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Biochemical Pharmacology 63 (2002) 2019–2023

Biochemical Pharmacology

Short communication

# Evidence for the involvement of L-citrulline but not nitric oxide in the proconvulsant action of the precursor L-arginine on picrotoxin-induced convulsions in rats

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Received 15 February 2001; accepted 2 October 2001

**Abstract**

To determine the role of the metabolites of L-arginine in its actions on picrotoxin-induced convulsions in rats, the concentrations of nitric oxide (NO) and L-citrulline were measured in the brain 30 and 60 min after the administration of L-arginine (1000 and 2000 mg/kg) or of *N*-nitro-L-arginine methyl ester (L-NAME, 30 mg/kg), an inhibitor of NO synthase. Animals treated similarly were challenged 30 and 60 min later with picrotoxin (5 mg/kg), and the time of onset of myoclonus and clonic convulsions and the frequency of convulsions were determined. These parameters were also determined 30 and 60 min after administering L-arginine in L-NAME-pretreated (30 min) animals. Thirty minutes after the administration of L-arginine, the concentrations of both NO and L-citrulline were raised, the onset of myoclonus and clonic convulsions was delayed, and the frequency of convulsions was decreased, indicating the anticonvulsant property of L-arginine. A 60-min treatment of L-arginine produced a further increase in the concentration of L-citrulline but not that of NO and promoted the frequency of picrotoxin-induced convulsions. Pretreatment with L-NAME prevented L-arginine from raising the concentrations of both NO and L-citrulline; it also promoted the anticonvulsant actions and prevented the proconvulsant actions of L-arginine. These results lead to the conclusion that NO has no involvement in the time-dependent anti and proconvulsant actions of L-arginine on the picrotoxin convulsion model, and that L-citrulline seems to have a role in the proconvulsant action of L-arginine.


*Keywords:* NO; L-Citrulline; L-Arginine; L-NAME; Anticonvulsant; Proconvulsant; Picrotoxin

## 1. Introduction

NO, a gaseous chemical messenger proposed to have a neurotransmitter/neuromodulator role in the brain [1], is formed enzymatically with L-citrulline as a co-product by NOS from L-arginine with calcium, calmodulin, and NADPH as cofactors [2]. Systemically administered, L-arginine has been reported to modulate experimentally induced convulsions. Investigators who observed an inhibition of convulsions induced by kainate [3], pentylenetetrazole [4], and sound [5] in L-arginine-pretreated animals have suggested that NO mediates the anticonvulsant action of L-arginine. Others have proposed a proconvulsant action for NO because in their studies L-arginine potentiated *N*-methyl-D-aspartate-induced convulsions in rodents [6,7]. Although these findings are available in the literature, the factors responsible for the production of the anticonvulsant and proconvulsant actions of L-arginine have not been assessed. Furthermore, biochemical evidence for these actions of L-arginine has also not been determined. With this aim in mind, we tested the dose- and time-dependent effects of L-arginine against picrotoxin-induced convulsions in rats. In addition, the concentrations of NO and L-cirtulline were measured in the brains of animals treated with L-arginine doses that modulated the convulsant action of picrotoxin. To establish further evidence for the involvement of its metabolic products, the effects of L-arginine were tested in animals pretreated with L-NAME, an inhibitor of NOS [8].

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*Abbreviations:* NO, nitric oxide; NOS, nitric oxide synthase; L-NAME, *N*-nitro-L-arginine methyl ester

0006-2952/02/$ – see front matter © 2002 Published by Elsevier Science Inc.
PII: S0006-2952(02)00891-2

## 2. Materials and methods

### 2.1. Animals

Colony bred, adult male Wistar rats weighing 150–180 g were used. Male animals were chosen because in a previous study in this laboratory females exhibited a greater response than male rats to picrotoxin-induced convulsions and died [9]. Control ($N = 10$) and experimental ($N = 10$) animals were chosen randomly. The animals were housed in groups (3 or 4 in a cage) at room temperature (22–26°) on a 12/12 hr light/dark cycle and were fed a balanced diet (Gold mohur) and tap water *ad lib*. Guidelines for the Breeding of and Experiments on Animals defined in 1998 by the Ministry of Social Justice & Empowerment, Government of India, were followed.

### 2.2. Chemicals

L-Arginine (S.D. Fine Chemicals), L-NAME, and picrotoxin (Sigma Chemical Co.) were dissolved in normal saline and injected intraperitoneally at 0.2 mL/100 g body weight. Freshly prepared solutions were used every time. Control animals received an equivalent volume of the vehicle at the appropriate time.

L-Arginine at 1000 mg/kg, but not at smaller doses, raised the concentration of NO in the brain significantly in a previous study in this laboratory [10]. Therefore, 1000 mg/kg and a larger dose (2000 mg/kg) were chosen for the present study. In our previous studies, L-NAME at 30 and 50 mg/kg doses inhibited the activity of NOS [11] and decreased brain NO concentration significantly [10]. The smaller dose was used in the present study to decrease NO formation in the brain. Picrotoxin was injected at a dose (5 mg/kg) that previously produced clonic convulsions but not tonus and mortality in rats [9].

### 2.3. Determination of convulsion responses

Groups of animals were treated with L-arginine, L-NAME, or saline. Another group was treated with L-NAME 30 min prior to L-arginine or saline. All of the animals were challenged 30 or 60 min later with picrotoxin. The time of onset of myoclonus (time between the injection of picrotoxin and the appearance of the first myoclonic movement of the body) and clonic convulsions (time between the injection of picrotoxin and the appearance of severe and repeated clonic convulsive movements of the whole body) were determined in these animals. Since convulsions resulted in movement of the whole body, the frequency of these movements was measured using a previously described convulsion monitor [12]. The capacitance sensors mounted in the floor of the chamber picked up the vibrations caused by the clonic convulsion movements of the animal and converted them into electric signals that activated the counter. Each animal was placed in the chamber after injection of picrotoxin, and the instrument was switched on immediately after clonic convulsions appeared. The consulsive movements were recorded as long as (50–60 min) clonic convulsions persisted after picrotoxin challenge.

### 2.4. Determination of NO and L-citrulline

The concentrations of NO and L-citrulline were measured in the brain 30 and 60 min after L-arginine treatment; 30 and 60 min after treatment with L-NAME; and 30 and 60 min after L-arginine treatment in L-NAME-pretreated (30 min) animals.

Animals were decapitated, and brains were removed and processed immediately for biochemical determinations. NO was determined using a hemoglobin-trapping method described previously [13]. The method is based on the quantitative reaction of NO and not other free radicals with oxyhemoglobin to form methemoglobin. The conversion of oxyhemoglobin to methemoglobin was measured at 401 nm in a spectrophotometer.

The formation of L-citrulline from L-arginine was determined in crude homogenates, in the presence of NADPH, calmodulin, tetrahydrobiopterin, and calcium, as described previously [2]. Different groups were used for NO and L-citrulline determinations.

The percent difference from the respective control was determined in order to distinguish clearly the dose- and time-related changes produced by L-arginine and L-NAME on the concentrations of NO and L-citrulline in the brain.

The convulsion study and killing of the animals for biochemical determinations were carried out between 11:00 a.m. and 1:00 p.m. at ambient housing temperature. Biochemical determinations were done in a cold room (4°).

### 2.5. Statistical analysis

The convulsion and biochemical data were analyzed using three-way ANOVA and Tukey's multiple comparison test.

## 3. Results

### 3.1. Thirty-minute effects of L-arginine

Thirty minutes after administration, L-arginine increased the concentrations of both NO (Fig. 1A) and L-citrulline (Fig. 1B) in a dose-dependent manner. A dose-dependent delay in the time of onset of myoclonus (Fig. 2A) and clonic convulsions (Fig. 2B) and a reduction in clonic convulsion frequency (Fig. 2C) were observed in these animals.

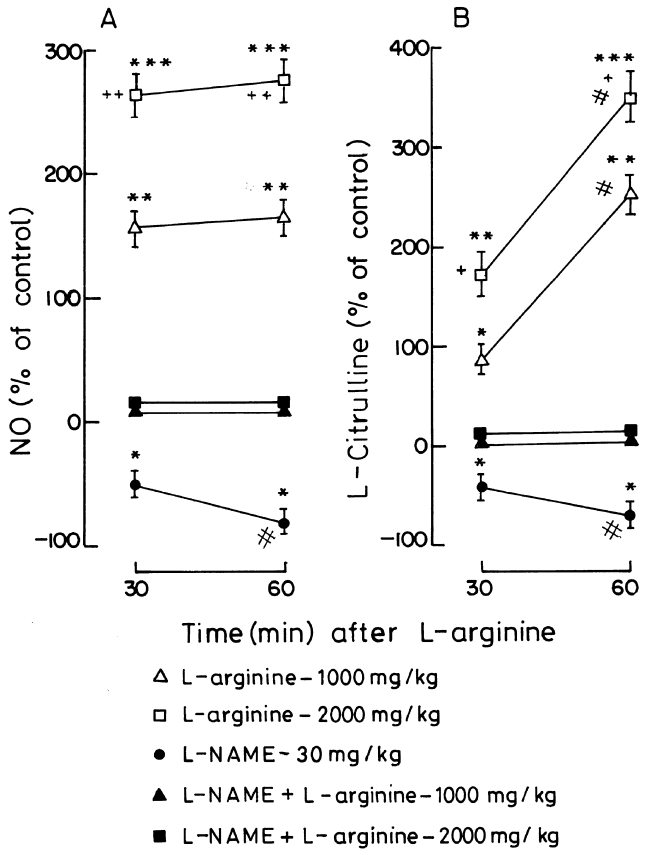


Fig. 1. Independent and combined action of L-arginine (1000 and 2000 mg/kg) and L-NAME (30 mg/kg) on NO (A) and L-citrulline (B) concentrations in the brain. Each point represents the mean ± SEM of 10 animals. Control values: NO, 25.5 ± 4.0 μmol/g tissue; L-citrulline, 0.6 ± 0.05 nmol/min/mg protein. Key: (*) $P < 0.05$, (**) $P < 0.01$, and (***) $P < 0.001$ as compared with the saline-pretreated control; (+) $P < 0.05$, and (++) $P < 0.01$ as compared with 1000 mg/kg of L-arginine; (#) $P < 0.05$ as compared with the 30-min effect (three-way ANOVA and Tukey's multiple comparison test).

### 3.2. Sixty-minute effects of L-arginine

Sixty-minute treatment with L-arginine produced a further significant increase in the concentration of L-citrulline (Fig. 1B) but not that of NO (Fig. 1A). The time of onset of myoclonus (Fig. 2A) and clonic convulsions (Fig. 2B) induced by picrotoxin was not altered in these animals. However, clonic convulsion frequency in response to L-arginine treatment was increased significantly as compared with the control data (Fig. 2C).

### 3.3. Effects of L-NAME

L-NAME decreased the concentrations of both NO (Fig. 1A) and L-citrulline (Fig. 1B) in a time-dependent manner. In addition, L-NAME pretreatment delayed the time of onset of myoclonus (Fig. 2A) and clonic convulsions (Fig. 2B) and reduced the frequency of clonic convulsions (Fig. 2C) in a time-dependent manner.

### 3.4. Thirty-minute effects of L-arginine in L-NAME-pretreated animals

Neither dose of L-arginine, 30 min after administration, produced changes in the concentrations of either NO (Fig. 1A) or L-citrulline (Fig. 1B) in L-NAME-pretreated animals. The effect of 1000 mg/kg of L-arginine on picrotoxin-induced convulsions was enhanced by L-NAME pretreatment. As a result, the protection observed in these animals was significantly greater in comparison to that produced by the same dose of L-arginine independently. L-arginine at a 2000 mg/kg dose produced total protection in L-NAME-pretreated animals. Myoclonus and convulsions were not observed in these animals (data not shown).

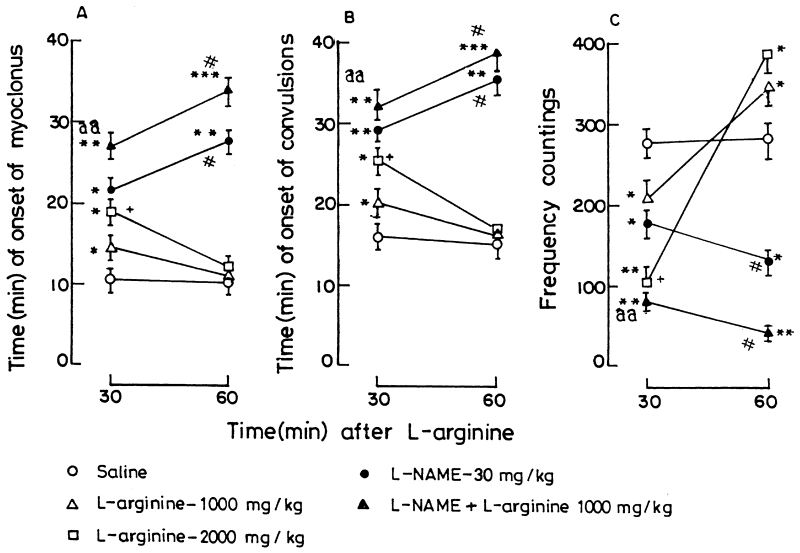


Fig. 2. Independent and combined actions of L-arginine (1000 and 2000 mg/kg) and L-NAME (30 mg/kg) on the time of onset of myoclonus (A), clonic convulsions (B), and convulsion frequency (C) induced by picrotoxin (5 mg/kg) in rats. Each point represents the mean ± SEM of 10 animals. Key: (*) $P < 0.05$, (**) $P < 0.01$, and (***) $P < 0.001$ as compared with the saline-pretreated control; (+) $P < 0.05$ as compared with 1000 mg/kg of L-arginine; (aa) $P < 0.01$ as compared with the independent effect of L-arginine; and (#) $P < 0.05$ as compared with the 30-min effect (three-way ANOVA and Tukey's multiple comparison test).

### 3.5. Sixty-minute effects of L-arginine in L-NAME-pretreated animals

L-Arginine did not alter the concentration of either NO (Fig. 1A) or L-citrulline (Fig. 1B) in these animals. The effects of 1000 mg/kg of L-arginine on the time of onset of myoclonus (Fig. 2A), clonic convulsion (Fig. 2B), and convulsion frequency (Fig. 2C) were much greater than its 30-min effect in L-NAME-pretreated animals. The larger dose of L-arginine (2000 mg/kg) produced total protection in L-NAME-pretreated animals. Neither myoclonus nor convulsions were observed in these animals (data not shown).

## 4. Discussion

In the present study, L-arginine raised the concentration of NO in the brain in a dose-dependent manner with no significant difference between the 30- and 60-min data. On the other hand, the concentration of its co-product, L-citrulline, was increased in a dose- as well as a time-dependent manner in these animals. Therefore, the saturation of NOS activity cannot account for the failure of L-arginine to produce a linear increase in NO concentration 30–60 min after its administration. A degradation of NO to nitrite, nitrate [14], and peroxynitrite [15] and the scavenging of it [15] in the brain tissue may have prevented further elevation of NO 60 min after administration of its precursor, L-arginine. However, 30- and 60-min pretreatment of L-arginine produced distinct actions on picrotoxin-induced convulsions.

A delay in the time of onset of both myoclonus and clonic convulsions and a reduction in the frequency of clonic convulsion movements were observed in 30-min pretreated animals, indicating an anticonvulsant effect of L-arginine on picrotoxin-induced convulsions. On the other hand, a marked increase in clonic convulsion frequency was observed in 60-min pretreated animals These results provide evidence that depending upon the time of its pretreatment, L-arginine produces both anticonvulsant and proconvulsant effects on the picrotoxin convulsion model.

In the present study, a time-dependent anticonvulsant effect was observed in animals also pretreated with a NO decreasing dose of L-NAME. Thus, both a NO precursor and a NO synthesis inhibitor have been found to be protective against picrotoxin-induced convulsions. Further, an indistinguishable increase found in NO concentration 30 and 60 min after L-arginine treatment was accompanied by anticonvulsant and proconvulsant effects. Together, these results suggest that NO has no convulsion-inhibiting properties. In consonance with this suggestion, previous investigators who found biochemical evidence for the anticonvulsant action of L-arginine reported that NO has no anticonvulsant action on experimentally induced convulsions [16].

The data showing no difference between the 30- and 60-min effects of either dose of L-arginine on NO concentration also indicate that NO cannot have a proconvulsant property. The present suggestion is supported by a previous report [17] that NO has neither neuroprotective nor neurotoxic properties in the brain.

L-NAME has been found to inhibit pentylenetetrazole- and strychnine-induced convulsions [18]. Further study in this field has shown that L-NAME produces anti or proconvulsant action on the same convulsion model depending upon the dose and time of its administration [19,20]. *N*-Nitro-L-arginine, an inhibitor of NOS, increased the convulsion threshold at 10 mg/kg and induced a proconvulsant effect at 40 mg/kg in a cortical stimulation model in rats [21]. However, the dose and time of L-NAME pretreatment employed in the present study were protective against the picrotoxin convulsion model.

In the present study, an increase in the concentrations of brain NO and L-citrulline in L-arginine-treated animals is a clear indication that the availability of L-arginine increases in the brain following its administration systemically. L-NAME is known to increase the concentration of L-arginine in the brain as a result of its NOS-inhibiting property [22]. These results and the data showing a dose-dependent inhibition of sound-induced convulsions by intracerebroventricularly injected L-arginine, but not an equimolar dose of D-arginine in GEP rats and DBA/2 mice, suggest that L-arginine is responsible for the independent anticonvulsant effect of systemically administered L-arginine and L-NAME. Thus, an enhancement of the concentrations of exogenous and endogenous L-arginine may account for the greater and the total protection produced by 1000 and 2000 mg/kg of L-arginine in L-NAME-pretreated animals, respectively.

An increase in the clonic convulsion frequency induced by picrotoxin in animals pretreated with L-arginine indicates that a metabolite that accumulated 60 min after its administration may have a convulsion-inducing action. In the present study, the concentration of L-citrulline was raised markedly 60 min after L-arginine treatment in comparison with that observed in 30-min treated animals, indicating that L-citrulline formed from a systemically administered precursor may have been degraded at a slow rate in the brain. This has been attributed to the failure of L-citrulline to convert to urea due to the absence of a complete urea cycle in the brain [23]. An elevation of the concentration of L-citrulline in the brain following its intracerebroventricular injection (250–831 μg) resulted in a dose-dependent induction of convulsions in DBA/2 mice [5]. In the present study, a more than 200% increase in the concentration of L-citrulline (but not below this level) was accompanied by a promotion of convulsion frequency induced by picrotoxin. Together, these results suggest that L-citrulline has the potential to induce convulsions or to promote picrotoxin-induced convulsions, if it occurs in a very high concentration in the brain following

its intracerebroventricular injection at doses greater than 250 μg or systemic administration of its precursor, L-arginine. The convulsion-inducing property of L-citrulline has also been observed clinically. Infants with citrullinemia were reported to develop convulsions, and multiple spikes were seen in their EEGs at all times of crisis [24].

The results of the present study, showing a reversal of L-citrulline concentration to control level and a prevention of the proconvulsant action of L-arginine in animals pretreated with L-NAME, provide further support to the suggestion that L-citrulline is involved in the proconvulsant action of L-arginine.

In conclusion, the results of the present study provide evidence that NO has neither anticonvulsant nor proconvulsant properties. However, the co-product, L-citrulline, acts as a proconvulsant with picrotoxin if its concentration is raised markedly in the brain following systemic administration of its precursor, L-arginine.

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