitric oxide synthase (nNOS) inhibitor, 7-nitroindazole (7-NI) provided protection against the METH-induced dopaminergic neurotoxicity [7,8] which implicated the role of nitric oxide radicals in METH neurotoxicity. Furthermore, it has also been demonstrated that nNOS knockout mice are protected from METH-induced DA neurotoxicity [9]. It is well known that NO is a molecule that plays many important roles in signal transduction pathways and it is produced during the conversion of L-arginine to L-citrulline, a reaction catalyzed by an enzyme nitric oxide synthase (NOS) [10–14]. The activation of NOS depends upon the activation of N-methyl-D-aspartate

*Lecture presented at the 4th Congress of Toxicology in Developing Countries (4th CTOX-DC), Antalya, Turkey, 6–10 November, 1999. Other presentations are published in this issue, pp. 973–1066.

(NMDA) receptors which leads to an increased influx of calcium into the cells which bind to calmodulin and in turn activates NOS. Blockade of NMDA type of glutamate receptors by dizoclipine (MK-801) attenuated METH-induced neurotoxicity [15]. The protective role of nNOS inhibitors and the blockers of NMDA receptors in case of METH-induced neurotoxicity suggests that this might be mediated via the activation of the nNOS resulting in the overproduction of NO thus interfering with and altering some important signal transduction cascade events causing METH-induced neurotoxic damage. Recent studies showed an increased free-radical formation in the striatal area after METH treatment and protection by free-radical scavengers [16–20]. METH treatment also resulted in the formation of peroxynitrite, an indication of over production of NO [20]. These studies showed protection against METH with a peroxinitrite decomposition catalyst [20]. The purpose of the present study was to understand the dose-dependent effect of METH on nNOS activity in various brain regions of young rats. Further, it was to show a reduction of nNOS activity by known inhibitor, 7-NI, involved in the protection.

MATERIALS AND METHODS

Chemicals

L-[2,3-^{3}H]-Arginine (7-C_i/mmol) was purchased from New England Nuclear Corp., Boston, MA, USA. All other chemicals used in this study are from Sigma Chemicals, St. Louis, MO, USA.

Animals and treatment

One-month-old male Sprague-Dawley rats were obtained from the NCTR, Jefferson, AR, USA breeding colony. All animals were maintained on a 12 h light/dark lighting schedule at normal room temperature and housed in groups of 3/cage with free access to food and water. Animals received ip injection of 5, 10, 20, and 40 mg METH/kg body weight and were sacrificed after 4 h. The brains were rapidly removed and dissected on ice into frontal cortex, hippocampus, and cerebellum and stored at –80 °C until the analysis of nitric oxide synthase.

In a follow-up experiment, another four groups of animals were injected ip with 7-NI (25 mg/kg) 30 min before the injection of METH (20 and 40 mg/kg). Animals were sacrificed 4 h after the injection of METH, brains were removed and dissected on ice into frontal cortex, hippocampus, and cerebellum and stored at –80 °C until the analysis of nitric oxide synthase.

Preparation of cytosolic fractions

Each brain region was homogenized using a Polytron Omni 5000 homogenizer at a setting of 7. The homogenizing buffer (pH 7.2) contained 20 mM HEPES, 0.32 M sucrose, 0.5 mM EDTA, and 1 mM dithiothreitol. The brain tissue was homogenized in ice-cold buffer and centrifuged at 20 000 g for 15 min. The supernatant was passed through a Dowex AG 50 Wx-8 (Na^+ form) column to remove the endogenous arginine. The arginine-free eluent was used to assay the nitric oxide synthase activity. Protein content of these samples was determined using the BioRad protein assay kit.

Determination of nitric oxide synthase activity

NOS activity in each sample was determined according to the method of Bredt and Snyder [21] as described previously [22]. Briefly, the NOS assay reaction medium of 400 mL contained 100 mM HEPES, pH 7.2; 2 mM NADPH; 0.45 mM $CaCl_2$; 6 mM L-[2,3-^{3}H]-arginine (0.2 mC_i/mL), and 400 mg of brain cytosolic protein. The reaction mixture was incubated for 45 min at 37 °C and stopped by the addition of stop buffer containing 20 mM Hepes and 10 mM EGTA at pH 5.5. The entire reaction mixture was passed through a column packed with Na^+ form of Dowex AG 50 Wx-8 resin. The flow-through fraction containing [^{3}H]-citrulline was counted for radioactivity using a Beckman 6000 liquid scintillation counter. The NOS activity was expressed as pmole citrulline/mg protein/min.

Data analysis

Each data point is the mean ± SE of three different samples each assayed in triplicate. Significant differences between treatment groups and controls were calculated using Student's 't' test. A p value < 0.05 was considered significant and represented by an asterisk in the figures.

RESULTS AND DISCUSSION

METH-activated nNOS activity in different brain regions of young adult rats dose dependently. The data in Fig. 1 show that the rats treated with METH exhibited a dose-dependent activation of nNOS in cerebellum. The increase of nNOS was more pronounced and dose-dependent in hippocampus (Fig. 2). However, in frontal cortex a maximum 70% increase of nNOS was evident in 5 mg/kg dosed rats with no further increase with higher doses. (Fig. 3). These data suggest that METH activates nNOS in all three brain regions tested. Such an increase leads to overproduction of NO, which may interact with other oxy-radicals forming more active free radicals such as peroxynitrite resulting in neurotoxicity. Several previously reported studies support our observations [16–20]. Recently, Imam *et al* [20] showed that increased formation of 3-nitrotyrosine in METH-treated mice was blocked by peroxynitrite decomposition catalyst. The nNOS-deficient mice were reported to be resistant to METH neurotoxicity [9]. These studies, along with our present data, support the contention that METH-induced dopaminergic neurotoxicity involves oxidative stress and NO is one of the free radicals contributing to such mechanism.

These observations are further strengthened with our additional experiments. Rats treated with 7-nitroindazole 30 min prior to METH dosing were protected against mortality (data not shown). However, the nNOS profile in these groups of rats showed a reversal of activated nNOS to normal levels in all three brain regions tested (Figs. 4–6). The data indicated that 7-NI treatment resulted in the reduction of nNOS activity, confirming 7-NI is an inhibitor of nNOS *in vivo*. Rats injected with 7-NI 30 min prior to 20 and 40 mg/kg METH showed a complete reversal of METH-induced nNOS to basal level in cerebellum (Fig. 4), hippocampus (Fig. 5), and frontal cortex (Fig. 6). These data support our contention as well as the previously reported studies that NO is one of the contributing factor for METH induced neurotoxicity. 7-NI is a known inhibitor of nNOS activity, and it was shown to provide protec-

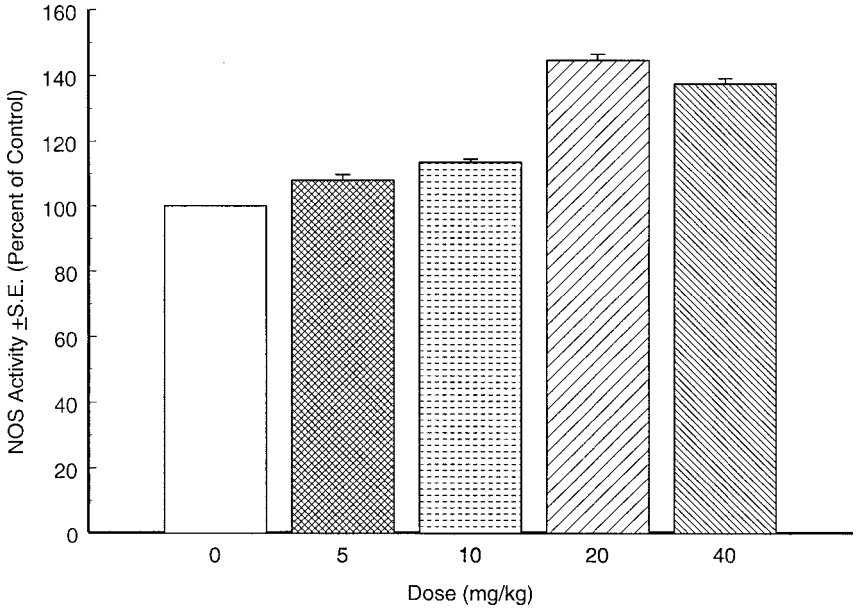

Fig. 1 *In vivo* effect of methamphetamine on neuronal nitric oxide synthase activity in rat cerebellum.

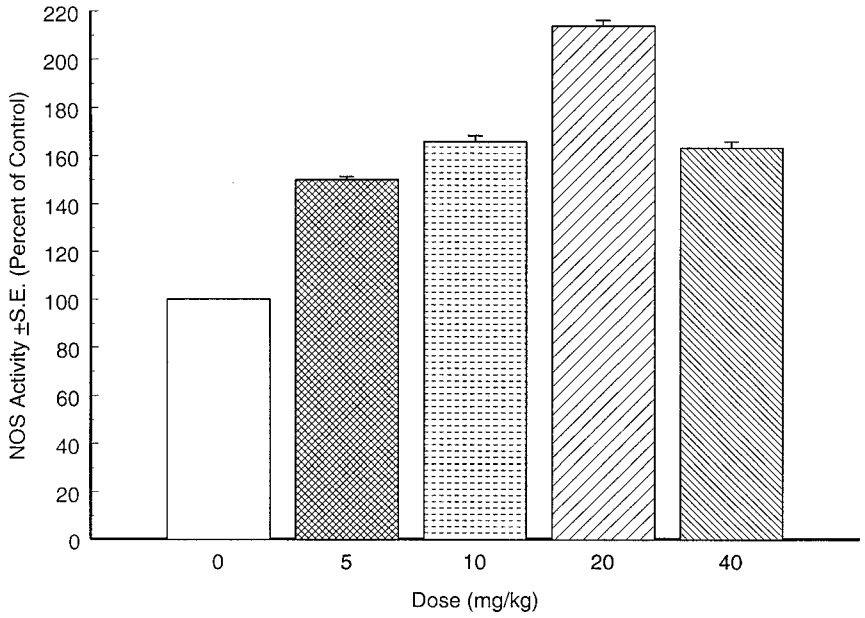

Fig. 2 *In vivo* effect of methamphetamine on neuronal nitric oxide synthase activity in rat hippocampus.

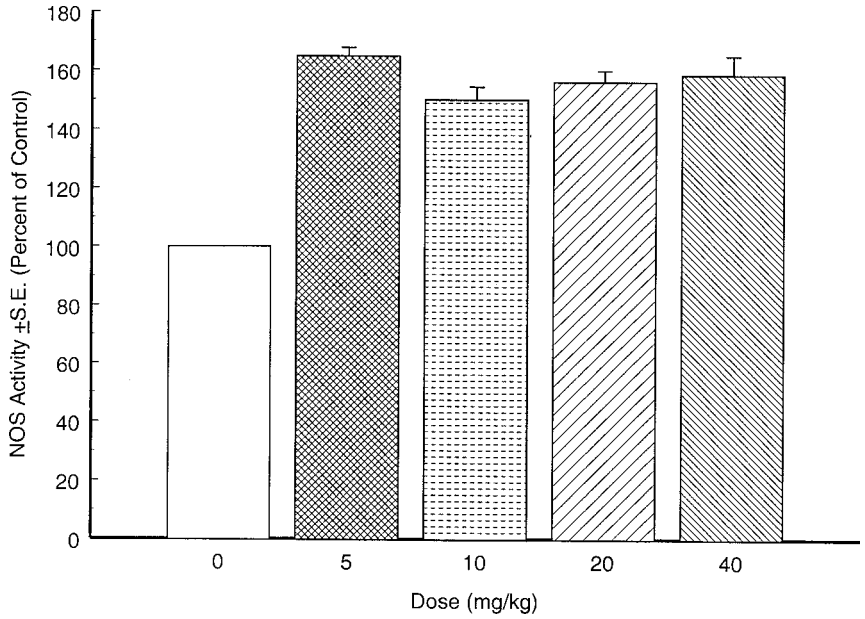

Fig. 3 *In vivo* effect of methamphetamine on neuronal nitric oxide synthase activity in rat frontal cortex.

tion against METH neurotoxicity in whole animals and in cell culture [6–8]. The protection afforded by 7-NI must be due to the reduction of METH-induced nNOS in different regions of the brain.

METH may cause neurotoxicity in all the regions of brain as evidenced by our present studies. The dopaminergic system may be particularly sensitive to NO. Our future studies will be directed towards the quantification of NOS in striatal region to understand the direct involvement of NO in dopaminergic neurotoxicity of METH.

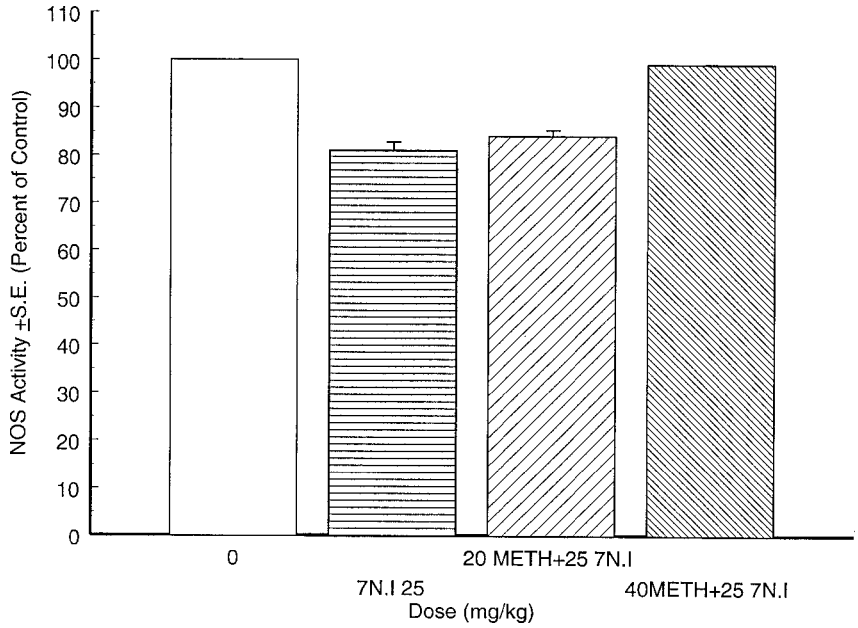

Fig. 4 Effect of 7-NI on methamphetamine-induced neuronal nitric oxide synthase activity in rat cerebellum.

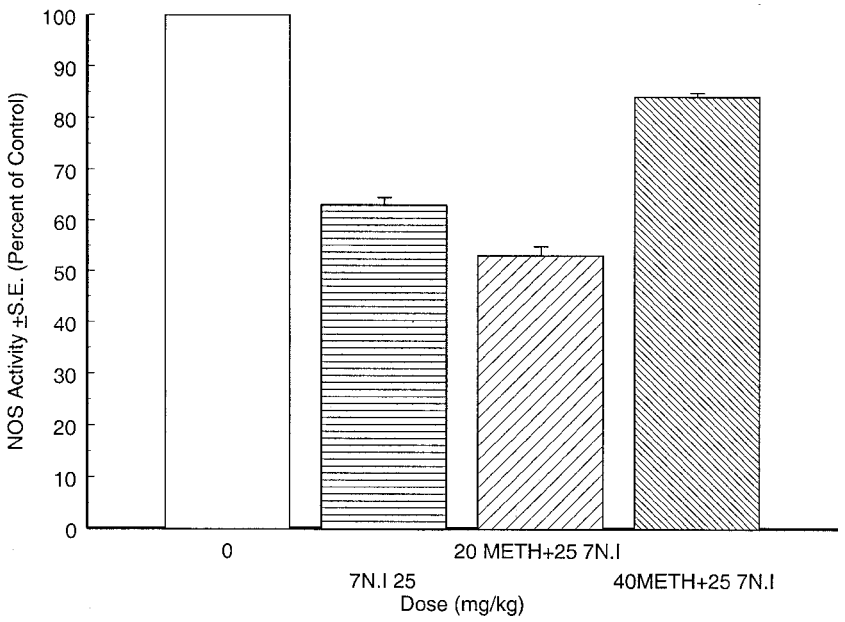

Fig. 5 Effect of 7-NI on methamphetamine-induced neuronal nitric oxide synthase activity in rat hippocampus.

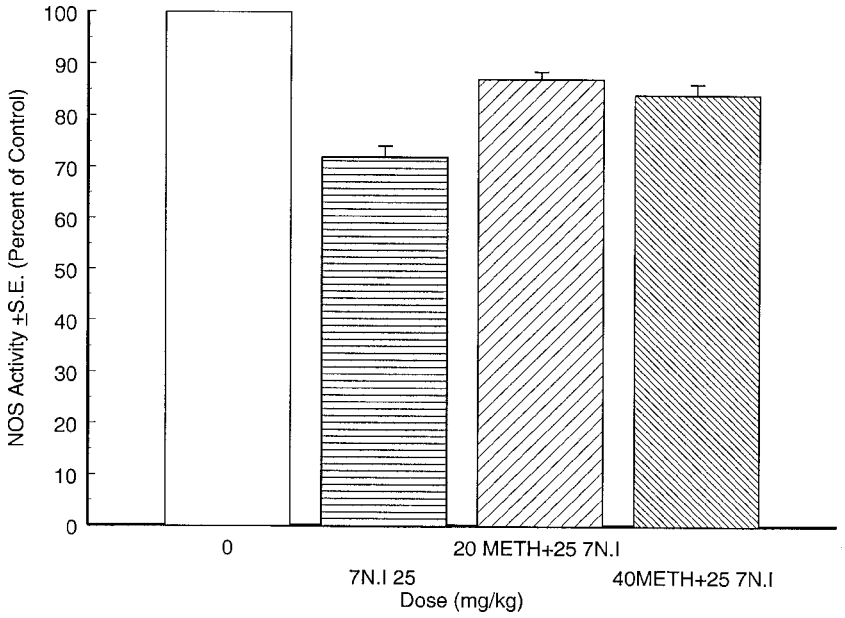

Fig. 6 Effect of 7-NI on methamphetamine-induced neuronal nitric oxide synthase activity in rat frontal cortex.

REFERENCES

1. F. J. Kogan, W. K. Nichols, J. W. Gibb. *Eur. J. Pharmacol*. **36**, 363–371 (1976).
2. R. W. Fuller, S. K. Hemrick-Luecke, P. L. Ornstein. *Neuropharmacol*. **31**, 1027–1032 (1992).
3. J. S. Gibb, M. Johnson, G. R. Hanson. *Neurotoxicol*. **11**, 317–21 (1990).
4. G. J. Marek, G. Vosmer, L. S. Seiden. *Brain Res*. **513**, 274–279 (1990).
5. J. M. Wilson, K. S. Kalasinsky, A. I. Levy, C. Bergeron, G. Reiber, R. M. Anthony, G. A. Schmunk, K. Shannak, J. W. Haycock, S. J. Kish. *Nature, Medicine* **2**, 699–703 (1996).
6. P. Sheng, C. Cerruti, S. Ali, J. L. Cadet. *Ann. N.Y. Acad. Sci*. **801**, 174–186 (1996).
7. Y. Itzhak and S. F. Ali. *J. Neurochem*. **67**, 1770–1773 (1996).
8. D. A. DiMonte, J. E. Royland, M. W. Jakowee, J. W. Langston. *J. Neurochem*. **67**, 2443–2450 (1996).
9. Y. Itzhak, C. Gandia, P. L. Huang, S. F. Ali, J. Pharmacol. *Exp. Ther*. **284**, 1040–1047 (1998).
10. J. G. Gathwaite. *Trends Neurosci*. **14**, 60–67 (1991).
11. S. H. Snyder and D. S. Bredt. *Sci. Amer*. **266**, 68–71 (1992).
12. J. Zhang and S. H. Snyder. *Ann. Rev. Pharmacol. Toxicol*. **35**, 213–233 (1995).
13. S. Moncada, R. M. J. Palmer, E. A. Higgs. *Biochem. Pharmacol*. **38**, 1709–1715 (1989).
14. M. A. Marletta. *J. Biol. Chem*. **268**, 12231–12234 (1993).
15. P. K. Sonsalla, D. E. Riordan, R. E.Heikkila. *J. Pharmacol. Exp. Ther*. **256**, 506–512 (1991).
16. O. Acikgoz, S. Gonene, B. M. Kayatekin, N. Uysal, C. Pekcetin, I. Semin, A. Gure. *Brain Res*. **813**, 200–202 (1998).
17. M. Z. Wrona, Z. Young, F. Zhang, G. Dryhurst. *NIDA Res. Monogr*, **173**, 146–174 (1997).
18. S. F. Ali, G. D. Newport, W. Slikker, Jr. *Ann. N.Y. Acad. Sci.* **801**, 187–198 (1996).
19. S. Z. Imam, g. D. Newport, F. Islam, W. Slikker, Jr., S. F. Ali. *Brain Res*. **818**, 575–578 (1999).
20. S. Z. Imam, J. P. Crow, G. D. Newport, F. Islam, W. Slikker, Jr. S. F. Ali. *Brain Res*. **837**, 15–21 (1999).
21. D. S. Bredt and S. H. Snyder. *Proc. Natl. Acad. Sci*. USA. **87**, 682–685 (1990).
22. P. Joshi, P. J. S. Vig, V. Veerisetty, J. A. Cameron, B. S. Sekhon, D. Desaiah. *Pharmacol. Toxicol*. **78**, 99–103 (1996).