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GASEOUS NEUROTRANSMITTER NITRIC OXIDE: ITS ROLE IN EXPERIMENTAL MODELS OF EPILEPSY

D. HRNČIĆ[1], ALEKSANDRA RAŠIĆ-MARKOVIĆ[1], JELICA BJEKIĆ-MACUT[2], VESELINKA ŠUŠIĆ [3], D. MLADENOVIĆ[4], D. DJURIC[1] and OLIVERA STANOJLOVIĆ[1†]

[1] *Laboratory of Neurophysiology, Institute of Medical Physiology "Richard Burian", Faculty of Medicine, University of Belgrade,* 11000 Belgrade, Serbia
[2] *CHC Bežanijska kosa, Faculty of Medicine, University of Belgrade,* 11000 Belgrade, Serbia
[3] *Serbian Academy of Sciences and Arts,* 11000 Belgrade, Serbia
[4] *Institute of Pathophysiology, Faculty of Medicine, University of Belgrade,* 11000 Belgrade, Serbia

Abstract - Epilepsy is one of the leading neurological disorders and affects 1-2% of the world's population. Generally, it is a result of an imbalance between excitatory and inhibitory phenomena in the central nervous system (CNS), but the mechanisms of its initiation and propagation still require further investigations. Experimental models represent one of the most powerful tools to better understand the mechanisms of epileptogenesis. Nitric oxide (NO) is gaseous molecule with pleiotropic physiological and pathological effects in almost all organ systems and intriguing biological relevance, especially in the CNS where it acts as a gaseous neurotransmitter. The role of NO in the generation of epilepsy is highly contradictory, since there is evidence of its anticonvulsive, as well as proconvulsive properties. Therefore, we will discuss in this review the involvement of NO-mediated signaling pathways in the mechanisms of epileptogenesis, taking into account the findings revealed in experimental studies on animal models of epilepsy.

Key words: Epilepsy, nitric oxide, animal models, gasotransmitters, homocysteine, lindane

INTRODUCTION

Nitric oxide (NO) is a gaseous molecule with distinct functions in almost all organ systems and intriguing biological relevance, especially in the central nervous system (CNS). From the discovery of the endothelial-derived relaxing factor in 1980 and its subsequent determination as NO (Furchgot and Zawadski, 1980; Murad, 1998), NO has become one of the leading research targets and the field of its research is one of the fastest growing areas in contemporary biosciences. Together with hydrogen sulfide (H_2S) and carbon monoxide (CO), NO belongs to the family of gaseous transmitters (also called gasotransmitters). Their common characteristics are as follows: a) small molecules of gas; b) freely permeable to membranes; c) endogenously and enzymatically generated in processes that can be regulated; d) well-defined physiological functions at physiologically relevant concentrations; e) their cellular effects may or may not be mediated by second messengers (Wang, 2002). Beside its function as a vasorelaxant, NO displays pleiotropic physiological and pathological effects (Guix et al., 2005). Due to its properties, NO plays a key role in a recently described form of interneuronal communication, characterized by the absence of synaptic contacts and lack of specific membrane receptors (Vizi, 2000). Although NO is involved in some major physiological process in the CNS such as learn-

ing and memory through long-term potentiation, it could also contribute to a number of neurological disorders including epilepsy (Guix et al., 2005). Its role in the generation of epilepsy is highly contradictory, since there is evidence of its anticonvulsive, as well as proconvulsive properties.

This review has focused on the contribution of NO-mediated signaling pathways in the initiation and propagation of epilepsy and it is based on the findings of experimental studies on animal models of epilepsy.

Epilepsy and its experimental models

Epilepsy is a chronic neurological disorder encompassing a diverse group of seizure disorders caused by a variety of morphological, cellular and molecular alterations of the brain primarily affecting the cerebral cortex and leading to recurrent epileptic seizures (Badway et al., 2009). Epileptogenesis, i.e. processes of alteration of a normal neuronal network into a network of synchronized hyperexcitable neurons, has been associated with an imbalance between inhibitory (gamma-amininobutyric acid (GABA)-mediated) and excitatory (glutamate-mediated) neurotransmission systems, in favor of the latter (McCormick and Contreras, 2001; Dalby and Mody, 2001; Avoli et al., 2005; Badway et al., 2009).

In developed countries, the incidence of epilepsy is around 50 per 100,000 people per year (Duncan et al., 2006). Thus, it is one of the major health problems. Despite an extensive search for adequate therapy, epilepsy remains poorly controlled in almost 40% of the patients (Loscher, 2002). Therefore, a better understanding of epileptogenesis is the only way for the development of new antiepileptic treatments and strategies.

Experimental models represent one of the most powerful tools to better understand the mechanisms of epileptogenesis. A number of very useful chronic experimental models of epilepsy that served to evaluate potential therapeutic treatments and drugs, has been developed and it is highly likely that no single model system could be useful for all types of epilepsy (Stanojlović and Živanović, 2004). These animal models involve experimental manipulation in which the epileptic condition is produced by different chemical compounds or electrical stimulation. Among others, commonly used experimental epilepsy models are those induced by N-methyl-D-aspartate (NMDA), pentylenterazole, pilocarpine, kainic acid, 4-aminopiridine, amygdala kindling, etc. (for review see Stanojlović and Živanović, 2004). In our laboratory, different animal models have been developed, including metaphit-induced audiogenic seizures (Stanojlović et al., 2002, 2004, 2007; Hrnčić et al., 2006, 2008), lindane- (Vučević et al., 2008, Mladenović et al., 2007, Hrnčić et al., 2009) and homocysteine-induced seizures (Stanojlović et al., 2009). We have shown that these experimental rat models of generalized epilepsy are suitable for the studies of epilepsy mechanisms and preclinical evaluation of potential avenues in antiepileptic treatment (Rašić et al., 2009a, 2011a).

Lindane (gamma-hexachlorocyclohexane), an organochloride extensively used as an insecticide, pesticide and scabicide (Li, 1999), interacts with the picrotoxin site within the $GABA_A$ receptor chloride channel leading to epileptic activity (Nyitrai et al., 2002). Seizures induced by lindane in rats represent an experimental model of generalized epilepsy (Vučević et al., 2008). On the other hand, homocysteine is a sulfur-containing amino acid endogenously generated during the metabolism of methionine (Hoffer, 2004). It has recently been recognized, together with its highly reactive thioester homocysteine thiolactone (Hct), as one of the most potent excitatory agents in the CNS (Jakubowski 2004; Perla-Kajan, 2007; Herrmann and Obeid, 2011). The primer mechanism of its convulsive properties has been assigned to the activation of NMDA and group I metabotropic glutamate receptors (Throen, 2005). Stanojlović et al. (2009) showed recently that acute administration of Hct to adult rats significantly alters the neuronal circuits, leading to epileptogenic activity in the electroencephalogram (EEG) with characteristic

spike-and-wave discharges (SWD), and convulsive episodes in animal behavior. Hct-induced seizures are accepted as a suitable model of generalized epilepsy in which the coexistence of convulsive and absence-like seizures has been proven (Rašić et al., 2009b, 2011b).

The aforementioned experimental models of epilepsy have been extensively used to investigate the role of NO-mediated signaling pathways in the process of epileptogenesis.

Biosynthesis of NO

NO is produced from L-arginine by the activity of the family of enzymes known as NO synthases (NOS). Three different forms of NOS have been identified as a product of distinct genes. Neural (nNOS) and endothelial NOS (eNOS) are Ca^{2+}/calmodulin-dependent enzymes, while inducible NOS (iNOS) shows Ca^{2+}-independent properties. Mitochondrial NOS (mtNOS) is actually an isoform of nNOS located in the inner mitochondrial membrane (Elfering et al., 2002). Each NOS peptide consists of a C-terminal reductase domain and an N-terminal oxygenase domain that forms the active site of the enzyme (Hemmens and Mayer, 1998). Its catalytic activity depends on the cofactors nicotinamide adenine dinucleotide phosphate (NADPH), tetrahydrobiopteridin (BH_4), flavin adenine dinucleotide (FAD), flavin mononucleotide (FMN), oxygen (O_2) and protoporphyrin IX (Stuehr, 1999).

Constitutive isoforms are responsible for the synthesis of physiologically vital amounts of NO (Bredt and Snyder, 1990), while iNOS produces high amounts of NO lasting hours or days (Aktan, 2004). All three NOS isoforms are expressed in various brain cells and may affect brain function. Neurons produce NO mostly by the activation of nNOS (Knowles and Moncada, 1994), which is found in the cerebral cortex, hippocampus, corpus striatum and cerebellum, as well as in some ganglion cells of the autonomic nervous system (Zhou and Zhu, 2009). iNOS is found to be expressed in the brains of humans with epilepsy, as well as in some spontaneously epileptic mice (González- Hernández et al., 2000; Murashima, 2000).

Pharmacological modulation of NO levels could be achieved through the application of NO donors, non-selective and selective NOS inhibitors. Of the donors, L-arginine, as an NO precursor, and sodium nitroprusside (SNP), have been used the most extensively in pharmacological studies of NO effects. N-nitro-L-arginine methyl ester (L – NAME) is a non-selective NOS inhibitor widely used to decrease NO levels. 7-nitroindazole and aminoguanidine are selective inhibitors of nNOS and iNOS, respectively, and are commonly used to investigate the role of nNOS and iNOS in brain functioning (Babbedge et al., 1993; Griffiths et al., 1993).

Modulation of NO-signaling in experimental models of epilepsy

Numerous reports have indicated an anticonvulsive activity of NO in different experimental models of epilepsy. NOS inhibitors have been reported to increase seizure severity induced by an intramygdaloid injection of kainate, and to increase the amygdala-kindling rate (Rondouin et al., 1992, 1993). It has also been found that L-arginine (150-600 mg/kg, i.p.) increased dose-dependently the dose of kainate necessary to produce clonic convulsions in 50% of the animals (Przegalinski et al., 1994). Results obtained on the status epilepticus induced by the systemic administration of kainic acid are consistent with the above-mentioned data (Alabadi et al., 1999). L-NAME caused an increase in both duration and seizure severity in a mice model of generalized epilepsy induced by intracerebroventricular (i.c.v.) administration of NMDA (Buisson et al., 1993), while NOS inhibition has also been reported to potentiate the seizures induced by an i.c.v. injection of quinolinate to rats (Haberny et al., 1992). NOS inhibitors also facilitated focal seizures induced by aminopyridine in rats (Boda and Szente, 1996). Tsuda et al (1997) showed an aggravation of DMCM (full inverse agonist of $GABA_A$)-induced seizures by NOS inhibitors in mice. The anticonvulsive role of NO has also been showed in picrotoxin-

induced convulsions (Paul and Ekambaram 2003; Paul et al., 2002; Paul and Ekambaram, 2005) and penicillin-induced epileptiform activity in rats (Marangoz and Bagirici, 2001; Ayyildiz et al., 2007). The involvement of NO in convulsions induced by nicotine in mice has been reported (Tutka et al 2007). L-arginine protected genetically epilepsy-prone rats and DBA/2 mice from sound-induced convulsions (Smith et al., 1996).

In the case of pentylenterazole (PTZ), a $GABA_A$ -mediated convulsive agent, NOS inhibitors may inhibit seizures (Osonoe et al., 1994; Hara et al., 1996; Kaputlu and Uzbay, 1997; Bashkatova et al., 2000; Han et al., 2000), although several reports have indicated no effect on PTZ-induced seizures (Przegaliñski et al., 1996; Urbanska et al., 1996; Han et al., 2000). However, a recent study has revealed that mice lacking the nNOS gene ($nNOS^{-/-}$) exhibited severe seizures following the administration of a sub-convulsive dose of PTZ and that a convulsive dose was lethal in all of the mice, following tonic convulsions (Itoh and Watanabe, 2009). Treatment of pilocarpine-induced seizures with NOS inhibitors has been reported to augment (Del-Bel et al., 1997; Starr and Starr, 1993), to inhibit (Van Leeuwen et al. 1995) or to be without effects (Noyan et al., 2007) on the epileptic activity. In the lithium-pilocarpine-induced seizure model, L-arginine at a dose of 300 mg/kg has been found to act as an anticonvulsant (Noyan and Gulec, 2000).

NO has been reported as a proconvulsant agent in several seizure models (Bagetta et al., 1992; De Sarro et al.,1991, 1993; Mollace et al., 1991; Urbanska et al., 1996; Proctor et al., 1997; Lu et al 1998; Borowicz et al., 2000; Yasuda et al., 2001; Sardo et al., 2006). In line with this, in a model of "maximal dentate activation" (MDA), the inhibition of nNOS caused an increase of the MDA onset time and a decrease of MDA-induced spiking afterdischarges duration. On the contrary, the administration of L-arginine caused opposite effects: a decrease in the MDA onset time and an increase of MDA and afterdischarges duration (Ferraro et al., 2004).

NO in an experimental model of homocysteine thiolactone-induced epilepsy: mechanisms of its anticonvulsive properties

Recently, we showed that the systemic administration of increasing doses of L-arginine in a dose-dependent manner significantly decreased seizure incidence and the prolonged latency time to the first seizure elicited by a convulsive dose of Hct (Hrnčić et al., 2010). On the other hand, pretreatment with L-NAME, in a dose-dependent manner, increased seizure incidence and severity and shortened the latency time to the first seizure following injection with a sub-convulsive dose of Hct. In the same study, L-arginine decreased and L-NAME increased the median number of SWD per rat, while the duration of individual SWD was not altered. These results showed the functional involvement of NO in the Hct-induced convulsive activity.

Furthermore, the involvement of nNOS in Hct-induced seizures was determined using a pharmacological inhibition of this enzyme by 7-nitroindazole (Hrnčić et al., 2012). Congruent results with those obtained using non-selective inhibition were obtained. Namely, systemic administration of 7-nitroindazole, a selective nNOS inhibitor, showed a tendency to increase seizure incidence, decrease latency time to the first seizure, increase the number of seizure episodes per rat and increase the severity of seizures induced by Hct in rats. The contribution of iNOS-derived NO in epileptogenesis caused by Hct was recently demonstrated using aminoguanidine (Hrnčić et al., 2012).

The anticonvulsive activity of NO in Hct-induced seizures could be explained by several mechanisms, including the relationship of NO with the NMDA and GABA receptors.

NO could modulate the NMDA receptor activity by interacting with the -SH group of the NMDA redox modulatory site via the process of S-nitrosylation. This results in the downregulation of this receptor complex (Lipton et al., 1993), preventing the neurotoxic effects of an excessive Ca^{2+} influx during homo-

cysteine-induced "overstimulation" of NMDA and mGluRs I receptors. Moreover, in cultured rat cortical neurons Kim et al. (1999) demonstrated that NO could ameliorate the effects of adverse excitotoxicity by S-nitrosylation. In addition, it should be pointed out that NO induces the reduction of glutamate by the activation of the glial cells (Nanri et al., 1996).

The co-localization of NOS and GABA has been demonstrated in experimental studies (Wang et al., 1997). Paul and Ekambaram (2005) suggested that NO inhibits GABA transaminase. The basal NO levels induce depression, while high concentrations of NO increase the GABA release (Getting et al., 1996). This could explain the anticonvulsive NO properties. Unlike to homocysteine, which increases oxidative stress by the production of reactive oxygen species (Ramakrishnan, 2006), NO can act as a neural protector, due to the formation of S-nitroso-L-glutathione, an antioxidant (Rauhala et al., 1998).

It is known that homocysteine and Hct can cause neurodegeneration, synaptic dysfunction and neuronal death by promoting DNA damage and the activation of apoptotic signaling (Mattson and Shea, 2003), which contribute to the observed high lethality upon Hct administration in higher doses. Inhibition of caspases by S-nitrosylation (Mannick et al., 1999) and the expression of cytoprotective genes (Hao et al., 1999) by NO could explain the neuroprotective effects of NO demonstrated in our experiments by a reduction in lethality (Hrnčić et al., 2010).

NO in an experimental model of lindane-induced seizures: mechanisms of its proconvulsive properties

Investigating the role of NO in lindane seizures, we showed that the systemic application of L-arginine in a dose-dependent manner significantly increased seizure incidence and severity and shortened the latency time to the first seizure elicited by a subconvulsive dose of lindane (Hrnčić et al., 2011). On the contrary, pretreatment with L-NAME decreased seizure incidence and severity, and prolonged the latency time to the first seizure following injection with a convulsive dose of lindane. Hence, the study elucidated the functional involvement of NO in lindane convulsive activity, i.e. it revealed NO as an endogenous mediator in this seizure model.

No signs of ictal activity were recorded in the EEG of rats treated with the highest applied doses of L-arginine or L-NAME (Hrnčić et al., 2010, 2011). Contrary findings to ours have been reported by Ferraro et al. (1999), showing that NOS inhibition elicited ictal activity in rat brain in the form of spikes, polyspikes and spike and waves. We showed an increase in the number and duration of the ictal periods in the EEG of rats receiving L-arginine prior to lindane and a decrease of this number in rats pretreated with L-NAME (Hrnčić et al., 2011). These results support the proconvulsive role of NO in lindane seizures.

Different mechanisms could be involved in the proconvulsant effects of NO in lindane seizures. Increased levels of NO activate guanylate cyclase (GC) and markedly increase the production of cyclic guanosine-3,5-monophosphate (cGMP), which, as a secondary messenger, stimulates protein kinase G, phosphorylates the $GABA_A$ receptor and inactivates it (Robello et al., 1996). Lindane evokes seizures mainly through the blockade of the $GABA_A$ receptor complex (Anand et al., 1998; Nyitrai et al., 2002; Sunol et al., 1997). Therefore, the proconvulsant effect of NO could be explained by its cooperation with lindane in $GABA_A$ receptor inactivation. NO increases Ca^{2+} intracellular levels (Willmott et al., 2000, Horn et al., 2002) and by this mechanism could potentiate the activity of lindane in Ca^{2+} mobilization. The engagement of excitatory amino acids has been proven in lindane seizures (Blaszczak et al., 1998), while NO is documented to enhance the release of glutamate, the main excitatory neurotransmitter, in different brain regions according to the study of Marcoli et al. (2006). Therefore, the synergistic effect of NO and lindane is also possible in this activity.

CONCLUSIONS

Although numerous attempts have been made to precisely define the role of NO in the mechanisms

of epileptogenesis, this remains an open question. We demonstrated both the anticonvulsive and proconvulsive properties of NO-signaling in different experimental models of epilepsy and reviewed the possible mechanisms of its activity. Observed differences in the effects of NO could, at least in part, be explained by the diversity of the molecules interacting with NO, the seizure models employed, the type and dose of drugs used in order to modify brain NO levels and the strain of animal. The growing evidence for the involvement of other members of the gaseous neurotransmitter family (H_2S and CO) in the process of epileptogenesis and their multiple interactions with NO will contribute to a better understanding of the NO-mediated regulation of neural excitability.

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