Inducible nitric oxide synthase (NOS-2) in subarachnoid hemorrhage: Regulatory mechanisms and therapeutic implications

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Abstract:
Aneurysmal subarachnoid hemorrhage (SAH) typically carries a poor prognosis. Growing evidence indicates that overabundant production of nitric oxide (NO) may be responsible for a large part of the secondary injury that follows SAH. Although SAH modulates the activity of all three isoforms of nitric oxide synthase (NOS), the inducible isoform, NOS-2, accounts for a majority of NO-mediated secondary injuries after SAH. Here, we review the indispensable physiological roles of NO that must be preserved, even while attempting to downmodulate the pathophysiologic effects of NO that are induced by SAH. We examine the effects of SAH on the function of the various NOS isoforms, with a particular focus on the pathological effects of NOS-2 and on the mechanisms responsible for its transcriptional upregulation. Finally, we review interventions to block NOS-2 upregulation or to counteract its effects, with an emphasis on the potential therapeutic strategies to improve outcomes in patients afflicted with SAH. There is still much to be learned regarding the apparently maladaptive response of NOS-2 and its harmful product NO in SAH. However, the available evidence points to crucial effects that, on balance, are adverse, making the NOS-2/NO/peroxynitrite axis an attractive therapeutic target in SAH.

Key words:
Heparin, nitric oxide (NO), NOS-1, NOS-2, NOS-3, subarachnoid hemorrhage (SAH)

Introduction
Subarachnoid hemorrhage (SAH) causes profound morbidity and mortality in approximately 30,000, often otherwise healthy, Americans yearly. While the hemorrhage is responsible for severe, frequently fatal initial brain injury, many surviving patients undergo a secondary decline in the days following the insult. This well-established phenomenon of delayed neurologic deficit (DND) accounts for approximately one-third of the morbidities and mortalities associated with SAH. Importantly, because of the delay between the ictal event and development of DND, this secondary injury is thought to be preventable. A comprehensive mechanistic understanding of DND would allow for the development of targeted therapies to improve the poor outcomes of many patients afflicted with SAH.

Despite years of study, the mechanisms of DND remain incompletely understood. Early surgical and radiographic observations of pathological blood vessel narrowing following SAH supported a vasospasm theory of DND whereby blood products in the subarachnoid space lead to vessel narrowing, resulting in reduced blood flow, cerebral ischemia, and ultimately infarction. While vasospasm undoubtedly accounts for some aspects of DND, recently obtained clinical evidence challenges its predominance. Nimodipine, the only formally approved therapy to prevent DND following SAH, is largely ineffective at preventing angiographic vasospasm. Conversely, clazosentan, a medication that is highly effective at preventing radiographic vasospasm, does not improve clinical outcome. Other mechanisms of DND include microthrombosis, free radical injury, loss of cerebral autoregulation, and inflammation. The modern view is that DND,
rather than being a simple problem of vascular dysregulation, actually results from a confluence of multiple pathological processes.

One molecule that has emerged at the center of this process is nitric oxide (NO).[6] Originally characterized as “endothelium-derived relaxing factor,” NO is a ubiquitous signaling molecule that has a crucial role in a variety of physiological processes.[7-9] In the vasculature, NO is important not only as a vasodilator but also for maintaining endothelial function, preventing vascular smooth muscle cell growth, inhibiting platelet aggregation, and blocking leukocyte adhesion. Furthermore, NO is an important part of the inflammatory process — phagocytes generate large quantities of NO in response to inflammatory stimuli, with NO acting as a potent cytotoxic agent. Moreover, NO reacts with superoxide to form peroxynitrite, a powerful biological oxidant with the potential to mediate significant bystander tissue injury.[10] Given that disordered vessel contractility, endothelial dysfunction, inflammation, and free radical injury are all considered to be central to the broader process of DND, abnormal NO metabolism is uniquely positioned to serve as a central mediator of DND in SAH.

The potentially harmful effects of NO in SAH have emerged from a broader trend in studies of neuropathology, which have shown deleterious effects of NO in neurodegeneration,[11] traumatic brain injury,[12] and stroke.[13] The generation of NO in excess, destructive quantities following a brain injury results largely from activation of the inducible isoform of nitric oxide synthase (iNOS or NO-2), which occurs in response to inflammatory stimuli. Given its broad role in various CNS pathologies, the mechanisms controlling NO-2 upregulation have been studied extensively. The object of this review is to situate the regulation of NO-2 within the broader pathology of SAH to discuss the molecular mechanisms that result in its upregulation and to explore methods to ameliorate the effects of NO-2 upregulation, with the hope of fostering the development of novel therapeutics to target the NO-2/NO/peroxynitrite axis.

Physiologic Roles of Nitric Oxide: Overview

Nitric oxide in vascular tone
NO is best known for its role in vasodilation. In response to a wide variety of physiologic stimuli including shear stress, platelet-derived factors and cytokines, vascular endothelial cells upregulate NOX, which generates NO from the amino acid, L-arginine.[14] Once generated, NO readily diffuses into adjacent smooth muscle cells of the vasculature where it interacts with soluble guanylate cyclase to generate cyclic GMP, initiating a chain of secondary signaling events that culminate in smooth muscle cell relaxation and dilation of blood vessels. This mechanism of action is present throughout the body but is of particular importance in the vasculature of the brain where NO signaling tightly controls cerebral blood flow.[15,16]

Apart from its direct vasodilatory actions, NO plays a significant role in modulating endogenous vasoconstrictive agents. Endothelin-1 (ET-1), an endogenous small peptide vasoconstrictor of particular importance in SAH, is one such molecule.[17] Pharmacologic blockade of NO production in both rats[18] and humans[19] induces significant systemic vasoconstriction and concurrent hypertension that is partially reversed by the blockade of endothelin signaling, suggesting an important antagonistic role between these two molecules in normal physiology. Mechanisms for this interaction are complex, including both direct and downstream inhibitions of ET-1 signaling[20] and modulation of ET-1 transcription.[21,22] Similarly, NO has important antagonistic interactions with a variety of small molecule vasoconstrictors. Experimental evidence suggests that endothelial NO production counteracts adrenergic signaling in the peripheral vasculature of both rats[23,24] and humans.[25] In the pulmonary vasculature, NO inhibits the vasoconstrictive actions of serotonin via chemical modification of its receptor[26] Finally, NO inhibits cerebral endothelial production of thromboxane, a potent vasoconstrictor.[27] Thus, NO not only acts as a vasodilator but also provides a physiologic counterbalance to a variety of vasoconstrictors, a set of findings particularly relevant in diseases of increased vasoconstrictive tone such as SAH.

Nitric oxide in inflammation
NO plays an important role in inflammation. Generated by phagocytes such as macrophages and neutrophils in response to inflammatory stimuli, NO is a potent antimicrobial, both on its own and as a precursor to the powerful oxidant, peroxynitrite, which mediates a variety of antimicrobial effects.[28,29] Multiple lines of evidence derived from preclinical and clinical researches suggest a significant role of NO in the defense against a variety of pathogens including bacteria, protozoa, and fungi.[30] Although essential for the host defense, NO can induce bystander damage to the body’s own tissues, as occurs in the excessive inflammatory response of sepsis. Similar to its role in vasodilation, the role of NO in inflammatory processes is ubiquitous throughout the body.

Nitric Oxide Synthases

Throughout the body, cells such as macrophages, neurons, and endothelial cells generate NO primarily through a class of enzymes known collectively as nitric oxide synthases (NOS). Three isoforms of this enzyme exist, NOS-1, NOS-2, and NOS-3. Although all share a common synthetic mechanism, the three isoforms differ in the mechanisms of expression, predominant cell type of expression, and physiologic roles.[31] Starting from the precursor arginine, the NOS enzymes catalyze serial nicotinamide adenine dinucleotide phosphate (NADPH)-mediated oxygenation of the guanidine residue to yield citrulline and NO. Both the NOS-1 and NOS-3 isoforms require physiologically elevated levels of calcium to function. In contrast, NOS-2 uniquely binds to calmodulin to allow continued function even in cellular environments with low Ca²⁺ concentrations, allowing it to function as a “calcium independent” isozyme.

The NOS isoforms differ significantly in both the predominant cell type that expresses them and the mechanism of expression.[32] NOS-1, or neuronal NOS (nNOS), is widely expressed by neurons of both the central and peripheral nervous systems in humans and other vertebrates. A constitutively expressed enzyme, its activity is modulated primarily by intracellular calcium. The NO generated by NOS-1 is important for long-term potentiation, neuronal plasticity, and central regulation of blood pressure.
NOS-3, or endothelial NOS (eNOS), is primarily expressed by vascular endothelial cells. NOS-3, like NOS-1, is constitutively expressed and depends primarily on the modulation of intracellular calcium for control of its activity. The NO generated by NOS-3 acts as a vasodilator, playing important roles in both systemic and cerebral blood flows but it also inhibits platelet aggregation and leukocyte adhesion.

NOS-2, or inducible NOS (iNOS), differs from its counterparts as it is not constitutively expressed. Instead, expression of NOS-2 relies on various signaling events such as cytokines to induce its expression. Although principally expressed in phagocytes such as macrophages, NOS-2 can be induced in a wide variety of cell types including microglia and astrocytes. Once induced, NOS-2 continues to produce NO regardless of the calcium concentration, allowing for NO production 100-1,000 times greater than the constitutive isoenzymes. NOS-2 activity is ultimately terminated only by degeneration of the enzyme. This ability to generate large quantities of NO is consistent with the role of NOS-2 in inflammatory processes as a mediator of cytotoxicity. Thus, while all NOS isoenzymes generate the same product, NO, the different isoenzymes play decisively different physiologic roles.

**Nitric Oxide and Pathologic Vasoconstriction Following Subarachnoid Hemorrhage**

One hallmark of aneurysmal SAH is vasospasm, the delayed development of marked pathologic constriction of the intracerebral vessels. A number of molecular signals are thought to mediate this constriction. The endothelin molecules, particularly ET-1, represent the best studied class of molecules in this regard. Initial studies of ET-1 identified a strong correlation between elevated cerebrospinal fluid (CSF) concentrations of ET-1 and the development of vasospasm in patients with SAH. Subsequent work identified a number of plausible mechanisms to explain the observed rise in ET-1 including hemoglobin-triggered synthesis in vascular smooth muscle cells, ischemia-induced synthesis in astrocytes, and inflammation-provoked production in infiltrating leukocytes. Clinical trials of the ET-1 antagonist, clazosentan, confirm the primacy of ET-1 in mediating vasospasm. Although ultimately ineffective at improving outcomes in SAH, ET-1 blockade nevertheless led to a marked reduction in vasospasm in patients with SAH. In addition to ET-1, a number of other molecular signals may contribute to vasospasm. Thromboxanes present an important class of platelet-derived vasoconstrictors with a potential role in vasospasm. Following SAH, vascular endothelial cells demonstrate increased contractility in response to thromboxane receptor activation and platelet release of thromboxane is augmented in patients with vasospasm following SAH. Consistent with these findings, inhibition of thromboxane synthesis improves vasospasm in a canine SAH model. Other lines of experimental evidence suggest that serotonin, another platelet-derived vasoconstrictor, plays a role in the development of vasospasm following SAH. Intracisternal injection of platelet-rich plasma in dogs induces vasospasm in a manner analogous to SAH, a finding correlated with an increase in perivascular serotoninergic neurons. In rats, experimental SAH upregulates arterial expression of serotonin receptors, with a concurrent increase in contractility in response to serotonin. Consistent with these experimental observations, use of selective serotonin uptake inhibitors is a risk factor for severe vasospasm in patients with SAH. Thus, SAH induces arterial vasospasm by modulating a number of vasoconstrictive signaling pathways.

Given the importance of NO as a physiologic counterbalance to endogenous vasoconstrictors, the marked augmentation of vasoconstrictive signals following SAH implies that even a mild decrease in NO in the perivascular compartment could lead to significant vessel contraction. In consistence with this hypothesis, experimental evidence derived from goat models suggests that loss of endothelial NO availability partially explains the observation of increased ET-1-induced vasoconstriction following experimental SAH. Analogous experiments evaluating enhanced serotonin vasoconstriction following SAH demonstrate similar findings. Blood products in the subarachnoid space are thought to reduce NO bioavailability by a variety of mechanisms including malfunction of NO-generating enzymes, production of NO-inhibiting metabolites, and sequestration of NO by the vast amounts of extracellular hemoglobin acting as an NO “sink.” In accordance with this hypothesis, NO-based therapies such as intracarotid infusion of NO donors in primates, intracisternal injection of NO donors in rabbits, and intrathecal administration of the NO precursor nitroprusside in humans all ameliorate SAH-associated vasospasm.

Although a loss of NO bioavailability fits well with the aforementioned data, direct experimental examinations of NO production following SAH suggest a more complex metabolism than simple loss of basal production. Although NO production does appear to be globally decreased immediately following experimental SAH, there is a relatively rapid restoration toward normal levels in most areas of the brain in the hours following hemorrhage. Studies evaluating the effects of inhibition of NO synthesis on cerebral blood flow following SAH suggest similar kinetics, namely, an immediate loss and relatively rapid restoration of endothelial NO production. A three-phase model has emerged in which SAH initially results in a hyperacute loss of NO availability followed by restoration to normal levels and then augmentation of production over the next 72 h. This model challenges the hypothesis that vasospasm following SAH results in the simple loss of NO availability.

**Nitric Oxide and its Metabolites Following Subarachnoid Hemorrhage In Humans**

Given that the early prevailing theories of DND were dominated by vasospasm, reports of an apparent “increase” in NO bioavailability in SAH were unexpected. Although direct measurement of NO production following SAH is not practical, given its reactivity and short biological half-life, several groups evaluated the levels of its metabolic products, nitrates, and nitrates, along with the levels of these metabolites in CSF and clinical outcomes. Studies of CSF almost uniformly demonstrated elevated levels of NO metabolites. When evaluating CSF levels of NO metabolites in patients with aneurysmal SAH, Suzuki et al. noted an increase at all time points compared to healthy controls, with the degree of increase correlating with the quantity of cisternal blood. Several other
studies yielded similar findings.\cite{56-58} Only one study to date identified marginally lower levels of NO metabolites in the CSF of SAH patients compared to healthy controls\cite{39}, although the timing and means of CSF collection in this study were not well-documented.

The finding of elevated NO metabolites in the CSF following SAH is now relatively well-established but the relationship between this finding and vasospasm or DND is still to be resolved. While some groups report an increase in NO products in the CSF that correlates with vasospasm,\cite{56,59} others report a relative decrease in NO products that correlates with vasospasm,\cite{57,58} and there are still others who report no clear relationship.\cite{57,58} In a rat model of SAH, evidence of peroxynitrite-mediated perivascular injury was found on a pathological review of rats demonstrating vasospasm but not in those without vasospasm,\cite{60} a finding consistent with a role of excessive NO in mediating vasospasm. To our knowledge, a comparable finding has not been reported in human arteries.

At the very least, the finding of elevated NO metabolites in the CSF suggests that early hypotheses postulating reduced bioavailability of NO as being responsible for DND were overly simplistic. Recent theories of DND postulate that the presence of blood and blood products in the subarachnoid space initiates multiple pathophysiological events including not only vasospasm but also inflammatory and free radical injury to the adjacent cortex. NO is an important mediator of the inflammatory response, and in the context of inappropriate or exaggerated inflammation, excess NO production can be harmful. Reactive nitrogen species (RNS), especially peroxynitrite, are capable of disrupting cellular metabolism, irreversibly damaging DNA,\cite{62} and ultimately causing cell death.\cite{63}

Apart from examining CSF, another approach to determine the role of NO in DND has been to examine NO metabolites in microdialysis samples of cortical tissues. Microdialysis of parenchymal tissues has successfully demonstrated a relationship between NO metabolites and brain oxygenation.\cite{64} Some microdialysis studies report an association between locally elevated NO metabolites and poor outcomes,\cite{65} whereas others fail to find such a relationship.\cite{66}

The aforementioned conflicting findings in humans regarding NO metabolites and DND should perhaps not be surprising. The baseline characteristics of patients including the severity and location of SAH, comorbidities and the medications used to treat them, the timing of CSF or dialysate sampling, cohort sizes, and numerous other variables were invariably poorly controlled. It is likely that different brain compartments are differentially affected by NO — the vascular smooth muscle may well experience a reduced bioavailability, whereas inflamed cortical tissues may well experience an overabundance of NO. CSF represents a distant and imprecise proxy for intraparenchymal activity of NO. As a bulk substrate, CSF cannot capture important compartmental, regional, cellular, and temporal variations in NO production that may be crucially important for biological activity. Similarly, the volume of tissue sampled in microdialysis studies is necessarily small and similar to CSF; dialysate cannot capture important compartmental, regional, and cellular variations in NO production. The resolution of these discrepant findings and the advancement in understanding of the undoubtedly complex roles of NO in SAH await technical advances in methodologies that will permit global and regional assessments of NO or its metabolites at high spatial and temporal resolutions in the human brain.

**Nitric Oxide Synthase Expression Following Subarachnoid Hemorrhage**

The constitutive NOS isoforms (NOS-1, NOS-3) undergo complex changes in regulation following SAH. NOS-1 is primarily downregulated following SAH,\cite{67,68} with loss of function in adventitial nerve fibers correlating with the degree of vasospasm. NOS-3, in contrast, exhibits a more complex pattern of expression following SAH, with decreases in endothelial expression\cite{69,70} and increases in parenchymal expression.\cite{65,69,71} The clinical ramifications of these changes are unclear, as both NOS-1 and NOS-3 are primarily controlled at the posttranslational level by intracellular calcium. The metabolic environment of the CNS following SAH may induce malfunction of these enzymes, particularly NOS-3.\cite{46} The general consensus is that loss of enzymatic function of these constitutive isoforms contributes to the pathophysiology of SAH via impaired vasodilation, a topic that is the subject of an excellent recent review.\cite{72}

In contrast to the constitutive isoforms of NOS, inducible NOS (NOS-2) undergoes unambiguous upregulation following SAH. In a rat model of SAH induced by endovascular perforation, NOS-2-positive mononuclear cells and neutrophils infiltrate the subarachnoid space as early as 24 h after SAH.\cite{73} The areas of highest NO-2 expression correlate closely with histological evidence of peroxynitrite-mediated cell injury and vasospasm.\cite{74} In the same model, blockade of NOS-2 with the selective inhibitor, aminoguanidine, prevents the development of post-SAH vasospasm. A murine endovascular model of SAH produces similar results, with upregulation of NOS-2 in both the vasculature and cortex.\cite{75} Overexpression of CuZn superoxide dismutase in this model protects against SAH-induced vasospasm and results in decreased NOS-2 expression,\cite{76} further supporting the pathological role of NOS-2. In a rat model of vasospasm induced by double injection of blood into the cisterna magna, the degree of vasospasm correlates with the degree of NOS-2 expression.\cite{77}

The findings in these models of SAH are consistent with the limited clinical data available. In an evaluation of NOS isoform expression in cerebral tissues taken during clipping of a ruptured aneurysm, NOS-2 expression was significantly increased compared to unruptured controls and unique among NOS isoforms; NOS-2 hyperexpression alone correlated with vasospasm.\cite{78} Thus, SAH rapidly induces NOS-2, best accounting for clinical studies demonstrating elevated NO production following SAH.

**Mechanisms of Nos-2 Upregulation Following Subarachnoid Hemorrhage**

Under normal conditions, NOS-2 upregulation represents an appropriate response to infectious organisms, allowing for the
clearance of pathogens via the production of cytotoxic levels of NO. Proinflammatory agents such as lipopolysaccharide, free radicals, and cytokines are principal inducers of NOS-2 upregulation. SAH results in the appearance of numerous proinflammatory molecules derived from blood and blood breakdown products such as thrombin, methemoglobin, heme, and Fe⁵⁺. In addition, the abrupt increase in intracranial pressure following aneurysmal SAH can induce global cerebral ischemia, with possible reperfusion injury. These events ultimately contribute to NOS-2 expression through several mechanisms, as given below.

**Toll-like receptors**

Toll-like receptors (TLRs) are a first-line of defense against pathogens and play an essential role in mediating innate and adaptive immune responses.[78,79] TLR-4 is expressed by microglia and to a lesser extent, by neurons and astrocytes following SAH.[80] Hemoglobin breakdown products including methemoglobin,[81-83] heme,[84,85] and hemin[86] can activate TLR-4. Of these, methemoglobin alone, due to its high solubility, rapidly diffuses throughout the CSF, enabling global activation of TLR-4 throughout the CNS. Experiments in rats,[87] rabbits,[88] and mice[89,90] all demonstrate evidence of TLR-4 activation following experimental SAH. In ischemic CNS injury, TLR-4 activation induces the expression of NOS-2, and TLR-4 knockout mice demonstrate decreased expression of NOS-2 following experimental stroke.[91,92] In a rat model of hypoxia, knockdown of TLR-4 results in reduced NOS-2 expression following hypoxic injury.[79] Given the presence of TLR-4 ligands in the form of hemoglobin metabolites, upregulation of TLR-4 receptors, and the capacity of TLR-4 to induce NOS-2, activation of TLR-4 forms a plausible mechanism to account for NOS-2 upregulation following SAH. However, direct experimental confirmation of this phenomenon is lacking.

**Cytokines**

Multiple inflammatory cytokines are upregulated and are thought to mediate delayed injury following SAH including interleukin-1β (IL-1β)[92,93] and tumor necrosis factor (TNF).[94,95] These proinflammatory cytokines can upregulate NOS-2.[94] IL-1β may be particularly relevant to SAH — in a cell culture model of SAH employing rat vascular smooth muscle cells, the combination of hemoglobin and IL-1β induces NOS-2 transcription, with hemoglobin markedly augmenting IL-1β induction of NOS-2 in spite of hemoglobin’s relatively poor ability to upregulate the enzyme by itself.[97] The inflammatory response to SAH rather than the blood products themselves may be responsible for the upregulation of NOS-2 following SAH[97] although further work along these lines is required for clarification.

**Reactive oxygen species**

The reactive oxygen species (ROS) and free radicals generated following SAH play an important role in NOS-2 upregulation. ROS derive from several sources following SAH including disrupted cellular respiration, extracellular methemoglobin in the subarachnoid space, and upregulation of certain enzymes including NOS-2 itself.[98] Several lines of evidence demonstrate the importance of oxidative stress in NOS-2 induction following SAH. In a mouse cell culture model of SAH, cerebral endothelial cells exposed to ferrous citrate complexes upregulate NOS-2.[99] The addition of the antioxidant N-acetyl cysteine to the cultures blocks NOS-2 upregulation, arguing for the importance of free radicals in inducing NOS-2. More direct evidence for the role of ROS in mediating NOS-2 induction following SAH derives from transgenic mice engineered to overexpress the potent antioxidant enzyme CuZn superoxide dismutase (CuZn-SOD).[75] Immunohistochemical studies 24 h after SAH demonstrate decreased NOS-2 expression in the vascular wall of SOD-transgenic mice compared to wild-type controls.[75] Taken together, these experiments demonstrate the importance of ROS in NOS-2 regulation following SAH.

**Ischemia**

Immediately following aneurysmal rupture, the brain may experience transient global ischemia, as intracranial pressure equilibrates with mean arterial pressure.[100] Clinically, this period of globally diminished perfusion corresponds to loss of consciousness, and has been shown to be highly predictive of mortality and delayed cerebral ischemia following SAH.[101] Transient global cerebral ischemia upregulates NOS-2 in rodents.[102-104] Although direct examination of the effects of ischemia on NOS-2 expression in the context of SAH does not exist, ischemic injury presents a plausible source for NOS-2 elevation following SAH.

**Proinflammatory transcription factors that upregulate NOS-2 after subarachnoid hemorrhage**

While multiple agents and conditions present in the CNS contribute to upregulation of NOS-2 following SAH, the upregulation of NOS-2 ultimately depends on activation of one or more transcriptional pathways, the most pertinent of which are reviewed here.

**Nuclear factor-κB**

Nuclear factor-κB (NF-κB) is a transcription factor closely associated with NOS-2. The promoter region for the human NOS-2 gene contains multiple canonical NF-κB binding sites.[105] Furthermore, small molecule and peptide inhibitors of NF-κB prevent upregulation of NOS-2 in response to known stimuli, consistent with a central role of this transcription factor in NOS-2 expression.[106,107] As a constitutively expressed factor, NF-κB is bound in the cytosol of cells by its inhibitor, IκB. IκB is degraded as a result of a variety of inflammatory signaling pathways, thereby activating NF-κB and allowing it to translocate to the nucleus. Activation of NF-κB occurs in multiple models of SAH,[98,108,109] Multiple triggers including TLR-4 activation, cytokine signaling, and ROS induce NOS-2 expression via activation of NF-κB in various models of SAH.[103] In consistency with this understanding, blockade of NF-κB by various agents including estradiol[110] N-acetylcysteine,[109] and overexpression of superoxide dismutase[73] leads to decreased expression of NOS-2 following SAH.

**Hypoxia-inducible factor 1**

Hypoxia-inducible factor 1 (HIF-1) is a transcription factor that is activated in response to hypoxia.[111,112] A heterodimeric DNA-binding complex that consists of one α and one β subunit, HIF-1 binds to the promoter elements of target genes that promote an adaptive response to hypoxia, such as erythropoietin and vascular endothelial growth factor.[113-115] While constantly degraded under aerobic conditions, under hypoxic conditions degradation of the HIF-1α subunit ceases, allowing formation of the active heterodimer and
transcriptional activation of HIF responsive genes.\cite{116,117} NOS-2 transcription is under the control of HIF-1.\cite{118,119} Given the possibility of hypoxia secondary to hypoperfusion immediately following the ictus as well as the possibility of later ischemia secondary to vasospasm, upregulation of HIF-1 following SAH seems likely. Experimental evidence is largely consistent with this hypothesis, with multiple models demonstrating an increase in cerebral HIF-1 activity following SAH.\cite{120,121} Thus, elevated levels of HIF-1 may contribute to the observed elevation of NOS-2 after SAH.

**Nuclear factor of activated T-cells**

Closely related to NF-κB, nuclear factor of activated T-cells (NFAT) is a transcription factor most commonly associated with adaptive immunity and T cell activation.\cite{122} During ischemic brain injury, there is an increase in intracellular calcium concentration leading to activation of calcineurin, a calcium-dependent phosphatase, which subsequently activates NFAT via dephosphorylation.\cite{123} NFAT isoform expression has been documented in diverse cell populations of the CNS such as neurons, astrocytes, macrophages, and microglia.\cite{124-127} While the relationship between NFAT and NOS-2 is poorly explored in the CNS, experimental evidence in other cell types suggests a close connection between the activation of NFAT and NOS-2 induction. Activation of calcineurin in cardiomyocytes potently induces NOS-2 via a NFAT-mediated mechanism\cite{128} while inhibition of NFAT blocks NOS-2 upregulation in activated macrophages.\cite{129} Although activation of calcineurin-NFAT activity has not been directly studied in SAH, it may plausibly contribute to upregulation of NOS-2.

**Nos-2 Therapeutics After Subarachnoid Hemorrhage**

**Blockade of NOS-2 upregulation**

Given NOS-2’s essential lack of posttranslational controls, prevention of NOS-2 upregulation represents an attractive therapeutic strategy. Several therapeutic targets are available to block NOS-2 upregulation following SAH.[Figure 1]. Blockade of TLR-4 represents one such strategy, as TLR-4 ligation activates several pathways culminating in NOS-2 upregulation including the activation of NF-κB through proinflammatory signaling cascades, induction of cytokines in microglia, and activation of phagocytes with subsequent generation of ROS. Blockade of TLR-4 can be achieved through a variety of small...
molecule antagonists such as eritoran\textsuperscript{[130]} and TAK-242.\textsuperscript{[131]} The opioid antagonist, naloxone, which also blocks TLR-4,\textsuperscript{[132]} may represent an interesting possibility as it is well-established clinically and has shown efficacy in treating vasospasm in a small case series.\textsuperscript{[133]} Clinically approved peroxisome proliferator-activated receptor γ (PPAR-γ) agonists such as rosiglitazone downregulate TLR-4 in both cell culture\textsuperscript{[134]} and in animal models\textsuperscript{[135]} of SAH.

Cytokines produced by activated microglia and infiltrating inflammatory cells are also possible targets for reducing NOS-2 expression. Blockade of inflammatory cytokines such as TNF using antibodies\textsuperscript{[136]} or IL-1β using small peptides\textsuperscript{[137]} has demonstrated preclinical efficacy in preventing neurologic injury following SAH.

Given the variety of elements in the CNS that upregulate NOS-2 following SAH, targeting any single one may not be entirely effective in preventing NOS-2 upregulation. As such, blockade of the transcription factors responsible for NOS-2 upregulation may present a viable strategy. Given its role as the principal upstream mediator of NOS-2 expression in SAH, NF-κB seems an obvious target. Several compounds demonstrating preclinical benefit in SAH including the hormone estradiol\textsuperscript{[138]} and the antioxidant N-acetyl cysteine\textsuperscript{[139]} exploit this mechanism to block NOS-2 upregulation. However, one potential problem with NF-κB blockade is timing. Statins, for example, reduce NOS-2 expression \textit{via} a NF-κB dependent mechanism.\textsuperscript{[140]} However, while statin therapy effectively blocks NF-κB activation\textsuperscript{[141]} and NOS-2 upregulation\textsuperscript{[142]} when given as a pretreatment, its effects on NOS-2 are lost when given immediately following SAH. Also, in cerebral ischemia and most likely other forms of acute CNS injury, NF-κB is “Janus-faced,” responsible not only for early adverse effects but also delayed beneficial effects,\textsuperscript{[143]} rendering broad inhibition potentially deleterious.

Blockade of HIF-1 may also be considered since HIF-1 activators enhance NOS-2 production.\textsuperscript{[138]} However, it is unlikely that targeting HIF-1 will be a viable therapeutic strategy due to off-target effects on potentially beneficial molecules under control of HIF-1 such as erythropoietin.\textsuperscript{[144]} Also, increased HIF-1 activity is required for the therapeutic effects of agents such as isoflurane\textsuperscript{[145]} and deferoxamine\textsuperscript{[146]} in preclinical models of SAH. The use of NOS-2 inhibitors in conjunction with HIF-1 activators or attempts to uncouple NOS-2 production from HIF-1 activation could be of potential benefit although such approaches remain untried.

Blockade of NFAT activation \textit{via} calcineurin inhibition provides another potentially attractive approach, given the regular use of calcineurin inhibitors such as cyclosporine in transplant medicine. These same drugs block NOS-2 upregulation through an NF-κB-independent mechanism.\textsuperscript{[146,147]} Cyclosporin prevents SAH-induced neurologic injury and vasospasm in several preclinical models.\textsuperscript{[148,149]} However, clinical experience with cyclosporine is conflicting, as one early trial reported no apparent benefit of cyclosporin for the treatment of vasospasm\textsuperscript{[150]} while another pilot study reported some benefit in neurologic status with early cyclosporine administration following SAH.\textsuperscript{[151]} Further research is needed to determine if this mode of action can be successfully translated to the clinic.

Inhibition of NOS-2 activity
Given its pernicious role in SAH, direct blockade of NOS-2 presents an attractive therapeutic option. One such experimental agent is aminoguanidine, a selective NOS-2 inhibitor originally developed for preventing diabetic nephropathy.\textsuperscript{[152]} Experimental work suggests that aminoguanidine may be an effective therapeutic agent in SAH. Employing an endovascular perforation model of SAH in rats, Sayama et al.\textsuperscript{[153]} found that aminoguanidine ameliorated vasospasm following SAH. Similarly, treatment of rabbits with aminoguanidine following experimental SAH significantly reduced the severity of vasospasm.\textsuperscript{[153]} Other NOS-2 inhibitors remain essentially unstudied in the context of SAH but the success of aminoguanidine in experimental studies suggests that NOS-2 inhibition may be a valid strategy to prevent secondary injury following SAH.

Apart from preventing excess NO formation \textit{via} NOS-2 blockade, counteracting the deleterious effects of ROS and RNS may present additional useful therapeutic strategies. Given the prominent role of oxidative stress in SAH, a number of antioxidants have been studied as potential therapeutics.\textsuperscript{[154]} While a review of antioxidants in SAH is beyond the scope of this article, one class of RNS agents, peroxynitrite scavengers, bears note. In theory, this class of agents could potentially neutralize peroxynitrite while allowing NO to perform important physiologic roles such as vasodilation. Although poorly studied in the context of SAH, peroxynitrite scavengers such as uric acid have demonstrated efficacy in other pathologies involving NOS-2 including MS\textsuperscript{[155]} and stroke.\textsuperscript{[156]}

Pleiotropic Therapeutics
Given the complex nature of NOS-2 upregulation following SAH, approaches targeting any single component of the NOS-2/NO/peroxynitrite axis in SAH may be inadequate. Statins, a class of medications traditionally associated with cardiovascular disease, present one such therapeutic class. Apart from preventing NF-κB activation and concurrent NOS-2 upregulation, statins also reduce ROS and peroxynitrite formation\textsuperscript{[142]} and downregulate IL-1β production\textsuperscript{[158]} following SAH. Despite their ability to address multiple aspects of NOS-2-mediated pathology, statins have had a disappointing clinical record in SAH. Although initial clinical studies suggested improved outcomes, more recent randomized trials have failed to show a benefit with regard to the rates of death or poor neurologic outcome.\textsuperscript{[157-159]} One reason for this failure may be the timing of statin therapy — although preclinical studies demonstrate the benefit of statin therapy following experimental SAH, the effects on vasospasm and neurologic injury were diminished when compared to treatment prior to SAH despite the relatively rapid (30 min) dosing.\textsuperscript{[45,160]} Whether these effects persist with dosing at later times is not clear.

Another pleiotropic agent of interest is heparin, which mediates a variety of anti-inflammatory and antioxidant effects. Heparin can bind numerous inflammatory cytokines and chemokines, and block the activation of NF-κB.\textsuperscript{[161]} In a rat model of SAH, treatment with a nonanticoagulating dose of heparin
provides a significant reduction in neural inflammation and cytokine production including IL-1β. Given its potent anti-inflammatory effects, the ability of heparin to reduce NOS-2 induction in response to inflammation both in vitro and in vivo should not be surprising. In a rat model of SAH, low-dose heparin is a powerful inhibitor of NOS-2 expression (Simard et al., unpublished). Moreover, heparin can act as an antioxidant and could theoretically prevent ROS/RNS-mediated injury through a variety of mechanisms. In consistence with these findings, early clinical evaluation of heparin in patients with SAH at high risk for DND demonstrated significant promise with regard to preventing vasospasm, infarction, and neurologic deterioration. Thus, heparin and heparinoids provide a promising class of therapeutic intervention for NOS-2-mediated pathology in SAH, addressing both the mechanisms that increase NOS-2 production as well as ameliorating the effects of increased ROS/RNS production.

**Summary and Perspectives**

Excessive NO production plays a significant role in secondary injury following SAH. However, given the diametrically opposed functions of NO following SAH, including its indispensable role as vasodilator versus its cytotoxic effects, complete blockade of NO production is inadvisable. Fortunately, the pathological increase in activity of NOS-2 in NO following SAH can be attributed largely to the activity of NOS-2, making this isoform a preferred target.

The increase in activity of NOS-2 is due to transcriptional upregulation of the enzyme induced by the proinflammatory environment of SAH. Once upregulated, NOS-2 is essentially free of posttranslational controls, and continues to generate cytotoxic amounts of NO until the enzyme is degraded. Several attractive strategies present themselves for targeting NOS-2 [Figure 1] although to date none has been successfully translated. Among the more promising approaches are blockade of NOS-2 upregulation, for example using TLR-4 antagonists, blockade of the enzyme itself using specific NOS-2 inhibitors, prevention of NO-induced bystander injury using peroxynitrite scavengers, and the use of pleiotropic agents such as heparin. As mechanistic understanding of NOS-2 upregulation following SAH improves, newer innovative therapies acting on the NOS-2/NO/peroxynitrite axis are anticipated.

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**Conflicts of interest**

There are no conflicts of interest.

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