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# The effect of *N*-nitro-L-arginine methyl ester posttreatment on the anticonvulsant effect of phenobarbitone and diazepam on picrotoxin-induced convulsions in rats

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**Abstract**

The present study has investigated the effect of posttreatment of the nonspecific inhibitor of nitric oxide (NO) synthase (NOS), *N*-nitro-L-arginine methyl ester (L-NAME), on the anticonvulsant effect of phenobarbitone and diazepam on picrotoxin-induced convulsions in rats. Phenobarbitone, which is known to inhibit convulsions by potentiating γ-aminobutyric acid (GABA) activity in the brain, did not inhibit the convulsive action of picrotoxin in L-NAME-posttreated animals. L-NAME produced no such interaction with diazepam, which inhibits convulsions through GABA potentiation as well as by a GABA-independent mechanism. L-arginine, the precursor of NO, increased the protective effect of both phenobarbitone and diazepam. These results suggest that a decreased synthesis of NO in the brain by the nonspecific inhibitors of NOS is likely to result in an impairment of the anticonvulsant effect of antiepileptics that inhibit convulsions solely by potentiating GABA activity in the brain.


*Keywords:* L-NAME; Phenobarbitone; Diazepam; Picrotoxin; Endogenous NOS inhibitors

## 1. Introduction

Nitric oxide (NO), which is known to play a neurotransmitter/neuromodulator role in the brain, is synthesized from L-arginine by the enzyme NO synthase (NOS) (Bredt et al., 1991). The analogs of L-arginine, methyl arginine (Kotani et al., 1992) and agmatine (Galea et al., 1996) and protein-like substances (Jaffrey and Snyder, 1996) that are present normally in the brain have been reported to decrease NO production by inhibiting NOS activity. Intracerebroventricular injection of α-guanidinoglutaric acid (GGA), an endogenous inhibitor of NOS in the mammalian brain (Yokoi et al., 1994), produced convulsions in rats (Shiraga et al., 1986), suggesting that an inhibition of NOS and a decreased synthesis of NO in the brain may result in an induction of convulsions. The concentrations of endogenous inhibitors of NOS have been found to be enhanced in pathological conditions like chronic renal failure (Vallance et al., 1992; Kielstein et al., 1999) and essential hypertension (Surdacki et al., 1999). Although these information are available in the literature, it has never been investigated whether the inhibitors of NO synthesis can alter the anticonvulsant effect of antiepileptics on picrotoxin-induced convulsions. In view of this, the present study has been aimed to investigate the effect of posttreatment of *N*-nitro-L-arginine methyl ester (L-NAME), a synthetic analog of L-arginine having NOS-inhibiting property (Rees et al., 1990), on the anticonvulsant effect of phenobarbitone and diazepam on picrotoxin-induced convulsions in rats. In order to investigate further evidence for the involvement of NO in this interaction, the anticonvulsant effects of phenobarbitone and diazepam were tested in animals posttreated with an NO increasing dose of NO precursor, L-arginine. The data were compared with the protective effects of phenobarbitone and diazepam in saline-posttreated animals.

## 2. Methods

Colony-bred adult male Wistar rats weighing 130–150 g were used. Since estrous cycle is known to alter the response of female rats to the convulsant action of picrotoxin (Paul and Krishnamoorthy, 1988), male animals were used for the

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study. Test ($n = 10$) and control ($n = 10$) groups were chosen randomly. Fresh animals were used for each experiment. Animals were housed in groups (3 or 4 in a cage) at room temperature (22–25 °C) to which they were acclimatized and were supplied with a balanced diet and tap water ad libitum. Guidelines for Breeding of and Experiments on Animals defined in 1998 by the Ministry of Social Justice and Empowerment, Government of India, were followed.

Solutions of picrotoxin, L-NAME (Sigma, St. Louis, MO, USA), L-arginine (SRL Chemicals, Mumbai, India) and commercially available phenobarbitone sodium (Samarth Pharma, Mumbai, India) and diazepam (5 mg/ml in benzyl alcohol, Ranbaxy, Mumbai, India) were made in saline and injected intraperitoneally 0.2 ml/100 g body weight.

Picrotoxin was administered at a dose (5 mg/kg) previously demonstrated to produce clonic convulsions and not tonus and death of animals (Paul and Krishnamoorthy, 1988). A dose of L-NAME (50 mg/kg) that inhibited NOS activity and decreased NO concentration significantly in the brain of rats in previous studies (Iadecola et al., 1994; Rajasekaran and Paul, 1999) and a smaller dose (25 mg/kg) that failed to decrease NO synthesis in our previous study (unpublished data) were chosen for the present study. L-Arginine was found in a previous study in this laboratory (Rajasekaran and Paul, 1999) to raise the concentration of NO in the brain with 1000, not 500, mg/kg dose. The effective dose (1000 mg/kg) of L-arginine was chosen for the present study. The doses of phenobarbitone (20 mg/kg) and diazepam (0.25 mg/kg) used in the present study were demonstrated to produce anticonvulsant action on picrotoxin-induced convulsions in rats (Albertson et al., 1981).

Fifteen minutes after the administration of phenobarbitone, diazepam or saline, the animals were treated with L-NAME or saline. Thirty minutes after L-NAME or saline treatment, these animals were challenged with picrotoxin. In another study, phenobarbitone, diazepam or saline pretreated (15 min) animals were injected with L-arginine or saline. Thirty minutes after L-arginine or saline treatment, the animals were challenged with picrotoxin.

The time between the injection of picrotoxin and the appearance of the first twitching movement of head or limbs was determined for the onset of convulsive action of picrotoxin. About 3 min later, clonic convulsions involving whole body appeared. The frequency of clonic convulsive movements was measured using a convulsion monitoring apparatus (Paul and Kazi, 1994). The floor of the apparatus was mounted with capacitance sensors, which picked up the vibrations caused by the clonic convulsive movements of the animal and converted them into electric signals. The signals activated the counter. Soon after injection of picrotoxin, the animal was placed in the chamber and the instrument was switched on when clonic convulsions appeared. Since convulsions appeared intermittently with normal motor activity in between the episodes, the instrument was switched off when the animal was not convulsing and switched on soon after the convulsions reappeared. The frequency of convulsive movements was recorded until the animal exhibited clonic convulsions (55–60 min after the time of its onset).

The tests were carried out between 11:00 and 13:00 h under the same temperature condition as the housing. The data of drug-treated animals were compared with the respective saline-treated control groups using one-way or two-way ANOVA followed by Tukey's multiple comparison test.

## 3. Results

Twitching movements appeared in the head and limbs 11.2 ± 1.2 min after the administration of picrotoxin in

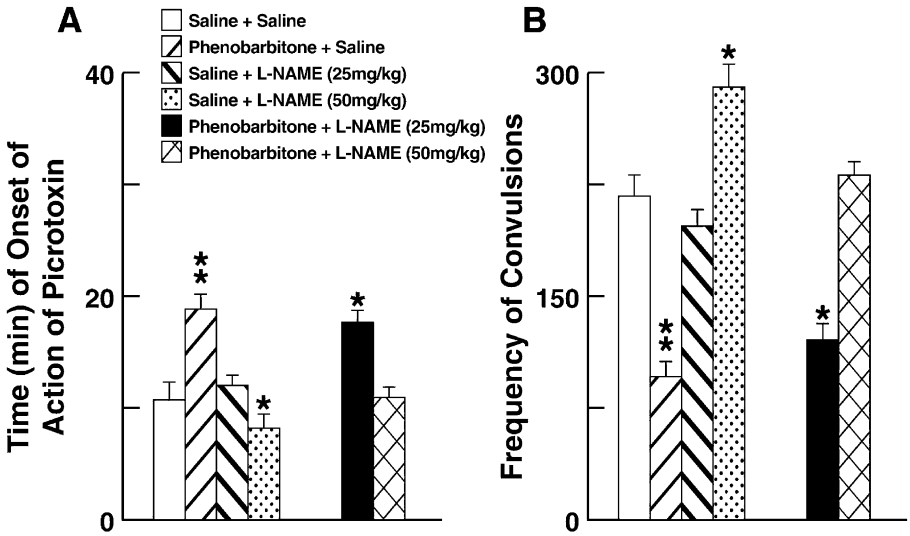


Fig. 1. Effect of phenobarbitone (20 mg/kg) or saline on the time of onset of the convulsive action of picrotoxin (5 mg/kg) (A) and the frequency of convulsive movements (B) in animals posttreated (15 min) with L-NAME (25 and 50 mg/kg) or saline. Each bar represents mean ± S.E.M. of 10 animals. * $P < .05$, ** $P < .01$, as compared to saline + saline-treated control group (two-way ANOVA and Tukey's multiple comparison test).

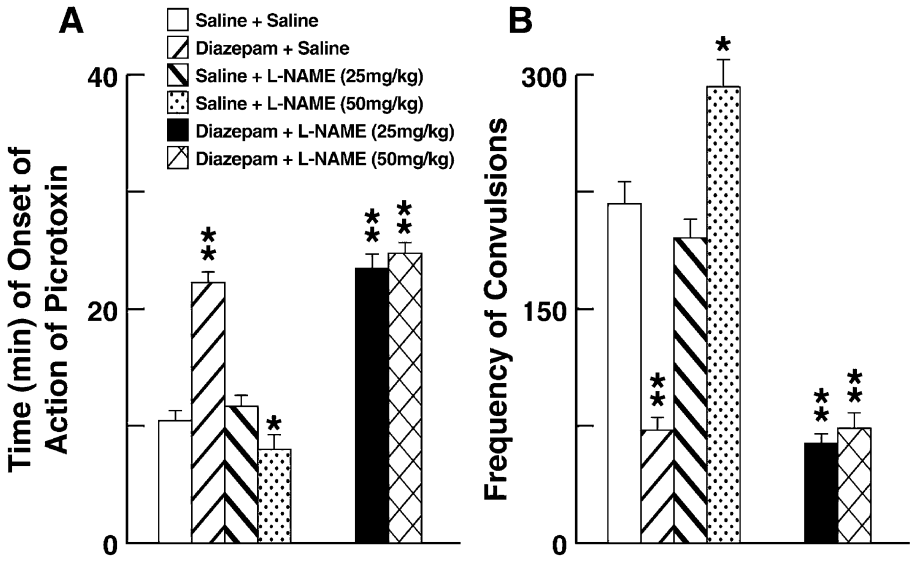


Fig. 2. Effect of diazepam (0.25 mg/kg) or saline on the time of onset of the convulsive action of picrotoxin (5 mg/kg) (A) and the frequency of convulsive movements (B) in animals posttreated (15 min) with L-NAME (25 and 50 mg/kg) or saline. Each bar represents mean ± S.E.M. of 10 animals. * $P < .05$, ** $P < .01$, as compared to saline + saline-treated control group (two-way ANOVA and Tukey's multiple comparison test).

saline + saline-treated control animals (Fig. 1A). No mortality occurred and all animals in this group regained normal activity 50–60 min after the onset of convulsive action of picrotoxin.

The time of onset of the convulsive action of picrotoxin and the convulsion frequency were not altered by the smaller dose of L-NAME (25 mg/kg) in saline-pretreated animals. The larger dose of L-NAME (50 mg/kg) shortened the time of onset of action of picrotoxin (Fig. 1A) and increased the frequency of convulsions in saline-pretreated animals (Fig. 1B).

Fifteen minutes of pretreatment of phenobarbitone (Fig. 1A and B) and diazepam (Fig. 2A and B) delayed the onset of the convulsive action of picrotoxin and decreased the frequency of convulsions in saline-posttreated animals. Posttreatment of the larger (50 mg/kg) and not the smaller dose (25 mg/kg) of L-NAME prevented phenobarbitone to delay the onset of the convulsive action of picrotoxin (Fig. 1A) and to decrease the frequency of convulsions (Fig. 1B). Posttreatment of either dose of L-NAME (25 and 50 mg/kg) did not alter the protective effect of diazepam on picrotoxin-induced convulsions (Fig. 2A and B).

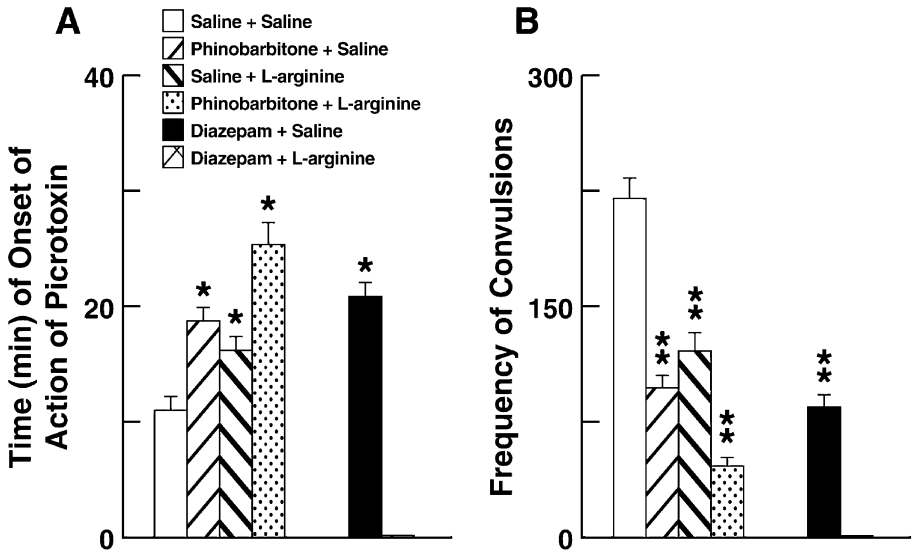


Fig. 3. Effect of phenobarbitone (20 mg/kg), diazepam (0.25 mg/kg) or saline on the time of onset of the convulsive action of picrotoxin (5 mg/kg) (A) and the frequency of convulsive movements (B) in animals posttreated (15 min) with L-arginine (1000 mg/kg) or saline. Each bar represents mean ± S.E.M. of 10 animals. * $P < .05$, ** $P < .01$, as compared to saline + saline-treated control group. $^{+}P < .05$, $^{++}P < .01$, as compared to phenobarbitone + saline group (two-way ANOVA and Tukey's multiple comparison test).

Phenobarbitone delayed the time of onset of convulsions and decreased the frequency of convulsions in animals posttreated with a dose of L-arginine that inhibited the convulsive action of picrotoxin. The effects were significantly greater than that produced by phenobarbitone in saline-posttreated animals (Fig. 3A and B).

The convulsive action of picrotoxin was completely inhibited by diazepam in L-arginine-posttreated animals. No convulsive responses were observed in these animals (Fig. 3A and B).

## 4. Discussion

In the present study, a dose of L-NAME (50 mg/kg) that decreased NO formation (Iadecola et al., 1994; Rajasekaran and Paul, 1999) and not a smaller dose (25 mg/kg) that did not inhibit NO synthesis in the brain (unpublished data of the authors) increased the frequency of picrotoxin-induced convulsions. In support of this observation, NOS-inhibiting doses of L-NAME and L-nitro-L-arginine (NNA), another nonspecific inhibitor of NOS (Rees et al., 1990), increased kainic acid (Penix et al., 1994) and cortical stimulation (Rundfeldt et al., 1995) induced convulsions in rats. Systemic administration of GGA, an endogenous NOS inhibitor (Yokoi et al., 1994), increased cobalt-induced convulsions in cats (Mori et al., 1980). These results suggest that the inhibitors of NOS have proconvulsant action. A decreased formation of NO accounted for the proconvulsant action of these agents, because L-arginine, the precursor of NO (Bredt et al., 1991), reverted NNA-induced facilitation of kainic acid-induced convulsions in rodents (Penix et al., 1994). These results and the previous report of Buisson et al. (1993) strongly support the hypothesis that NO has an anticonvulsant action in the brain.

NO seems to inhibit experimentally induced convulsions by activating γ-aminobutyric acid (GABA) mechanism in the brain because NO has been found to increase the concentration of GABA (Paul and Jayakumar, 2000), the inhibitory neurotransmitter with anticonvulsant property (Silvilotti and Nistri, 1991). An inhibition of GABA transaminase (GABA-T) was suggested for the GABA increasing action of NO because, in this study, the NO-increasing and NO-decreasing actions of L-arginine and L-NAME coincided with an inhibition and activation of brain GABA-T activity, respectively. Studies demonstrating an interaction between NO and GABA showed that a rise in the concentration of NO resulted in an increased release of GABA in the cerebral cortex (Ohkuma et al., 1995; Kuriyama and Ohkuma, 1995), striatum (Segovia and Mora, 1998) and hippocampus (Lonart et al., 1992). Further, an activation and inhibition of GABA-A receptor activity increased and decreased the activity of NOS and the concentration of NO in the brain, respectively (Paul et al., 2001).

Thus, because a functional interaction occurs between NO and GABA in the brain, the posttreatment of an NO-increasing dose of L-arginine, the precursor of NO, has increased the anticonvulsant action of diazepam in previous studies (Paul and Jayakumar, 1999; Jayakumar et al., 1999), and in the present study, the effects of both phenobarbitone and diazepam are known to inhibit convulsions by potentiating GABA activity in the brain (Haefely, 1980) against picrotoxin-induced convulsions. Under this circumstance, the anticonvulsant effect of these drugs can be decreased if GABA activity is impaired following a reduction in the production of NO in the brain by the inhibitor of NOS, L-NAME. In support of this suggestion, L-NAME pretreatment decreased the anticonvulsant action of phenobarbitone on electroshock-induced convulsions in mice (Borowicz et al., 1998).

Since a decreased formation of NO by L-NAME in the vascular endothelium is known to produce vasoconstriction (Macrae et al., 1993), a prevention of penetration of phenobarbitone into the brain can account for its inability to inhibit convulsions in these animals. In order to clarify whether simply a pharmacokinetic mechanism or a neuronal action resulting from an inhibition of NO synthesis is responsible for this interaction, in the present study, L-NAME was administered 15 min after the animals received an anticonvulsant dose of phenobarbitone or diazepam. Interestingly, the anticonvulsant effect of phenobarbitone and not that of that of diazepam was completely abolished by L-NAME posttreatment. This result indicates that diazepam inhibits convulsions by a GABA-independent mechanism also. In support of this suggestion, Hoogerkamp et al. (1996), who tested the action of diazepam on seizures induced by cortical stimulation, have emphasized that an interaction of the GABA–benzodiazepine receptor complex cannot be fully accountable for the anticonvulsant action of diazepam.

L-NAME prevented the anticonvulsant action of not only phenobarbitone but also of sodium valproate (Borowicz et al., 1998), a GABA potentiating anticonvulsant agent (Macdonald and Bergey, 1979). Conversely, L-NAME failed to inhibit the effects of diazepam as well as that of carbamazepine and diphenylhydantoin (Borowicz et al., 1998), which inhibit convulsions by blocking sodium channels (Catterall, 1987). These results strongly suggest that the anticonvulsant action mediated by GABA potentiation and not other mechanisms is impaired if NO synthesis is inhibited in the brain by the inhibitors of NOS.

Interestingly, 7-nitroindazole (7-NI), a neuronal specific inhibitor of NOS (Babbedge et al., 1993), did not impair the anticonvulsant effect of phenobarbitone in rats (Borowicz et al., 1997) and in mice (DeSarro et al., 2000). In another study, 7-NI potentiated the anticonvulsant effect of flurazepam on electrically induced hind limb extension in mice (Deutsch et al., 1995). In the present study, L-NAME impaired the anticonvulsant effect of phenobarbitone and not that of diazepam on picrotoxin-induced convulsions. Thus, the neuronal specific and nonspecific inhibitors of NOS seem to interact distinguishably with the anticonvuls-

ant effect of phenobarbitone and benzodiazepine compounds against electrically and chemically induced convulsions. If these results suggest that only nonspecific inhibitors of NOS have the property of inhibiting the anticonvulsant effect of GABA potentiating drugs, then the endogenous inhibitors of NOS are likely to interfere with the effectiveness of phenobarbitone, since agmatine (Galea et al., 1996), dimethylarginine (Kotani et al., 1992), GGA (Yokoi et al., 1994) and a protein-like substance (Moore et al., 1990) have been found to be nonspecific in their NOS-inhibiting action in the brain. L-NAME, the nonspecific inhibitor of NOS, decreased the anticonvulsant effect of phenobarbitone against picrotoxin and electroshock-induced convulsions in the present and in a previous study (Borowicz et al., 1998), respectively. Taken together, these results lead to a conclusion that the anticonvulsant effect of GABA potentiating antiepileptic agents may be decreased if NO synthesis is impaired in the brain by the nonspecific inhibitors of NOS.

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