

Role of Nitric Oxide Synthases in Cerebral Ischemia

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Nitric oxide (NO) is a gaseous molecule which plays many important roles in the central nervous system and cardiovascular system. NO is generated by the activation of three distinct nitric oxide synthases (NOS); neuronal NOS (nNOS), inducible NOS (iNOS) and endothelial NOS (eNOS). Each of these enzymes is activated in response to cerebral ischemia which produces NO at different stages of ischemia promoting either beneficial or detrimental effects. In this review we attempted to document the role of NOS isoforms, NOS inhibitors and NO donors in experimental cerebral ischemia. We highlight the studies concerning the pathological and beneficial roles of NO produced by the different NOS isoforms in ischemic brain injury. The obvious contradiction about both pathological and beneficial roles of NO can be explained by the type of NOS producing it, cell type responsible, the amount produced and whether the NO undergoes further oxidation. Due to the dual effect of NO in brain ischemia there is necessity of developing new therapeutic strategies for selective and controlled inhibition of iNOS and nNOS activity and stimulation of eNOS activity.

Introduction:

Nitric oxide (NO), a free radical, generated by the nitric oxide synthase (NOS) enzyme is implied in many biological processes; such as vascular homeostasis, neurotransmission and inflammation¹. NO is a critical player in pathological and physiological processes in the central nervous system and also an important mediator which regulates the cellular and molecular events involved in cerebral ischemia². NO has both protective and deleterious effects which is dependent on many factors such as the type of NOS isoform secreting it, the cell type in which it is secreted and the stage at which it releases during ischemic brain injury³. There are three isoforms of NOS (nNOS or NOS-1, iNOS or NOS-2 and eNOS or NOS-3) developed in distinct cells which produce NO at different stages of ischemia promoting either beneficial or detrimental effects. The nNOS and eNOS, expressed in neuronal and endothelial cells respectively, are the constitutive enzymes which are dependent on calcium or calmodulin for their activity whereas iNOS, mainly expressed in astroglia and microglia, is the inducible isoform insensitive to calcium and requiring proinflammatory stimuli like endotoxins and cytokines for its activation⁴. NO released by the iNOS and nNOS mainly promote the detrimental effects and eNOS promotes the beneficial effects in cerebral ischemia. Considering these different roles of NOS isoforms, many inhibitors were developed which showed various effects in cerebral ischemia. These inhibitors work through several mechanisms like reduced formation of peroxynitrite and reactive oxygen species⁵, reduced vascular damage^{6,7} and

inhibition of apoptosis and necrosis^{8,9}. Some NOS inhibitors also work through additional neuroprotective mechanisms that do not involve inhibition of NO synthesis. Many selective inhibitors show therapeutic effects but they may not discriminate efficiently between iNOS and nNOS¹⁰. Also, these inhibitors are able to inhibit the detrimental effects in the animal models but they are not clinically efficacious in humans suggesting that there is need for further development of these inhibitors¹¹. Moreover due to the dual effect of NO in brain ischemia there is necessity of developing new therapeutic strategies for selectively inhibiting iNOS and nNOS and increasing eNOS activity. In this review we documented the present understanding of the NOS inhibition strategies used for neuroprotection against cerebral ischemia.

NO as a signaling agent:

NO was first identified as a endothelium-dependent relaxing factor (EDRF) by Furchgott and Zawadzki¹². The vasodilatory effects of NO are mediated by the relaxation of vascular smooth muscle. NO activates guanylate cyclase by binding to its heme moiety, resulting in increased cGMP levels and vasodilation. In addition, NO modulates other important aspects of vascular function, including leukocyte activation, platelet aggregation, interactions between the endothelium and circulating cells, and vascular smooth muscle cell proliferation. NO is also produced by nonvascular cells, including neurons, skeletal myocytes, and monocytes/macrophages. NO produced as a neurotransmitter in the autonomic nervous system innervates the gastrointestinal tract, urinary tract, and the respiratory tract, mediates smooth muscle relaxation in these tissues. It has diverse

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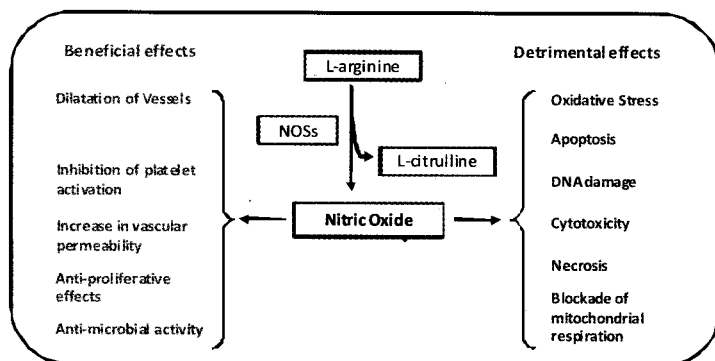


Fig. 1. The physiological and pathological effects of nitric oxide. NOS: nitric oxide synthase

role in the central nervous system. Nitric oxide (NO) is a critical player in pathological and physiological processes^{13,14}. The summary of various physiological and pathological effects of NO are depicted in the Figure 1.

NOSs and cerebral ischemia:

nNOS expression in cerebral ischemia:

nNOS (NOS-1) is the constitutive enzyme expressed in the brain, peripheral nervous system and skeletal muscles¹⁵. nNOS is the dimeric protein with one reductase domain and other oxygenase domain. In ischemia, anoxic depolarization leads to release of excessive amount of glutamate. Glutamate is an excitotoxic neurotransmitter, increases cellular calcium which is required for nNOS activity. Calcium mediates the binding of calmodulin to the nNOS. Calmodulin joins the two monomers of enzyme and activates it. Thus nNOS is inactive in the absence of calmodulin¹⁶. As calcium play key role in the activation of nNOS, this enzyme has many consequences in the pathological conditions where calcium level is increased like ischemia, excitotoxicity, depression and parkinson's

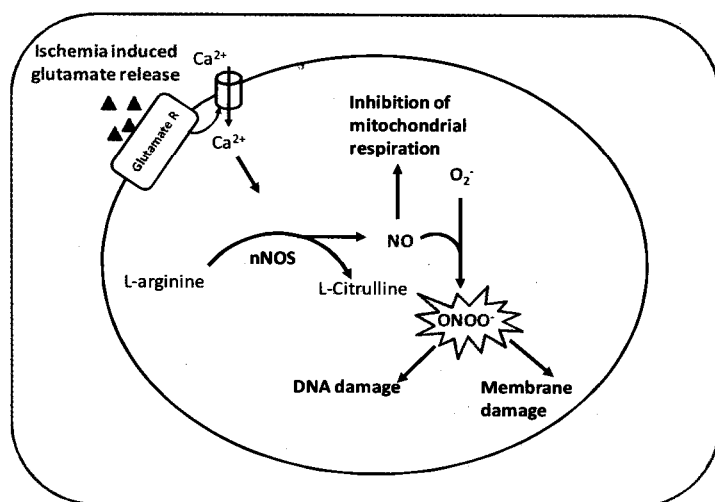


Fig. 2. The pathological effects of nitric oxide generated by nNOS in ischemia. R: receptor; NO: nitric oxide; O_2^- : superoxide, $ONOO^-$: peroxynitrite

disease. NO synthesized from nNOS can produce wide range of actions. The pathological roles of NO induced by nNOS in cerebral ischemia is shown in Figure 2.

The NO generated by this isoform mediates synaptic plasticity and neuronal signaling in normal brain but promotes neurotoxicity following ischemic damage¹⁷. The role of nNOS in ischemia has been investigated using different animal models. After transient ischemia in rats, the number of NOS-1 immunoreactive neurons in the cortex increased markedly as early as 15 min, and expression persisted for 24 h¹⁸. The superoxide and peroxynitrite formed after transient cerebral ischemia mediates the neurotoxic effects of nNOS¹⁵. The nNOS gene deletion conferred neuroprotection in mice with transient cerebral ischemia¹⁹. Increased basal and ischemia induced neurogenesis is observed in nNOS knockout mice depicting that NO released from nNOS is a negative regulator of neurogenesis in ischemic brain. 7-nitroindazole, a selective nNOS inhibitor increased neurogenesis demonstrating that inhibition or knockout of nNOS is involved in both reducing infarct volume and enhancing neurogenesis in ischemia²⁰. It is reported that NO production in the striatum after reperfusion is mainly related to activity of nNOS. Also, cerebral blood flow was significantly higher in nNOS (-/-) mice than in control mice at 70 minutes and 90-120 minutes after reperfusion²¹. Thus the transgenic mice lacking gene for nNOS were found to be more resistant to ischemic damage. This was again proved by giving the specific inhibitors of nNOS including 7-nitroindazole (7-NI) and ARL 17477 reduce the infarct size in ischemia²¹. These reports indicate that nNOS overexpression occurs in early time points of ischemic injury and it contributes towards neurodegeneration.

nNOS inhibitors:

Considering the role of nNOS in ischemic damage many inhibitors have been developed to prevent the harmful effects of nNOS [Table 1]. Many nonselective NOS inhibitors like L-NAME, L-NAME and L-NNA showed a reduction in infarct volume and also lead to exacerbation of injury. This exacerbation of injury resulted from increased dose of nonselective NOS inhibitors which resulted in the reduction of eNOS activity producing reduced cerebral blood flow and increased infarction volume^{2,22,23}. In order to preclude this situation selective nNOS inhibitors like 7-nitroindazole (7-NI), tri (fluoromethylphenyl) imidazole (TRIM), ARL which 17477, AR-R18512, BN 80933 and PPBP¹¹ are developed which do not influence the eNOS activity but diminish nNOS activity. However, some of these inhibitors can lead to the activation of NF- κ B leading to iNOS induction²⁴, which depicts that there is a need for cautious use of these inhibitors.

Table 1 nNOS inhibitors used for neuroprotection in cerebral ischemia

nNOS inhibitor	Animal Model	Time, route of administration and dose	Neurological efficacy finding
7-Nitroindazole (7-NI)	Transient focal cerebral ischemia (rat) ²⁵	Immediately after 5 minute of occlusion (60 mg/kg i.p.)	Reduced lesion volume at 22 hours of reperfusion
BN 80933	Transient middle cerebral artery occlusion (MCAO) (rat) ²⁶	1, 4, 6, 8 and 24 hours after MCAO (0.3-10 mg/kg i.v.)	Reduced infarct volume and enhanced behavioral recovery at 48 hour and 7 day reperfusion and treated after 8 hours at MCAO
ARL 17477	Transient global ischemia (rat) ²⁷	Immediately post-occlusion (50 mg/kg i.p.)	Reduced ischemia induced hippocampal damage at 1, 3 day
	Endothelin-1 model of focal ischemia (rat) ²⁷	0h, 1h or 2h post ischemia (1 mg/kg i.v.)	Reduced infarct volume
Sigma 1-Receptor ligand 4-Phenyl-1-(4-Phenylbutyl) Piperidine (PPBP)	Transient Middle cerebral artery occlusion (rat) ²⁸	After 90 minutes of occlusion (1 µmol/kg i.v.)	Reduced infarct volume at 22 hours of reperfusion
Tri(fluoromethyl phenyl)imidazole (TRIM)	Transient global ischemia (gerbils) ²⁹	Before or immediately after occlusion (50 mg/kg i.p.)	Prevents ischemia induced cell death in CA1 of hippocampus at 4 and 24 hours after occlusion
	Transient global ischemia (rat) ²⁹	Immediately after occlusion (50 mg/kg i.p.)	Prevents ischemia induced cell death in CA1 and dorsolateral striatum at 15 min of 4-VO
	Transient focal ischemia (rat) ²⁹	30 min after occlusion 50 mg/kg i.p.)	Reduction in infarct volume at 4 and 24 h post occlusion

iNOS expression in cerebral ischemia:

iNOS (NOS-2) is the inducible form of NOS expressed in different cells in response to various proinflammatory stimuli like cytokines and lipopolysaccharides³. This isoform is independent of calcium for its activity and it is regulated at transcriptional level. In the nervous system iNOS generated NO has both neuroprotective and neurotoxic effects⁴. The iNOS expression is not found in either astroglia or microglia of healthy brain but following ischemic, traumatic, neurotoxic or inflammatory damage there is substantial increase in iNOS resulting in the harmful effects³⁰. The expression of NOS-2 is induced in both resident and infiltrating cells in response to experimental cerebral ischemia³¹ and in human stroke³². Also, iNOS mRNA, protein and enzymatic activity are increased in the brain after transient or permanent ischemia³³. It is reported that the activity of NOS-2 following transient ischemia increases progressively over time, with maximal levels after 24 h in the striatum and 48 h in the cortex^{34,35}. Following cerebral ischemia there is marked inflammatory reaction resulting in the release of several

cytokines mediating the expression of iNOS in various cells like astroglia and microglia of central nervous system³⁶. Using in vitro model of brain ischemia it is demonstrated that neurons also express iNOS³⁷. In ischemic core the iNOS activity and NO content are higher than in penumbra region demonstrating that core region is more vulnerable to damage³⁵. iNOS produces excess NO which causes the tissue damage by producing reactive nitrogen species⁴. The NO produced by iNOS reacts with superoxide and generates peroxynitrite causing the neuronal loss in ischemia³⁰. Evidences also report that NO produced from iNOS contributes to the late stages of cerebral ischemic damage^{38,39}. Altogether it is evident that iNOS plays detrimental role in cerebral ischemia and effective inhibition could provide neuroprotection.

iNOS inhibitors:

As iNOS is involved in promoting the detrimental effects in cerebral ischemia many inhibitors of this isoform have been developed to prevent the neurodegenerative effect [Table 2]. The regulation of NO production via iNOS can

Table 2 iNOS inhibitors used for neuroprotection in cerebral ischemia

nNOS inhibitor	Animal Model	Time, route of administration and dose	Neurological efficacy finding
Aminoguanidine	Transient focal cerebral ischemia (rat) ³⁸	24h after ischemia (100 mg/kg i.p.)	Reduction in ischemic brain damage at 4 days
1400W	Transient focal cerebral ischemia (rat) ⁴⁷	Every 8 h after ischemia-7 times (20 mg/kg s.c.)	Reduction in lesion volume at 3 days after ischemia
N6-(1-iminoethyl)-lysine	Transient focal cerebral ischemia (rat) ⁴⁸	15 minutes before ischemia (10 mg/kg i.p.)	Reduction in infarction volume 24h reperfusion

be controlled mainly at the transcriptional and translational levels, because once iNOS is activated it produces huge amount of NO until substrate depletion⁴⁰. There are many stimulus/agonist which activates iNOS transcription like endotoxin lipopolysaccharide (LPS), pro-inflammatory cytokines like TNF α , IL-1 α , and IFN α , phorbol esters and stimulus like ischemia. However, the inhibition of iNOS activity by various inhibitors has been well studied approach. Many amino acid based small molecule iNOS inhibitors like modified L-arginine compounds viz. N-iminoethyl-L-ornithine, N6-(1-iminoethyl)-lysine are developed which are highly specific to iNOS. Also non-amino acid based inhibitors like guanidines (aminoguanidine), benzoxazolones, 2-aminopyrimidines and isothioureas have been developed to inhibit the iNOS activity and they have various levels of selectivity and potency⁴. Other inhibitors include N^G-iminoethyl-lysine, bis-isothioureas, 1400W (N-[3-(aminomethyl) benzyl] acetamide, GW273629 and GW274150^{41,42}. Amongst these, aminoguanidine is the selective iNOS inhibitor has exhibited beneficial effects in a rodent model of endotoxic shock⁴³ and cerebral ischemia^{44,45}. Other potent iNOS inhibitors include bicyclic amidine inhibitors, perhydro-iminopyridine and perhydro-iminoquinoline with excellent selectivity for iNOS over other NOS isoforms and have been shown to be orally active in rats⁴⁶.

In addition to these inhibitors many agents like statins, sphingolipid metabolites, antioxidants (N-acetyl cysteine, glutathione, phosphodiesterase inhibitors, curcumin and resveratrol) has been shown to inhibit iNOS and induce neuroprotection in cerebral ischemia. The PPAR ligands belonging to thiazolidinediones (pioglitazone, troglitazone and rosiglitazone) and non thiazolidinediones (cyclopentanone prostaglandin 15-deoxy-D12,14-prostaglandin-J2 (15d-PGJ2), L-796,449, or conjugated linoleic acid (CLA) are also iNOS suppressive and has been found to be neuroprotective in ischemia⁴.

eNOS expression in cerebral ischemia:

The eNOS (NOS-3) is another constitutive enzyme which is dependent on calcium for its activity. This isoform of NOS is mainly expressed in vascular endothelial cells and to some extent in neurons of the central nervous system, platelets, smooth muscle cells, bone cells and

cardiomyocytes⁴⁹. After ischemia the eNOS activity and protein expression is upregulated which takes place during the early stages of ischemia³. eNOS expression and activity is enhanced after the induction of transient cerebral ischemia⁵⁰. The detailed mechanism of upregulation of eNOS in cerebral ischemia is unknown but it is thought to be mediated by various inflammatory reactions, kinase signaling pathway and transcription factors like hypoxia inducible factor-1 and activator protein-1^{51,52}. eNOS has been shown to be neuroprotective in cerebral ischemic conditions. This isoform is different from the other two isoforms in that it promotes a beneficial effect through vasodilatation causing the increase in cerebral blood flow, inhibiting the platelet and leukocyte adhesion, preventing inflammation, oxidative damage, thrombosis and apoptosis^{17,23,53}, mobilization of stem and progenitor cells⁵⁴ and improving neovascularization⁵⁵. NO generated by eNOS enhances levels of cGMP and causes vasodilation, thus enhancing the regional blood flow¹⁹. Some data demonstrates that eNOS also produces its protective effects by promoting arteriogenesis after stroke causing the increase in cerebral blood flow⁵⁶.

eNOS stimulation in cerebral ischemia:

eNOS inhibition has been detrimental in experimental cerebral ischemia. N^G-nitro-L-arginine methyl ester (L-NAME), N^G-monomethyl-L-arginine (L-NMMA) and N (5)-(1-iminoethyl)-L-ornithine (L-NIO) has been associated with inhibition of eNOS activity and thereby stimulates platelet aggregation^{49,57}. Several modalities, including HMG-CoA reductase inhibitors (statins), steroid hormones, nutrients, and physical activity, that up-regulate eNOS expression and/or activity have been identified to lead to enhanced cerebral blood flow and protection from ischemic stroke⁵⁸ [Table 3]. The beneficial enzyme eNOS is regulated by multiple mechanisms like transcription factors, regulation of mRNA stability, protein-protein interaction and cellular localization.

NO and neuroprotection in cerebral ischemia

Apart from the detrimental effects of the NO, it has many beneficial effects in terms of neuroprotection in cerebral

Table 3 eNOS activators used for neuroprotection in cerebral ischemia

nNOS inhibitor	Animal Model	Time, route of administration and dose	Neurological efficacy finding
Corticosteroids ⁵⁹	Transient cerebral ischemia (mice)	2 h of ischemia (20 mg/kg i.p.)	Increase in eNOS activity, reduction in infarct volume and increase in regional cerebral blood flow within 2h of ischemia
Simvastatin ⁶⁰	Permanent middle cerebral artery occlusion (rats)	Immediately after ischemia (20 mg/kg s.c.)	Increase in eNOS protein level, reduction in infarct volume 24 h and 48 h after occlusion
Atorvastatin ⁶¹	Embolic focal cerebral cerebral ischemia (C57BL/6 mice)	Everyday for 14 days before ischemia (20 mg/kg s.c.)	Increase in eNOS mRNA level, reduction in infarct volume 24h after ischemia
Nitro-L-arginine ⁶²	Focal cerebral ischemia (mice)	5 min, 3h, 6h after ischemia (6 mg/kg i.p.)	Reduction in infarct volume 24h after occlusion

ischemia. As discussed earlier NO produced from the eNOS activity plays important role by its actions like antithrombosis, anti-inflammation and increased cerebral perfusion through vasodilatation. NO plays important role in ischemia preconditioning induced neuroprotection. Mice having nNOS and eNOS knockouts were not protected to severe ischemic insults given after brief ischemic preconditioning insults⁶³. Using *in-vitro* study it is shown that NO donor can reproduce preconditioning effect and NOS inhibitors can reverse it⁶⁴. Moreover, NO can precondition neurons to withstand against oxygen glucose deprivation insult⁶⁵.

It is observed that rate of neurogenesis following ischemic insult is increased in the brain⁶⁶. It is shown that administration of NO donor increased neurogenesis after focal ischemia in rats and significant improvements in neurological outcome. Further, NO donors in combination with marrow stromal cells significantly enhanced angiogenesis and neurogenesis following cerebral ischemia⁶⁷. NO generated by iNOS is also involved in ischemia induced neurogenesis as evidenced in the dentate gyrus of rat brain⁶⁸. The efficacy of the various NO donors like organic nitrates, sodium nitroprusside, sydnonimines and S-nitrosothiols has been investigated in experimental ischemia. Many have shown benefit, although within a relatively short therapeutic time window. Although NO donors have shown promising results following experimental ischemia⁶⁹, its effectiveness in clinical trials just recently initiated. A Phase III clinical trial [ENOS (Efficacy of Nitric Oxide in Stroke) is under way to consider the benefits (at 3 months) of reducing hypertension through application of transdermal glyceryl trinitrate (5 mg/day) for 7 days following stroke (<http://www.enos.ac.uk>).

Future strategies

The nNOS mediated NO is involved in promoting detrimental effects in cerebral ischemia so there is a need for the development of new therapies for preventing these harmful effects. Selective inhibitors for nNOS have to be developed which does not influence the activity of eNOS. The agents which block the activity of receptors and calcium accumulation are also the targets for inhibiting nNOS. Phosphorylation of nNOS is critical for its activity in various ways depending on the enzyme and site of phosphorylation^{70,71}. Protein phosphatase 1 decreases the phosphorylation of nNOS at the same site (Ser⁸⁴⁷) and enhances the activity of nNOS. Phosphorylation of nNOS at Ser⁸⁴⁷ by the calcium-calmodulin protein kinase II (CaMKII) inhibits nNOS activity⁷¹. Another site of phosphorylation is Ser¹⁴¹² at which phosphorylation by kinase decreases whereas dephosphorylation increases activity of nNOS⁷⁰. The inhibition of nNOS at transcriptional level could be another approach. Transcription factors involved in the transcription of nNOS are activator protein-2 (AP-2), the transcriptional enhancer factor-1/MCAT binding factor (TEF-1/MCBF), cAMP response element-binding protein/activating transcription factor/c-Fos (CREB/ATF/c-Fos), nuclear respiratory factor-1 (NRF-1), Ets, nuclear factor-1 (NF-1) and NFκB⁷². Selective modulation of these transcription factors may be useful for inhibiting its transcription. Moreover, development of new inhibitors which partially inhibit the nNOS mediated NO production may be useful in the neurodegenerative conditions like ischemia.

Out of three isoforms of NOS, iNOS is transcriptionally active and the inhibition of the transcription of the iNOS is promising approach. The activity of iNOS is regulated by many signaling pathways at transcriptional level. Cerebral ischemia and inflammation trigger numerous cell specific signal transduction pathways out of which activation of the IκB/NF-κB transcription pathway is deemed virtually

indispensable for iNOS gene transcription. Alternative signaling pathways include the janus tyrosine kinase-signal transducers and activators of transcription (JAK/STAT) pathway, the mitogen-activated protein kinases (MAPK) pathway^{73,74}. These pathways leads to the activation of transcription factors such as activator protein 1 (AP1), activating transcription factor 2 (ATF2) and cAMP-responsive elements, which are closely linked with iNOS gene expression. So targeting these signaling pathways and finding out new therapeutic strategies will be a major advance in the regulation of iNOS mediated neurodegenerative diseases. The activation of eNOS is dependent on specific phosphorylation at Ser1177⁵⁸. New therapeutic strategies can be developed which increase the phosphorylation of eNOS thereby improving the beneficial effects.

Concluding remarks

Nitric oxide is considered to be a ubiquitous endogenous signaling agent, which is involved in both the pathological and neuroprotective processes following ischemia. This obvious contradiction can be explained by the type of NOS producing it, cell type responsible, the amount produced and whether the NO undergoes further oxidation. It is very important to consider both protective and detrimental actions of nitric oxide while inhibiting the particular NOS for therapeutic effect against cerebral ischemia.

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