REVIEWS

Possible Role of Nitric Oxide in Pathologies of the Central Nervous System

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Nitric oxide is a gaseous chemical messenger involved in the regulation of respiration, cardiovascular homeostasis, immunity, macrophagal activity, gene expression, morphogenesis, synaptic plasticity of the nervous tissue, memory, and release of neurotransmitters. This compound acts as a pathological factor in the states associated with neurodegeneration, ischemia, stroke and convulsions.

Key Words: *nitric oxide; NO-synthase; cerebral ischemia; convulsions*

The discovery that nitric oxide (NO) acts as a polyfunctional physiological regulator in a major breakthrough in modern biology [5,8,31]. This agent is involved in the regulation of respiration, cardiovascular homeostasis, immunity, macrophagal activity, gene expression, morphogenesis, synaptic plasticity of the nervous tissue, memory, and presynaptic release of neurotransmitters [14,18,31].

Nitric oxide also acts as a pathogenic factor in conditions associated with neurodegenerative diseases, ischemia, stroke, epilepsy, and other convulsive disorders. Activation of N-methyl-D-aspartate (NMDA)-receptors in a culture of cerebellar granular cells results in the release of NO [21]. Local administration of NMDA into the hippocampus induces neuronal death, i.e., elicits the effect of glutamate neurotoxicity [30]. Similar effects are produced by the NO donors sodium nitroprusside, (±)-S-nitroso-N-acetyl-D-penicillamine (SNAP), and S-nitrosoglutathione (SNOG).

The specificity of NO as a biological messenger is determined by its high lability, short life and high reactivity [3,7]. It has been generally accepted for a long time that the brain functions are mediated exclusively by excitatory and inhibitory neurotransmitters. Recent studies showed that biogenic amines, amino acids, and neuropeptides act as neurotransmitters.

Nitric oxide represents a novel family of unusual regulatory molecules with neurotransmitter activity [10,20,31]. Conventional neurotransmitters are synthesized by specific enzymes deposited in synaptic vesicles of nerve endings and are released into the synaptic cleft by exocytosis in response to depolarization of the synaptic membrane by nerve pulse. Neurotransmitter activity of NO is realized in a different way. Nitric oxide is synthesized by NO-synthase (NOS) from the amino acid L-arginine, implying that NO synthesis is the key component in the regulation of the functional activity of NO. Biological effects of NO are determined predominantly by the small size of the molecule, high reactivity, and ability to diffuse in biological tissues. Bearing in mind these properties, NO has been referred to as a retrograde messenger [14].

Here we review the data on a possible participation of NO in some CNS pathologies, such as ischemia, stroke, and convulsive disorders. It was hypothesized that NO is a key pathophysiological factor in these conditions [14,18,31,33,39].

Biosynthesis of NO and properties of NO-synthase. In humans and animals NO is synthesized by

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enzymatic oxidation of the guanidine group of L-arginine with formation of L-hydroxyarginine as an intermediate product. Hydroxylation is catalyzed by the cofactor tetrahydrobiopterin, it is calcium-calmodulin-dependent and is blocked by carbon monoxide. At the next (stoichiometric) stage of reaction L-citrulline and the free radical NO are formed. Constitutive (cNOS), which is always present in the tissue, and inducible (iNOS) isoforms of NOS have been identified. According to tissue localization, NOS has been defined as neuronal (nNOS), endothelial (eNOS), and macrophagal (macNOS). Neuronal and endothelial NOS are predominantly constitutional, while macrophagal NOS is inducible [10].

The enzyme generates NO in response to receptor stimulation, for instance, activation of NMDA receptors by cerebral ischemia or Ca^{2+}-modulating agents. Constitutive NOS is present predominantly in endothelial and nerve cells [41], while iNOS is generated in response to cytokines. Inducible NOS is localized in macrophages, endothelial cells, hepatocytes, and smooth muscle cells [10]. Induction of this isoform by cytokines increases macrophagal toxicity towards tumor cells, bacteria, fungi, and protozoa.

Since NO is a highly labile and rapidly diffusing compound, it cannot be deposited, released, and uptaken from synaptic cleft by presynaptic endings by the known mechanisms. The first attempts to purify NOS were unsuccessful, since the enzyme is rapidly inactivated during isolation. Neuronal NOS was purified from the brain after its coupling to calmodulin had been discovered [9,10]. Then NOS was isolated from endothelium (eNOS) and macrophages (macNOS) [10]. The structure of NOS implies the existence of several regulatory mechanisms. It was demonstrated that the enzyme is inhibited by the structural analogs of its substrate: nitro- and alkyl derivatives of L-arginine. In the presence of an arginine excess the activity of these inhibitors decreases, indicating a competitive mechanism associated with the action on the active center of the enzyme. NOS isoforms differ in the sensitivity to inhibitors: N^{ω}-nitro-L-arginine inhibits neuronal and endothelial isoforms (K_i=200-500 nM), while L-arginin-N^{G}-amine is a potent blocker of macNOS (K_i=1-5 nM). Inhibitors with high specificity to NOS isoforms are prospective candidates for the use in clinical practice. Since NO may participate in a number of CNS pathologies, NOS inhibitors have been extensively studied as potential neuroprotectors in ischemia, epilepsy, and some other states.

A new group of selective inhibitors of neuronal NOS — 7-nitroindazole derivatives — has been recently described [32].

It was demonstrated that 7-nitroindazole selectively inhibits nNOS (IC_{50}=0.71±0.001 μM, for rat cerebellum homogenate), producing antinociceptive and anticonvulsive effects [33] without antihypertensive effect typical of other NOS inhibitors.

Calcium ions and calmodulin play an important role in the regulation of NOS activity. Activation of brain NMDA-glutamate receptors triggers Ca^{2+} influx into the cell and activation after calcium binding to calmodulin. Thus, NO acts as a component of intracellular glutamatergic transduction. Nitric oxide interacts with guanylate cyclase [14]. It was demonstrated that injection of NMDA into the hippocampus produces neurotoxic effect which is abolished by NOS inhibitors [30]. The involvement of NO in this effect is indirectly confirmed by the observation that metabolic donors of NO, for example, sodium nitroprusside, also produce this effect. In the vascular wall, generation of NO is triggered by activation of endothelial muscarinic receptors by acetylcholine with subsequent initiation of the phosphoinositide cycle, and Ca^{2+} generation, which stimulates NO formation. This cascade of biochemical reactions leads to vasodilation. Neuronal and endothelial NOS are sensitive to calmodulin inhibitors, specifically, to the phenothiazine neuroleptics aminazine and trifluoroperazine (IC_{50}=10 μM).

By contrast, inducible NOS of macrophagal or any other origin is not stimulated by Ca^{2+} and is not blocked by calmodulin inhibitors, although it bears the calmodulin-recognizing site. Generation of iNOS is triggered by γ-interferon or *E. coli* lipopolysaccharide [6].

Phosphorylation involving cAMP-, cGMP, or Ca^{2+}-calmodulin-dependent protein kinases or protein kinase C is an important mechanism of NOS regulation. It was demonstrated that phosphorylation inhibits the enzyme activity [8]. On the other hand, the protein phosphatase calcineurin dephosphorylates NOS, thus increasing its activity [15]. Thus, phosphorylation provides a multilevel regulation of the enzyme activity. It was reported that tetrahydrobiopterin is involved in the regulation of NOS activity [27]. Both nNOS and eNOS are constitutive isoforms, i.e., their activation requires no protein synthesis. However, in some pathologies (trauma or stroke) *de novo* protein synthesis was observed [18]. Human nNOS and eNOS genes are located in the 12th and 7th chromosome, respectively [35].

In addition to macrophages, iNOS was identified in human hepatocytes and chondrocytes. The enzyme was cloned [23]; its gene is located in the 17th chromosome [35].

NO in the nervous system. Nitric oxide has been regarded as neuronal messenger since the discovery

that it is produced by the cerebellum granular cells in response to the glutamate receptor agonists [21, 22]. The short life of NO (several seconds) hampered quantitation and identification of NO-producing neurons in the brain. Therefore, researchers focused their attention on nNOS. Localization of this enzyme in the brain was studied by immunohistochemical methods and *in situ* hybridization [9]. Neuronal NOS is localized predominantly in glutamatergic granular cells and GABAergic basket cells of the cerebellum and cortical neurons, where it is colocalized with somatostatin, neuropeptide Y, or GABA. A similar situation was observed in the corpus striatum, where NOS-positive neurons contained the same peptides. It is noteworthy that all NOS-positive neurons also contain NADPH-diaphorase [13,24]. The brain cortex and corpus striatum contain 1-2% nNOS-positive neurons of the total neuronal population. Pyramidal cells in CA1 area practically do not contain nNOS, while high levels of eNOS were detected in the *gyrus dentatus* granular cells and CA3 area [17].

NO and brain ischemia. Association of NO with neuromediatory function of glutamate excited the interest into the role of NO in pathophysiological mechanisms underlying cerebral circulation disorders, brain ischemia, and stroke [18,34]. It should be noted that not only nNOS but also eNOS can participate in the development of these pathologies. Both enzymes have similar properties, the major difference consisting in their localization: nNOS is localized in the cytosol, while eNOS is a membrane-bound protein [19]. Soluble guanylate cyclase, which is localized in vascular smooth muscle cells, is the primary target of NO. During deficiency of L-arginine (normal substrate), nNOS generates superoxide anion and hydrogen peroxide, which produce neurotoxic effect in ischemia [38]. Presumably, iNOS plays even more important pathophysiological role in the development of brain ischemia and stroke, since after stimulation with cytokines this enzyme generates an excess of NO which is toxic for neurons [27,31]. The cytotoxic effect of NO is probably guanylate cyclase-independent [40]. With the use of immunochemical methods it was demonstrated that NOS is present in the endothelium but not in the smooth muscle cells of cerebral blood vessels. *In situ* hybridization revealed the cNOS mRNA [28]. Vasodilating activity of NO toward cerebral blood vessels has been extensively investigated [18]. NOS inhibitors act as vasoconstrictors, reducing basal cerebral blood flow [25]. In this review we do not discuss the participation of NO in the regulation of cerebral circulation in detail. We shall mention some attempts of using NO donors and NOS inhibitors as potential modulators of ischemic damage to the brain in modeled ischemia and neuronal death.

During ischemia, increased production of NO associated with NMDA-glutamate receptors may produce both negative (enhanced formation of free radicals, for example, peroxynitrite) and positive effect. Positive effect results not only from direct dilation of cerebral blood vessels but also from a possible retrograde blockade of NMDA-receptors [26].

EPR-spectroscopy showed that NO concentration in ischemic brain (bilateral occlusion of the common carotid arteries followed by reperfusion) markedly increases [39]. Administration of the NO donors sodium nitroprusside or 3-morpholinosydnonimine after occlusion of the middle cerebral artery increased local brain flow and reduced the infarction zone [43]. This effect was not observed after administration of the NO precursor L-arginine [37].

There is controversy over the neuroprotecting effect of NOS inhibitors (nitro and methyl derivatives of L-arginine) in brain ischemia. The NOS inhibitor L-NAME reduced the infarction zone, i.e., had a protective effect, in a rat model of focal ischemia [37]. However, other researches reported an opposite effect of this compound in a similar model [12,30]. It was hypothesized that NO produces a positive effect at the early stages (up to 8 min) and a negative (neurotoxic) effect at late stages of ischemia [37].

Mouse lacking NOS isoform provided a new model for the investigation of molecular mechanisms operating in brain ischemia. The finding that mice with genetically determined nNOS deficiency are more resistant to brain ischemia indicates that NO plays an important role in the development of ischemia-induced brain infarction.

NO and convulsive disorders. The data on functional association between NO generation in the brain and activation of glutamatergic neurotransmission [20] imply that NO is involved in pathophysiological mechanisms underlying convulsive disorders. It was reported that NO is involved in the development of epileptic convulsions [16,24], i.e., the brain content of NO increases in convulsions induced by kainic acid, an agonist of glutamate receptors [33]. There is evidence that NO acts both as pro- and anticonvulsant [1,2,33]. Generally, indirect approaches (the use of metabolic precursors, NO donors, or NOS inhibitors) to the evaluation of the role of NO in convulsion have been employed. These studies were hampered by the absence of adequate methods for quantitative determination of NO.

A direct quantitative method [5] enabled us to determine the rate of NO generation in rat brain by formation of paramagnetic mononitrosyl iron complexes between NO and diethyldithiocarbamate (MNIC—DETC). The formation of MNIC in rats was provided by administration of Na-DETC (500 mg/kg)

and Fe^{2+} citrate. After a 30-min period, measurements of NO content were carried out in frozen brain (liquid nitrogen). The concentration of the MNIC—DETC complex measured by the EPR signal reflected NO content in the brain [1,2,6,33]. In our studies convulsions were induced by the maximum electric shock, corazol, and the glutamic decarboxylase inhibitor thiosemicarbazide which lowers the GABA level in the brain [1,2].

The brain content of the MNIC—DETC complex during convulsions induced by the maximum electric shock was about 4-fold higher than in the control rats: 5±1 *vs.* 1.5±0.5 nmol/g wet tissue. Production of NO in the brain during convulsions was prevented by administration of N^{ω}-nitro-L-arginine, a NOS competitive inhibitor, although clonic convulsions were observed.

Brain content of NO during thiosemicarbazide-induced clonic convulsions was considerably higher than in control rats. Thus, the NO concentration markedly increases in convulsions of various origins. This finding is consistent with the results of experiments in which convulsions were induced with kainic acid [33]. Our data provide no answer to the question whether enhanced generation of NO from L-arginine in rat brain cortex is associated with activation of neuronal or endothelial NOS.

It should be noted that during tonic convulsions induced by electric shock the NO level was significantly higher than during corazol- or thiosemicarbazide-induced clonic convulsions, implying that there is no direct relationship between the nature of convulsions (clonic or tonic) and increase in the brain content of NO. Presumably, NO is not always necessary for the development of convulsive attacks, judging from the finding that the MNIC—DETC signal is not detected during convulsion induced by electric shock in rats treated with the NOS inhibitor N^{ω}-nitro-L-arginine.

In a corazol model, convulsions result from blockade of chlorine channel of the $GABA_A$-receptor complex. In this model, the intensity of lipid peroxidation, which reflects corazol neurotoxicity, was measured in parallel with NO determination. The NO precursor L-arginine and the NOS-inhibitor N^{ω}-nitro-L-arginine were used. Experiments were performed on male albino rats weighing 180-300 g. The times of the following parameters of convulsive attack were recorded: first convulsion, first false attack of clonic convulsions, true clonic convulsion, tonic convulsions with extension of the fore limbs, and full tonicoextensive attack. Our goal was to find out whether corazol-induced convulsions are influenced by the excess of L-arginine and blockade of NO synthesis with a NOS inhibitor. Both arginine (300 mg/kg) and the inhibitor (10 or 250 mg/kg) were injected intraperitoneally. The NO content was measured by EPR spectrometry [4-6]. The content of secondary products of lipid peroxidation was determined spectrophotometrically by measuring the concentration of thiobarbituric-acid reactive substances (TBARS) [36].

L-Arginine had no appreciable effect on any phase of corazol-induced convulsive attack. At 10 mg/kg N^{ω}-nitro-L-arginine significantly decreased the latency of the first convulsion and prolonged (2-fold) the period of true clonic convulsions. At 250 mg/kg the inhibitor produced a similar but more pronounced effect and delayed clonic convulsions.

The NO content in brain cortex increased almost 5-fold compared with the control, indicating that NO has a major role in this process. A considerable increase in the TBARS concentration (by 60%) was observed during convulsions. This increase was not prevented by N^{ω}-nitro-L-arginine (250 mg/kg), suggesting that elevation of NO is not directly related to lipid peroxidation.

At 300 mg/kg L-arginine had no effect on the parameters of convulsive attack, while the NOS inhibitor N^{ω}-nitro-L-arginine (10 mg/kg) markedly prolonged the latency of true clonic convulsions. A similar but more pronounced effect this inhibitor produced at 250 mg/kg and delayed clonic convulsions. Interpretation of these observations is difficult particularly in the light of the evidence that inhibition of NOS enhances convulsions of various origins [11]. It is interesting to note that this is consistent with our observation that NOS inhibition shortens the latency of the first convulsion in response to corazol. It can be suggested that NO exhibits its protective (in our case anticonvulsive) activity only at the initial phase of convulsive attack, without affecting its subsequent phases. The possibility that N^{ω}-nitro-L-arginine inhibits neuronal excitability, thus slowing down generalization of convulsions, cannot be ruled out. It was reported that this inhibitor has neuroprotective activity [43].

Thus, our results are consistent with the concept on the triggering role of NO in convulsive states [33]. According to this concept, NO acts as a retrograde neurotransmitter and triggers the cascade of reactions that limit generalization of convulsions, i.e., fulfills the function of an endogenous anticonvulsant [42]. In fact, reduction in the latency of the first corazol-induced convulsion caused by NOS inhibitor may be due to the fact that the mechanisms of retrograde blockade of NMDA-receptors do not operate in the absence of NO [26]. Molecular mechanisms underlying NO generation so far remain obscure. It can be suggested than the initial stages of the "triggering" reaction can be common and include, for example,

activation of NMDA-glutamate receptors located in various brain structures and participating in a number of reactions, such as Ca^{2+} inflow, guanylate cyclase activation, and generation of free radicals with subsequent neurotoxic effect [20,29].

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REFERENCES

1. V. G. Bashkatova, G. Yu. Vitskova, V. B. Narkevich, *et al.*, *Neirokhimiya*, **13**, No. 2, 110-115 (1996).
2. V. G. Bashkatova, E. S. Kosacheva, V. D. Mikoyan, *et al.*, *Dokl. Ross. Akad. Nauk*, **348**, No. 1, 119-121 (1996).
3. A. A. Boldyrev, *Neirokhimiya*, **12**, No. 1, 3-13 (1995).
4. A. F. Vanin, *Biofizika*, **38**, 751-761 (1993).
5. A. F. Vanin, P. I. Mordvintsev, and A. L. Kleshchev, *Stud. Biophys.*, **102**, 135-137 (1984).
6. V. D. Mikoyan, L. N. Kubrina, and A. F. Vanin, *Biofizika*, **39**, 915-918 (1994).
7. V. P. Reutov, *Usp. Biol. Khimii*, **35**, 189-228 (1995).
8. D. S. Bredt, C. D. Ferris, S. H. Snyder, *et al.*, *J. Biol. Chem.*, **267**, 10976-10981 (1992).
9. D. S. Bredt, C. E. Glatt, P. M. Hwang, *et al.*, *Neuron*, **7**, 615-624 (1991).
10. D. S. Bredt and S. H. Snyder, *Annu. Rev. Biochem.*, **63**, 175-195 (1994).
11. A. Buisson, N. Lakhmeche, C. Verrecchia, *et al.*, *Neuroreport*, **4**, No. 4, 444-446 (1993).
12. D. A. Dawson, K. Kusumoto, D. I. Graham, *et al.*, *Neurosci. Lett.*, **142**, 151-154 (1992).
13. T. M. Dawson, D. S. Bredt, M. Fotuhi, *et al.*, *Proc. Natl. Acad. Sci. USA*, **88**, 7797-7801 (1991).
14. T. M. Dawson and V. L. Dawson, *Neuroscientist*, **9**, 9-20 (1994).
15. T. M. Dawson, J. P. Steiner, V. L. Dawson, *et al.*, *Proc. Natl. Acad. Sci. USA*, **90**, 9808-9812 (1993).
16. V. L. Dawson, T. M. Dawson, E. D. London, *et al.*, *Ibid.*, **88**, 6368-6371 (1991).
17. J. L. Dinerman, T. M. Dawson, M. J. Schell, *et al.*, *Ibid.*, **91**, 4214-4218 (1994).
18. F. M. Faraci and J. E. Brian, *Stroke*, **25**, 692-703 (1994).
19. U. Forstermann, H. H. H. W. Schmidt, J. S. Pollack, *et al.*, *Biochem. Pharmacol.*, **42**, 1849-1857 (1991).
20. J. Garthwaite, *Trends Pharm. Sci.*, **14**, 60-67 (1991).
21. J. Garthwaite, S. L. Charles, and R. Chess-Williams, *Nature*, **336**, 385-388 (1988).
22. J. Garthwaite, G. Garthwaite, R. M. Palmer, *et al.*, *Eur. J. Pharmacol.*, **172**, 413-416 (1989).
23. D. A. Geller, C. J. Lowenstein, R. A. Shapiro, *et al.*, *Proc. Natl. Acad. Sci. USA*, **90**, 3491-3495 (1993).
24. B. T. Hope, G. J. Michael, K. M. Knigge, *et al.*, *Ibid.*, **88**, 2811-2814 (1991).
25. A. G. B. Kovach, C. Szabo, Z. Benyo, *et al.*, *J. Physiol. (Lond.)*, **449**, 183-196 (1992).
26. O. Manzoni, L. Prezeau, P. Marin, *et al.*, *Neuron*, **8**, 653-662 (1992).
27. M. A. Marletta, *J. Biol. Chem.*, **268**, 12231-12234 (1993).
28. P. A. Marsden, K. T. Schappert, H. S. Chen, *et al.*, *FEBS Lett.*, **307**, 287-293 (1992).
29. S. Moncada and A. Higgs, *N. Engl. J. Med.*, **329**, No. 27, 2003-2011 (1993).
30. S. Moncada, D. Lekieffre, B. Arvin, *et al.*, *Neuroreport*, **3**, 530-532 (1992).
31. S. Moncada, R. M. Palmer, and E. A. Higgs, *Pharmacol. Rev.*, **43**, 109-142 (1991).
32. P. K. Moore, P. Wallace, Z. Gaffen, *et al.*, *Br. J. Pharmacol.*, **110**, 219-224 (1993).
33. A. Mulsch, R. Busse, P. Mordvintsev, *et al.*, *Neuroreport*, **5**, 2325-2328 (1994).
34. P. R. Myers, R. L. Minor, R. Guerra, *et al.*, *Nature*, **345**, 161-164 (1990).
35. C. Nathan and Q.-W. Xie, *J. Biol. Chem.*, **19**, 13725-13728 (1994).
36. H. Ohkawa, N. Ohishi, and K. Yagi, *Anal. Biochem.*, **95**, 351-358 (1979).
37. D. A. Pelligrino, *Neurosurg. Anesthesiol.*, **5**, 221-231 (1993).
38. S. Pou, W. S. Pou, D. S. Bredt, *et al.*, *J. Biol. Chem.*, **267**, 24173-24176 (1992).
39. S. Sato, T. Tominaga, T. Ohnishi, *et al.*, *Biochim. Biophys. Acta*, **1181**, 195-197 (1993).
40. H. H. H. W. Schmidt, S. M. Lohmann, and U. Walter, *Biochim. Biophys. Acta*, **1178**, 153-175 (1993).
41. E. M. Schuman and D. V. Madison, *Annu. Rev. Neurosci.*, **17**, 153-183 (1994).
42. M. A. Theard, V. L. Baughman, Q. Wang, *et al.*, *Neuroreport*, **6**, 921-924 (1995).
43. F. Zhang and C. Iadecola, *Ibid.*, **4**, 559-562 (1993).