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PERSPECTIVE

# Inhibition of the AMPK/nNOS pathway for neuroprotection in stroke

Stroke ranks fourth among all causes of death and is the major cause of long-term disability in the United States. Furthermore, it is associated with significant morbidity/mortality and a direct/indirect cost of approximately $65 billion annually (Roger et al., 2012). Other than thrombolysis by tissue plasminogen activator (tPA), which offers only a short window of treatment (~3–4 hours), an effective neuroprotective or functional recovery therapy is not available mainly because of limited understanding of the signaling mechanisms of stroke disease (Moskowitz et al., 2010).

Stroke is a complex disease with significant components of excitotoxcity, inflammation and redox, leading to neuronal cell death and neurological deficits. After stroke, the nitric oxide (NO) metabolome is derailed due to aberrant activities of nitric oxide synthases (NOS). In neurons, peroxynitrite production comes to dominate the metabolome rather than S-nitrosoglutathine (GSNO) (**Figure 1**). In spite of peroxynitrite's major causal role in stroke, neuronal peroxynitrite- or neuronal nitric oxide synthase (nNOS)-targeted therapy does not exist due to limited mechanistic understanding of nNOS regulation/signaling and peroxynitrite-modified targets.

While GSNO is a natural and potent S-nitrosylating (*trans*-nitrosylating) agent and regulates enzymatic activity *via* S-nitrosylation of cysteine residue, peroxynitrite invokes its effect mainly *via* nitrotyrosination (3-NT formation) of the tyrosine residue of a protein/enzyme. In order to determine the opposing roles of GSNO and peroxynitrite in stroke and their potential links to outcomes after cerebral ischemia and reperfusion (IR), research needs to elucidate the complex interplay between phosphorylation/dephosphoryaltion and S-nitrosylation/denitrosylation of nNOS and its regulatory enzymes. Undoubtedly, these reactions determine the role of nNOS and the potential effects of excess peroxynitrite and GSNO on outcomes following stroke.

Both wild type mice treated with nNOS specific inhibitors and nNOS knockout (KO) mice show reduced levels of IR injury and improved neurological functions following IR (Huang et al., 1994), supporting the need to investigate the nNOS-mediated injury mechanisms and to develop an nNOS targeted stroke therapy for neuroprotection and recovery of functions. Indirect inhibition of nNOS activity after stroke by NA-1, an inhibitor of postsynaptic scaffolding protein (PSD-95), provides neuroprotection, confirming a deleterious role of nNOS activity in stroke (Instrum and Sun, 2013).

nNOS activity is regulated by several mechanisms/mediators, including S-nitrosylation (NO/GSNO), adenosine monophosphate activated protein kinase (AMPK) and peroxynitrite. Therefore, the focus of this perspective is to examine both mechanisms of the NOS-mediated stroke disease and its amelioration by novel S-nitrosylation mechanisms using GSNO in an animal model of transient cerebral ischemia reperfusion (IR) (Khan et al., 2015). Perhaps discoveries in this realm could offer new opportunities for drug development that could widen or open new therapeutic options for stroke.

Of the three known NOS, nNOS activity plays a critical role in neuronal loss during the acute IR phase, likely contributing ~90% to NOS activity. Recent reports document that S-nitrosylation of NOS regulates its activity (Khan et al., 2012), in addition to phosphorylation/dephosphorylation (Rameau et al., 2007). In resting neurons, the nNOS is inhibited by phosphorylation of $Ser^{847}$ and S-nitrosylation of $Cys^{331}$; however, immediately upon stroke injury, nNOS is activated by NMDA receptor-mediated excitotoxicity and calcium influx through site specific phosphorylation ($Ser^{1412}$), denitrosylation ($Cys^{331}$) and dephosphorylation ($Ser^{847}$). In this environment, nNOS-derived NO is converted to peroxynitrite by an instantaneous, diffusion limited reaction with superoxide. Peroxynitrite is reported to activate LKB1 (an upstream kinase to AMPK), which activates AMPK andAMPK, in turn, activates nNOS (phosphorylation at $Ser^{1412}$), thus maintaining a nNOS/peroxynitrite/AMPK vicious cycle (Zou et al., 2002; Khan et al., 2015). Our initial studies show that treatment of IR animals with the trans-nitrosylating agent GSNO attenuates nNOS activity, likely *via* S-nitrosylation of $Cys^{331}$ of nNOS. The S-nitrosylation of nNOS causes an increased phosphorylation at $Ser^{847}$ and adecreased phosphorylation at $Ser^{1412}$, resulting in the decreased peroxynitrite formation and thus reduced LKB1 and AMPK activities (Khan et al., 2015).

The interplay between nNOS and neuronal AMPK during the acute phase of stroke is now recognized to contribute to neuronal loss (Manwani and McCullough, 2013). AMPK is therefore an important potential target for stroke treatment. However, timing, duration and degree of its activation are critical for the outcome of stroke injury (Manwani and McCullough, 2013). AMPK is activated during decreased cellular energy supply. It is highly expressed in neurons (AMPKα2) and is rapidly activated during an energy deprived status such as stroke (McCullough et al., 2005). Its activation during the acute phase of IR is deleterious; both pharmacological inhibition (using AMPK inhibitor compound c) and gene deletion of AMPK were found to be neuroprotective (McCullough et al., 2005; Khan et al., 2015). Possibly, AMPK activation during IR's acute disease hyperactivates nNOS via increased phosphorylation at $Ser^{1412}$ of "aberrant" nNOS, which results in $Cys^{331}$ denitrosylation and $Ser^{847}$ dephosphorylation, leading to peroxynitrite formation.

Peroxynitrite has been documented to be injurious because its scavenger provides neuroprotection following IR (Khan et al., 2015). In contrast, 3-morpholinosyndnonimine (SIN-1), a peroxynitrite forming agent, was found to be deleterious in animal models of stroke (Khan et al., 2006). Unlike peroxynitrite, GSNO was found to decrease not only peroxynitrite levels but also to protect the neurons from peroxynitrite-mediated cell death and functional deficits (**Figure 2**). GSNO is also reported to inhibit NF-κB, STAT-3, caspase-3 and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase which contribute to its neuroprotective activities (Broniowska et al., 2013).

Advantage associated with the use of GSNO is that it is an endogenous non-toxic component of the human body and is mainly responsible for secondary modification via protein S-nitrosylation. S-nitrosylation is a newly characterized redox-based mechanism for regulation of cellular function. S-nitrosylated proteins (PSNO) and GSNO are in dynamic equilibrium in the human body, and their dysregulation hampers cellular functions.

In stroke pathology, the levels of GSNO and the consequent PSNO are believed to decrease due to four major reasons: i) Decreased oxygen supply under an ischemic/hypoxic condition reduces GSNO biosynthesis; ii) Excessive superoxide formed during reperfusion instantaneously reacts with nitric oxide synthase (NOS)-derived nitric oxide (NO), forming peroxynitrite and thus reducing NO bioavailability for GSNO biosynthesis; iii) Decreased biosynthesis of GSNO as a result of reduced levels of Glutathione (GSH) (redox imbalance) and NO (due to its reaction with superoxide) under IR condition. Furthermore, NO reacts slowly with GSH as compared with superoxide; and iv) In the inflammatory environment, the expression of GSNO degrading enzyme GSNO reductase (GSNOR) is increased, leading to reduced levels of GSNO.

Our recent stroke studies use GSNO to maintain the equilibrium of S-nitrosylation and to regulate the nNOS/peroxynitrite/AMPK vicious cycle, leading to neuroprotection and functional recovery (Khan et al., 2015). A recent report showing neuroprotection in stroke by microparticles loaded GSNO further supports the efficacy of GSNO (Parent et al., 2015). Exogenous administration of GSNO has not shown any evident toxicity or side effects in humans (de Belder et al., 1994). In clinical settings, GSNO is of even greater relevance to stroke therapy because it additionally shows antiplatelet, (Radomski et al., 1992) anti-embolization, (Molloy et al., 1998) and vasodilatory properties in humans (de Belder et al., 1994). Based on the efficacy of GSNO in our preclinical studies using animal models of stroke and absence of toxicity in human uses, we submit that GSNO is a promising drug candidate to be evaluated for human stroke therapy.

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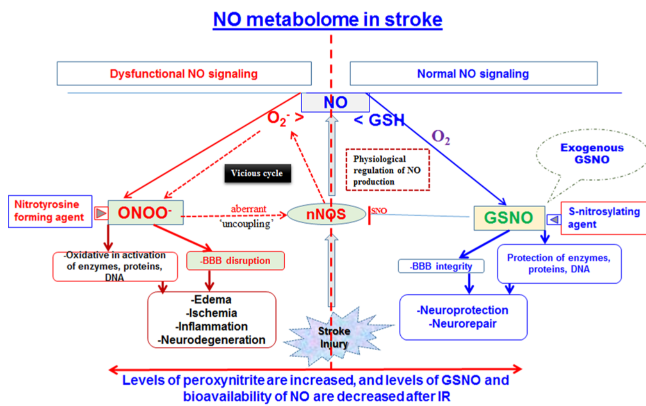


**Figure 1 Hypothesized dysregulation of nitric oxide (NO) metabolome in stroke.**
Neuronal NO metabolome is maintained by neuronal nitric oxide synthase (nNOS) activity and targeted NO functions under physiological condition. NO has three major targets (receptors): metal (mainly iron and copper) protein, *e.g.*, soluble guanylyl cyclase (sGC), cysteine (G-SH and Protein-SH) and superoxide ($O_2^-$). S-nitrosoglutathine (GSNO) is formed by a reaction of NO with Glutathione (GSH) (when GSH > NO) in presence of oxygen. GSNO, in turn, inhibits nNOS activity *via* the mechanism of reversible S-nitrosylation of nNOS, thus maintains NO metabolome (right half of the figure). Under pathological conditions such as stroke, NO metabolome is dysregulated due to excessive formation of $O_2^-$ from various sources including nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, lipoxygenases, cyclooxygenases and nNOS. Under such a pathological condition (when $O_2^-$ > NO), NO forms peroxynitrite ($ONOO^-$) by a diffusion limited reaction with $O_2^-$. $ONOO^-$ is a strong oxidizing agent and reduces the levels of tetrahydrobiopterin (a cofactor of nNOS) and L-arginine by oxidizing them and thus causing aberrant nNOS activity. Aberrant nNOS activity produces more $O_2^-$ than NO in the same compartment thus making excessive $ONOO^-$ and causing a vicious cycle of aberrant nNOS/$O_2^-$/$ONOO^-$ (left side of the figure). $ONOO^-$ has been implicated in blood-brain barrier (BBB) disruption, neuroinflammation and neuronal cell death, leading to neurological deficits. Exogenously administered GSNO is anticipated to inhibit the aberrant activity of nNOS and thus decreasing $ONOO^-$ production, leading to reduced neuronal loss and improved neurological functions.

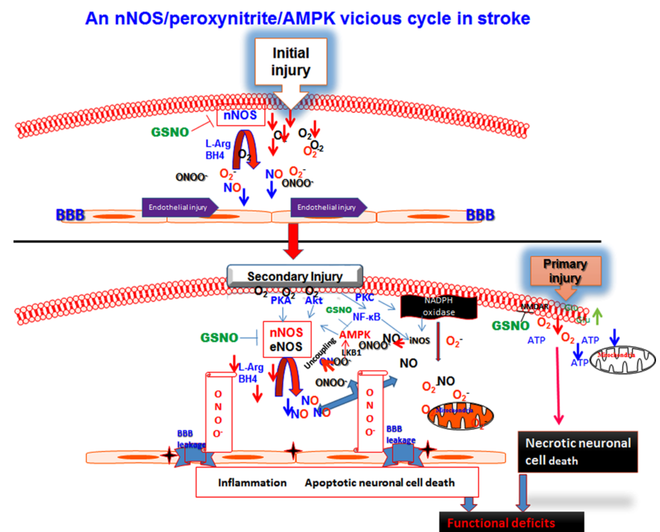


**Figure 2 Role of the nNOS/peroxynitrite/AMPK vicious cycle in stroke.**
Stroke causes necrotic neuronal cell death in the core area immediately after the injury. Necrotic neuronal death is fast and irreversible. However, secondary injury in larger penumbra area due to nitroxidative stress and inflammation is reversible and may be blocked by targeting the nNOS/peroxynitrite/AMPK vicious cycle. The injurious cycle is activated by aberrant activity of nNOS due to increased neuronal $ONOO^-$ formation. Peroxynitrite activates LKB1, a kinase which activates AMPK *via* phosphorylation. AMPK maintains the nNOS in aberrant activity state by phosphorylating nNOS and/or endothelial nitric oxide synthase (eNOS), leading to sustained production of $O_2^-$/$ONOO^-$ and thus maintaining the nNOS/peroxynitrite/AMPK vicious cycle. GSNO invokes neuroprotection and aids functional recovery by inhibiting aberrant activities of nNOS and AMPK and thus reducing the activities of the nNOS/peroxynitrite/AMPK vicious cycle. AMPK: Adenosine monophosphate activated protein kinase; ATP: adenosine triphosphate; BBB: blood-brain barrier; BH4: tetrahydrobiopterin; GSNO: S-nitrosoglutathine; L-Arg: L-arginine; LKB1: an upstream kinase to AMPK; NADPH: nicotinamide adenine dinucleotide phosphate; NMDAR: N-methyl-D-aspartate receptor; nNOS: neuronal nitric oxide synthase; $ONOO^-$: peroxynitrite; PKA: protein kinase A; PKC: protein kinase C.

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