

Differential action of 7-nitro indazole on rat brain nitric oxide synthase

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Abstract

We examined the dose-response characteristics of brain nitric oxide synthase (NOS) inhibition following intraperitoneal administration of 7-nitro indazole (7-NI). 7-NI inhibited striatal, hippocampal, cortical, cerebellar and nigral NOS activity in a dose-dependent manner. NOS activity in the striatum and hippocampus could not be inhibited more than 60% while cerebellar and nigral activity was depleted by at least 85%, indicating that 7-NI has differential effects in different brain regions. ED₅₀ values obtained from the 7-NI dose-response curves of the striatum and hippocampus were significantly higher than the ED₅₀ values obtained from the cortex, cerebellum and substantia nigra, further confirming the differential actions of 7-NI. In addition, inhibition of NOS activity 4.5 h following a maximal dose of 7-NI demonstrated differential recovery. At this time point, the cerebellum and hippocampus were more inhibited than the striatum, cortex and substantia nigra. Therefore, the extent of recovery from this inhibition was independent of the level of maximal NOS inhibition in the different brain regions. We suggest determining the extent and duration of NOS inhibition resulting from 7-NI administration prior to using it to study the role of neuronal nitric oxide (NO) in various systems.

Keywords: 7-Nitro indazole (7-NI); Brain nitric oxide synthase; Differential inhibition

It has been postulated that nitric oxide (NO) synthesis through activation of nitric oxide synthase (NOS) plays a role in neuronal processes such as long-term potentiation [2], nociceptive processing [13], regulation of local cerebral blood flow [9], autonomic regulation [10] and *N*-methyl-D-aspartate (NMDA) receptor-mediated excitotoxicity [6]. Most of the evidence supporting a role for NO in central processes has been based on the use of inhibitors of NOS. The most commonly used inhibitors of NOS, the arginine analogs (i.e. *N*^ω-nitro-L-arginine methyl ester and *N*^ω-monomethyl-L-arginine), may be inappropriate as *in vivo* pretreatment regimens due to their lack of isozyme specificity (for review, see [7]).

There has been recent interest in agents that selectively inhibit different NOS isozymes *in vivo*. Several investigators have demonstrated that 7-nitro indazole (7-NI) acted as a selective inhibitor of neuronal NOS when administered systemically [11,14–17] despite the fact that it was found to inhibit all NOS isozymes *in vitro* [1]. This was

based on the findings that 7-NI did not cause an increase in mouse or rat mean arterial blood pressure when administered systemically [14,15], did not inhibit acetylcholine-induced relaxation of rabbit aortic rings [15] and did not alter acetylcholine-induced vasodilation in rats *in vivo* [16,17]. In addition, 7-NI was approximately 50 times more potent at inhibiting neuronal NOS than the inducible isoform which was measured in spleen homogenates of endotoxin pretreated rats [1]. Recently, MacKenzie et al. [12] investigated the effects of a single dose of 7-NI on regional brain NOS activity and concluded that 7-NI inhibited NOS activity to the same extent in all the brain regions examined. However, preliminary experiments in our laboratory demonstrated that submaximal doses of 7-NI inhibited cerebellar NOS activity, but failed to inhibit the activity in the striatum, indicating differential sensitivity. Based on these findings and the potential experimental applications of 7-NI, we investigated the dose-response characteristics and degree of neuronal NOS inhibition produced by 7-NI in rat striatum, hippocampus, cortex, cerebellum and substantia nigra.

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7-NI was obtained from Lancaster Synthesis Inc. (Windham, NH) and prepared each day by sonicating and heating in peanut oil. There was no evidence for a different maximal effect of 7-NI on NOS activity with or without heating of 7-NI, however, heating and sonication of 7-NI was necessary in order to obtain consistent levels of NOS inhibition. [^{14}C]L-arginine (0.319 Ci/mmol, 50 $\mu\text{Ci/ml}$) was purchased from New England Nuclear Research Products (Mississauga, ON). The dye reagent for the protein assay was supplied by Bio-Rad Lab. (Mississauga, ON). All other drugs were obtained from the Sigma Chemical Co. Ltd. (St. Louis, MO). Dowex 50WX8-400 was purchased in H^+ form and converted to the Na^+ form by soaking in 1 N NaOH.

Male Sprague–Dawley rats weighing 250–300 g, receiving food and water ad libitum, were used in all experiments. Animals were given a single injection of 7-NI (20–75 mg/kg i.p.) or vehicle and sacrificed 0.5 or 4.5 h thereafter. These time points were chosen based on reports that maximal inhibition of NOS activity occurs 0.5 h following systemic 7-NI administration [12] and that recovery from this inhibition is occurring, but not complete, 4 h after i.p. injection of 7-NI [12,16]. Following pretreatment, the cerebellum, striatum, hippocampus, cortex and substantia nigra were quickly dissected and homogenized in ice-cold 50 mM HEPES buffer (pH 7.4) containing 1 mM EDTA, 1 mM DTT and 10 $\mu\text{g/ml}$ leupeptin. The brain regions were homogenized in the following volumes (ml) of buffer: striatum 0.20; hippocampus 0.30; cortex 1.0; cerebellum 1.0; substantia nigra 0.175. Homogenates were centrifuged at $20\,000 \times g$ for 30 min at 4°C and the supernatants retained for the NOS assay.

NOS activity was measured by monitoring the conversion of [^{14}C]L-arginine to [^{14}C]citrulline using a modification of the method of Bredt and Snyder [4]. Each supernatant sample (25 μl) was incubated with 25 μl of 100 μM [^{14}C]L-arginine and 100 μl of reaction buffer for 1 h at 37°C . The reaction buffer consisted of 1 mM DDT, 1.25 mM CaCl_2 , 1 mM valine (arginase inhibitor), 2 mM NADPH and 1 mM EDTA in 50 mM HEPES buffer at pH 7.4. The reaction was stopped by the addition of 2 ml ice-cold 20 mM HEPES buffer containing 2 mM EDTA at pH 5.5. Each sample was passed through a 0.75 ml Dowex 50WX8-400 (Na^+) column to separate [^{14}C]L-arginine from [^{14}C]citrulline. The columns were subsequently washed with 2 ml of distilled water and the [^{14}C]citrulline in the resulting eluate was counted using a Beckman scintillation counter. Protein content of the tissue supernatant was determined by the method of Bradford [3].

NOS activity was expressed as nmol [^{14}C]citrulline formed/mg protein/h. The effects of 7-NI are expressed as the percentage of NOS activity compared with results from brain regions of vehicle treated rats. Data are presented as the mean \pm SD. Statistical analysis was performed by one-way ANOVA followed by Tukey's test to determine which groups were significantly different from

each other. Differences in mean values were considered significant when $P < 0.05$.

Baseline NOS activity in vehicle (peanut oil) treated animals was as follows: striatum 3.7 ± 1.9 ; hippocampus 1.9 ± 0.9 ; cortex 2.5 ± 1.2 ; cerebellum 10.1 ± 1.7 ; substantia nigra 3.5 ± 1.0 (nmol [^{14}C]citrulline/mg protein/h; $n = 17$). These values were not different from those obtained from untreated animals ($n = 10$, compared by student's *t*-test).

Following both 50 and 75 mg/kg treatments, 7-NI administration produced sedative behavior in rats. The dose-response relationships for the effect of a single injection of 7-NI on regional NOS catalytic activity are presented in Fig. 1. Following treatment of animals with 7-NI, NOS activity in all the brain regions examined was dose-dependently inhibited. Maximal inhibition of NOS activity was obtained following a dose of 50 mg/kg 7-NI in all five brain regions, however, the degree of inhibition, expressed as a percent of control, differed in some of these areas: striatum 40.3 ± 11.6 ; hippocampus 43.7 ± 8.6 ; cortex 33.7 ± 6.1 ; cerebellum 16.6 ± 2.1 substantia nigra 21.4 ± 6.4 ($n = 5$). Striatal and hippocampal NOS activities were not decreased to the same degree as nigral and cerebellar NOS activity following injection of 40, 50 and 75 mg/kg 7-NI (Fig. 1). Residual cortical NOS activity fell between these values and was not significantly different from any of the other brain regions at all doses of 7-NI

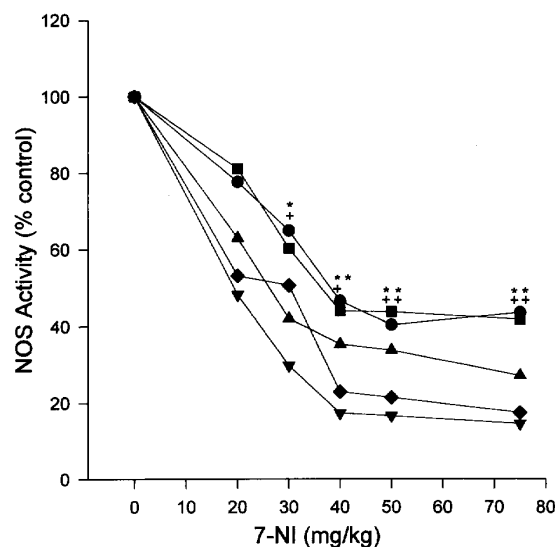


Fig. 1. Dose-response relationship between the dose of 7-NI (i.p.) and regional NOS activity measured 30 min post-injection. NOS activity was assessed in the striatum (●), hippocampus (■), cortex (▲), cerebellum (▼) and substantia nigra (◆). Values are expressed as the mean percentage of NOS activity compared with vehicle injected rats ($n = 4-6$ for each point). SDs were omitted for clarity but lie within the range of 1.0 to 22.7% of control. All differences are significant at $P < 0.05$; *striatum versus cerebellum, **striatum versus substantia nigra, +hippocampus versus cerebellum, ++hippocampus versus cerebellum and substantia nigra.

Table 1
Regional ED₅₀ values (mg/kg) obtained from 7-NI dose-response curves

Brain Region	ED ₅₀ ± SD
Striatum	25.33 ± 2.4*
Hippocampus	25.02 ± 2.2*
Cortex	19.80 ± 2.1
Cerebellum	17.70 ± 2.1
Substantia nigra	18.87 ± 2.1

Values were derived by sigmoidal regression and expressed as the mean ± SD ($n = 4$). A significant difference from cortex, cerebellum and substantia nigra is indicated by * $P < 0.05$.

tested. The respective ED₅₀ values for each 7-NI regional dose-response curve (Fig. 1) were calculated by sigmoidal regression and are presented in Table 1. The ED₅₀ values obtained from the striatal and hippocampal dose-response curves were significantly higher than those obtained from the dose-response curves of the cortex, cerebellum and substantia nigra.

The level of regional brain NOS inhibition measured 4.5 h following a maximal dose of 7-NI (50 mg/kg, i.p.; based on dose response curves in (Fig. 1.) is depicted in Fig. 2. Cerebellar NOS activity was significantly more inhibited than striatal, cortical and nigral NOS activity measured 4.5 h following 7-NI injection. Conversely, striatal NOS activity was less inhibited than hippocampal, cortical and cerebellar NOS activity at this time point.

It has previously been found that maximal inhibition of neuronal NOS activity occurs 0.5 h after systemic injection of 7-NI [12]. Therefore, in the present study, the level of dose-dependent 7-NI-induced inhibition of regional brain NOS activity was examined at this time point. At doses of 7-NI producing maximal NOS inhibition, the extent of NOS inhibition was greater in the cerebellum and substantia nigra than in the striatum and hippocampus, indicating a differential inhibitory effect of 7-NI on enzyme activity in various brain regions. The magnitude of 7-NI-induced NOS inhibition was not related to the baseline NOS activity measured in these regions. It is unlikely that these differences are the result of the levels of endothelial versus neuronal NOS in these brain areas since neuronal NOS knock out mice have a greater than 90% depletion of cytosolic NOS catalytic activity in various brain regions [8]. The ED₅₀ values reported in the present study further confirm the differential actions of 7-NI in the brain regions examined. The striatum and hippocampus have significantly higher ED₅₀ values than the cortex, cerebellum and substantia nigra. These data may reflect differences in exposure of individual brain regions to 7-NI via differential bloodflow and/or uptake of 7-NI.

The level of maximal NOS inhibition obtained in the striatum, hippocampus and cortex at 40, 50 or 75 mg/kg 7-NI was lower than that achieved at a dose of 30 mg/kg in a previous report [12]. MacKenzie et al. [12] tested this single dose of 7-NI and found it to be an equally effective

and maximal inhibitor in all brain regions examined (including the striatum and hippocampus). In the present study, a dose of 30 mg/kg 7-NI did not maximally inhibit brain NOS activity and did not affect all brain regions to the same extent. The reason for these discrepancies could be due to methodological differences. However, this does not appear to be the case, since cerebellar NOS activity in the present study was decreased to the same maximal level as in the report by MacKenzie et al. [12]. Since Sprague-Dawley rats were used in the present study and Wistar rats were used by MacKenzie et al. [12], it is possible that the two different rats strains exhibited different sensitivities to the actions of 7-NI.

To examine the possibility of differential recovery from 7-NI-induced inhibition of regional brain NOS activity, we examined the level of NOS inhibition following a single dose of 7-NI (50 mg/kg) 4.5 h after treatment. At the 0.5 h time point, both striatal and hippocampal NOS activity were inhibited to the same degree following systemic 7-NI yet at 4.5 h post-injection striatal NOS activity recovered to a greater extent than did hippocampal activity. Similarly, at 0.5 h, both cerebellar and nigral NOS activity were equally inhibited following 7-NI treatment yet nigral NOS activity recovered to a greater extent 4.5 h post-injection than did cerebellar activity. These results suggest that there may be differential recovery of regional brain NOS activity following exposure to 7-NI which occurs independently of the level of NOS inhibition. Differential recovery

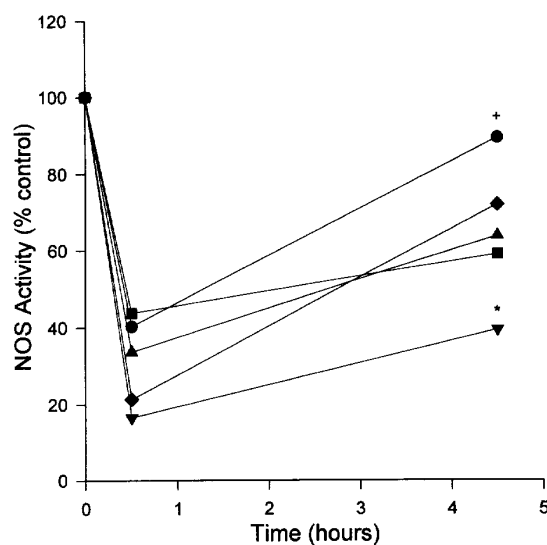


Fig. 2. Recovery of NOS activity in various brain regions following inhibition with 7-NI. Rat striatal (●), hippocampal (■), cortical (▲), cerebellar (▼) and nigral (◆) NOS activity measured 0.5 and 4.5 h following injection of 7-NI (50 mg/kg, i.p.). Values are expressed as the mean percentage of NOS activity compared with vehicle injected rats ($n = 4-5$ for each point). SDs were omitted for clarity but lie within the range of 2.1 to 11.6% of control. All differences are significant at $P < 0.05$; *cerebellum versus striatum, cortex and substantia nigra, †striatum versus hippocampus, cortex and cerebellum.

of regional NOS activity following 7-NI administration may be due to such mechanisms as increased extrusion or enhanced metabolism of 7-NI in certain brain areas. Although these processes have not been elucidated, we previously reported that the enzyme indolamine 2,3-dioxygenase (IDO) may be involved in 7-NI metabolism since it was found that an IDO inhibitor, norharmane, increased the duration of 7-NI action [5]. Future studies on the metabolism or sequestration of 7-NI in different brain areas may provide insight into the differential effects of 7-NI in the central nervous system.

The present study is the first to demonstrate dose-response effects and ED₅₀ values of intraperitoneal 7-NI on NOS activity in the striatum, hippocampus, cortex, cerebellum and substantia nigra. The magnitude of inhibition produced by 7-NI is dependent on the brain area examined. Different brain regions also recover differentially from the effects of 7-NI. It is therefore important to examine the extent and duration of NOS inhibition following administration of 7-NI before using it as a tool to study the proposed role of NO in central processes.

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