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Distinct Effects of Nω-Nitro-L-Arginine on Seizures Induced by Several Drugs in Mice

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HARA, S., F. KURIIWA, N. IWATA, T. MUKAI, S. KANO AND T. ENDO. *Distinct effects of Nω-nitro-L-arginine on seizures induced by several drugs in mice.* PHARMACOL BIOCHEM BEHAV 53(3) 673–677, 1996.—A potent nitric oxide (NO) synthase inhibitor, Nω-nitro-L-arginine (L-NA), suppressed tonic seizure elicited by pentylenetetrazol (PTZ; 100 mg/kg, SC) in a dose-related manner (25 to 100 mg/kg, IP), but had no effect on clonic seizure. The effect was most potent at 1 h after the administration of L-NA. L-NA (100 mg/kg, IP) suppressed clonic seizure as well as tonic seizure in bicuculline-treated (3.0 or 4.5 mg/kg, SC) mice. However, it did not affect seizures elicited by picrotoxin (2.0 to 6.0 mg/kg, SC). On the other hand, *N*-methyl-DL-aspartate (NMDLA; 300 mg/kg or 350 mg/kg, IP) induced clonic seizure, but tonic seizure was not always noted. All mice with clonic and tonic seizures died, and some mice with clonic seizure died without accompanying tonic seizure. L-NA did not influence NMDLA-induced seizures, but it appeared to enhance NMDLA lethality, though without statistical significance. These findings suggest distinct roles of NO in seizures induced by different drugs in mice.

Nitric oxide Nω-Nitro-L-arginine Pentylenetetrazol Picrotoxin Bicuculline *N*-Methyl-DL-aspartate Mouse

NITRIC OXIDE (NO) is a free radical gas, synthesized from L-arginine (L-Arg) by NO synthase (NOS) in various tissues including the brain (2). It is believed to be a novel neuronal messenger or a neurotransmitter in the peripheral and central nervous systems (2,23). It has been well established that one of the pathways for NO signaling begins with activation of guanylate cyclase, resulting in an increase of an intracellular second messenger, cGMP (14,23,26). Various convulsants including pentylenetetrazol (PTZ), picrotoxin (PIX), and excitatory amino acids (EAAs) such as *N*-methyl-D-aspartate (NMDA), kainate (KA), and quisqualate (QA) increase the cGMP level in the cerebellum, and drugs that have an anticonvulsant potency decrease it (28). Thus, it is likely that the NO-cGMP pathway plays an important role in the central excitatory actions of convulsants. However, there are conflicting reports on the role of NO in the convulsive behavior. An endogenous NO precursor, L-Arg, possesses a proconvulsant effect (18), and potentiates seizures elicited by NMDA (5,18). In addition, NOS inhibitors, Nω-nitro-L-arginine methyl ester (L-NAME) and Nω-monomethyl-L-arginine (L-NMMA), prevent seizures elicited by KA (4,20), NMDA (4), and tacrine, an acetylcholinesterase inhibitor (in LiCl-treated rats) (1). However, L-NAME and L-NMMA potentiate NMDA-induced wild running (3) and pilocarpine-induced seizures (24). Another NOS inhibitor, Nω-nitro-L-arginine (L-NA), also aggravates KA-induced seizures (19,21). On the other hand, a putative brain-selective NOS inhibitor, 7-nitroindazole (7-NI), had no effect on KA-induced seizures (19).

EEA receptors participate in convulsive behavior, because their antagonists, especially NMDA receptor antagonists, can suppress seizures elicited by $GABA_A$ receptor-ionophore complex antagonists such as PTZ, PIX, and (+)-bicuculline (BIC), electroshock, barbital withdrawal, kindling, and so on, except for strychnine-induced seizures (6). On the other hand, stimulation of NMDA receptors leads to NO generation and the generated NO modifies the functions of the NMDA receptors (23). Therefore, most studies on the roles of NO in convulsive behavior have been done in respect to the association with EAA receptors, as described above. Penix et al. (19) recently reported that L-NA (0.5 to 15 mg/kg, IP) enhanced seizure activity and increased mortality in KA-treated mice and it also increased mortality in mice treated with picrotoxin,

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but not PTZ or BIC, although it had no effect on the seizures induced by these compounds.

In the present study, we examined the role of NO in seizures induced by PTZ, PIX, and BIC, by pretreating mice with L-NA at higher doses (up to 100 mg/kg), and compared the results with those in mice treated with *N*-methyl-DL-aspartate (MNDLA). We used L-NA as the NOS inhibitor, because it penetrates into the brain after peripheral administration (7) and is more potent than L-NAME and L-NMMA (26). Furthermore, this inhibitor does not generate the NO precursor, L-Arg, though other inhibitors such as L-NMMA do (11).

METHOD

Animals

Male ICR mice, weighing 22–30 g, were purchased from Charles River Japan (Kanagawa, Japan). Animals were housed in an air-conditioned (22–24°C) room with a 12 L : 12 D cycle. They were given food and water ad lib. The experimental protocol had been reviewed by the Tokyo Medical College Animal Care Committee and is in accordance with the Japanese Animal Research Association standards as defined in the Guideline for Animal Experiments.

Chemicals

L-NA, PTZ, PIX, BIC, and NMDLA were purchased from Sigma Chemical Co. (St. Louis, MO). All chemicals except BIC were dissolved in sterile physiological saline immediately before use. BIC was dissolved in 1 N HCl, and the solution was diluted with the above saline, then adjusted to pH 4 to 5 with 1 N NaOH. PTZ (100 or 125 mg/kg), PIX (2.0 to 6.0 mg/kg), and BIC (3.0 or 4.5 mg/kg) were administered subcutaneously. L-NA (25 to 100 mg/kg) and NMDLA (300 or 350 mg/kg) were administered intraperitoneally. All drugs were administered in a volume of 0.1 ml/10 g body weight. Mice without L-NA were given the same volume of the vehicle. L-NA was administered 60 min before the convulsants, unless otherwise stated.

Observation of Animals

Animals were observed for 60 min immediately after the administration of convulsants in a quiet air-conditioned (22–24°C) room. The incidence and the time to onset of clonic (forelimb clonus) and tonic (hindlimb extension) seizures were recorded. In NMDLA-treated mice, clonic seizure was not exactly the same as that elicited by PTZ, PIX, and BIC. We recorded forelimb clonus-like behavior (often with partial or full rolling of the body) as clonic seizure. Mice that underwent tonic seizure were killed by excess ether inhalation immediately after tonic hindlimb extension, except for NMDLA-treated mice. We had found in a preliminary result that NMDLA (350 mg/kg, IP) elicited clonic seizure in seven out of eight mice and tonic seizure in six out of eight mice, but that mice treated with NMDLA in combination with L-NA underwent clonic seizure, and then died without tonic seizure. Therefore, we determined the time to death to examine whether L-NA had any effect on tonic seizure or NMDLA toxicity. All experiments were carried out between 1000 and 1600 h.

Statistics

The incidence of each seizure was expressed as the number of animals convulsed per the total animals in individual treated groups. The time to onset of each seizure component was expressed as the mean ± SE obtained from animals that had the episode. To analyze the effects of L-NA on the incidence of each seizure and the latency to the seizure, or on mortality and the time to death, together, we used the Mann–Whitney *U*-test, in which we assigned the last order to animals with no seizure or death. Furthermore, to verify the effect of L-NA on tonic seizure, the interval between clonic and tonic seizures was calculated, when both seizures appeared in all animals, according to the report of Weiss et al. (27). The interval was expressed as the mean ± SE and was statistically analyzed by using the Mann–Whitney *U*-test.

RESULTS

As shown in Table 1, L-NA decreased the incidence of PTZ (100 mg/kg)-induced tonic seizure in a dose-dependent manner. Significant suppression of PTZ-induced tonic seizure by L-NA was obtained at doses of 50 and 100 mg/kg. There was no significant effect of L-NA at any dose used on PTZ-induced clonic seizure, although the highest dose slightly prolonged the mean time to onset of clonic seizure. The suppres-

TABLE 1

EFFECT OF L-NA ON PTZ-INDUCED CLONIC AND TONIC SEIZURES IN MICE

PTZ (mg/kg, SC)	L-NA (mg/kg, IP)	Time to PTZ After L-NA (min)	Time to Onset of Seizures (min)		Interval (min)
			Clonic Seizure	Tonic Seizure	
100	0	60	3.7 ± 0.5 (8/8)	15.8 ± 2.8 (6/8)	
	25	60	3.1 ± 0.4 (7/8)	11.4 ± 3.2 (4/8)	
	50	60	3.1 ± 0.3 (6/8)	22.5 (1/8)*	
	100	60	6.8 ± 2.8 (7/8)	– (0/8)*	
100	0	30	4.0 ± 0.4 (7/8)	10.5 ± 1.5 (5/8)	
	100	30	4.0 ± 0.7 (7/8)	13.7 ± 5.3 (3/8)	
100	0	120	3.4 ± 0.6 (8/8)	16.8 ± 2.6 (7/8)	
	100	120	6.7 ± 2.6 (8/8)	5.2 (2/8)	
125	0	60	2.6 ± 0.3 (8/8)	6.6 ± 1.1 (8/8)	4.0 ± 1.2
	100	60	3.8 ± 0.9 (8/8)	12.2 ± 2.3 (8/8)	8.4 ± 2.2*

Data are expressed as means ± SE. Figures in parentheses represent the number of mice with seizures per the total number of mice. An asterisk indicates a significant ($p < 0.05$) difference from the corresponding group without L-NA.

TABLE 2

EFFECT OF L-NA ON SEIZURES ELICITED BY BIC IN MICE

BIC (mg/kg, SC)	L-NA (mg/kg, IP)	Time to Onset of Seizures (min)		Interval (min)
		Clonic Seizure	Tonic Seizure	
3.0	0	3.7 ± 0.4 (8/8)	7.4 ± 0.7 (8/8)	
	100	10.1 ± 3.8 (6/8)*	16.5 ± 5.7 (4/8)*	
4.5	0	3.5 ± 0.5 (8/8)	5.7 ± 1.0 (8/8)	2.2 ± 0.6
	25	3.1 ± 0.4 (8/8)	6.2 ± 0.6 (8/8)	2.9 ± 0.6
	50	3.9 ± 0.5 (8/8)	8.5 ± 1.7 (8/8)	4.6 ± 1.3
	100	4.7 ± 0.5 (8/8)*	11.1 ± 1.2 (8/8)*	6.3 ± 0.9*

L-NA was administered 1 h before BIC. Data are expressed as means ± SE. Figures in parentheses represent the number of mice with seizures per the total number of mice. An asterisk indicates a significant difference ($p < 0.05$) from the corresponding group without L-NA.

sive effect of L-NA was most potent when it was administered 60 min before PTZ (Table 1). So, the following experiments were performed under this condition. The time to onset of clonic seizure in the group treated with L-NA (100 mg/kg) plus PTZ (125 mg/kg) was comparable with that in the control. L-NA prolonged the time to onset of tonic seizure, but the difference from the control was not statistically significant ($p = 0.052$). However, L-NA significantly prolonged the interval between clonic and tonic seizures (Table 1).

L-NA at the highest dose significantly suppressed both clonic and tonic seizures in mice treated with BIC (3.0 or 4.5 mg/kg) (Table 2). The interval between onset of the two components of the seizures at 4.5 mg/kg BIC was prolonged in parallel with increasing dose of L-NA (Table 2).

As shown in Table 3, there was no significant effect of L-NA on PIX-induced seizures, although L-NA at 100 mg/kg prolonged the mean values of clonic and/or tonic seizures elicited by 4.0 and 6.0 mg/kg of PIX and the interval between the seizures at the latter dose.

L-NA at the doses tested did not cause seizures or death by itself. All mice without tonic seizure survived until the end of the observation period after treatment with PTZ, BIC, or PIX and in combination with L-NA.

In NMDLA-treated mice, we found unusual phenomena. Following clonic seizure, which appeared in most mice after NMDLA, some mice underwent tonic seizure, and thereafter died. However, mice with clonic seizure did not always develop tonic seizure. In addition, forelimb and hindlimb extension, though much weaker than typical extension, sometimes occurred before death in some mice with clonic seizure. We did not regard this extension as tonic seizure. As shown in Table 4, we did not find any significant or dose-related effect of L-NA on NMDLA-induced clonic and tonic seizures. On the other hand, increasing doses of L-NA increased mortality in mice treated with 300 mg/kg NMDLA (Table 4). The mortality of mice treated with L-NA plus 350 mg/kg NMDLA was higher than that without L-NA (Table 4). However, the increased lethality obtained by combining L-NA with NMDLA was not statistically significant.

DISCUSSION

Because NO dilates blood vessels including cerebral arteries (8,25), it seems likely that the inhibition of NO production restricts the transfer of centrally acting drugs from the blood to the brain tissues, resulting in reduction of the drugs' effects. Penix et al. (19) reported that L-NA enhanced KA seizures, but a putative brain-selective NOS inhibitor, 7-NI, did not, suggesting that L-NA might modify the drug disposition by inhibiting endothelial NOS. If this is the case, the effect of

TABLE 3

EFFECT OF L-NA ON SEIZURES ELICITED BY PIX IN MICE

PIX (mg/kg, SC)	L-NA (mg/kg, IP)	Time to Onset of Seizures (min)		Interval (min)
		Clonic Seizure	Tonic Seizure	
2.0	0	17.0 ± 1.5 (3/4)	– (0/4)	
	100	14.7 ± 1.8 (3/4)	– (0/4)	
4.0	0	11.4 ± 0.7 (6/6)	27.7 ± 4.7 (4/6)	
	100	16.6 ± 5.1 (6/6)	34.9 ± 2.3 (5/6)	
6.0	0	8.9 ± 0.4 (8/8)	21.2 ± 1.5 (8/8)	12.5 ± 1.2
	25	8.9 ± 0.6 (8/8)	22.1 ± 2.0 (8/8)	13.2 ± 1.5
	50	10.3 ± 0.8 (8/8)	21.6 ± 1.2 (8/8)	11.4 ± 1.2
	100	9.7 ± 0.6 (8/8)	25.8 ± 3.4 (8/8)	16.1 ± 3.1

L-NA was administered intraperitoneally 1 h before PIX. Data are expressed as means ± SE. Figures in parentheses represent the number of mice with seizures per the total number of mice.

TABLE 4

EFFECT OF L-NA ON NMDLA-INDUCED SEIZURES AND MORTALITY IN MICE

NMDLA (mg/kg, IP)	L-NA (mg/kg, IP)	Time to Onset of Seizures (min)		Time to Death (min)
		Clonic Seizure	Tonic Seizure	
300	0	13.6 ± 2.6 (7/8)	4.4 (1/8)	5.4 (2/8)
	25	17.5 ± 3.9 (7/8)	28.7 (1/8)	21.6 ± 6.7 (4/8)
	50	11.5 ± 1.6 (7/8)	– (0/8)	25.7 ± 4.6 (7/8)
	100	13.7 ± 3.6 (8/8)	18.8 ± 6.5 (4/8)	21.0 ± 6.0 (7/8)
350	0	12.7 ± 2.1 (8/8)	14.6 (2/8)	11.4 ± 2.4 (5/8)
	25	8.5 ± 1.4 (8/8)	8.5 ± 2.8 (3/8)	18.6 ± 4.8 (8/8)
	50	12.6 ± 1.9 (7/8)	30.2 (1/8)	27.8 ± 2.6 (7/8)
	100	7.8 ± 1.0 (8/8)	9.5 ± 0.8 (3/8)	13.5 ± 1.5 (8/8)

L-NA was administered 1 h before NMDLA. Data are expressed as means ± SE. Figures in parentheses represent the number of mice with seizures per the total number of mice.

L-NA on seizures elicited by convulsants should be similar. However, we observed diverse effects of L-NA on seizures elicited by PTZ, BIC, PIX, and NMDLA; e.g., a) L-NA suppressed PTZ-induced tonic seizure, but not clonic seizure, while it suppressed BIC-induced clonic and tonic seizures; b) L-NA had no effect on seizures elicited by PIX and NMDLA. Yonekawa et al. (30) demonstrated that PTZ-induced clonic seizure depended on the brain PTZ level in mice, whereas tonic seizure was induced in a manner independent of the brain PTZ levels. In addition, L-NA prolonged the interval between clonic and tonic seizures by PTZ or BIC, suggesting a central effect of L-NA on their tonic component (17). Thus, the suppressive effect of L-NA on PTZ and BIC seizures may be due to NOS inhibition in the brain rather than reduced or delayed disposition of the convulsants to the brain. However, the lack of 7-NI effect on KA seizures cannot be explained.

Gale (9) proposed a classification of seizures into three components based on qualitatively distinct and independent anatomical circuits; e.g., a) facial and forelimb clonus (often with rearing and falling); b) explosive, running/bouncing clonus; and c) tonic convulsion. She suggested that the first component and the latter two components might be associated with forebrain and hindbrain neuronal circuits, respectively, and that the GABAergic system in the substantia nigra (SN) might play a role as a key station to control progression or generation of the above three seizure components. In the SN, EEA receptors may play an opposite role (9). It has been elucidated that PTZ, BIC, and PIX exert their central excitatory effects by reducing the inhibitory activity via $GABA_A$ receptor channels (15). A subtype of EEA receptors, NMDA receptors, participates in seizures elicited by PTZ, BIC and PIX, because NMDA receptor antagonists suppress these seizures (6). On the other hand, it has been demonstrated that activation of NMDA receptors enhances NO formation, which activates guanylate cyclase, resulting in an increase of one of the intracellular second messengers, cGMP, in the cerebellum and hippocampus (14,23,26,28,29). This augmentation of the cGMP level was antagonized by NOS inhibitors (26,28, 29). Various convulsants, including PTZ and PIX, increase the cerebellar levels of cGMP as well (28,29). The increase in cGMP by PTZ is reversed by NOS inhibitors as well as NMDA receptor antagonists, including noncompetitive NMDA and competitive glycine receptor antagonists (28,29). This suggests that the NO-cGMP pathway mediated through NMDA receptors may be involved in the central actions of PTZ. Therefore, it is likely that NO production in SN and/or neuronal circuits plays a progressive role in seizures. However, this may not always be the case, based on the present finding that NOS inhibition leads to suppression of PTZ and BIC seizures, but had no remarkable effect on PIX and NMDLA seizures. Furthermore, the NO pathway may not always be associated with the central GABAergic system. The different effects of L-NA on these antagonists of $GABA_A$ receptor channels also suggest different mechanisms for induction of seizures, beyond the reduction of the GABAergic activity. This may be at least in part supported by the previous reports that drugs modified PTZ and PIX seizures in different manners (10,22). In addition, distinct potentiation by a neurosteroid, pregnenolone sulfate, of NMDA and PTZ seizures has been reported (16). Thus, further studies remain necessary to clarify the mechanisms of seizures. The finding that the antagonists partially block PTZ and BIC seizures and completely block PIX and NMDA seizures (6) might provide a clue to the mechanisms involved.

On the other hand, L-NA somewhat increased the mortality when combined with NMDLA, although the increment did not reach statistical significance. Penix et al. (19) reported that L-NA potentiated the lethal effect of KA in rats and mice, although 7-NI did not. They also reported that L-NA increased mortality in mice treated with pilocarpine and PIX, but not PTZ and BIC, without affecting the seizure activities (19). As we killed mice treated with PTZ, BIC, and PIX if they showed tonic seizure, the effect of L-NA on the lethality of these agents is unknown. However, our finding was that all mice, except for those with tonic seizure, survived until the end of the observation period after the administration of PTZ, BIC, or PIX alone and in combination with L-NA. Thus, it is unlikely that L-NA potentiates the lethality of PTZ, BIC, and PIX. Lei et al. (12) demonstrated a protective effect of donors of NO such as sodium nitroprusside and nitroglycerine on NMDA-mediated cell death in cultured cortical neurons. Therefore, NO formation might attenuate the EEA receptor-mediated toxicity. However, a potentiating role or a negligible role of NO in EEA-mediated neurotoxicity has been also suggested (23). Lipton et al. (13) suggested that NO generated by NMDA receptor stimulation could lead to neurotoxicity, at least in part, by reacting with superoxide anion and that reaction of NO with thiol group(s) of the redox modulatory sites

of NMDA receptors might account for its neuroprotective potency. These results suggest complex participation of NO in the central nervous system.

In conclusion, inhibition of NO formation led to suppression of PTZ-induced tonic, but not clonic seizure, and of BIC-induced clonic and tonic seizures, in mice. In contrast, inhibition of NOS had no effect on either seizure elicited by PIX and NMDLA. No remarkable enhancement of seizures was seen as a consequence of inhibiting NOS in our experimental models. However, the lethal effect of NMDLA was increased by NOS inhibition. These results suggest complex roles of NO in the central nervous system, that is, NO might play a proconvulsant role in some cases, no role in some cases, and a protective role in EEA-mediated neurotoxicity in some cases.

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