Different effects of nitric oxide synthase inhibitors on convulsions induced by nicotine in mice

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Abstract:
Acute intraperitoneal (ip) administration of N\textsuperscript{G}-nitro-L-arginine (NNA, 10, 20 and 40 mg/kg), a non-selective nitric oxide synthase (NOS) inhibitor, significantly and dose-dependently decreased the incidence of convulsions induced by ip nicotine (NIC) in mice, whereas 7-nitroindazole (7NI, 50 and 100 mg/kg ip), a selective neuronal NOS inhibitor, had a proconvulsant effect. Aminoguanidine (100 mg/kg ip), a specific inducible NOS inhibitor, remained without an effect on convulsive behavior. L-arginine, a nitric oxide (NO) precursor, which independently has no effect on convulsions, markedly reversed the anticonvulsant effect of NNA; yet only partially reversed the proconvulsant effect of 7NI when injected at 500 mg/kg ip. Convulsions evoked by intracerebroventricular injection of NIC were significantly suppressed by ip NNA (40 mg/kg ip) and enhanced by ip 7NI (100 mg/kg ip); however, these effects of NNA and 7NI were less potent than those seen when NIC was administered ip.

The present study revealed essential differences in the action of NOS inhibitors in NIC-induced convulsions. It appears that only NO produced by constitutive NOS is involved in the mechanism of NIC-induced convulsions. The proconvulsant effect of 7NI may result from the mechanisms unrelated to NOS inhibition.

Key words: nicotine, nitric oxide, seizures, N\textsuperscript{G}-nitro-L-arginine, 7-nitroindazole, aminoguanidine

Introduction

Evidence in support of the central functions of nitric oxide (NO) has been accumulated over the past 20 years [21]. Apart from playing the role as a potent smooth muscle relaxant involved in cerebral blood flow control [6, 26, 27, 50], NO has been implicated in the neuronal hyperexcitability and thus in the pathophysiology of epilepsy [11, 20, 22, 59].

It has been demonstrated that NO is released upon activation of receptors for N-methyl-D-aspartic acid (NMDA), an excitatory amino acid considered to play an important role in excitability, and exerts proconvulsant effects [12, 43, 44]. Other studies have indicated the opposite effect of NO. It has been demonstrated that endogenous NO causes a relatively persistent blockade of NMDA receptors by an interaction with the redox modulatory site of these receptors [35]. Moreover, NO can modulate NMDA receptors through...
a feedback inhibition of the receptors [41]. It explains that NO has anticonvulsant activity.

NO has the ability to evoke the release of several neurotransmitters including acetylcholine, catecholamines, neuroactive amino acids and γ-aminobutyric acid (GABA) [24, 33]. For example, an increase in NO concentration in the brain is associated with the release of GABA in the cerebral cortex, hippocampus, and striatum [38, 57]. The effect of NO in the release of GABA is biphasic, depending on the NO concentration. Basal NO levels induce a depression of GABA release but high concentrations of NO increase GABA release [23].

With the use of animal models of epilepsy, it has been shown that NO may modulate seizure activity induced by various stimuli [7, 29, 51, 58, 61, 63, 66]. Pharmacological strategies for interfering with the NO level are being developed as potential interventions for the treatment of seizure disorders. The compounds influencing NO pathway have been tested as potential adjunctive anticonvulsive agents; they have been found to affect the anticonvulsant activity of certain antiepileptic drugs [5, 9, 13, 39, 40, 62].

Despite considerable progress in understanding the role of NO in epileptiform activity, it remains difficult to make a clear distinction between NO as a pro- or anticonvulsant. The results from many animal models of seizures provided evidence that the effects of NO on seizure phenomena depend on the model of seizures used, the dose, the timing, the type of NO pathway modulators used, the examined brain structures and the age of animals [2, 31, 49, 54, 61].

The systemic or central administration of high doses of nicotine (NIC), a compound responsible for the tobacco dependence [4], induces clonic-tonic convulsions in animals [14]. The convulsant action of NIC is well documented [3, 10]; in spite of this, relatively little is known about the pharmacological mechanisms of these convulsions. It has been demonstrated that NIC-induced convulsions are centrally mediated and involve the activation of α4, α3, and α7 nicotinic acetylcholine receptor subunits [10, 30, 55]. An involvement of NMDA receptor-mediated events associated with these convulsions has been also reported [19, 56]. Fedele et al. [19] proposed a hypothesis explaining a role of NMDA receptor activation, the production of NO and cyclic GMP in the hippocampus in the convulsive action of NIC. Damaj et al. [10] have suggested that NIC enhances the release of glutamate, which in turn stimulates NMDA receptors and triggers the cascade of events leading to NO formation and seizure production.

The contribution of GABA receptors to the mechanism of NIC-induced convulsions was postulated more than 30 years ago [1]. Relatively recently, Dobelis et al. [15] have investigated a potential relationship between increased GABA neurotransmission and high doses of NIC which induce synchronization of interneurons in the hippocampus and then synchronous activity in a large population of pyramidal neurons and thus, convulsions. It seems that the development of NIC-induced convulsions might result from the increased glutamate/NMDA pathway activation and/or be mediated through the GABAergic system.

The studies reported here attempt to determine whether NO is involved in NIC-induced convulsions. For this purpose, we used three inhibitors of nitric oxide synthase (NOS) activity with different selectivity for different isoforms of the enzyme. We investigated the effects of N⁵-nitro-L-arginine (NNA), a non-selective NOS inhibitor (inhibiting both, endothelial and neuronal isoform), 7-nitroindazole (7NI), a selective inhibitor of neuronal NOS, and aminoguanidine, a specific inhibitor of inducible NOS, on clonic convulsions evoked by intraperitoneally (ip) or intracerebroventricularly (icv) administered NIC in mice.

**Materials and Methods**

**Animals and experimental conditions**

Male Swiss mice, 6 weeks old, weighing 20–26 g were used. The animals were housed in colony cages under standard laboratory conditions (ambient temperature of 20 ± 1°C, free access to chow pellets and tap water, natural light/dark cycle, relative humidity of 55 ± 3%). Standard laboratory food and water were available *ad libitum*. The experimental groups, consisting of 8 mice, were chosen by means of a randomized schedule. All experiments were performed following a 7-day period to allow for adaptation to laboratory conditions, and were carried out between 10:00 and 15:00 h. The experimental protocol was approved by the Medical University of Lublin Ethics Committee for the Use of Experimental Animals and conformed to the Guide for the Care and Use of Laboratory Animals.

**Drugs**

For *ip* administration, nicotine [NIC, (–)-nicotine hydrochloride, (–)-nicotine] and L-arginine hydrochloride (LARG)
(both from Sigma, St. Louis, MO, USA), N\textsuperscript{G}-nitro-L-arginine (NNA) and N\textsuperscript{G}-nitro-D-arginine (DNNA) (both from RBI, Natick, MA, USA) were dissolved in 0.9% NaCl. Aminoguanidine (Sigma, St. Louis, MO, USA) was suspended in a 1% aqueous solution of Tween 81 (Loba Chemie, Vienna, Austria) whereas 7-nitroindazole (7NI) was dissolved in dimethyl sulfoxide (DMSO) (both from Sigma, St. Louis, MO, USA). Fresh drug solutions or suspensions were prepared \textit{ex tempore} on each day of experimentation. All compounds were administered in a volume of 10 ml/kg at pH = 7.0. The control mice received adequate amounts of 0.9% NaCl, Tween 81 or DMSO.

For \textit{icv} injection, NIC was dissolved in sterile saline and administered in a volume of 5 μl into the lateral brain ventricle, according to the method of Lipman and Spencer [36]. The pH of the NIC solution for \textit{iv} injection was buffered and adjusted to 7.4 with 0.2 M NaOH. The control mice received adequate amounts of 0.9% NaCl.

**Nicotine-induced convulsions**

The mice were injected \textit{ip} with different doses of NIC (2–12 mg/kg). After receiving NIC, the animals were placed in Plexiglas cages and observed for the occurrence of clonic convulsions for 30 min. The number of mice convulsing out of the total number of mice tested was recorded for each treatment condition. At least 4 groups of animals, consisting of 8 mice per group, were injected with different doses of NIC to yield 10–30%, 30–50%, 50–70%, and 70–90% of mice with convulsions. A dose-response curve was constructed on the basis of the percentage of convulsing animals. The convulsant effect of NIC was evaluated as the CD\textsubscript{50} (convulsive dose50, i.e. the dose of NIC which produced convulsions in 50% of mice).

Convulsions were also induced when NIC was administered \textit{icv} at doses of 6–14 μg/mouse over 30 s. The injection syringe (Hamilton microsyringe type 75 N) with a nylon stop to attain a depth of 3.2 mm was placed perpendicular to the surface of the skull. After injecting, the needle was left in place for a further 30 s to minimize backflow of the solution. The mice were observed for the occurrence of clonic convulsions for 30 min. The CD\textsubscript{50} value for NIC was calculated. None of the vehicles used to prepare solutions of the tested compounds had any anticonvulsant/proconvulsant effect when tested against NIC.

**Effects of nitric oxide synthase inhibitors and L-arginine on nicotine-induced convulsions**

To investigate the effect of NOS inhibitors on clonic convulsions, each inhibitor was administered \textit{ip} before \textit{ip} or \textit{icv} injection of different doses of NIC. The doses of the NOS inhibitors and pretreatment times were as follows: NNA (15, 30, and 60 min prior to the test; 1–40 mg/kg), 7NI (15, 30, 60, and 90 min; 25–100 mg/kg), and aminoguanidine (15, 30, and 60 min; 100 mg/kg). DNNA, an inactive isomer of NNA, was administered at a dose of 40 mg/kg 30 min prior to injection of NIC. LARG, an NO precursor, was administered \textit{ip} at doses of 50–500 mg/kg 15 and 60 min before NIC. Pretreatment times of the NOS inhibitors and LARG were based on data provided by drug manufacturers and on our previous studies [61, 63]. They were also confirmed by pilot experiments.

After the injection of NIC, each animal was placed in a Plexiglas cage and observed for 30 min. The percentage of mice exhibiting clonic convulsions was recorded for each dose, and a dose-response curve was constructed for each compound administered in combination with NIC. The CD\textsubscript{50} of NIC for mice pretreated with the NOS inhibitors or LARG were calculated and compared with the CD\textsubscript{50} of NIC administered separately. At least 32 mice (8 mice per group) were used to calculate each CD\textsubscript{50} value.

**Statistics**

To construct dose-effect curves for each condition, NIC was tested at several doses. The CD\textsubscript{50} values (with their respective 95% confidence limits) were calculated from dose-response curves and analyzed statistically by computer-assisted log-probit analysis according to the method described by Litchfield and Wilcoxon [37]. The index of probability of less than 0.05 (p < 0.05) was considered significant in comparative analysis.

**Results**

**Nicotine-induced convulsions**

The \textit{ip} NIC administration produced jerks and Straub tail followed by clonic convulsions of all four limbs with a loss of righting reflexes. In many animals, con-
vulsions were preceded by sudden locomotor activation with violent jumps. Typically, when NIC was injected at the dose of 8.0 mg/kg convulsions occurred within 5 min. After continuous clonic convulsions, the majority of mice showed normal behavior by the end of the 30 min observation period. The CD$_{50}$ of NIC for clonic convulsions was 7.8 (6.3–9.6) mg/kg.

The icv injection of NIC also produced a loss of righting reflexes in mice followed by clonic convulsions. The occurrence of convulsions was dose-dependent and the CD$_{50}$ of NIC was 8.3 (6.5–10.7) µg/mouse.

*Effects of nitric oxide synthase inhibitors and L-arginine on convulsions caused by ip administration of nicotine*

The effects of NNA on convulsions were observed when NNA was administered 15, 30 or 60 min before injection of the convulsant. The most potent effect was seen with NNA given 30 min before NIC (Tab. 1). NNA administered at 10, 20, and 40 mg/kg significantly and dose-dependently increased the CD$_{50}$ value of NIC. Lower doses of NNA (1 and 5 mg/kg) did not significantly change the CD$_{50}$ of NIC. The CD$_{50}$ value was not markedly affected by DNNA (40 mg/kg). LARG administered (50, 250, and 500 mg/kg) 15 or 60 min prior to the test also did not have significant effect on the CD$_{50}$ of NIC (Tab. 1).

To test whether we could reverse the anticonvulsant effect of NNA, animals of the NNA group (40 mg/kg) were pretreated with LARG at dose, which itself had no effect on convulsions (500 mg/kg). The CD$_{50}$s of NIC were 11.7 (9.9–14.0) for NNA (40 mg/kg) given separately and 7.2 (5.7–9.2) mg/kg for the combination of NNA and LARG (p < 0.01 vs. NNA 40 group) (Tab. 1).

7NI (100 mg/kg) increased an incidence of NIC-induced convulsions at 15, 30, and 60 min, but not at 90 min after administration. The CD$_{50}$ of NIC at the time of peak (30 min) proconvulsant effect of 7NI was 3.7 (2.9–4.8) mg/kg, and was significantly lower than the CD$_{50}$ of NIC administered separately [6.7 (5.8–7.7) mg/kg] (p < 0.001). The significant proconvulsant effect of 7NI was also observed when 7NI was administered at 50 mg/kg but not at 25 mg/kg (Tab. 2).

The pretreatment of 7NI (100 mg/kg)-injected mice with LARG resulted in an increase in the CD$_{50}$ of NIC from 3.7 (2.9–4.8) (7NI + NIC group) to 5.1 (4.1–6.4) µg/mouse.

<table>
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<tr>
<th>Treatment (mg/kg)</th>
<th>Time before nicotine administration (min)</th>
<th>CD$_{50}$ of nicotine (mg/kg)</th>
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<tr>
<td>Saline 30</td>
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<td>7.8 (6.3–9.6)</td>
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<td>NNA 1 30</td>
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<td>8.4 (7.2–9.7)</td>
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<td>NNA 5 30</td>
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<td>8.5 (7.6–9.7)</td>
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<td>NNA 10 30</td>
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<td>10.7 (8.8–13.0)*</td>
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<td>NNA 20 30</td>
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<td>10.9 (8.7–13.1)*</td>
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<td>NNA 40 15</td>
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<td>8.5 (7.1–9.9)</td>
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<td>DNNA 40 30</td>
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<td>7.5 (6.2–9.0)</td>
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<td>L-ARG 50 60</td>
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<td>6.9 (6.0–8.0)</td>
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<td>L-ARG 250 60</td>
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<td>L-ARG 500 15</td>
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<td>NNA 40 + L-ARG 500 30</td>
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<td>7.2 (5.7–9.2)***</td>
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The data are presented as CD$_{50}$ values with 95% confidence limits in parentheses. The calculation of CD$_{50}$ values and their statistical evaluation were based upon the method of Litchfield and Wilcoxon [37] but modified in that dose-effect curves were calculated on a computer. At least 4–5 groups consisting of 8 mice each, were used to calculate each CD$_{50}$ value. * p < 0.05, ** p < 0.01 vs. saline group; *** p <0.01 vs. NNA 40 group

<table>
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<tr>
<th>Treatment (mg/kg)</th>
<th>Time before nicotine administration (min)</th>
<th>CD$_{50}$ of nicotine (mg/kg)</th>
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<tr>
<td>Vehicle 30</td>
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<td>6.7 (5.8–7.7)</td>
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<td>7-NI 25 30</td>
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<td>7-NI 50 30</td>
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<td>5.2 (4.5–6.0)*</td>
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<td>7-NI 100 15</td>
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<td>L-ARG 500 60</td>
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<td>7.4 (6.1–9.1)</td>
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<td>7-NI 100 + L-ARG 500 30</td>
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<td>5.1 (4.1–6.4)*</td>
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<td>L-ARG 60</td>
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The data are presented are CD$_{50}$ values with 95% confidence limits in parentheses. The calculation of CD$_{50}$ values and their statistical evaluation were based upon the method of Litchfield and Wilcoxon [37] but modified in that dose-effect curves were calculated on a computer. At least 4–5 groups consisting of 8 mice each were used to calculate each CD$_{50}$ value. * p < 0.05, ** p < 0.01, *** p < 0.001 vs. vehicle group.
mg/kg (LARG + 7NI + NIC group) (not significant vs. 7NI + NIC group, p < 0.05 vs. NIC alone group).

Aminoguanidine did not affect convulsions. The CD50s of NIC in aminoguanidine (100 mg/kg)-pretreated mice at 15, 30 or 60 min before injection of NIC were 6.8 (5.9–7.8), 6.8 (5.8–7.9) and 6.7 (5.8–7.7) mg/kg, respectively (not significant vs. NIC alone; data not shown).

**Effects of nitric oxide synthase inhibitors on convulsions caused by icv administration of nicotine**

The CD50s of icv administered NIC showed significant differences when mice were pretreated with different doses of NIC. As shown in Figure 1, the anticonvulsant effect of NNA was dose-dependent, and statistically significant at a dose of 40 mg/kg. The CD50 of NIC for this dose of NNA was 11.5 (9.5–13.7) µg/mouse, and was significantly higher than the CD50 of NIC given separately, which was 8.3 (6.5–10.7) µg/mouse (p < 0.05). NNA at doses 1, 10, and 20 mg/kg caused a 5, 17, and 29% increase in the CD50 values of NIC but statistical analysis revealed no significant changes between these CD50s and the control value. DNNA remained without effect on the CD50 of NIC.

The pretreatment with 7NI resulted in dose-dependent reduction of the CD50. However, the statistical significance was observed only when 7NI was administered at the highest dose (100 mg/kg) (Fig. 1). At this dose, the value of CD50 was 4.8 (3.6–6.6) µg/mouse, so it was significantly lower in comparison with the value of 8.3 (6.5–10.7) µg/mouse for the group treated with NIC alone (p < 0.01).

In the case of aminoguanidine, the dose of 100 mg/kg did not significantly affect convulsions and the CD50 of NIC was 7.3 (5.6–9.5) µg/mouse (data not shown).

**Discussion**

The present study revealed essential differences in the action of NOS inhibitors in NIC-induced convulsions. NNA, a non-selective NOS inhibitor, attenuated the development of convulsions, 7NI, a selective neuronal NOS inhibitor, exerted a proconvulsant effect, whereas aminoguanidine, a specific inducible NOS inhibitor, was without effect.

The effects of NOS inhibitors, mainly NNA and 7NI, on the development of convulsions have been investigated in many animal models of seizures. A majority of the studies have suggested that NNA is a proconvulsant. Proconvulsant properties of NNA have been documented in kainic acid- [48, 51, 53, 61], aminophylline- [64], and aminopyridine-induced convulsions [63] in rodents. In other studies, NNA suppressed icv glutamic acid-induced convulsions [61] and has been found to be an anticonvulsant. Finally, NNA did not influence bicuculline- [48, 61], pentetrazole- [48, 61], and pilocarpine-induced seizures [61] in mice and rats.

Among specific NOS inhibitors, most of the attention has been focused on 7NI. 7NI suppressed convulsions evoked by enoxacin [42], kainic acid [28, 46], pentetrazole [17], picrotoxin [65], pilocarpine [66], and sound-induced seizures [58]. Proconvulsant effect of 7NI has been documented in soman-induced convulsions in rats, where 7NI enhanced the severity of clonic convulsions and increased lethality produced by soman [34].

In the present study, NNA given at the dose of 40 mg/kg, known to decrease the level of NO in in vitro and in vivo models [16], dose-dependently suppressed clonic convulsions evoked by ip NIC. The inactive isomer DNNA did not prevent the development of convulsions. LARG, a NO precursor, at a large dose of 500 mg/kg, reversed the anticonvulsant effect of NNA.
Our findings confirm and extend a previous report of the ability of NNA to prevent NIC-induced convulsions in mice [10]. There are two arguments supporting a possible involvement of NO in NIC-induced clonic convulsions: (1) NNA but not its inactive isomer DNNA inhibits convulsions, (2) pretreatment with LARG reverses the antiseizure effect of NNA. So, one can conclude that convulsions induced by NIC are related to the increased level of endogenous NO. However, some results of our study speak against the hypothesis attributing NIC convulsions to the increased NO formation. First, LARG, a precursor of NO, administered at neither relatively small nor large dose affected the occurrence of NIC convulsions. Second, 7NI, a selective neuronal NOS inhibitor, at a dose of 25 mg/kg, known to significantly inhibit NOS activity in the cortex, diencephalon, brainstem and cerebellum [25], had little effect on convulsions. The lack of its effect was observed both in animals given NIC ip or icv. Third, in contrast to NNA, 7NI at doses of 50 and 100 mg/kg potently enhanced convulsions. The proconvulsant effect of 7NI was time-related (15 to 60 min), being the highest 30 min before the ip administration of the convulsant. The attempt to reverse the proconvulsant effect of 7-NI with LARG was only partially successful. Taken together, these results do not permit us to make clear conclusions whether NO is pro- or anticonvulsant. Moreover, we cannot rule out that mechanisms underlying the development of NIC-induced convulsions in mice may be unrelated to the NO pathway.

Although many reports have supported similar role of selective and non-selective NOS inhibitors in chemically-induced seizures [17], several investigators have described their contradictory effects upon seizure susceptibility. Evidence supporting different effects of NOS inhibitors include findings that 7NI, but not NNA, attenuated kainate-induced convulsions in mice and rats [44, 46, 48, 51, 61]. Moreover, 7NI, in contrast to another non-selective inhibitor, NNA methyl ester, had little effect on these convulsions in rats [29]. 7NI, but not NNA and its methyl ester, suppressed the development of convulsions evoked by pilocarpine [60, 61, 66]. In a previous study, we have shown the differences in the effects of NNA and 7NI using ip or icv 4-aminopyridine-induced clonic and tonic convulsions in mice: NNA exerted a proconvulsant effect and 7NI remained without effect [63].

An interpretation of data deriving from the seizure studies using NOS inhibitors is complicated by the variety of their central and peripheral effects. It is well known that NO may play an important role in controlling cerebrovascular blood flow [26, 27, 50]. Non-selective inhibition of NO synthesis considerably attenuated an increase in hippocampal blood flow during convulsions induced by kainate in rats [44, 52]. Non-selective NOS inhibitors, like NNA and its methyl ester, may modify the disposition of convulsant by inhibiting endothelial NO. Although 7NI, in contrast to non-selective NOS inhibitors, does not seem to influence blood pressure [45], several investigators have suggested that 7NI may alter local blood flow in the brain [18, 32]. Altered local blood flow could contribute to the effects of 7NI on seizures. In the present study, the effects of both NNA and 7NI on convulsions were more potent when NIC was injected ip than icv. We cannot exclude that the differences between the actions of two inhibitors, dependent on the route of NIC administration, might result from the changes in the blood flow caused by inhibitors which can modify the disposition of the convulsant.

It has been documented that the differences between actions of various NOS inhibitors during convulsions may also result from mechanisms unrelated to NO inhibition [58, 63, 65]. For example, Vanaja and Ekambaram [65] found that 7NI, at 50 and 100 mg/kg (doses that did not produce significant changes in NOS activity and NO concentration in the brain), inhibited picrotoxin-induced convulsions in rats. LARG, given at the same dose as used in our study, did not reverse the anticonvulsant effect of 7NI. The larger doses of 7NI (150 and 200 mg/kg), which inhibited both the activity of NOS and the NO concentration, potentiated picrotoxin-induced convulsions and their effect was reversed by LARG [47, 65]. It was concluded that the margin between the protective and proconvulsant doses of 7NI was relatively narrow. Further, the study by Vanaja and Ekambaram [65] showed that the doses of 7NI up to 100 mg/kg could exert the action on seizure development by a mechanism(s) unrelated to the NOS inhibition. As a possible explanation, we should take into account the sedative effect of a high dose (100 mg/kg) of 7NI that was described in another study [25] or its negative effect on spontaneous locomotor activity [40]. Furthermore, we cannot exclude the possibility of the inhibition of monoamine oxidase (MAO) B by 7NI as it was reported by Castagnoli et al. [8]. The changes of MAO activity have been shown both in epileptic humans and in some animal models of seizures. Finally, an in-
volvement of the pharmacokinetic mechanisms in the different effects of NOS inhibitors cannot be ruled out.

The findings of this study do not fully support an involvement of the glutamate/NMDA/NO pathway in the development of NIC-induced convulsions. Therefore, in our opinion, the hypothesis of a role of the GABAergic system as proposed by Dobelis et al. [15] might be an attractive explanation of the mechanism of NIC convulsions. The potential relationship between these convulsions and GABA system function is worthy of further investigation.

In summary, it appears that only NO produced by constitutive NOS is involved in the mechanism of NIC-induced convulsions. The lack of effect of aminoguanidine excludes the role of inducible NOS in these convulsions. The proconvulsant effect of 7-NI may result from mechanisms unrelated to NOS inhibition.

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