Role of Nitric Oxide in the Convulsions Following the Coadministration of Enoxacin With Fenbufen in Mice

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Received December 15, 1997 Accepted February 4, 1998

ABSTRACT—Effects of nitric oxide (NO) synthase inhibitors on the enoxacin-induced convulsions were examined in mice pretreated with fenbufen. 7-Nitroindazole markedly suppressed the incidence of convulsions, whereas L-arginine did not modify the convulsions at all. The suppression of the convulsions by 7-nitroindazole was not reversed by the pretreatment of L-arginine. Brain NO synthase activity was significantly raised at 30 min after enoxacin when combined with fenbufen. The increased NO synthase activity was found to be suppressed by the pretreatment of 7-nitroindazole. These findings suggest that endogenous NO may be involved as a proconvulsant substance in the development of enoxacin-induced convulsions in mice pretreated with fenbufen.

Keywords: Convulsion, Enoxacin, Nitric oxide synthase

Fluoroquinolones have a broad spectrum of antibacterial activity, but they are known to exhibit undesirable adverse reactions and interactions with other drugs following their administration. The fluoroquinolones are routinely coadministered with acidic nonsteroidal anti-inflammatory drugs for the therapy of infectious diseases with inflammation. Recently, convulsive seizure has been reported to occur after the coadministration of some fluoroquinolones and an acidic nonsteroidal antiinflammatory drug, fenbufen, in both humans (1) and experimental animals (2); and the combination of enoxacin and fenbufen had a high potency for producing the convulsions (2). It is now generally accepted that the inhibitory effect of the fluoroquinolones on GABA binding and the potentiation of fenbufen on the inhibitory effect may be associated with the expression of the convulsive seizure (3). Recently, we have reported that there was a circadian variation in the susceptibility to enoxacin-induced convulsions in mice pretreated with fenbufen (4).

Nitric oxide (NO) has been regarded as a neuronal messenger in the central nervous system and a modulator of several brain functions (5). There have been some reports on the role of NO in the convulsive phenomena in different experimental models, while the reported results are far from being unequivocal (6–11). However, there has been no report dealing with whether the L-arginine-NO pathway has some role in the convulsive expression

by coadministration of the fluoroquinolones with fenbufen.

To elucidate a possible role of the L-arginine-NO pathway in the development of convulsions by enoxacin when combined with fenbufen, we investigated whether the convulsions produced in mice coadministered with enoxacin and fenbufen were modified by various inhibitors of NO synthase and whether NO synthase activity in the brain was changed during the development of convulsions.

Enoxacin, fenbufen and L-arginine hydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 7-Nitroindazole and *N*^G-nitro-L-arginine methylester (L-NAME) were from Dojindo Laboratories (Kumamoto). [³H]L-Arginine (36.8 Ci/mmol, 1 Ci=37 GBq) was obtained from DuPont/NEN (Wilmington, DE, USA). Other chemicals used were of analytical grade. Male ddY mice (age 6- to 7-week-old) were obtained from Japan SLC, Inc. (Hamamatu). Mice were housed in a room controlled at 24°C under a 12-hr dark-light cycle (light 0700 to 1900 hr) for a week before initiating the experiments and allowed free access to food and water. Enoxacin, fenbufen and 7-nitroindazole were suspended in 1.0% sodium carboxymethylcellulose (CMC), and other compounds were dissolved in saline. Enoxacin (40–100 mg/kg) was administered orally 5 min after fenbufen (100 mg/kg, p.o.), the animals were put

singly into plastic cages (15×10×13 cm) and observed for 180 min for the occurrence of clonic and tonic convulsions. Animal survival was recorded within 24 hr after enoxacin. NO synthase inhibitors and L-arginine were injected intraperitoneally 30 min and 60 min before enoxacin, respectively. The administration of these drugs were done throughout 1400 and 1500 hr.

NO synthase activity was measured by determining the conversion of [³H]L-arginine to [³H]L-citrulline by the method of Bredt and Snyder (12). The brain without cerebellum was removed from mice following decapitation and homogenized in 10 vol. of 20 mM TrisHCl buffer (pH 7.4) containing 0.25 M sucrose, 0.1 mM dithiothreitol, 2.5 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, and 0.01% bacitracin. The homogenate was centrifuged at 20,000×g for 20 min, and the resulting supernatant was applied to columns of Dowex AG50WX-8 (Na⁺ form) for removal of endogenous L-arginine, and the eluent was used for determination of the NO synthase activity. The reaction mixture routinely contained enzyme preparation (20–30 mg protein), 10 nM [³H]L-arginine, 50 mM Hepes (pH 7.4), 1 mM NADPH, 1 mM EDTA, 1.25 mM CaCl₂ and 1 mM dithiothreitol in a total

vol. of 1 ml. After incubation for 20 min at 37°C, the reaction was stopped by adding 1 ml 20 mM Hepes buffer (pH 5.5) containing 2 mM EDTA, and 200 ml of reaction mixture was applied to 1 ml columns of Dowex AG50WX-8 (Na⁺ form), followed by elution with 2 ml of water. [³H]L-Citrulline was determined by liquid scintillation spectroscopy.

Data are shown as the mean±S.E.M. Comparisons of the mean values were made by analysis of variance followed by Ryan's multiple range test. Incidence of clonic and tonic convulsions and percent mortality in the different groups were compared with Fisher's exact probability test.

When combined with fenbufen (100 mg/kg, p.o., 5 min prior to enoxacin), enoxacin (40–100 mg/kg, p.o.) produced clonic convulsions about 30 min after administration, which thereafter proceeded to tonic convulsions, leading to death. Meanwhile, surviving mice showed no tonic convulsions, although they exhibited the decrease in locomotor activity. Both the incidence of clonic and tonic convulsions and mortality were increased in a dose-dependent manner by enoxacin, with both the CD₅₀ (50% convulsive dose) and LD₅₀ (50% lethal dose) of enoxacin being 56.5 (47.4–64.3) mg/kg in fenbufen-pretreated

Table 1. Effect of NO synthase inhibitors on the convulsions induced by the combination of enoxacin with fenbufen in mice

Treatment (mg/kg, i.p.)		Convulsions (Positive/Total)						Mortality (Dead/Total)	
		Clonic			Tonic				
Exp. 1									
Control		54.4±	8.3 ^a	9/10	59.4±	8.4 ^a	9/10	70.0±10.1 ^b	9/10
7-Nitroindazole	100	65.5±	8.6	4/10*	74.0±	9.0	4/10*	84.3±11.8	4/10*
L-NAME	100	55.4±	7.0	9/10	56.6±	7.7	8/10	80.8± 8.6	8/10
Aminoguanidine	100	58.4±	11.9	8/10	72.0±	13.1	7/10	78.0±12.4	8/10
Exp. 2									
Control		63.6±	6.8	9/12	50.9±	6.7	8/12	48.5± 7.0	8/12
L-NAME	100	66.8±	8.2	10/12	54.6±	7.1	9/12	55.3± 5.5	9/12
	300	73.3±	11.0	10/13	69.8±	9.3	10/13	69.3± 7.9	10/13
Exp. 3									
Control		51.3±	9.4	10/10	59.9±	12.0	9/10	65.6±10.8	10/10
7-Nitroindazole	25	49.5±	12.7	8/10	58.8±	13.4	8/10	62.8±13.9	8/10
	50	79.3±	17.4	8/10	77.7±	15.3	7/10	94.5±17.3	8/10
	100	66.8±	8.0	4/10**	73.5±	8.5	2/10**	76.8± 9.0	4/10**
Exp. 4									
Control		47.8±	6.7	10/10	54.5±	6.8	10/10	58.4± 6.9	10/10
7-Nitroindazole	100	87.6±	18.3**	5/10*	96.4±	20.0**	5/10*	99.8±20.2**	5/10*
L-Arginine	300	40.9±	9.5	9/10	50.6±	10.0	9/10	57.4± 9.5	9/10
7-Nitroindazole + L-Arginine		64.3±	9.5	7/10	72.0±	12.9	6/10*	81.7±12.0*	7/10

Enoxacin (80 mg/kg) was administered orally 5 min after fenbufen (100 mg/kg, p.o.). NO synthase inhibitors and L-arginine were injected intraperitoneally 30 min and 60 min before enoxacin, respectively. ^a: latency up to onset (min), ^b: survival time (min). *P<0.05, **P<0.01: compared with the control group.

Table 2. Effect of 7-nitroindazole and L-arginine on the convulsions induced by the combination of low dose enoxacin with fenbufen in mice

Treatment (mg/kg, i.p.)	Convulsions (Positive/Total)		Mortality (Dead/Total)
	Clonic	Tonic	
Control	69.5 ^a 2/10	55.0 ^a 1/10	59.0 ^b 1/10
7-Nitroindazole 50	55.2 1/10	61.4 1/10	63.1 1/10
100	98.5 2/10	— 0/10	— 0/10
L-Arginine 150	51.7 1/10	55.8 1/10	57.0 1/10
300	34.0 2/10	28.0 1/10	29.0 1/10

Enoxacin (40 mg/kg) was administered orally 5 min after fenbufen (100 mg/kg, p.o). 7-Nitroindazole and L-arginine were injected intraperitoneally at 30 min and 60 min before enoxacin, respectively. ^a: onset time (min), ^b: survival time (min).

Table 3. Brain NO synthase activity in mice coadministered with enoxacin and fenbufen, and the effect of 7-nitroindazole

Treatment	NO synthase (nmol citrulline/mg protein/20 min)
Control	2.59 ± 0.16 (5)
Enoxacin, 80 mg/kg + Fenbufen, 100 mg/kg	4.12 ± 0.28** (5)
Enoxacin + Fenbufen + 7-Nitroindazole, 100 mg/kg	3.65 ± 0.08**,# (5)

Enoxacin (80 mg/kg) was administered orally 5 min after fenbufen (100 mg/kg, p.o.). 7-Nitroindazole (100 mg/kg) was injected intraperitoneally 30 min before enoxacin. Mice were sacrificed at 30 min after enoxacin. Number of mice is shown in parentheses. **P < 0.01: compared with the control group, #P < 0.05: compared with the enoxacin + fenbufen group.

mice (4).

Possible involvement of the L-arginine-NO pathway in the development of convulsions was investigated in mice coadministered with enoxacin and fenbufen. To clarify whether either NO synthase inhibitors or L-arginine may exhibit a suppressive effect against the convulsions, experiments were carried out with the higher dose (80 mg/kg) of enoxacin, which produced submaximal convulsions in mice. As shown in Table 1, a selective neuronal NO synthase inhibitor, 7-nitroindazole (100 mg/kg, i.p., 30 min prior to enoxacin) markedly suppressed both the incidence of convulsions and convulsive death. However, L-NAME (a nonselective NO synthase inhibitor) and aminoguanidine (an inducible NO synthase inhibitor) did not exhibit any suppressive effect. L-Arginine (300 mg/kg, i.p., 60 min prior to enoxacin) did not modify the convulsions at all. The suppression of the convulsions with 7-nitroindazole was not reversed by the pretreatment of L-arginine. The latency up to onset of the convulsions and to death tended to be prolonged by 7-nitroindazole. In contrast, when using a subconvulsive dose of enoxacin (40 mg/kg), neither the incidence of

convulsions nor mortality was affected by the pretreatment of 7-nitroindazole or L-arginine (Table 2). These findings suggest that endogenous NO in the brain may be involved in the potentiation of the convulsions, but not in their suppression. Since experimental conditions (species, doses, injection route, timing of dosing, etc.) and brain concentration of endogenous L-arginine may significantly influence the effect of injected L-arginine, the lack of effects of L-arginine on the convulsions and the decreased convulsions by 7-nitroindazole does not exclude an involvement of the L-arginine-NO pathway in the enoxacin-induced convulsions in mice pretreated with fenbufen. Furthermore, although unlike 7-nitroindazole, the reason behind the lack of effect of L-NAME is unclear, it may be due to differences in the characteristics of both compounds such as inhibitory potency against brain NO synthase and the presence of hypertensive activity, as well as to the experimental conditions described above.

Next, we investigated whether NO synthase activity in the brain was increased in response to coadministration of enoxacin with fenbufen. Brain NO synthase activity was measured at 30 min after enoxacin (80 mg/kg) in mice pretreated with fenbufen. The administration of enoxacin or fenbufen alone did not influence the brain NO synthase activity, but 7-nitroindazole markedly suppressed it to about 60% of the control. The brain NO synthase activity in mice coadministered with enoxacin and fenbufen was markedly increased as compared to normal control mice (Table 3). The pretreatment of 7-nitroindazole (100 mg/kg, i.p., 30 min prior to enoxacin) was found to suppress the increased NO synthase activity. In the experiments, since typical clonic convulsions had not appeared within 30 min after enoxacin, the time when NO synthase activity was measured was thought to be a pre-convulsive period. Thus, it is likely that the increase in NO synthase activity in the brain may lead to the development of convulsions.

The present results indicate that 7-nitroindazole at

tenuated the convulsions induced by enoxacin, when combined with fenbufen in mice, and that the development of convulsions in response to enoxacin was accompanied by a significant increase of brain NO synthase activity and the increased activity was suppressed by the pretreatment of 7-nitroindazole. Since the increased NO formation via NO synthase is thought to occur in the pre-convulsive period, NO may play a role as a proconvulsant substance in enoxacin-induced convulsions in mice pretreated with fenbufen. These findings are in agreement with recent reports (6–9) indicating that NO has proconvulsant activity. According to Mulsch et al. (6), brain NO increased during kainate-induced convulsions, and the NO formation and kainate-induced convulsions were attenuated by 7-nitroindazole pretreatment. Furthermore, various NO synthase inhibitors such as 7-nitroindazole, L-NAME and *N*^G-monomethyl-L-arginine have been reported to attenuate pilocarpine (7)-, pentylentetrazole (8)- and quinolinic acid (9)-induced convulsions.

Contrary to these reports, there was some information that NO was an anticonvulsant (10, 11). In the present study, L-arginine did not suppress the incidence of convulsions induced by a submaximal dose of enoxacin (80 mg/kg), and 7-nitroindazole also could not enhance the incidence of convulsions induced by a subconvulsive dose (40 mg/kg) of enoxacin. Therefore, NO does not function as an anticonvulsive substance in the enoxacin-induced convulsions.

It is possible that the excessive enhancement of excitatory and/or the reduction of inhibitory neurotransmissions may produce convulsions. Halliwell et al. (13) have reported that the proconvulsant effects of ciprofloxacin when combined with biphenyl acetic acid (an active metabolite of fenbufen) may be related to antagonism of GABA_A receptors but not to the interaction at ionotropic glutamate receptors in cultured hippocampal neurons using the whole cell patch clamp technique.

The activation of NMDA receptor is thought to be an especially effective mechanism for triggering the increase of brain NO synthase activity. However, under the present conditions, MK801 (0.03, 0.1 mg/kg, i.p., 30 min prior to enoxacin, NMDA-receptor antagonist) did not exhibit any effect on the enoxacin-induced convulsions (data not shown). Thus, it is hard to conceive that the increase of NO synthase activity via the NMDA receptor may occur in the development of the convulsions by enoxacin when combined with fenbufen. Since the synthesis and release of NO occurs in response to a variety of neurotransmitters, possible mechanisms other than NMDA-receptor activation may probably contribute to the activation of brain NO synthase.

According to Proctor et al. (14), NO and L-arginine

potentiated the seizures evoked by focal intracerebral application of bicuculline (GABA_A-receptor antagonist), but L-NAME did not attenuate the seizures, suggesting that the L-arginine-NO pathway did not normally contribute to the seizure expression. Although the interrelationship between the L-arginine-NO pathway and GABA_A-receptor activity is unknown in detail, it seems likely that endogenous NO may interact with GABA_A-receptor activity in mice coadministered with enoxacin and fenbufen. Thereafter, further investigations are necessary to explore the mechanisms of the interrelationship between the L-arginine-NO pathway and GABA_A-receptor functions underlying the development of convulsions.

In conclusion, 7-nitroindazole, a selective neuronal NO synthase inhibitor, attenuated enoxacin-induced convulsions in mice pretreated with fenbufen. The increased NO synthase activity in the brain was found to precede the appearance of typical clonic convulsions, and this increase was suppressed by the pretreatment of 7-nitroindazole. These findings suggest that endogenous NO in the brain may play a role as a proconvulsant substance in the convulsions.

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