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Short communication

Effect of N^G-nitro-L-arginine on the anticonvulsant action of four second-generation antiepileptic drugs in pentetrazole-induced clonic seizures in mice

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Abstract:
The exact role of compounds modulating nitric oxide (NO) content in the brain during seizure phenomena is under intensive investigation. This study was aimed at determining the effect of N^G-nitro-L-arginine (L-NA; a non-selective NO synthase inhibitor) on the anticonvulsant activity of four second-generation antiepileptic drugs (AEDs: gabapentin [GBP], oxcarbazepine [OXC], tiagabine [TGB] and vigabatrin [VGB]) in the mouse pentetrazole (PTZ)-induced seizure model. The acute adverse-effect liability of the studied AEDs in combinations with L-NA were evaluated in the chimney test (motor coordination).
Results indicate that L-NA (40 mg/kg; *ip*) significantly reduced the anticonvulsant activity of OXC in the PTZ test, by increasing its ED_{50} from 20.9 to 29.8 mg/kg ($p < 0.05$). Similarly, L-NA at doses of 20 and 40 mg/kg considerably attenuated the antiseizure effects of VGB by raising its ED_{50} from 595 to 930 mg/kg ($p < 0.05$), and 1022 mg/kg ($p < 0.01$), respectively. L-NA at lower doses of 10 and 20 mg/kg did not affect significantly the anticonvulsant effects of VGB and OXC in PTZ-induced seizures. Likewise, the co-administration of L-NA (40 mg/kg; *ip*) with GBP and TGB was associated with no significant changes in their anticonvulsant activities in PTZ-induced seizures in mice. Moreover, none of the examined combinations of L-NA (40 mg/kg; *ip*) and second-generation AEDs (at their ED_{50} values) affected motor coordination in the chimney test.
Based on this preclinical study, one can conclude that L-NA reduced the anticonvulsant activities of VGB and OXC in the mouse PTZ-induced seizure model. Only, GBP and TGB were resistant to the action of L-NA in this model.

Key words:
N^G-nitro-L-arginine, nitric oxide, oxcarbazepine, vigabatrin, pentetrazole-induced seizures, tiagabine, gabapentin, mice

Abbreviations: 7-NI – 7-nitroindazole, AED – antiepileptic drug, ETS – ethosuximide, GABA – γ-aminobutyric acid, GBP – gabapentin, L-Arg – L-arginine, L-NA – N^G-nitro-L-arginine, L-NAME – N^G-nitro-L-arginine methyl ester, MES – maximal electroshock seizure test, NO – nitric oxide, NOS – nitric oxide synthase, OXC – oxcarbazepine, PTZ – pentetrazole, TGB – tiagabine, VGB – vigabatrin

Introduction

Overwhelming evidence indicates that nitric oxide (NO), a small diffusible gaseous messenger, synthesized from the amino acid L-arginine (L-Arg) by the enzyme NO synthase (NOS), appears to play a crucial role in a number of physiological and pathophysiological processes in the brain, including the modulation of neuronal plasticity, cerebral blood-flow, cognitive and behavioral functions, as well as, its involvement in neurological disorders such as ischemia and epilepsy [10, 23, 24, 26, 33].

The synthetic L-Arg analogues have been found to inhibit NOS activity, which allowed for the examination and clarification of the role of NO in the brain functioning, especially in seizure phenomena. N^G-nitro-L-arginine (L-NA) is considered to be a non-selective NOS inhibitor responsible for suppression of activity of both endothelial and neuronal NOSs [7, 23]. Experiments performed using synthetic L-Arg analogues have yielded conflicting results, reporting both pro- and anticonvulsant properties of NO [5, 6, 31, 34–37]. Hence, the exact role of NO in the pathophysiology of seizures is debated and remains unclear.

Previously, it has been reported that L-NA administered systemically (*ip*) at a dose of 40 mg/kg reduced the anticonvulsant activity of ethosuximide (ETS), having had no impact on the antiseizure effects of valproate, phenobarbital and diazepam against pentetrazole (PTZ)-induced seizures in mice [5]. Similarly, it has been documented that N^G-nitro-L-arginine methyl ester (L-NAME) attenuated the anticonvulsant action of valproate and phenobarbital, but not that of carbamazepine and diphenylhydantoin against maximal electroshock (MES)-induced seizures in mice [1, 4]. Moreover, L-NA reduced the antiseizure effects of felbamate and lamotrigine, but not those of oxcarbazepine (OXC) and topiramate in the MES test in mice [17].

The aim of this study was to assess the effects of L-NA on the anticonvulsant effects of four second-generation AEDs (gabapentin [GBP], OXC, tiagabine [TGB] and vigabatrin [VGB]) against PTZ-induced clonic seizures in mice. Generally, it is accepted that PTZ-induced seizures are thought to be an experimental animal model of myoclonic seizures in man [14]. The potential adverse-effect profiles of AEDs co-administered with L-NA were determined in the chimney test. In rodents, this test allows for the determination of acute adverse effects produced by drugs administered alone or in combination as regards their ability to affect motor coordination in experimental animals [15].

Materials and Methods

Animals and experimental conditions

All experiments were performed on adult male Swiss mice weighing 22–26 g. The mice were kept in colony cages with free access to food and tap water, under standardized housing conditions (natural light-dark cycle, temperature of 21 ± 1°C). After 7 days of adaptation to laboratory conditions, the animals were randomly assigned to experimental groups consisting of 8 mice. Each mouse was used only once. All tests were performed between 9.00 a.m. and 2.00 p.m. Procedures involving animals and their care were conducted in conformity with current European Community and Polish legislation on animal experimentation. Additionally, all efforts were made to minimize animals' suffering and to use only the number of animals necessary to produce reliable scientific data. The experimental protocols and procedures listed below conformed also to the *Guide for the Care and Use of Laboratory Animals* and were approved by the Local Ethics Committee at the Medical University of Lublin.

Drugs

The following drugs were used in this study: L-NA (RBI, Natick, MA, USA), GBP (Neurontin, Parke-Davis, Freiburg, Germany), OXC (Trileptal, Novartis Pharma AG, Basel, Switzerland), TGB (Gabitril, Sanofi Winthrop, Gentilly, France), and VGB (Sabril, Marion Merrell S.A., Puteaux, France). All drugs were suspended in a 1% aqueous solution of Tween 80 (Sigma, St. Louis, MO, USA) and administered intraperitoneally (*ip*) in a volume of 0.005 ml/g of body weight. Fresh drug solutions were prepared on each day of experimentation and administered as follows: VGB at 240 min, GBP – 60 min, OXC, L-NA – 30 min, and TGB – at 15 min, before PTZ-induced seizures and all behavioral tests used in this study. These drug administration times were based on literature data on their biological activity [5, 29] and were confirmed in our

previous experiments [17, 21]. The times of peak maximum anticonvulsant effects for AEDs were used as reference times in all experimental tests. PTZ (Sigma, St. Louis, MO, USA) was dissolved in distilled water and administered subcutaneously (*sc*) into a loose fold of the skin in the midline of the neck in a volume of 0.005 ml/g of body weight.

Pentetrazole (PTZ)-induced convulsions

Clonic convulsions were induced in mice by *sc* administration of PTZ at the doses ranging between 70–120 mg/kg. Following the injection of PTZ, mice were placed separately into transparent Plexiglas cages (25 × 15 × 10 cm) and observed for 30 min for the occurrence of clonic seizures. The clonic seizure activity was defined as clonus of whole body lasting over 3 s, with an accompanying loss of righting reflex. The number of animals convulsing out of the total number of mice tested was recorded for each treatment condition. The convulsive action of PTZ was evaluated as the CD_{50} (the dose of PTZ that produced clonic seizures in 50% of the mice tested). To determine the CD_{50} value, four or five various doses of PTZ were used (8 mice per group). Subsequently, an intensity-response curve was constructed from the percentage of mice convulsing according to log-probit method by Litchfield and Wilcoxon [12]. Afterwards, from the equation of intensity-response curve for PTZ, both CD_{50} and CD_{97} values were calculated. Noteworthy, the CD_{97} value reflects the dose of PTZ required to produce seizures in 97% of animals tested. This experimental procedure has been described in more detail in our earlier study [21].

The anticonvulsant activities of GBP, OXC, TGB and VGB against PTZ-induced clonic seizures were determined after *sc* administration of PTZ at its CD_{97} (110 mg/kg). The animals were treated with increasing doses of the AEDs, and the anticonvulsant activity of each drug was evaluated as its ED_{50} value (protecting 50% of mice against PTZ-induced clonic convulsions). At least four groups of animals were used to estimate each ED_{50} value, calculated from the respective dose-response curves, according to Litchfield and Wilcoxon [12].

Chimney test

The effects of combinations of L-NA with second-generation AEDs on motor coordination impairment were quantified with the chimney test of Boissier et al. [2]. In this test, animals had to climb backwards up the plastic tube (3 cm inner diameter, 25 cm length). Motor impairment was indicated by the inability of the animals to climb backward up the transparent tube within 60 s. Data were presented as a percentage of animals that failed to perform the chimney test.

Statistical analysis

Both, CD_{50} and ED_{50} values with their 95% confidence limits were calculated by computer log-probit analysis according to Litchfield and Wilcoxon [12]. The obtained 95% confidence limits were transformed to standard errors (SE) as described previously [18, 19]. The ED_{50} values were statistically analyzed using one-way analysis of variance (ANOVA) followed by the *post-hoc* Tukey/Kramer test for multiple comparisons. A p value < 0.05 was considered statistically significant. Qualitative variables from the chimney test were compared with Fisher's exact probability test.

Results

Effect of L-NA on the threshold for PTZ-induced clonic seizures

L-NA (40 mg/kg) administered *ip*, 30 min before the test, did not affect statistically significantly the threshold for PTZ-induced clonic seizures. The CD_{50} value of PTZ for L-NA-treated animals was 62.1 (54.6–70.5) mg/kg and did not differ significantly from the CD_{50} value of PTZ for control animals, which was 73.0 (64.9–82.2) mg/kg.

Influence of L-NA on the anticonvulsant activity of GBP, OXC, TGB and VGB against PTZ-induced clonic seizures

All AEDs studied i.e., GBP, OXC, TGB and VGB displayed clear-cut antiseizure effects against PTZ-induced clonic seizures in mice. The ED_{50} values for all AEDs, calculated from their dose-response curves according to the log-probit method are presented in Table 1.

L-NA administered systemically (*ip*) at a dose of 40 mg/kg decreased significantly the anticonvulsant

activity of OXC in the PTZ test by elevating its ED_{50} value by 43% ($p < 0.05$; Tab. 1). L-NA at the lower dose of 20 mg/kg had no significant effect on the antiseizure effects of OXC against PTZ-induced clonic seizures (Tab. 1). Similarly, L-NA at 20 and 40 mg/kg significantly attenuated the anticonvulsant action of VGB, increasing significantly the ED_{50} value for VGB by 56% and 72%, respectively ($p < 0.05$ and $p < 0.01$; Tab. 1). Only, L-NA at 10 mg/kg had no significant impact on the antiseizure activity of VGB in the PTZ test, although a 26% increase in the ED_{50} value was observed for the combination of L-NA with VGB (Tab. 1). In contrast, L-NA at 40 mg/kg did not alter significantly the anticonvulsant effects of TGB and GBP in the PTZ test in mice (Tab. 1).

Tab. 1. Effect of L-NA on the anticonvulsant activity of some second-generation AEDs against PTZ-induced seizures

Treatment (mg/kg)	ED_{50} (mg/kg)	N	SE	R (%)
GBP + vehicle	289 (215–387)	24	43.2	–
GBP + L-NA (20)	321 (240–429)	24	52.1	11
GBP + L-NA (40)	396 (291–538)	32	62.0	37
OXC + vehicle	20.9 (17.9–24.4)	16	1.65	–
OXC + L-NA (20)	22.8 (18.8–27.7)	24	2.26	9
OXC + L-NA (40)	29.8 (24.7–35.9) *	24	2.85	43
TGB + vehicle	0.7 (0.4–1.3)	16	0.23	–
TGB + L-NA (20)	0.8 (0.5–1.3)	24	0.28	14
TGB + L-NA (40)	1.0 (0.6–1.8)	24	0.30	43
VGB + vehicle	595 (458–774)	32	79.8	–
VGB + L-NA (10)	748 (615–911)	16	75.2	26
VGB + L-NA (20)	930 (797–1085) *	16	73.0	56
VGB + L-NA (40)	1022 (877–1191) **	16	79.8	72

Data are presented as median effective doses (ED_{50}s in mg/kg, with 95% confidence limits in parentheses) of AEDs that protected 50% of animals against PTZ-induced clonic seizures. The PTZ-induced seizures were produced by the *sc*-injection of PTZ at its CD_{97} (110 mg/kg). All AEDs were administered *ip*, as follows: VGB – 240 min, GBP – 60 min, OXC and L-NA – 30 min, and TGB was given 15 min prior to the PTZ test. Statistical evaluation of the data was performed with log-probit method, followed by one-way ANOVA with the *post-hoc* Tukey/Kramer test for multiple comparisons . N – total number of animals used at those doses whose expected anticonvulsant effects ranged between 4 and 6 probits; SE – standard error of ED_{50}; R – percentage of reduction in ED_{50} value after L-NA co-administration; L-NA – N^G-nitro-L-arginine; GBP – gabapentin; OXC – oxcarbazepine; TGB – tiagabine; VGB – vigabatrin. * $p < 0.05$, and ** $p < 0.01$ *vs.* the respective control group (AED + vehicle-treated animals)

Influence of L-NA in combination with GBP, OXC, TGB and VGB on motor performance in the chimney test

None of the combinations of L-NA (40 mg/kg) with four second-generation AEDs (at doses corresponding to their ED_{50} values from the PTZ test) impaired significantly motor coordination in animals subjected to the chimney test (results not shown).

Discussion

Here we showed that L-NA (at the dose of 40 mg/kg) did not affect the CD_{50} value of PTZ in mice, and this result is consistent with previous studies, documenting that L-NA (up to 40 mg/kg) administered alone had no significant effect on the threshold for PTZ-induced clonic seizures in mice [29, 34]. Moreover, it was found that L-NA significantly reduced the anticonvulsive action of OXC and VGB against PTZ-induced clonic seizures in mice. In contrast, L-NA did not alter the antiseizure effects of GBP and TGB in the PTZ test in mice. The L-NA-induced reduction in the antiseizure effects of OXC and VGB seems to be similar to previous findings, showing that L-NA significantly decreased the anticonvulsant activity of ETS in the PTZ test in mice [5]. Interestingly, L-NA had simultaneously no impact on the anticonvulsant activity of valproate, phenobarbital and diazepam against PTZ-induced clonic seizures in mice [5]. Moreover, in our previous study, it has been documented that L-NA at 40 mg/kg attenuated the anticonvulsant effects of felbamate and lamotrigine, having had no effect on the antiseizure activity of OXC and topiramate in the MES test [17]. Moreover, L-NAME (an unspecific NOS inhibitor) impaired the anticonvulsant activity of valproate and phenobarbital, but not that of carbamazepine and diphenylhydantoin in the MES-induced seizures in mice [1, 4].

To explain the observed interactions between VGB, OXC and L-NA, one should consider their molecular mechanisms of action. With respect to VGB, the drug binds to neuronal and glial GABA-α-oxoglutarate aminotransferase (GABA-transaminase), and irreversibly inhibits the enzyme, thus increasing GABA levels and enhancing GABAergic neurotransmission in the brain [11]. In the case of OXC, the drug and its rapidly formed 10-monohydroxy derivative reduce

high frequency repetitive firing of neurons by an action on Na^+ channels and enhance K^+ current [22]. Moreover, OXC and its 10-monohydroxy derivative inhibit high-voltage activated N-type Ca^{2+} channels and reduce glutamatergic transmission at cortical synapses in rat brain slices [22]. As mentioned in the Introduction, L-NA is the non-selective NOS inhibitor, which reduces the activity of both, endothelial and neuronal NOSs and thus, it produces a decrease in NO content in the brain [7]. Some data suggest that NO plays a modulatory role in the release or uptake of GABA in the brain [30]. It has been postulated that NO can directly activate $GABA_A$ receptors through the interaction with their γ_2 subunits [9]. NO has been found to increase release of GABA from the cerebral cortex [25], hippocampus [13] and striatum [30]. Additionally, it has been observed that NOS inhibitors decreased release of GABA from cortical and striatal synaptosomes [25]. Previously, it has been reported that L-Arg (a natural precursor of NO) inhibited GABA-transaminase activity and thus, increased GABA concentration in the brain [28]. Conversely, in L-NAME-treated animals, a decreased NO content in the brain has been accompanied with an activation of GABA-transaminase and decrease in GABA concentration in the brain [28].

It is important to note that pharmacokinetic characteristics of interactions between L-NA, VGB and OXC were not evaluated in this study. However, based on previous studies showing that L-NA had no significant impact on plasma concentrations of four conventional AEDs (ETS, valproate, diazepam, and phenobarbital) [5], one can presume that L-NA affected neither VGB nor OXC plasma and brain concentrations and the observed attenuation of the antiseizure effects of VGB and OXC in the PTZ test in mice was of pharmacodynamic nature. In the case of VGB, only one report exists showing that VGB increased total brain concentrations of ETS and phenobarbital, having simultaneously no effect on total brain concentrations of valproate and clonazepam in the PTZ test in mice [21]. With regard to OXC, no pharmacokinetic interactions have been documented for the drug combined with other AEDs in preclinical studies. Since L-NA did not alter plasma concentrations of conventional AEDs, one can accept that the existence of pharmacokinetic interactions between L-NA and VGB or OXC is improbable, but not entirely excluded.

Another fact deserves more explanation while interpreting the results of this study. The L-NA-induced attenuation of the anticonvulsant effects of ETS in the PTZ test has been partially reversed by the pretreatment with L-Arg at a dose of 500 mg/kg [5]. However, in the present study, the effects of L-NA were not reversed by using L-Arg because there has recently appeared a suggestion that co-administration of L-Arg with NOS inhibitors may activate alternative pathways for metabolic transformation of L-Arg [27]. In such a case, L-Arg may be transformed into agmatine and CO_2 (by arginine decarboxylase); ornithine and urea (by arginase); citrulline and NH_4^+ (by arginine deiminase); or ornithine and guanidinoacetate (by arginine:glycine amidinotransferase) [27]. Accumulating evidence indicates that agmatine produces *per se* the anticonvulsant effects in both MES and PTZ-induced seizures in rodents [8, 32]. Therefore, to avoid transformation of L-Arg into agmatine and other active metabolites that could change the anticonvulsant effects of the AEDs tested, the action of L-NA on VGB and OXC anticonvulsant activities was not reversed by L-Arg.

It is worthy of mentioning that 7-nitroindazole (7-NI – a preferential neuronal NOS inhibitor) did not significantly alter the antiseizure effects of GBP, OXC, TGB and VGB in the PTZ test in mice (unpublished data). However, there has recently appeared a suggestion that 7-NI is able to produce itself the antiseizure effects in experimental models of epilepsy in rodents and these effects seem to be independent of 7-NI-induced modulation of NO content in the brain [3, 6, 16, 20, 36, 37]. In such a case, the evaluation of the role of NO in seizure phenomena after pretreatment with 7-NI and newer AEDs might reflect not only the modulation of NO content in the brain, but also a direct antiseizure action of 7-NI on PTZ-induced seizures in mice. Detailed discussion concerning the role of 7-NI in seizure phenomena and its influence on the antiseizure potential of conventional and newer AEDs has been presented elsewhere [6, 16, 20, 29, 31, 33].

Based on this preclinical study, one can ascertain that L-NA attenuated the antiseizure effects of some newer AEDs in the PTZ test. On the other hand, results of this study suggest that the effect of L-NA on the anticonvulsant activity of VGB in the PTZ test may be dependent on the modulation of GABA-transaminase activity in the brain. To elucidate this phenomenon, more advanced neurochemical and electrophysiological studies are required.

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