JMB 2007; 26 (3) DOI: 10.2478/v10011-007-0026-4

UDK 577.1 : 61 **ISSN 1452-8258**

JMB 26: 220–226, 2007

Original paper
Originalni naučni rad

SPERMINE AND L-NAME PRETREATMENT EFFECTS ON POLYAMINE AND NITRIC OXIDE METABOLISM IN RAT BRAIN DURING SEIZURES

UTICAJ SPERMINA I L-NAME NA METABOLIZAM POLIAMINA I AZOT MONOKSIDA U MOZGU PACOVA U TOKU KONVULZIJA

Ivana Stojanović[1], Ankica Jelenković[2], Ivana Vasiljević[3], Dušica Pavlović[1], Gordana Bjelaković[1]

[1]Institute of Biochemistry, Faculty of Medicine, Niš, Serbia
[2]Institute for Biological Research »Siniša Stanković«, Belgrade, Serbia
[3]Military Medical Academy, Belgrade, Serbia

Summary: In the CNS polyamines can exert opposite effects, depending on the concentration and conditions in the cell. Protective or neurotoxic polyamine effects were documented during seizures and repeated CNS excitation. Intensive research of exogenous polyamines effects during seizures induced by numerous agents did not clear up confusions about the duality of effects and the role of polyamines in seizures. In order to understand polyamine modulatory effects in seizures, the importance of NO and polyamine metabolism interdependence and the possible implication of changes of postulated NO and polyamine equillibrium in seizures, the effects of spermine alone and in combination with L-NAME (NOS inhibitor) on seizures induced by pentazol (PTZ) were investigated. To compare the obtained results, the effects of anticonvulsant midazolam on NO production during seizures were also investigated. Seizures were induced by i.p. application of pentazol (100 mg/kg b.w.). Spermine and L-NAME were administered i.p. before PTZ. In the striatum and hippocampus, spermine induced increased NO production ($p<0.001$) related to values in the group treated by PTZ. Application of L-NAME before spermine and PTZ caused decrease of NO production in comparison with animals treated only by PTZ or spermine and PTZ. L-NAME given before spermine exerts protective effects related to seizures induced by PTZ and to the group treated by spermine, extending the time of seizure symptoms appearance, thus confirming the NO signaling system involvement in spermine effects during seizures. Highly significant PAO activity increase caused by spermine points out the intensified interconversion of spermine into putrescine, in order to maintain the intracellular putrescine concentration. The obtained results prove a strong relationship between the NO signaling system and polyamine metabolism in the brain during seizures and the importance of their changes in this kind of CNS injury.

Keywords: polyamines, nitric oxide, L-NAME, convulsions

Kratak sadržaj: U CNS-u poliamini mogu imati dijametralno suprotne efekte, zavisno od njihove koncentracije i uslova u ćeliji. U toku konvulzija i pri ponavljanoj ekscitaciji CNS-a dokazani su i protektivni i neurotoksični efekti poliamina. Intenzivna istraživanja efekata egzogenih poliamina u toku konvulzija izazvanih brojnim agensima *in vivo* nisu još uvek razjasnila nedoumice o dualitetu efekata i ulozi poliamina u konvulzijama. U cilju razjašnjenja modulatornih efekata poliamina u konvulzijama, značaja povezanosti metabolizma NO i poliamina i mogućnosti implikacije promena postulirane ravnoteže između NO i poliamina u konvulzijama, ispitivani su efekti spermina na konvulzije izazvane pentazolom (PTZ) i produkciju NO, kao i efekti spermina u kombinaciji sa L-NAME (inhibitorom NOS). Radi poređenja dobijenih rezultata, ispitivani su i efekti antikonvulziva midazolama na produkciju NO u toku konvulzija. Konvulzije su izazivane i.p. aplikacijom pentazola (100 mg/kg TM). Spermin i L-NAME su aplikovani i.p. pre PTZ-a. U strijatumu i hipokampusu spermin izaziva povećanu produkciju NO ($p<0,001$) u odnosu na vrednosti u grupi tretiranoj PTZ-om. Aplikacija L-NAME pre spermina i PTZ-a izazvala je smanjenje produkcije NO u odnosu na životinje tretirane samo PTZ-om ili sperminom i PTZ-om. Primena spermina pre pentazola produbljuje kliničke efekte pentazola. L-NAME, dat pre spermina, ispoljava protektivne efekte u odnosu na konvulzije izazvane pentazolom i grupu tretiranu sperminom, produžavajući vreme pojave simptoma konvulzija, što potvrđuje uključenost NO signalnog sistema u efekte spermina u toku konvulzivne aktivnosti. Visokosignifikantni porast aktivnosti PAO izazvan sperminom ukazuje na intenzivnu interkonverziju poliamina u putrescin u cilju održanja intracelularne koncentracije putrescina. Dobijeni rezultati dokazuju povezanost NO signalnog sistema i metabolizma poliamina i značaj njihovih promena u ovoj vrsti oštećenja CNS-a.

Ključne reči: poliamini, azot monoksid, L-NAME, konvulzije

Address for correspondence:

Ivana Stojanović
Institute of Biochemistry, Faculty of Medicine, Niš, Serbia

Introduction

Epileptic seizures result from excessive discharge in a population of excitable neurons. Most of them are due to discharges generated in cortical and hippocampal structures, although subcortical structures are also involved in some seizure types. The changes in neuronal excitability not only induce abnormal activity of individual neurons, but also recruit a critical mass of hyperexcitable cells into highly synchronized activities which are propagated through normal or pathological pathways. The complex events underlying hyperexcitability, as well as the possibility of involvement of numerous factors, open a great range of potential candidates for initiators or modulators of convulsive activity.

Nitric oxide (NO), an enzymatic product of NO synthase (NOS), exerts significant neurophysiological functions. It acts as an intercellular messenger, also involved in intracellular signaling (1) and modulation of neuronal transmission. The activation of N-methyl-D-aspartic acid (NMDA) type of the glutamatergic receptor induces Ca^{2+}-dependent NOS activity and NO release, which then activates soluble guanylate cyclase for the synthesis of cGMP. Both compounds appear to be important mediators in long-term potentiation and long-term depression, and thus may play important roles in the mechanisms of learning and memory (2). It has been proved that NO is a mediator in glutamate-induced neurotoxicity.

Polyamines, putrescine, spermidine and spermine, play an important role in the regulation of cell proliferation and differentiation. In the CNS, polyamines have the role of synaptic transmission modulators and they are proved to be both neuroprotective and neurotoxic, depending on cell state and their concentrations (3). As intracellular messengers, they contribute to intracellular Ca^{2+} increase and modify NMDA receptor activity, increasing their affinity for the ligands (4). This undoubtly forms the relation between polyamines and seizures. The significant increase of ornithine decarboxylase activity and putrescine levels during seizures involves polyamines in molecular mechanisms of generation and propagation of convulsive activity (5). However, the data from the literature about their role in seizures are extremely contradictory.

It has been known that polyamines are important for CNS lesion recovery (6) and that exogenous polyamines have protective effects in numerous types of experimental CNS injury (7). However, the data that the blockade of the polyamine site on NMDA receptors was protective in some experiments, but unsuccessful in preventing excitotoxic effects of glutamate in others, made a confusion in the understanding of these neuromodulators roles in seizures.

Interactions of NO and polyamine metabolism, considering that they originate from the same substrate, L-arginine, and their functions in the CNS, are a very intriguing area in the neuropathology research. That is why the aim of this study was to examine the effects of exogenous spermine (Sp) on nitric oxide and polyamine metabolism during convulsions induced by chemical convulsant penthylene tetrazole (PTZ). Considering the possibility that the NO signaling system might be involved in polyamine effects in these conditions, we have tested this hypothesis using L-NAME, NO synthesis inhibitor, in combination with spermine. In order to compare the obtained results, the effects of antiepileptic drug Midazolam were also followed.

Materials and Methods

Animals

Male adult Wistar rats, body mass 300 ± 50 g, were used for the experiment. The animals were housed in an airconditioned room at a temperature of 23 ± 2 °C with 55 ± 10% humidity and with lights on 12 h/day (07:00–19:00 h). The animals were given a commercial rat diet and tap water ad libitum.

Experimental procedure

The animals were allocated into five experimental groups (each of them consisted of 8 animals): I group (control) – treated by saline, intraperitoneally (i.p.) applied; II group (PTZ) – seizures were induced by i.p. application of penthylenetetrazole (100 mg/kg bw); III group (Sp) – the animals were treated by i.p. application of spermine (1mg/kg bw 50 min before PTZ application); IV group (L-NAME + Sp) – spermine (1 mg/kg bw, i.p.) was applied in combination with NOS inhibitor – L-NAME (100 mg/kg bw, given i.p., 50 min before spermine, i.e. 1 h before PTZ application) and V group (Mid) – treated by antiepileptic midazolam in a dose of 100 mg/kg b.w. 45 min before PTZ.

All animals were decapitated and the heads were immediately removed and flash-frozen in liquid nitrogen. Striatum and hippocampus from individual animals were quickly isolated and homogenized in ice-cold buffer containing 0.25 mol/L sucrose, 0.1 mmol/L EDTA, 50 mmol/L K-Na phosphate buffer, pH 7.4. Homogenates were centrifuged at 3500 rot/min for 15 minutes, and each supernatant was then sonified (three times during 30 s at 10 kHz). The supernatants obtained by this procedure were then frozen and stored at –70 °C.

Biochemical analysis

After deproteinization, the production of NO was evaluated by measuring nitrite and nitrate concentrations. Nitrites were assayed directly, spectrophotometrically, at 543 nm, using the colorimetric method of Griess (Griess reagent: 1.5% sulfanilamide in 1 mol/L HCl plus 0.15% N-(1-naphthyl) ethylendiamine dihydrochloride in distilled water). However,

nitrates were previously transformed into nitrites by cadmium reduction (8)

DAO and PAO activities were determined using the spectrophotometrical method of Bachrach and Reches (9). Putrescin dihydrochloride and spermine tetrahydrochloride were used as substrates for DAO and PAO, respectively (10).

Arginase activity was determined according to the method of Porembska and Kedra (11).

Tissue protein content was measured according to the Lowry procedure, using bovine serum albumin (Sigma) as standard (12).

Chemicals were purchased from Sigma (St. Louis, MO, USA). All used chemicals were of analytical grade. All drug solutions were prepared on the day of the experiment. Animals used for procedure were treated in strict accordance with the NIH Guide for Care and Use of Laboratory Animals (1985).

Data presentation and analysis

Data are expressed as means ± S.D. Statistical significance was determined as $p<0.05$ using the Student's t-test.

Results

$NO_2^- + NO_3^-$ concentration in striatum and hippocampus

In both examined brain structures, PTZ induced statistically significant increase of nitrate and nitrite concentrations (37.43 ± 5.34; 46.93 ± 6.78 nmol/mg prot.) ($p<0.001$) compared to the control group (10.1 ± 2.58; 16.2 ± 4.82) (*Figures 1* and *2*). Midazolam reduced PTZ effects less efficiently in the striatum (23.93 ± 5.2) ($p<0.01$ vs. PTZ), and more pronouncedly in the hippocampus (24.6 ± 3.52) ($p<0.001$).

The application of spermine before PTZ led to augmented NO production (72.93 ± 5.16) (*Figure 1*), which was statistically highly significant ($p<0.001$) compared to values in the group treated only with PTZ (37.43 ± 5.34). The used dose of spermine induced a significant increase of nitrate + nitrite levels in the hippocampus (70.1 ± 16.37; $p<0.001$) in comparison with the group treated by PTZ (46.93 ± 6.78 nmol/mg prot.).

In the examined brain structures application of L-NAME before spermine and PTZ diminished NO production compared to the animals treated by PTZ only, or PTZ and spermine in combination, but the obtained values (27.45 ± 4.91; 23.26 ± 4.91) were still statistically significantly higher than control ones.

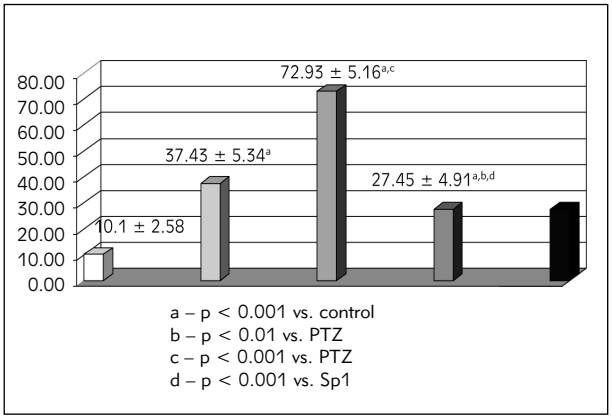

Figure 1 Spermine and L-NAME effects on $NO_2^- + NO_3^-$ concentration in rat striatum during seizures (nmol/L).

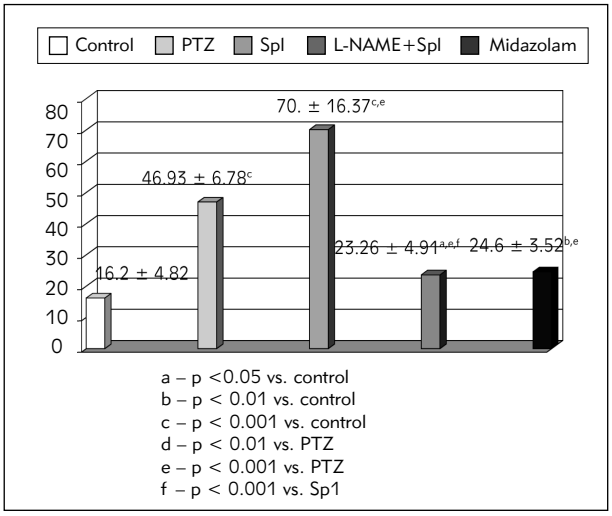

Figure 2 Spermine and L-NAME effects on $NO_2^- + NO_3^-$ concentration in rat hippocampus during seizures (nmol/L).

Arginase activity in striatum and hippocampus

In both structures, PTZ decreased arginase activity (*Figures 3* and *4*), which is more pronounced in the hippocampus. In the striatum of PTZ treated rats, spermin pretreatment increases arginase activity (0.45 ± 0.14 and 0.31 ± 0.09 U/mg prot.) ($p<0.01$ and $p<0.05$ vs. PTZ), but not to the control values. Almost identical effects of spermine were recorded in the hippocampus of treated animals (*Figure 4*).

L-NAME pretreatment diminished spermine effects on PTZ treated animals in the striatum. On the contrary, in the hippocampus the application of L-NAME intensified spermine effects (0.86 ± 0.48) pushing arginase activity to the control levels (0.78 ± 0.20 U/mg prot.).

Midazolam, applied before PTZ, exerted protective effects related to PTZ, correcting arginase activity nearly to the control values. However, there were no significant changes in the striatum.

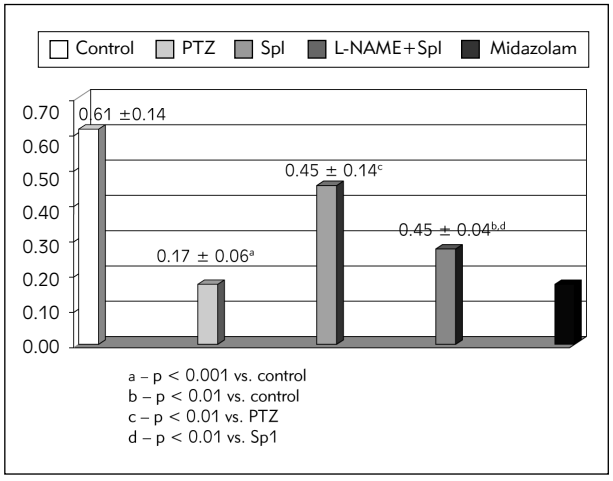

Figure 3 Spermine and L-NAME effects on arginase activity in rat striatum during seizures (U/mg prot.).

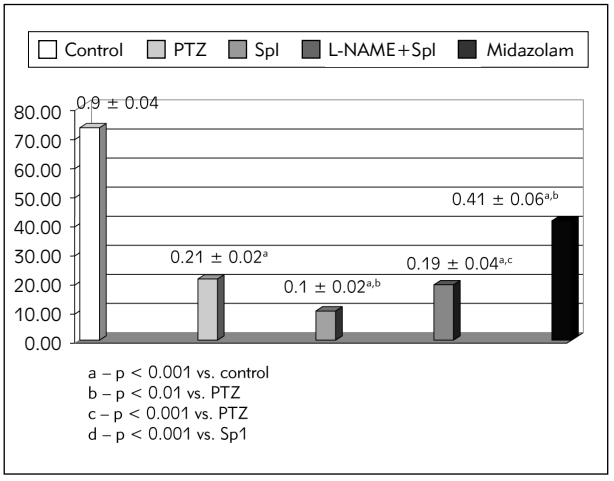

Figure 5 Spermine and L-NAME effects on DAO activity in rat striatum during seizures (U/mg prot.).

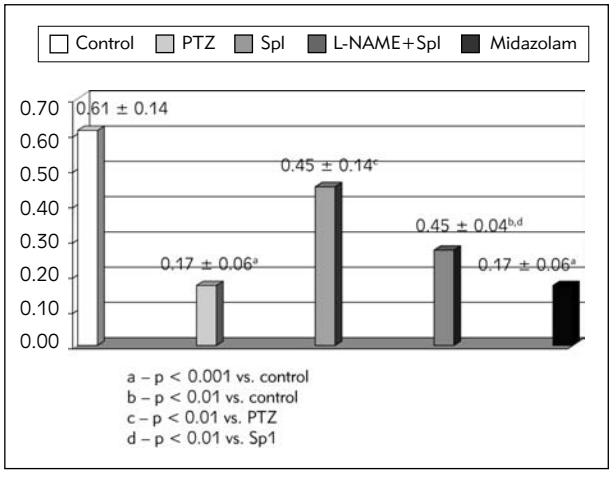

Figure 4 Spermine and L-NAME effects on arginase activity in rat hippocampus during seizures (U/mg prot.).

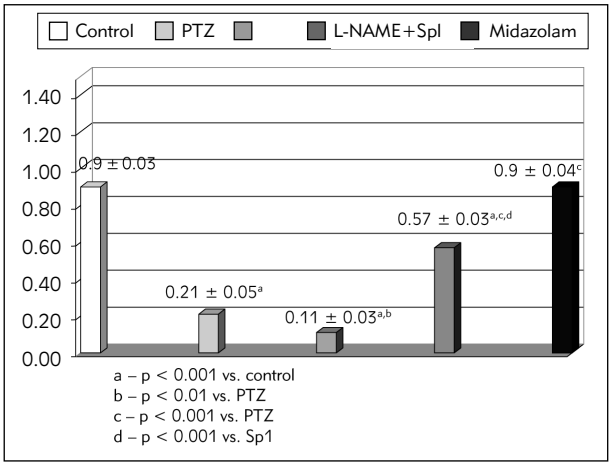

Figure 6 Spermine and L-NAME effects on DAO activity in rat hippocampus during seizures (U/mg prot.).

DAO activity in striatum and hippocampus

In the striatum and hippocampus PTZ induces significant decrease of DAO activity (0.21 ± 0.02; 0.21 ± 0.05 U/mg prot.) ($p<0.001$) compared to the values in the control group (0.73 ± 0.04; 0.9 ± 0.03) (*Figures 5* and *6*).

In both brain structures spermine intensified PTZ effects of this enzyme activity (0.1 ± 0.02; 0.11 ± 0.03) ($p<0.001$; $p<0.01$ vs. PTZ). Applied before Sp and PTZ, L-NAME induced the increase of DAO activity in the hippocampus (0.57 ± 0.03) ($p<0.001$), but not in the striatum.

Midazolam protective effects on DAO activity are noticed in both brain structures. In the hippocampus, this effect is more pronounced (0.90 ± 0.04 U/mg prot.) and statistically highly significant ($p<0.001$) compared to the PTZ group (0.21 ± 0.05 U/mg prot.), and the obtained values are at the control level.

PAO activity in striatum and hippocampus

Figures 7 and *8* show the effects of applied substances on PAO activity. In both examined structures PTZ caused significant increase of this enzyme activity (1.49 ± 0.2; 3.9 ± 0.39) ($p<0.001$) in comparison with control values (0.09 ± 0.03; 0.98 ± 0.1).

The used dose of spermine pronounced PTZ effects (1.82 ± 0.37; 4.97 ± 0.2). Pretreatment with L-NAME more strongly potentiates the effects of two other substances (2.1 ± 0.19; 5.45 ± 0.37). Midazolam pretreatment leads to diminished PAO activity compared to PTZ, but this decrease is statistically significant only in the hippocampus ($p<0.001$).

Discussion

In our experiment, the application of pentazol induced highly significant increase of NO production in both examined brain structures, which is in accor-

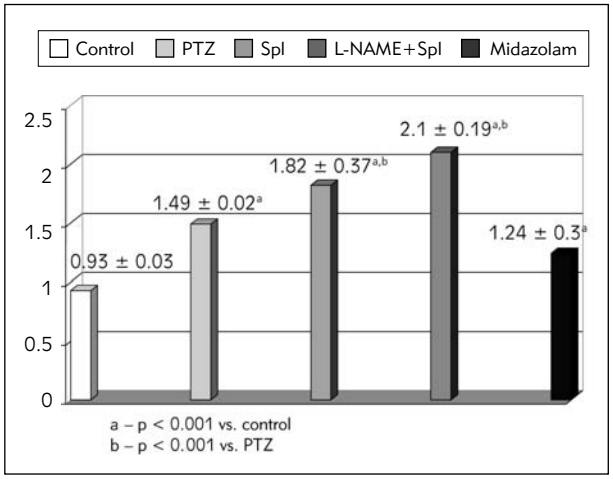

Figure 7 Spermine and L-NAME effects on PAO activity in rat striatum during seizures (U/mg prot.).

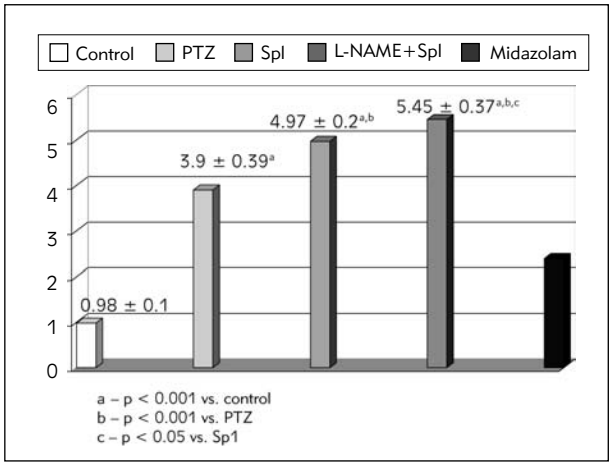

Figure 8 Spermine and L-NAME effects on PAO activity in rat hippocampus during seizures (U/mg prot.).

dance with the data from the literature (13, 14). The mechanism by which PTZ induces seizures leads to both inhibitory and excitatory neurotransmitter systems changes. Disinhibition of the inhibitory transmitter – GABA (15) or activation of NMDA receptors (16), or both, could be the factors involved in the initiation and generalization of seizures induced by PTZ. Since the main mechanism of these two factors' effects is the increase of intracellular Ca^{2+} and activation of Ca^{2+}/calmodulin-dependent enzymes, this could be the mechanism by which PTZ causes NOS activation.

It is well known that polyamines open calcium channels, releasing Ca^{2+}, which results in these channels' conformational changes. Polyamines can also mobilize intracellular Ca^{2+} bound to acidophilic sites of plasma membrane and mitochondrial and EPR membranes by cation exchange reaction. The increase of citosolar calcium leads to the activation of NOS, as a Ca^{2+}-calmodulin/dependent enzyme, and augmented NO production. Carter et al. (17) proved that polyamines increase cGMP synthesis through the activation of NMDA receptors.

In our experiment, spermine pretreatment induced highly significant increase of nitrate and nitrite levels compared to the group treated by PTZ. Taking in account that it has been suggested that NMDA receptor of glutamate subtype activation is the predominant mechanism leading to NO production in the CNS (18), these results correlate with the above-mentioned literature data.

Although it has to be mentioned that polyamine ability to interact with nNOS is under strong influence of their intra- and extracellular concentrations, L-arginine level and availability of NADPH, spermine undoubtly exerts its proconvulsive effects, at least partly, through the NO signaling system.

N^{ω}-nitro-L-arginine methyl ester (L-NAME) is a nonselective competitive NOS inhibitor, acting prevalently on its constitutive isoforms (nNOS and eNOS) (19). As it was expected, L-NAME, applied before Sp and PTZ, significantly reduced NO production in the examined structures. The obtained effects were more pronounced in the hippocampus, which could be explained by the already documented presence of both eNOS and nNOS in this brain structure (2). It should be emphasized that the L-NAME effects completely correlated with the midazolam effects.

The obtained results point out the significant roles of NO and polyamines and the importance of their interactions during seizures induced by PTZ. Despite this, the correlation of NO increase and clinically proved PTZ and Sp convulsive effects is not sufficient basis to confirm that NO generally exerts proconvulsive effects.

Arginase activity in seizures

Interest in arginase as a possible regulatory enzyme is growing due to its potential to regulate the availability of arginine for the synthesis of NO, polyamines, agmatine proline and glutamate. It has been proven that sufficient quantities of arginase can limit the availability of arginine for NO synthesis by intact cells, as well as that arginase and NOS can compete for arginine in some conditions. It should be clarified whether they compete for an intracellular pool of arginine, as well as for an extracellular one. All authors agree that, in reality, the basis of an interplay between arginase and NOS is more complex than the fact that they use a common substrate. So, although PTZ-induced decrease of arginase activity in our experiment correlates with diminished NO production, the question is whether this decrease is the consequence of reduced arginine availability, or the factor which regulates its availability for the reaction catalyzed by NOS during convulsive activity.

The hypothesis that arginase may also regulate arginine availability for polyamine synthesis is supported by observations that arginase activity is often co-induced with ODC and that the cells that are deficient in arginase cannot proliferate in serum free medium unless ornithine or polyamines are provided.

Spermine-induced increase of arginase activity in relation to increased NO production upon Sp treatment points out the complexity of the mechanisms of arginase activity regulation and the interrelationship between arginase and NOS, which cannot be considered as simple competition for the same substrate.

Since L-NAME inhibits the pathway of NO synthesis, it was expected that its application would lead to arginase activity increase, but this NOS inhibitor reduced the increase caused by Sp. The literature data about arginine decarboxylase inhibition by L-NAME (20) drove us to postulate the hypothesis about the possible inhibitory effect of this arginine analogue upon arginase, as well.

DAO and PAO activity in seizures

In a dose of 1 mg/kg spermine diminishes DAO activity which is already significantly reduced upon the action of PTZ. This is a protective effect, since the higher polyamines/putrescine ratio is disturbed in cells during the increase of Sp level. So, the mechanisms of maintaining the putrescine pool in the CNS, such as diminished catabolism, are triggered in these conditions.

Described spermine effects on DAO activity completely correlate with PAO activity changes after Sp pretreatment. Pronounced PAO activity increase points out the intensive interconversion of spermine into putrescine, which is suggested by Hayashi (21) to be protective, considering its involvement in the regulation of GABA and putrescine levels during seizures. It was shown (22) that GABA synthesis from putrescine was increased in astrocytes of epileptic mice. Taking in account these facts, as well as different putrescine effects related to higher polyamines, it seems that this kind of CNS injury induces the reaction of »incomplete polyamine response«, i.e. triggering of mechanisms for putrescine increase and other polyamines decrease, leading to specific metabolic alterations of the polyamine cycle.

The application of L-NAME caused a significant increase of DAO activity in our experiment. Considering the fact that NO increases GABA concentration (23) and release in the brain (24), it is possible that during the reduction of NO production an alternative pathway of GABA synthesis from putrescine, catalyzed by DAO, is activated, which offers a logical explanation for the increased activity of this enzyme in these conditions.

Applied before spermine, L-NAME caused highly significant increase of PAO activity compared to the group treated by Sp and PTZ in combination, pointing out an intensified polyamine interconversion pathway. In the conditions of diminished availability of ornithine for putrescine synthesis, disturbance of putrescine/higher polyamines ratio and existing convulsive activity, this highly pronounced increase of PAO activity could be the result of CNS efforts to substitute the intracellular putrescine pool by the activation of polyamine interconversion into putrescine.

The obtained results prove a strong relationship between the NO signaling system and polyamine metabolism in the brain during seizures and the importance of their changes in this kind of CNS injury.

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Received: December 22, 2006

Accepted: January 26, 2007