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NITRIC OXIDE (NO) AND AN NMDA RECEPTOR ANTAGONIST IN PENTYLENETETRAZOLE-INDUCED CONVULSIONS

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Controversy about proconvulsant and anticonvulsant nitric oxide (NO) effects and the place of oxidative stress in convulsions, are still a matter of research. We investigated the interaction between 2-amino-5-phosphonovaleric acid (APV), a competitive N-methyl-D-aspartate (NMDA) receptor antagonist, and Nω-nitro-L-arginine methyl ester (L-NAME), a nonselective nitric oxide synthase (NOS) antagonist, in pentylenetetrazole (PTZ)-induced convulsions. Pentylenetetrazole was applied to adult Wistar rats intraperitoneally (ip), in a single dose of 80 mg/kg, and L-NAME (10 µg/10 µl) or APV (20 µg/10 µl) intracerebroventricularly (icv), 30 and 10 minutes before PTZ, respectively. In the same manner, another group received both antagonists. Control animals were given 0.9% saline.

Nω-nitro-L-arginine methyl ester exerted a weak anticonvulsant effect, preventing generalized clonic (GCC) and clonic-tonic convulsions (CTC) in 17% of cases. With APV protection against GCC and CTC was 100%, forelimb dystonia (FLD) was decreased in 33% of cases, and time to onset of all convulsive patterns was prolonged ($p<0.05$ to 0.01). All effects of APV, except in CTC, were reversed by L-NAME applied prior to APV.

In APV-PTZ treated animals, superoxide anion content was increased in the forebrain cortex, striatum and hippocampus, without an overwhelmed antioxidative superoxide dismutase (SOD) defence system in the other treatments. When the APV-PTZ group was treated with L-NAME, both SOD activity and superoxide anion content were additionally decreased, indicating that the NOS-NO system was involved in the metabolism of superoxide anions.

It is suggested that clinical and biochemical effects of NO strongly depend upon the pretreatment and might lead to a wrong impression of NO contradictory activity.

Key words: convulsions, nitric oxide, NMDA antagonists, pentylenetetrazole, superoxide anion, superoxide dismutase

INTRODUCTION

The complex biochemical and structural heterogeneity of epilepsies and unsatisfactory understanding of their pathophysiological mechanisms, are a considerable scientific, clinical and pharmacological challenge. Better understanding of the cellular and molecular basis would make possible the development of more effective antiepileptics and antiseizure drugs, that would improve the prevention and treatment of epilepsy.

Imbalance between excitatory amino acids (EAK) and gamma aminobutyric acid (GABA) is the cornerstone in epileptogenesis. There are a number of data about convulsions induced by EAK, acting on different receptor sites (Tutka *et al.*, 1996), or induced by GABA antagonists, with some new insights in to the GABAergic role in the EAK-GABA interaction (Kohling, 2002).

There is no doubt that nitric oxide (NO), which is released by glutamate, is involved in the pathogenesis of convulsions, but the results remain conflicting. Proconvulsant (Gross *et al.*, 1994; Jayakumar *et al.*, 1999) and anticonvulsant effects (Lallement *et al*., 1996) have been registered. It was also found that NO does not influence epileptogenesis (Czuczwar *et al.*, 1999). Many of these disagreements came from unstandardized definitions of convulsions and differences in the choice of experimental model and experimental animals.

Besides unequivocal results about the role of NO in several animal convulsion models, data about the place of NO in pentylenetetrazole (PTZ)-evoked convulsions are different. According to the results obtained, NO could be anticonvulsant (Nidhi *et al.*, 1999), enhance some convulsive patterns (Del-Bel *et al.*, 1997), or be without any importance in clonic and tonic seizures (Przegalinski *et al.*, 1996; Hara *et al.*, 1996).

It is obvious that the dose and the route of administration of PTZ and nitric oxide synthase (NOS) antagonists, as well as the type of NOS isoenzyme antagonists, strongly affect the outcome of PTZ-evoked behaviour (Alexander *et al.*, 1998). That is why direct comparison between the convulsive responses of different animal species in different experimental models (*in vitro, ex vivo, in vivo*) and protocols is not suggested. Namely, great caution is required in order to escape the conclusion trap.

Glutamate and NO are involved in physiological transmission and in the metabolism of reactive oxygen and nitrogen species, such as superoxide anions, hydroxyl and NO radicals, and also in peroxynitrite (ONOO-) production (Crow and Beckman, 1995). This is a stable and long acting toxic metabolite of NO and superoxide anion that can produce a number of free radicals (Goss *et al.*, 1999). It may indicate that NO is a part of neuroprotective as well as neurotoxic processes.

In an attempt to investigate the potential clinical and biochemical interactions between EAK and NO in seizures, we used chemically induced convulsions in rats as an experimental model.

MATERIAL AND METHODS

The experiments were performed on 13 week-old male Wistar rats (*Rattus norvegicus*), housed in a temperature-controlled room (23±2°C), with a light/dark cycle regulated to 11:13 hours. The rats had free access to food and water. All experiments were conducted within two weeks, between 10 am and 3 pm.

Surgery was performed under pentobarbital sodium anaesthesia (45 mg/kg body weight-bw, Vetanarcol , Werfft-chemie, Wien). For intracerebroventricular (icv) drug application, a polyethylene plastic cannula was stereotaxically implanted into the left lateral ventricle (coordinates: 1.3 mm behind the bregma, 1.8 mm left from the midline suture, 3.7 mm ventral from the durra) (Paxinos and Watson, 1982). A cannula was fixed to the skull with dental cement and two jeweller screws. The postoperative recovery period lasted six days, after which the rats (five groups with 6-7 rats in each) were assigned randomly to different drug treatments. The route and the time of applying chemicals, dissolved in 0.9% saline, were selected according to their pharmacokinetic characteristics.

Chemicals for icv and ip application were given in a volume of 10 l and 1 ml/kg, respectively. The control group received 0.9% saline icv, 30 and 10 minutes before saline (ip). Pentylenetetrazole (PTZ, Sigma) was used as the chemoconvulsant. It was applied intraperitonally (ip) in a dose of 80 mg/kg bw 30 and 10 minutes after treatment with 0.9% saline (icv).

Thirty minutes before PTZ, 10 g of a nonselective NOS antagonist, N -nitro-L-arginine methyl ester (L-NAME, Sigma) was given to one group. Twenty minutes later, it also received saline (icv).

A competitive N-methyl-D-aspartate (NMDA) receptor antagonist, 2-amino-5-phosphonovaleric acid (APV, Sigma), was administered to another two groups, in the dose of 20 g, 20 minutes after 0.9% saline or L-NAME. Ten minutes after APV, both groups received PTZ.

During the four minutes after PTZ treatment, the appearance (incidence) and the time to onset of convulsive patterns were recorded, such as forelimb dystonia (FLD), generalized clonic (GCC) and clonic-tonic convulsions (CTC). Thereafter, the rats were sacrificed by decapitation. Heads were frozen in liquid nitrogen, and stored at –70°C until the brain structures were required for assay.

Forebrain cortex, striatum and hippocampus were dissected in the cold and prepared for the spectrophotometrical biochemical analyses.

Superoxide anion content was determined through the reduction of nitroblue-tetrazolium (Merck) in an alkaline, nitrogen saturated medium. Analysis was performed at 515 nm (Sun and Zigman, 1978).

Superoxide dismutase (SOD) activity was measured as inhibition of epinephrine autooxidation at 480 nm. After adding 10 mM epinephrine (Sigma), the kinetics were monitored in sodium carbonate buffer (Serva) containing 0.1 mM EDTA (Sigma) (Auclair and Voisin, 1985).

STATISTICS

The incidence of convulsive responses was expressed as a percentage of the total animals in the group. Student's t-test of proportion was performed with $p<0.05$ and $p<0.01$ as indicators of the statistical significance of differences. The time to convulsive response was expressed as the mean standard deviation (SD) and was analysed by the Mann-Whitney U-test, using the same levels of significance.

Biochemical data were expressed as the mean standard deviation (SD) and were compared by Students t-test and analysis of variance. Differences were considered statistically significant at $p<0.05$ and $p<0.01$.

RESULTS

Contrary to the saline group, which was without any convulsive pattern, the treated rats developed convulsions that appeared in a regular order: forelimb dystonia (FLD), generalized clonic convulsions (GCC) followed by clonic-tonic convulsions (CTC).

Pentylenetetrazole alone evoked convulsive patterns in all of the animals in the group (100%) (Figure 1). The NOS antagonist (L-NAME) demonstrated a very weak anticonvulsant activity, preventing the incidence of GCC and CTC in 17% of cases.

Figure 1. Influence of APV and L-NAME on PTZ-evoked convulsions (80 mg/kg)in rats (n=6-7)
The incidence of convulsive responses is expressed as the percentage of affected rats in the group; *(**): $p<0.05$ (0.01), significance *vs.* PTZ treated group (Student's t-test of proportion).
[1]FLD=forelimb dystonia, [2]GCC=generalized clonic convulsions, [3]CTC=clonic-tonic convulsions

The APV application was particularly effective. It prevented the appearance of GCC and CTC completely ($p<0.01$ for both). It was less successful with limbic convulsions, which were prevented only in 33% of cases. The anticonvulsant activity of APV was diminished by L-NAME administration before APV. This led to FPD augmentation by 16% and GCC by 50% ($p=0.07$). Such treatment did not affect CTC.

At the same time, when compared to the PTZ treated group, APV delayed the onset of all convulsive patterns ($p<0.05$, 0.01 and 0.01 for FLD, GCC and CTC, respectively) (Table 1). The time for FLD and GCC appearance was partly reversed by L-NAME.

Table 1. Time to convulsive onset in the PTZ (80 mg/kg)-treated rats (n=6-7)

Drug treatment (L-NAME and APV: 10 and 20 g; PTZ: 80 mg/kg)	Convulsive response latency time (seconds)		
	FLD[1]	GCC [2]	CTC [3]
PTZ	49.5±11.3	118.6±25.8##	131.7±35.6##
L-NAME+PTZ	57.7±11.9	125.3±68.3#	128.1±67##
APV+PTZ	140.8±89.6*	240.0±0**	240.0±0**
L-NAME+APV+PTZ	87.5±74.8	169.5± 82.4#	240.0±0**##

Values are expressed as means±SD. When there was no convulsive response, the latency was defined as 240 seconds. *(**): $p<0.05$ (0.01), significance of difference from corresponding PTZ treated group; #(##): p.05 (0.01), significance to corresponding APV+PTZ treated group (Mann-Whitney U-test).
[1]FLD=forelimb dystonia, [2]GCC=generalized clonic convulsions, [3]CTC=clonic-tonic convulsions.

Parameters of oxidative stress and antioxidative defence changed rapidly after PTZ application, taking a very short time course of alterations, i.e. four minutes only.

In the group treated with APV before PTZ, the content of superoxide anion radical was increased ($p<0.01$) in all structures (Figure 2), and did not change in parallel with SOD activity (Figure 3). The effects of APV on the superoxide anion were reversed with L-NAME.

The activity of SOD was not changed in the cortex, but it was decreased in the striatum and in the hippocampus of the APV treated group ($p<0.01$ for both stuctures) (Figure 3). Furthermore, SOD activity was additionally decreased after L-NAME pretreatment ($p<0.01$, compared to APV+PTZ treated group). The only enhancement was registered in the hippocampus after PTZ application.

Figure 2. Superoxide anion content in PTZ-evoked convulsions (80 mg/kg) in rats (n=5-7)
Values are expressed as means±SD; @: p<0.05, significante with respect to corresponding saline treated group; *(**): p<0.05 (0.01), significant wilt respect to corresponding PTZ treated group; #(##):p<0.05 (0.01), significance to corresponding APV+PTZ treated group (Student t-test)

Figure 3. Superoxide dismutase activity in PTZ-evoked convulsions (80 mg/kg) in rats (n=6-7)
Values are expressed as means±SD; @: p<0.05, significant in comparison to corresponding saline treated group; *(**): p<0.05 (0.01), significanct comparison to corresponding PTZ treated group ; #(##): p<0.05 (0.01), significant in comparison to corresponding APV+PTZ treated group (Student t-test)

DISCUSSION

In the applied experimental model and protocol, L-NAME only slightly reduced PTZ convulsive responses, in comparison to the very strong APV activity. It prevented the appearance of GCC and CTC by 17%. These results are partly confirm to our previous study (Jelenković *et al*., 2002), when, with some differences in the applied experimental design, we found much stronger, dose dependent, anticonvulsant effects of L-NAME on PTZ-evoked convulsions. CTC was influenced CTC the most. This finding is in agreement with the results of Han and coworkers (2000) of increased brain NO concentration during PTZ-evoked convulsions.

The appearance of GCC and CTC were totally prevented by APV. On the other hand, FLD was very resistant to APV. It has been proposed that clonic-tonic convulsions are generated in the brainstem (Willoughby, 1999), representing an animal model equivalent to grand mal epilepsy in humans. Activation of the hippocampus and other limbic, as well as extralimbic structures (thalamus), is responsible for the appearance and the spread of limbic convulsions, with forelimb dystonia as the part of their clinical characteristics. They correspond to complex partial seizures in people (Mraovitch and Calando, 1999), which are difficult to control. No treatment in our experiment could successfully prevent them.

Our results suggest that the anticonvulsant effects of APV are the result of two processes: NMDA receptor blockade and the activity of the NOS-NO system, since L-NAME pretreatment to some degree reversed the protective effects of APV.

As a competitive NMDA antagonist, APV blocks NMDA, but not non-NMDA and metabotropic receptors (Michaelis, 1998). Thus, in the APV treated group, increased glutamate activity evoked by PTZ could activate non-NMDA and metabotropic receptors in glial cells, as well in neurons, with L-arginine supply to neurons from the glial cells. Also, through these receptors, NO could be released from nitrosothiols (Yamada and Nabeshima, 1998), allowing neuronal transmission, including NO-mediated increase in GABA release from neurons (Ohkuma *et al.*, 1995). It was found that the GABA-releasing process in the hippocampus is L-NAME dose-dependent (Getting *et al*., 1996). In our experiment, L-NAME application could decrease NO production resulting in reduction of GABAergic activity, that would diminish some anticonvulsant effects of APV. The relative resistance of the hippocampus to the anticonvulsant effects of APV and L-NAME probably is due to its neurochemical organization and richnes sin glutamate transmission, since glutamate activity leads to NO synthesis through the glutamate-Ca-calmodulin-NOS system. Also, this brain region is the most sensitive to PTZ (Ben-Ari, *et al.,* 1981). At the same time, the response of the brain antioxidative system to the possible prooxidants (PTZ-glutamate-NO) was well balanced, followed by SOD activity decrease.

In the APV-treated group, superoxide anion production was enhanced and was dependent on the NOS-NO system. Besides the well known superoxide anion activity in NO scavenging, there is evidence about NO-evoked increases in superoxide anion content. A number of mechanisms are of interest, such as the me-

tabolism of peroxynitrite and hydrogen peroxide, and increase of free iron (Beckman 1991). Also, NO is involved in cytochrome c oxidase blockade, the last step in the respiratory chain, that is followed by superoxide anions leaking from mitochondria (Smoth *et al*., 1999).

Another important source contributing to the superoxide anion increase could be nitration of tyrosine residues in Cu, Zn superoxide dismutase with ONOO-, which would result in decline and insufficiency of the SOD defence mechanism (Souza, 1999). In our experiment, that was not the reason for the increase in superoxide anion content, as L-NAME additionally decreased it in the APV+PTZ treated group.

Not only metabolites of NO, but, in some conditions, NOS by itself can also produce superoxide anions (decrease of oxygen or arginine supply) (Vega-Agapito *et al.*, 1999). Whether directly, through NOS, or indirectly, through NO metabolism, SOD, a superoxide anion scavenging enzyme, was only overwhelmed in APV+PTZ treated rats in our experiment, and superoxide anion content was enhanced all brain regions.

In PTZ-evoked convulsions, APV exhibited a strong anticonvulsant effect and increased superoxide anion in the brain, indicating that NMDA receptors are involved in these kinds of convulsions. Some divergences in NO effects were registered at the clinical and biochemical level. Nitric oxide exerted a weak proconvulsant, and a stronger anticonvulsant activity. Also, NO did not influence superoxide anion and SOD activity when L-NAME was given before PTZ, but, when L-NAME was applied before APV+PTZ, both parameters were decreased. Such findings suggest that NO effects are closely dependent on the neurochemical changes induced by the substances applied. This gives basis for further investigations.

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Jelenković Ankica *et al.* Nitric oxide (NO) and an NMDA receptor antagonist in pentylenetetrazole-induced convulsions

AZOT OKSID (NO) I ANTAGONIST NMDA RECEPTORA U KONVULZIJAMA IZAZVANIM PENTILENTETRAZOLOM

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SADRŽAJ

Kontroverzni nalazi o prokonvulzivnim kao i antikonvulzivnim efektima azot oksida (NO) i značaju oksidativnog stresa u konvulzijama, i dalje su predmet istraživanja. U konvulzijama izazvanim primenom pentilentetrazola (PTZ), ispitivali smo interakciju između 2-amino-5-fosfovalerinske kiseline (APV), kompetitivnog antagoniste N-metil-D-aspartat (NMDA) receptora i N -nitro-L-arginin metil estra (L-NAME), neselektivnog antagoniste azot oksid sintaze (NOS). Odraslim pacovima Wistar soja, PTZ je ubrizgavan intraperitonealno (ip) u jednoj dozi od 80 mg/kg.

Ostale supstance, L-NAME (10 μg/10 μl) i APV (20 μg/10 μl), primenjivale su se intracerebroventrikularno (icv), i to L-NAME 30, a APV 10 minuta pre PTZ. Po istom vremenskom principu, jedna grupa dobila je oba antagonista, a kontrolna fiziološki rastvor NaCl.

N -nitro-L-arginin metil estar ispoljio je slabo antikonvulzivno dejstvo, smanjujući incidenciju generalizovanih kloničnih (GCC) i kloničnо-tоničnih konvulzija (CTC) za 17%. Za razliku od L-NAME, APV je sprečila nastanak GCC i CTC kod svih životinja (100%), a incidencija klonusa prednjih nogu (FLD) smanjena je za 33%. Istovremeno, primenom APV produženo je vreme od aplikacije PTZ do pojave svih konvulzivnih tipova ($p<.05$ do 0.01). Primenom L-NAME pre APV, umanjeni su efekti APV, pri čemu je došlo do povećanja incidencije FLD i GCC za 16%, odnosno 50%.

U kori prednjeg mozga, strijatumu i hipokampusu, životinja koje su dobile APV+PTZ, došlo do povećanja koncentracije superoksidnog anjona. Aktivnost superoksid dizmutaze ne prati ovaj skok. Njen dodatni pad u grupi tretiranoj sa L-NAME pre APV+PTZ, ukazuje da je sistem NOS-NO uključen u metabolizam superoksidnog anjona.

Dobijeni rezultati ukazuju da klinički i biohemijski efekti NO u velikoj meri zavise od prethodno primenjenih supstanci i promena izazvanih njima, što može da doprinose sticanju pogrešnog utiska o kontradiktornim dejstvima NO.