Endogenous Protection in Subarachnoid Hemorrhage

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Endogenous Protection in Subarachnoid Hemorrhage

by

Eric Milner

A dissertation presented to the Graduate School of Arts and Sciences of Washington University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

December 2015

St. Louis, Missouri
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December 2015
ABSTRACT OF THE DISSERTATION

Endogenous Protection in Subarachnoid Hemorrhage

by

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Doctor of Philosophy in Biology and Biomedical Sciences
Neurosciences
Washington University in St. Louis, 2015

Professor Gregory Zipfel, Chair

Vasospasm-induced delayed cerebral ischemia (DCI) remains a major source of morbidity in patients with aneurysmal subarachnoid hemorrhage (SAH). Moreover, cognitive dysfunction is the primary driver of poor long-term outcome in SAH survivors; modeling such deficits preclinically is thus key for mechanistic and translational investigation. We hypothesized that activating innate neurovascular protective mechanisms by conditioning may represent a novel therapeutic approach against SAH-induced DCI, short-term, neurological deficits, and long-term neurocognitive deficits; and, secondarily, that the neurovascular protection it provides is mediated by endothelial nitric oxide synthase (eNOS) and hypoxia-inducible factor 1α (HIF-1α).

In Experiment 1, wild-type C57BL/6 mice were subjected to hypoxic preconditioning (PC) or normoxia followed 24 hours later by endovascular-perforation SAH. Neurological function was analyzed daily via sensorimotor scoring; vasospasm was assessed on post-surgery day 2. Nitric oxide availability, eNOS expression, and eNOS activity were also assessed. In a separate experiment, wild-type and eNOS-null mice were subjected to hypoxic PC or normoxia
followed by SAH and assessed for vasospasm and neurological deficits. All experiments were performed in a randomized and blinded fashion.

PC nearly completely prevented SAH-induced vasospasm and neurological deficits. It also prevented SAH-induced reduction in nitric oxide availability and increased eNOS activity in mice with and without SAH. PC-induced protection against vasospasm and neurological deficits was lost in wild-type mice treated with the nitric oxide synthase inhibitor $N^{G}$-nitro-L-arginine methyl ester and in eNOS-null mice.

From these results, we conclude that endogenous protective mechanisms against vasospasm exist, are powerful, and can be induced by PC. eNOS-derived nitric oxide is a critical mediator of this neurovascular protection. These “proof-of-principle” results suggest that conditioning represents a promising new strategy to mitigate SAH-induced neurovascular dysfunction.

In Experiment 2, we sought to determine whether these innate protective mechanisms are induced by a more clinically relevant conditioning paradigm, and whether the cerebrovascular protection extends to non-vasospasm contributors to DCI, microthrombosis and microvessel dysfunction.

Adult male mice were subjected to sham surgery, SAH surgery, or SAH and subsequently postconditioned with isoflurane (2% for 1h, starting 1h after surgery). Contributors to DCI – vasospasm of the ipsilateral middle cerebral artery, cortical microthrombosis as assessed by fibrinogen immunohistochemistry, and cerebrovascular vasodilatory function was assessed in pial vessels through a closed cranial window – were measured 3 days post-SAH. Neurological outcome was assessed daily. Moreover, isoflurane-induced changes in HIF-1α-dependent genes (glucose transporter-1, GLUT1; BNIP3) and HIF-2α-driven erythropoietin
(EPO) were assessed via quantitative-PCR. HIF-1α was inhibited either pharmacologically (2-methoxyestradiol, 2ME2) or genetically in endothelial cells (EC-HIF-1-null). All experiments were performed in a randomized and blinded fashion.

We found the following: first, isoflurane postconditioning markedly reduced SAH-induced DCI in wild-type mice: vasospasm was attenuated, microthrombosis was significantly reduced, and microvessel function was restored. Neurological deficits were also significantly attenuated. Second, isoflurane modulated HIF-1α- and HIF-2α-dependent genes; these changes were abolished in 2ME2-treated wild-type mice and in EC-HIF-1-null mice (HIF-1α-dependent genes only in the latter). Third, postconditioning-induced protection against vasospasm and neurological deficits was attenuated in 2ME2-treated wild-type mice and in EC-HIF-1-null mice.

In Experiment 3, we sought to assess whether the protection afforded by conditioning extended to long-term neurocognitive outcomes. Whereas rat SAH causes long-term deficits in learning and memory, it remains unknown whether similar deficits are seen in the mouse, a species particularly amenable to powerful, targeted genetic manipulation. We thus subjected mice to SAH and assessed long-term cognitive outcome via the Morris water maze (MWM), the most commonly used metric for rodent neurocognition. No significant differences in MWM performance (by either of two protocols) were seen in SAH versus sham mice. Moreover, SAH caused negligible hippocampal CA1 injury. These results undercut the potential of commonly used methods (of SAH induction and assessment of long-term neurocognitive outcome) for use in targeted molecular studies of SAH-induced cognitive deficits in the mouse.

From these results, we concluded that endogenous protective mechanisms against DCI exist, are powerful, and can be induced by hypoxic PC or isoflurane postconditioning. This protection depends critically on eNOS-derived nitric oxide and endothelial cell–derived HIF-1α.
These studies provide strong evidence that conditioning – especially isoflurane postconditioning – represents a promising new strategy to reduce DCI after SAH. Future studies examining long-term neurocognitive deficits should utilize rat models of SAH.
CHAPTER 1: Endothelial Nitric Oxide Synthase Mediates Endogenous Protection Against Subarachnoid Hemorrhage-Induced Cerebral Vasospasm

INTRODUCTION

Aneurysmal subarachnoid hemorrhage (SAH) occurs in ~600,000 people worldwide each year,\textsuperscript{4} and in ~30,000 patients in the US.\textsuperscript{5} While it accounts for only 5-8\% of strokes,\textsuperscript{4, 6} it accounts for a quarter of cerebrovascular deaths and of stroke-related loss of life\textsuperscript{7, 8} due to its low median age (52 years, \textit{versus} 70-80 years for ischemic stroke\textsuperscript{4}). Its case fatality remains around 40\% despite improvements in diagnosis, immediate repair of aneurysms, and improved medical and ICU management.\textsuperscript{4} Among SAH survivors, only 25\% make a good recovery while 50\% suffer long-term cognitive deficits\textsuperscript{9, 10} that preclude their return to work and significantly impact their quality of life.\textsuperscript{11} Independent risk factors for poor outcome, as determined by a multivariate analysis of over 3500 patients,\textsuperscript{12} include the following: older age, worse neurological status or elevated blood pressure upon presentation; quantity of subarachnoid blood or the presence of intracerebral or intraventricular blood on admission; previous SAH; or history of hypertension, myocardial infarction, or liver disease. In contrast, other predictors of poor outcome may be amenable to therapeutic intervention: these include fever, cerebral infarction, and delayed cerebral ischemia (DCI).\textsuperscript{12, 13} Aneurysm rupture results in markedly perturbation of intracranial homeostasis: intracranial pressure rapidly rises to arterial levels resulting in global cerebral ischemia. This acute insult is thought to lead to secondary neurological injurious processes, delayed cerebral ischemia and early brain injury.\textsuperscript{14}
Delayed Cerebral Ischemia

Delayed cerebral ischemia (DCI) is the most common and most severe form of secondary brain injury to develop after SAH. It occurs with a stereotypical delay, with a peak incidence of four to twelve days post-ictus, as a result of which it is considered the prognostic factor most amenable to therapeutic intervention. Rates of DCI have remained stable over the last decades: prior to 1994, 33% of patients were afflicted, and the rate in patients between 1994 and 2009 was 29%. Its presence doubles the risk of poor patient outcome.

DCI was previously attributed directly and exclusively to cerebral vasospasm, a phenomenon characterized by angiographic narrowing of the large cerebral arteries 5-14 days post-SAH. Approximately two thirds of SAH patients develop angiographic vasospasm, and in one third it is moderate to severe. Vasospasm is thought to be a result of erythrocyte hemolysis in the subarachnoid space: rupture of red blood cells liberates hemoglobin and other molecules that initiate inflammation, production of oxygen free radicals, and an imbalance of vasodilatory and vasoconstrictive factors. Several observations support vasospasm’s central role in DCI: vasospasm and DCI coincide temporally; DCI-related symptoms occur within the territory of spastic arteries in many patients with vasospasm; and targeted endovascular treatment of vasospasm often improves patients’ neurological status.

The past decade has seen an evolution in understanding of DCI pathophysiology. The notion of vasospasm being the sole cause of DCI has been challenged by the results of clinical trials. First, only half of patients with angiographic vasospasm develop DCI. Second, drugs that reduce angiographic vasospasm do not improve outcome after SAH (e.g., the non-glucocorticoid 21-aminosteroid tirilazad or the endothelin receptor antagonist clazosentan). Third, the only drug that improves long-term outcome after SAH, the calcium antagonist nimodipine, has
minimal effect on vasospasm. Nonetheless, vasospasm has been repeatedly identified as an independent risk factor for both brain infarction and poor outcome after SAH.\textsuperscript{12,19,20,28-31}

As a result of these findings, the contribution of several additional pathophysiological processes have been linked to DCI; these include microvessel dysfunction (i.e., microcirculatory autoregulatory dysfunction), microvessel thrombosis, and cortical spreading ischemia.\textsuperscript{4} In fact, many believe a combination of these pathological events are required to produce SAH-induced DCI (for review, see Macdonald\textsuperscript{4}).

Cortical microthrombosis occurs in 70% of SAH patients as assessed by a prospective study employing transcranial Doppler ultrasonography;\textsuperscript{32} post-mortem, it is seen in over 90% of patients.\textsuperscript{33} Microthrombosis correlates strongly but non-significantly with symptomatic vasospasm,\textsuperscript{32} correlates significantly with DCI,\textsuperscript{33} and is more severe in patients who die of DCI compared to those who die of rebleeding or acute hydrocephalus.\textsuperscript{34} Experimentally, microthrombi are seen as early as 10 minutes after SAH but peak 1-2 days after.\textsuperscript{35,36} The pathophysiology of these fibrin– and activated platelet–rich thrombi\textsuperscript{37} is thought to involve a pro-coagulant state and altered cerebral hemodynamics. Within days of SAH, multiple measures of platelet activation are increased; serum levels of platelet-activating factor, von Willebrand factor, and tissue factor are elevated;\textsuperscript{37} and endothelial cell damage and increased expression of p-selectin\textsuperscript{38} all encourage platelet aggregation and conversion of fibrin to fibrinogen. Decreased blood flow further promotes clotting: this is due to transient ischemia secondary to increased intracranial pressure immediately after aneurysmal rupture, whereas days later it results from microvessel constriction.\textsuperscript{38} Unfortunately, treatments aimed at reducing microthrombosis have been disappointing: a randomized controlled trial examining the effect of aspirin in SAH patients was stopped because the probability of benefit became negligible.\textsuperscript{39} Moreover, a Cochrane
review found that antiplatelet therapy is not recommended in SAH, as a trend toward decreasing DCI and improving outcome was counterbalanced by increased risk of intracranial hemorrhage.\textsuperscript{27} As such, future interventions should target either target thrombosis more selectively (in order to avoid hemorrhagic complications) or target thrombosis along in combination with other deleterious processes.

Another contributor to DCI is \textit{microvessel dysfunction}. Under normal conditions, small cerebral arteries, pial arterioles, and penetrating arterioles dilate or constrict in response to a variety of physiological stimuli. However, SAH results in increased baseline vascular tone and compromises many of these auto-regulatory responses in patients\textsuperscript{40, 41} and in animal models.\textsuperscript{42-44} Altered vasoreactivity appears as early as 20 minutes after experimental SAH\textsuperscript{43} and persists for as long as 7 days.\textsuperscript{43} Similar to large-artery vasospasm, the underlying mechanism for this phenomenon appears to be a fundamental imbalance in vasodilation and vasoconstriction. Studies in preclinical models of SAH have documented attenuated vasodilation in response to adenosine, adenosine diphosphate and triphosphate, vasopressin, and nitroprusside, as well as increased responses to vasoconstrictive stimuli including endothelin-1 and basic pH.\textsuperscript{38} Arteriolar constriction was shown to correlate with microthrombosis in mouse SAH,\textsuperscript{45} supporting the notion that multiple deleterious processes interact to cause DCI. Indeed, alterations in microvascular reactivity have been linked to risk of developing DCI.\textsuperscript{46-49} Interestingly, the beneficial effect of nimodipine – which, as discussed above, appears to be independent of an effect on vasospasm – may result from attenuating the increase in myogenic tone after SAH.\textsuperscript{27, 50}
Early Brain Injury

The term early brain injury (EBI) refers to cerebral damage that occurring within the first 72 hours after ictus. It is considered to be a direct consequence of aneurysm rupture, which leads to transient global ischemia and attendant microcirculatory disturbance, as well as direct toxicity of subarachnoid blood. Its two principal contributing pathophysiological processes are cerebral edema and cell death.\textsuperscript{14}

Cerebral edema is an independent risk factor for poor outcome (especially poor cognitive outcome) after SAH.\textsuperscript{51} This condition generally begins in the first several hours after SAH and peaks 2 to 3 days later.\textsuperscript{52} Despite affecting as many as 20\% of patients\textsuperscript{53-55} and being identified as an independent risk factor for poor patient outcome,\textsuperscript{51} no intervention has been shown to mitigate it. Indeed, few studies have thus far examined its underlying pathophysiology; of those that have, most have identified breakdown of the blood-brain barrier (BBB) as a key contributing event.\textsuperscript{52,56-61} The causes of BBB disruption include widening of inter-endothelial spaces, apoptosis of endothelial cells and astrocytes, and degradation of extracellular matrix proteins including type IV collagen and tight junction proteins.\textsuperscript{38}

Another key contributor to EBI is neuronal cell death. Both direct consequences of aneurysm rupture – transient global ischemia and toxicity of subarachnoid blood – have been linked to neuronal cell apoptosis;\textsuperscript{57,62} moreover, neuronal cell death correlates with cerebral edema.\textsuperscript{63} Both caspase-dependent and –independent pathways have been implicated in neuronal cell apoptosis after SAH.\textsuperscript{64} Of note, one protein involved in the caspase-independent apoptosis after SAH is Bcl-2/adenovirus E1B 19kDa-interacting protein 3 (BNIP3).\textsuperscript{65}
Neuropsychiatric Outcomes

While relatively few SAH survivors have significant focal neurological deficits of the sort that are common following ischemic stroke, long-term cognitive dysfunction is seen in 50-60%, allowing only 33% to return to their previous level of employment despite good neurological outcome. The long-term cognitive deficits after SAH cross numerous cognitive domains, including memory, executive function, and language (for review, see Al-Khindi). Of the memory deficits, most but not all studies have documented visuospatial memory as being particularly affected. One genetic predictor of poor cognitive outcome after SAH has been identified – apolipoprotein E, a well-established risk factor for Alzheimer’s disease. Strikingly, no treatment has been shown to improve cognitive outcome after SAH. It is therefore critical to devise experimental methods to elucidate underlying mechanism and develop novel therapeutics for these deficits, as they are a principal driver of the long-term loss of quality of life in SAH survivors.

Experimental SAH research has thus sought to model long-term neurobehavioral outcomes and elucidate the mechanisms responsible. Morris water maze (MWM) deficits have been documented in all three principal rat models (see below) 3-5 weeks after SAH, along with T-maze deficits in endovascular perforation SAH, moreover, treatment effects have been seen with statins and minocycline. Despite these reports in rat models of SAH, to date there has been no characterization of longer-term neurobehavioral deficits in mouse models of SAH.

Animal Models of Subarachnoid Hemorrhage

Multiple animal models are used to study SAH (for a review, see Titova). These vary in terms of species (mouse, rat, rabbit, dog, primate) and method of inducing SAH (endovascular
perforation, cisterna magna injection, prechiasmatic cistern injection, clot placement); each produces a different constellation of SAH-induced deficits (timing and degree of vasospasm, BBB disruption, neuronal cell death, neurocognitive deficits, etc.). Endovascular perforation SAH in mice has several important advantages: the perforation is similar to the direct arterial damage seen with a ruptured aneurysm; cerebrovascular dysfunction occurs, at the level of the macrovessel and the microvessel; and, perhaps most importantly, the mouse is amenable to powerful genetic manipulation allowing incorporation of myriad transgenic lines to query mechanism precisely. One disadvantage to mouse models of SAH, however, is their apparent inability to recapitulate the neuronal cell death and long-term cognitive deficits seen in SAH.

Translation of Preclinical Interventions

The importance of including long-term outcomes in preclinical studies has been substantiated by disappointing results from recent therapeutic trials. Despite successfully reducing angiographic vasospasm after SAH, long-term patient outcome was not affected by administration of the non-glucocorticoid 21-aminosteroid tirilazad\(^25\) or the endothelin receptor antagonist clazosentan.\(^26\) One interpretation of these results is that, beyond examining short-term preclinical outcomes (such as early brain injury and cerebral vasospasm), the successful translation of putative therapies would be enhanced by evaluating longer-term neurobehavioral outcomes in preclinical models,\(^80\) as recommended by the Stroke Therapy Academic Industry Roundtable’s criteria.\(^81\) A second conclusion, given the failure of therapies targeting a single pathophysiological process, is that future therapies should ideally target multiple components of SAH-induced secondary brain injury.
Cerebral Conditioning

Cerebral conditioning describes the phenomenon wherein the brain’s endogenous protective mechanisms against a severe injury can be induced by exposure to a mildly stressful stimulus (for review, see Gidday\textsuperscript{82} or Iadecola and Anrather\textsuperscript{83}). Preconditioning and postconditioning are defined, by convention, according to whether the conditioning stimulus is delivered prior to or after injury, respectively. Initial investigations into cerebral conditioning focused on its beneficial effects on neuronal survival and function; research in recent years, however, has made it clear that the cerebrovasculature (as well as glial cells) is also an important effector of the resulting injury-tolerant phenotype.\textsuperscript{84} Support for a vascular contribution to cerebral conditioning comes primarily from the experimental stroke literature where conditioning has been shown to increase vascular patency;\textsuperscript{85} improve endothelium-dependent vasodilation\textsuperscript{86} and cerebral blood flow;\textsuperscript{85, 87} and reduce blood-brain barrier breakdown and vasogenic edema.\textsuperscript{88, 89} Given that the pathophysiological events that underlie DCI are primarily vascular (vasospasm, microcirculatory dysfunction, and microvessel thrombosis), a conditioning-based strategy capitalizing on endogenous protective cascades that protect the cerebrovasculature (as well as neurons and glia) would represent a powerful, novel intervention for SAH-induced DCI.

Due to a variety of unique clinical and pathophysiological factors related to SAH-induced DCI, we\textsuperscript{1} and others\textsuperscript{90} have long hypothesized that SAH represents a patient population that is perfectly suited to a conditioning-based therapy. First, a large portion (approximately one-third) of patients predictably develop DCI after SAH.\textsuperscript{15} Second, DCI stereotypically develops in delayed fashion (at least 4 days post-ictus), providing an ample therapeutic window of opportunity. Third, a conditioning-based strategy addresses one of the principle reasons previous therapeutic approaches against DCI have likely failed: the targeting of a single component (often
vasospasm) of what is now known to be a multifactorial process (including deleterious effects on the cerebral microcirculation). In contrast, conditioning is inherently pleiotropic, exhibiting robust beneficial effects on large cerebral arteries and the cerebral microcirculation, as well as on other important CNS cell types including neurons and glia. Preconditioning has myriad positive effects on cerebral vessels and cerebral blood flow in the setting of brain ischemia, including maintenance of the BBB, reduction in cerebral edema, improvements in cerebral blood flow and cerebral vessel reactivity, reduction in leukocyte-mediated inflammation, and reduction in endothelial cell apoptosis. Though the effect of conditioning on these deleterious processes has not been examined in SAH, the fact that each is seen following SAH raises the possibility that a conditioning-based intervention, via its impact on multiple injurious pathways, may succeed in improving outcomes where other therapies have failed. Moreover, several FDA-approved drugs that function as conditioning stimuli, raising the possibility of rapid clinical translation should one prove efficacious in SAH.

In the context of experimental SAH, Altay and colleagues showed that EBI after mouse SAH can be impacted by isoflurane postconditioning, as cerebral edema, neuronal cell death, and neurological deficits were all reduced 24 hours post-SAH. However, these investigators noted that isoflurane-induced protection in SAH appeared transient, as reductions in cerebral edema and neurological deficits were lost at 72 hours post-SAH (neuronal cell death was not examined beyond 24 hours).

The notion that the human brain can be preconditioning is substantiated by recent studies examining the issue of conditioning-induced protection in cerebrovascular patient populations: first, patients with transient ischemic attacks who later suffered ischemic strokes have smaller infarctions and lower rates of in-hospital mortality than patients without prior history of such
attacks. In the setting of SAH, one study of twelve patients found that brain tissue hypoxia during temporary arterial clamping for aneurysm clipping was attenuated by prior ischemic preconditioning (2-minute occlusion) of the proximal artery. More recently, in collaboration with Hoh and colleagues, we showed that SAH patients with pre-existing steno-occlusive cerebrovascular disease are less likely to develop angiographic vasospasm than those without such disease – the first evidence in humans of a potential preconditioning effect on SAH-induced vascular dysfunction. Although these reports do not demonstrate causation, they intriguingly suggest that the human cerebrovasculature may in fact benefit from non-injurious stressors in ways similar to what has been documented in animal models.

**Endothelial Nitric Oxide Synthase**

Nitric oxide (NO) is a potent vasodilator whose down-regulation after SAH has been linked to vasospasm as well as to microcirculatory dysfunction and microvessel thrombosis. Though the link of reduced NO signaling after SAH is well established – and that mitigating the drop is beneficial – the impact of SAH on the three isoforms of NO synthase (NOS) remains to be elucidated fully. The effect of SAH on neuronal NOS (nNOS) and inducible NOS (iNOS) remains poorly understood. For endothelial NOS (eNOS), the principal source of NO in the vasculature, the effect is controversial: past studies are divided among reporting no change, an increase, or a decrease in eNOS after experimental SAH.

Not only has the NOS-NO pathway been implicated in the pathophysiology of SAH’s sequelae, it has also been shown to underlie vascular conditioning in experimental cerebral ischemic injury. Ischemic preconditioning–induced protection against focal cerebral ischemia is lost with a pan-NOS antagonist but not with nNOS– or iNOS-selective pharmacologic
inhibitors. A similar loss of ischemic tolerance was noted in eNOS-null mice subjected to ischemic preconditioning and then focal stroke.

Hypoxia-Inducible Factor 1

One molecule that has been identified as a mediator of isoflurane-induced conditioning, at least in the context of cerebral ischemia (a fundamentally different form of experimental brain injury), is hypoxia-inducible factor 1 (HIF-1). The central function of this molecule relates to maintenance of cellular homeostasis during periods of low oxygen tension – a function that has been strongly conserved across evolutionary lines. Under normoxic conditions, hydroxylation of the cytosolically-located alpha subunit (HIF-1α) by members of the prolyl hydroxylase domain (PHD) family leads to its rapid proteasomal degradation. Under hypoxic conditions, PHD activity decreases, leading to accumulation of HIF-1α. It then associates with the beta subunit and translocates to the nucleus, where it modulates transcription of over 100 genes.

In SAH, HIF-1-driven transcription appears to have either a deleterious or a salubrious effect depending on the timing and extent of its activation. Several studies have explored the role of HIF-1 in early brain injury and DCI after SAH. HIF-1 has been repeatedly shown to be up-regulated following experimental SAH throughout the brain. Experimental modulation of HIF-1, however, appears to have different effects based on the timing of intervention. Some studies have shown that pharmacologic inhibition 1-2 hours after experimental SAH is salubrious: 2ME2 administration reduced mortality and improved neurological outcome; attenuated BBB disruption and cerebral edema; and reduced vasospasm. However, another study showed that inhibiting HIF-1 prior to (rather than after) SAH was deleterious, finding that YC-1 given 24 hours and 30 minutes before SAH increasing neuronal cell apoptosis.
in hippocampal CA1 and worsening cognitive outcome as assessed by the Morris water maze. Finally, one study showed that delayed up-regulation of HIF-1 was protective: administering desferoxamine four days after experimental SAH improved cerebral blood flow and reduced vasospasm. It is thus likely that in the context of SAH, the ultimate result of HIF-1 signaling – pro-survival or pro-death – is exquisitely dependent on the degree of up-regulation and its timing: a double-edged sword.

Despite these several studies examining HIF-1α in experimental SAH, none has assessed HIF-1α in the context of conditioning. In contrast, HIF-1α has been strongly implicated in protecting neurons from experimental ischemia (both in vivo and in vitro). Nonetheless, its protective role in the cerebrovasculature has thus far only been examined in in vitro studies with cultured vascular endothelial cells. As such, whether HIF-1α-mediated ischemic tolerance is driven (at least in part) by improved cerebral vessel function (e.g., microvessel function, cerebral blood flow, or vessel patency), and whether such protection extends to SAH, remain unknown.

MATERIALS AND METHODS

Animals: All experimental protocols were approved by the Animal Studies Committee at Washington University in St Louis. Three to four months old male wild-type mice (C57BL/6J) and homozygous eNOS−/− mice (B6.129P2-Nos3tm1Unc/J with a C57BL/6 genetic background) from Jackson Laboratories (Bar Harbor, ME) were used.

Hypoxic PC: Mice were placed in a hypoxic chamber and exposed to air containing 8% O₂/92% N₂ for 4 hours with access to food and water ad libitum; mice were then returned to their cages. Normoxic controls were placed in chambers containing room air.
Pharmacological Inhibition of NO Synthase: To examine the effect of NO synthase inhibition on PC-induced neurovascular protection in SAH, wild-type mice were administered the pan-NO synthase inhibitor L-N^G-nitroarginine methyl ester (L-NAME; 20 mg/kg intraperitoneally) or vehicle (normal saline) starting 1 hour before PC and continued once daily thereafter.

Experimental Protocols and Groups: The first set of experiments was performed to assess whether hypoxic PC attenuates SAH-induced vasospasm and neurological deficits. Three groups of mice were used: (1) normoxia followed 24 hours later by sham surgery (Norm:Sham), N=11; (2) normoxia followed 24 hours later by SAH (Norm:SAH), N=16; and (3) PC followed 24 hours later by SAH (PC:SAH), N=13. The second set of experiments was designed to explore the molecular pathways affected by PC and SAH. Four groups of mice were used: (1) Norm:Sham, N=6; (2) PC followed 24 hours later by sham surgery (PC:Sham), N=5; (3) Norm:SAH, N=10; and (4) PC:SAH, N=8. The third set of experiments was designed to examine whether pharmacological inhibition NO synthase blocks the neurovascular protective effects of PC in SAH. Six groups were used: (1) Vehicle:Norm: Sham, N=3; (2) Vehicle:Norm:SAH, N=5; (3) Vehicle:PC:SAH, N=5; (4) L-NAME:Norm:Sham, N=5; (5) L-NAME:Norm:SAH, N=11; and (6) L-NAME:PC:SAH, N=11. For statistical power, data from the vehicle-treated groups were combined with data from Experiment 1 once it was determined that the corresponding groups were statistically similar (data not shown). The fourth set of experiments was designed to directly assess the contribution of eNOS in PC-induced neurovascular protection. Six groups of mice were used: (1) eNOS^{+/+} Norm:Sham, N=14; (2) eNOS^{+/+} Norm: SAH, N=12; (3) eNOS^{+/+} PC:SAH, N=17; (4) eNOS^{-/-} Norm: Sham, N=12; (5)
eNOS\textsuperscript{-/-} Norm:SAH, N=19; and (6) eNOS\textsuperscript{-/-} PC:SAH, N=20. All assessments were performed by investigators blinded to the surgical procedure and genotypes.

**Experimental SAH:** Endovascular perforation SAH was performed as described. Briefly, mice were anesthetized with isoflurane (4% induction, 1.5% maintenance), and a 5-0 nylon suture was introduced into the external carotid artery and advanced through the internal carotid artery until the internal carotid artery bifurcation. The suture was then advanced to induce SAH, then removed and the external carotid artery ligated. Mice in the sham surgery groups underwent these procedures except for suture perforation.

**Neurobehavioral Tests:** Neurobehavioral outcome was examined daily using Neuroscore and Rotarod tests as described. Briefly, neurological function was graded based on a motor score (0 to 12) that evaluated spontaneous activity, symmetry of limb movements, climbing, balance, and coordination and a sensory score (4 to 12) that evaluated body proprioception and vibrissae, visual, and tactile responses. Balance and coordination were assessed by performance on the Rotarod (Rotamex-5; Columbus Instruments, Columbus, OH). Mice were pretrained on the Rotarod 1 day before surgery. Latency on 3 trials of 180 seconds was averaged daily.

**Vasospasm Assessment:** Vasospasm assessment was performed on post-surgery Day 2 through cerebrovascular casting, as described. Briefly, mice were anesthetized and transcardially perfused with phosphate-buffered saline, 10% formalin, and 3% gelatin–India ink solution. Brains were removed, SAH was graded as described, and blood vessels imaged under a microscope using a charge-coupled device camera. The narrowest diameter within the first 1000 µm of the middle cerebral artery was measured to assess vasospasm.

**NO Availability Assay:** NO availability was determined using DAF-2DA (fluorophore 4,5-diaminofluorescein-2-diacetate; EMD Chemicals, Gibbstown, NJ) as described. Briefly,
brain lysates were incubated with 50 µmol/L DAF-2DA for 30 minutes at 22°C in the dark. The reaction mixture was excited at 495 nm and emission read at 515 nm in a spectrophotometer (Biotek, Winooski, VT).

**Western Blot:** Western blots were performed as described. Briefly, brains were harvested 48 hours after sham or SAH surgery. Tissue from the hemisphere ipsilateral to endovascular perforation was lysed in a buffer containing 10 mmol/L 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid, 5 mmol/L MgCl₂, 1 mmol/L dithiothreitol, 2 mmol/L ethylenediaminetetra-acetic acid, 2 mmol/L ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′-tetra-acetic acid, 1 mmol/L phenylmethylsulfonyl fluoride, 1% Triton X-100, 0.5 mmol/L sodium orthovanadate, 0.1 µmol/L okadaic acid, 25 mmol/L β-glycerophosphate, and protease inhibitor cocktail (Sigma). The following primary antibodies were used: mouse antieNOS (BD Biosciences), rabbit anti-inducible nitric oxide (iNOS; Santa Cruz Biotechnology), mouse antineuronal nitric oxide synthase (nNOS; Santa Cruz Biotechnology), and mouse anti-α-tubulin (Sigma). Blots were subsequently incubated with anti-mouse or anti-rabbit horseradish peroxidase-conjugated IgG and visualized using an enhanced chemiluminescence kit (BioRad).

**eNOS Activity Assay:** NO synthase activity assay was performed as described. Briefly, brain tissue ipsilateral to endovascular perforation was lysed in a buffer containing 20 mmol/L 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid, pH 7.4, 320 mmol/L sucrose, 1 mmol/L dithiothreitol, and protease inhibitor cocktail. Brain lysates were incubated for 30 minutes in an assay buffer containing 20 mmol/L 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid, pH 7.4, 60 µmol/L [³H]-L-arginine 1 mCi/mL, 1 mmol/L ethylenediaminetetra-acetic acid, 1.2 mmol/L CaCl₂, and 2.5 mmol/L β-reduced nicotinamide-adenine dinucleotide phosphate. Samples were also incubated in this mixture along with 10
µmol/L L-NG-monomethyl arginine (competitive inhibitor of all NO synthase isoforms) or 10 µmol/L 1-(2-trifluoromethylphenyl) imidazole (selective inhibitor of nNOS and iNOS). Protein was removed by centrifugation and supernatant was passed through Dowex50WX-8 columns (Na+ form) and eluted. The radioactivity of [3H]-citrulline was measured within eluates by scintillation spectrometry (Packard Instrument Company, Meriden, CT). The activity in the mixture containing L-NG-monomethyl arginine was considered background and subtracted from the activity of the mixture containing 1-(2-trifluoromethylphenyl) imidazole to obtain activity specific to eNOS.

Statistical Analyses: Data are presented as the mean±SEM and were analyzed by analysis of variance followed by Newman-Keuls multiple comparison method unless otherwise indicated. P<0.05 was considered statistically significant.

RESULTS

PC Attenuates SAH-Induced Vasospasm

Significant vasospasm (27%±6% vessel constriction, P<0.05) was noted 2 days after SAH in nonpreconditioned mice (Figure 1). In contrast, preconditioned mice did not develop significant vasospasm (7%±6% vessel constriction, P>0.05; Figure 1). Hemorrhage grade (nonpreconditioned=3.9; preconditioned=3.7) and mortality (nonpreconditioned=5.8%, preconditioned=7.1%) were not significantly different between SAH groups.
PC Improves Neurological Outcome After SAH

SAH led to significant neurobehavioral deficits by Neuroscore and Rotorod latency test ($P<0.05$) in nonpreconditioned mice (Figure 2). These neurobehavioral deficits were significantly attenuated in preconditioned mice ($P<0.05$; Figure 2).

PC Attenuates SAH-Induced Reduction in NO Availability

NO availability was assessed 72 hours after PC (48 hours after sham or SAH surgery). SAH reduced NO availability by $42\%\pm20\%$ ($P<0.05$) in nonpreconditioned mice (Figure 3). However, in preconditioned mice, NO availability was unchanged after SAH (i.e., SAH-induced reduction in NO availability was prevented; Figure 3). PC also increased NO availability in sham-operated mice by $69\%\pm34\%$ ($P<0.05$).

NO Synthase Inhibition Blocks PC-Induced Neurovascular Protection in SAH

NO synthase inhibition with L-NAME was found to (1) reduce baseline middle cerebral artery diameter by $12\%\pm4\%$ ($P=0.03$); (2) have no impact on the degree of vasospasm after SAH ($29\%\pm5\%$ vessel constriction in vehicle-treated versus $32\%\pm8\%$ vessel constriction in L-NAME-treated nonpreconditioned mice, $P=0.7$); and (3) completely block PC-induced protection against vasospasm ($66\%\pm15\%$ vasospasm protection in vehicle-treated versus $−25\%\pm23\%$ vasospasm protection in L-NAME-treated mice, $P=0.003$) and neurological deficits by Neuroscore and Rotarod ($P>0.05$) after SAH (Figure 3). Hemorrhage grade was not significantly different between L-NAME-treated SAH groups (nonpreconditioned=3.5; preconditioned=4.1); however, mortality was different (nonpreconditioned=31%; preconditioned=0%, $P<0.05$ by Fisher exact test).
PC Selectively Increases eNOS Expression After SAH

eNOS, nNOS, and iNOS expression was assessed 72 hours after PC (48 hours after sham or SAH surgery). PC increased eNOS expression by 1.4-fold ($P=0.04$) in SAH-operated mice but had no effect on nNOS ($P=0.39$) or iNOS ($P=0.40$; Figure 4). SAH alone did not alter eNOS, nNOS, or iNOS expression (Figure 4).

PC Increases eNOS Activity After SAH

eNOS activity was assessed 72 hours after PC (48 hours after sham or SAH surgery). PC increased eNOS activity by 2.3-fold ($P<0.05$) in sham-operated mice and by 1.6-fold ($P<0.05$) in SAH-operated mice (Figure 4). SAH alone did not alter eNOS activity (Figure 4).

eNOS Contributes to PC-Induced Protection Against Vasospasm and Neurological Deficits After SAH

To directly assess the role of eNOS in PC-induced neurovascular protection, a separate group of wild-type (eNOS$^{+/+}$) and eNOS-null (eNOS$^{-/-}$) mice was examined. SAH caused significant ($P<0.05$) vasospasm in both eNOS$^{+/+}$ (21%±5% constriction) and eNOS$^{-/-}$ (24%±5% constriction) mice (Figure 5A). As expected, SAH-induced vasospasm was nearly completely prevented (94%±19% vasospasm reduction; $P<0.05$) in preconditioned eNOS$^{+/+}$ mice (Figure 5A–B). However, vasospasm protection by preconditioning was largely absent (24%±20% vasospasm reduction) in eNOS$^{-/-}$ mice (Figure 5A–B). A commensurate loss in PC-induced neurobehavioral protection (by Neuroscore) was also found in eNOS$^{-/-}$ mice (Figure 5C). Rotarod testing was not performed because naïve eNOS$^{-/-}$ mice had substantial Rotarod deficits at baseline (data not shown). Hemorrhage grade (eNOS$^{+/+}$ nonpreconditioned mice=3.2;
eNOS<sup>+/+</sup> preconditioned mice=3.7; eNOS<sup>/−</sup> nonpreconditioned mice=3.1; and eNOS<sup>/−</sup> preconditioned mice=3.7) and mortality (eNOS<sup>+/+</sup> nonpreconditioned mice=6.7%; eNOS<sup>/−</sup> preconditioned mice=10.3%; eNOS<sup>/−</sup> nonpreconditioned mice=5.0%; and eNOS<sup>/−</sup> preconditioned mice=4.7%) were not significantly different between SAH groups.

DISCUSSION

In the present study, we established the existence of endogenous protection against SAH-induced neurovascular deficits by demonstrating that preconditioned mice develop almost no vasospasm and exhibit reduced neurological deficits after SAH. This finding demonstrates, for the first time, that PC is efficacious in a fundamentally different form of stroke, SAH, and provides support to the evolving concept of vascular PC for brain ischemia. Moreover, we demonstrated that the neurological protection afforded by PC in the setting of SAH is due, in part, to decreased vasospasm, a well-recognized risk factor for poor outcome after SAH.

With respect to the molecular mechanism underlying PC-induced vasospasm protection, we examined NO given its influence on vascular function and its role in both vasospasm pathophysiology and PC-induced neuroprotection. We found that NO availability decreased after SAH and that PC increased NO levels in both sham- and SAH-operated mice. Both results are consistent with past studies, although the latter has not been previously reported in the setting of SAH. We also demonstrated that inhibition of NO synthesis abolished the protective effect of PC on SAH-induced vasospasm and neurological dysfunction. This result is similar to that reported in studies examining PC in the setting of cerebral ischemia and...
neonatal hypoxia–ischemia. Collectively, these data strongly suggest that NO synthase-derived NO is a critical mediator of PC-induced neurovascular protection in SAH.

To identify the NO synthase isoform that contributes to the observed protective effect of PC, we examined the effect of PC on the expression of all 3 NO synthase isoforms (eNOS, nNOS, and iNOS) in the setting of SAH. Given that eNOS expression was selectively upregulated after PC and that eNOS is the primary enzymatic source of NO in cerebral vessels and the most strongly implicated NO synthase isoform in vasospasm pathophysiology and vascular PC, we next examined the effect of PC on eNOS activity. Our finding that PC increased eNOS activity in both sham and SAH mice, when coupled with the aforementioned observation that NO synthase-derived NO is critical for PC-induced neurovascular protection, suggests that the vasculoprotective effects of PC likely occur through increased NO secondary to a persistent up-regulation in eNOS.

Our findings in eNOS-null mice provided further support for this hypothesis. That PC-induced abrogation of vasospasm and neurobehavioral deficits in wild-type mice was lost in eNOS-null mice (although the degree of vasospasm and neurological deficits after SAH was similar between the 2 groups when not preconditioned) indicates that the innate protective mechanisms by which PC reduces SAH-induced neurovascular deficits are dependent, to a significant extent, on eNOS. More broadly for the field of PC, our results lend additional support to the notion that eNOS is a key contributor to PC-induced neuroprotection. Other studies of PC-induced vasculoprotection in focal ischemic stroke have advanced similar conclusions regarding the participation of eNOS.

The effect of SAH on eNOS expression and/or activity is controversial. We found no effect of SAH on either metric, but previous studies report no change, a reduction, or an
increase\textsuperscript{107} in eNOS expression after SAH. Similarly, SAH was found to either have no effect\textsuperscript{105}, \textsuperscript{132} on phosphorylated eNOS (at Ser1177, one of many active forms of eNOS\textsuperscript{132}) or an increase.\textsuperscript{106-108} In addition, our experiments identified what could be considered a discrepancy between eNOS activity (no change) and NO availability (decreased) in response to SAH. Several possibilities could explain this observation. First, it is possible that a SAH-induced decrease in eNOS activity actually occurred in vivo but was undetected by our ex vivo assay. This could occur if availability of one or more substrates (e.g., L-arginine) or cofactors (e.g., reduced nicotinamide-adenine dinucleotide phosphate), which were artificially provided in our ex vivo assay, became limited; or if the endogenous NO synthase inhibitor asymmetrical dimethyl L-arginine was upregulated by SAH.\textsuperscript{103} Second, it is possible that NO availability was decreased after SAH despite unchanged eNOS activity. For example, this could occur through NO scavenging by oxyhemoglobin\textsuperscript{133} and/or leukocytes\textsuperscript{134} present in the subarachnoid space. In either case, PC-induced changes appear significant enough to overwhelm these factors and restore NO production to pre-SAH levels.

Some limitations of our study should be noted. First, PC was administered before SAH. This was by design, because our study was conceived as a “proof-of-concept” one to establish whether endogenous protective mechanisms against vasospasm are present and thus permit their exploration. Studies examining the efficacy of post-SAH conditioning paradigms have already been initiated in our laboratory. Second, although the clinical applicability of hypoxia as a preconditioning stimulus may be difficult to envision, several pharmacological agents with known PC effects are available for future testing.\textsuperscript{135} Third, because no animal model fully recapitulates human SAH, it is essential to validate our findings in complementary SAH models. Fourth, although vasospasm is a major contributor to delayed cerebral ischemia, other factors
may also contribute (for review, see Macdonald et al.⁸); whether PC also positively affects these factors will require future study. Fifth, our study does not exclude a contribution from nNOS or iNOS; however, it is very unlikely that either plays a major role given that neither was upregulated after PC in the setting of SAH. Finally, we recognize that the neurological deficits documented in our SAH model are likely multifactorial and that the observed neuroprotection could be explained in part by beneficial effects of PC on reducing neuronal apoptosis,¹³⁶ inflammation,¹³⁷ and/or blood–brain barrier breakdown.⁹¹ In fact, the robustness of the PC-induced protection noted in our study suggests a multifaceted underlying mechanism; therapeutic strategies for SAH that target several deleterious cascades would likely have great potential for clinical efficacy.

In conclusion, results from this “proof-of-principle” study indicate that powerful endogenous protective mechanisms against vasospasm exist, that they can be activated by a preconditioning stimulus, and that their underlying mechanism is at least partly dependent on eNOS-derived NO. Further studies to more fully elucidate the innate molecular pathways that prevent vasospasm, and to examine the therapeutic potential of postconditioning strategies for SAH, are warranted.
Figure 1: PC attenuates SAH-induced vasospasm.

Mice underwent hypoxic PC or normoxia (Norm) followed 24 hours later by SAH or sham surgery. On post-surgery Day 2, mice were perfused with gelatin–India ink. A, Representative images of gelatin-India ink casted vessels. Vasospasm of the middle cerebral artery (arrow) was evident in SAH- vs. sham-operated mice; PC attenuated this vasospasm. Scale bar: 200 microns. B, Vessel diameter in the proximal middle cerebral artery ipsilateral to suture perforation was determined. Data indicate mean±SEM *P<0.05.
Figure 2: PC improves neurological outcome after SAH.

Mice underwent hypoxic PC or normoxia (Norm) followed 24 hours later by SAH or sham surgery. Neurobehavioral assessment was performed on Days 0 to 2 by Neuroscore (A) and Rotarod latency (B) tests. Data indicate mean±SEM with Rotarod results normalized to Day 0. *P<0.05 vs. Norm:Sham; #P<0.05 vs. Norm:SAH. Statistical analysis was performed by repeated-measures analysis of variance of rank transformed variables followed by a Fisher's least significant difference post-hoc test.
Figure 3: NO synthase-derived NO mediates PC-induced protection against neurovascular dysfunction after SAH.

Mice underwent hypoxic PC or normoxia (Norm) followed 24 hours later by SAH or sham surgery. NO availability was quantified by fluorometric detection of DAF-2DA on brain homogenates obtained on post-surgery Day 2 (A). Data indicate mean±SEM *P<0.05 vs. Norm:Sham; #P<0.05 vs. Norm:SAH. In a separate experiment, mice were administered the pan-NOS inhibitor L-NAME and subjected 1 hour later to hypoxic PC or normoxia (Norm) followed...
24 hours later by SAH or sham surgery. Vasospasm was assessed in the middle cerebral artery on post-surgery Day 2 by the gelatin–India ink casting method (B). *P<0.05, n.s. P>0.05 by analysis of variance and Newman-Keuls multiple comparison test; neurobehavioral assessment was performed on Days 0 to 2 by Neuroscore (C) and Rotarod latency (D) tests. *P<0.05 vs Norm:Sham. Data indicate mean±SEM.
Figure 4: PC increases eNOS expression and activity.

Mice underwent hypoxic PC or normoxia (Norm) followed 24 hours later by SAH or sham surgery. eNOS, nNOS, and iNOS expression was assessed by Western blot and eNOS activity was assessed by [3H]L-arginine to [3H]L-citrulline conversion, both performed on brain homogenates obtained on post-surgery Day 2. (A) Representative immunoblot images (N indicates normoxia); (B) quantification data of eNOS, nNOS, and iNOS expression normalized to α-tubulin; and (C) quantitative data of eNOS activity. Data indicate mean±SEM (B) *P<0.05, n.s. P>0.05 by Student's t-test; (C) *P<0.05 vs. Norm:Sham, #P<0.05 vs. Norm:SAH by analysis of variance and Newman-Keuls multiple comparison test.
Figure 5: PC-induced protection against vasospasm and neurological deficits after SAH is dependent on eNOS.

Wild-type (eNOS\(^{+/+}\)) and eNOS-null (eNOS\(^{-/-}\)) mice were subjected to hypoxic PC or normoxia (Norm) followed 24 hours later by SAH or sham surgery. Vasospasm was assessed on post-surgery Day 2 by the gelatin–India ink casting method and shown as absolute diameter changes (A) or normalized to the extent of SAH-induced vasospasm in normoxic mice (B). Neurological outcome was evaluated on Days 0 to 2 by Neuroscore (C). Data represent mean±SEM. (A)
*P<0.05, n.s. P>0.05 by analysis of variance and Newman-Keuls multiple comparison test; (B) *P<0.05 for vasospasm protection in eNOS<sup>−/−</sup> vs. eNOS<sup>+/+</sup> mice using Student's t-test; (C) *P<0.05 vs. eNOS<sup>−/−</sup> Sham by repeated-measures analysis of variance of rank transformed variables and Fisher's least significant difference post-hoc test.
CHAPTER 2: Isoflurane Postconditioning Reduces Subarachnoid Hemorrhage–Induced Delayed Cerebral Ischemia via Hypoxia-Inducible Factor 1α

INTRODUCTION

Delayed cerebral ischemia (DCI) is the most common and most severe form of secondary brain injury to develop after aneurysmal subarachnoid hemorrhage (SAH). Occurring after a stereotypical delay (peak incidence 4-12 days post-ictus), it is thus the most likely to be amenable to therapeutic intervention. The hallmark of DCI is cerebrovascular pathology: Cerebral vasospasm is a primary driver of DCI, as supported by the following observations: vasospasm and DCI coincide temporally; DCI-related symptoms occur within the territory of spastic arteries in many patients with vasospasm; and targeted endovascular treatment of vasospasm often improves patients’ neurological status. Vasospasm has also been repeatedly identified as an independent risk factor for both brain infarction and poor outcome after SAH. In recent years, however, several additional pathophysiological processes have been linked to DCI; these include microcirculatory autoregulatory dysfunction, microvessel thrombosis, and cortical spreading ischemia. Indeed, many believe a combination of these pathological events are required to produce SAH-induced DCI (for review, see Macdonald).

Cerebral conditioning describes the phenomenon wherein the brain’s endogenous protective mechanisms against a severe injury can be induced by exposure to a mildly stressful stimulus (for review, see Gidday or Iadecola and Anrather). Preconditioning and postconditioning are defined, by convention, according to whether the conditioning stimulus is delivered prior to or after injury, respectively. Initial investigations into cerebral conditioning
focused on its beneficial effects on neuronal survival and function; research in recent years, however, has made it clear that the cerebrovasculature (as well as glial cells) is also an important effector of the resulting injury-tolerant phenotype. Support for a vascular contribution to cerebral conditioning comes primarily from the experimental stroke literature where conditioning has been shown to increase vascular patency, improve endothelium-dependent vasodilation and cerebral blood flow, and reduce blood-brain-barrier breakdown and vasogenic edema. Given that the pathophysiological events that underlie DCI are primarily vascular (vasospasm, microcirculatory dysfunction, and microvessel thrombosis), a conditioning-based strategy capitalizing on endogenous protective cascades that protect the cerebrovasculature (as well as neurons and glia) would represent a powerful, novel intervention for SAH-induced DCI. We were the first to apply such a strategy to the field of SAH, showing that hypoxic preconditioning (i.e., brief exposure to hypoxia prior to induction of SAH) prevented vasospasm and markedly improved neurological outcome. We also showed that this protection depended critically on endothelial nitric oxide synthase (eNOS), a molecule whose dysregulation after SAH is well known to contribute to vasospasm and has also been linked to microcirculatory dysfunction and microvessel thrombosis.

Due to a variety of unique clinical and pathophysiological factors related to SAH-induced DCI, we and others have long hypothesized that SAH represents a patient population that is perfectly suited to a conditioning-based therapy. First, a large portion (approximately one-third) of patients predictably develop DCI after SAH. Second, DCI stereotypically develops in delayed fashion (at least 4 days post-ictus), providing an ample therapeutic window of opportunity. Third, a conditioning-based strategy addresses one of the principle reasons previous therapeutic approaches against DCI have likely failed: the targeting of a single component (often
vasospasm) of what is now known to be a multifactorial process (including deleterious effects on the cerebral microcirculation). In contrast, conditioning is inherently pleiotropic, exhibiting robust beneficial effects on large cerebral arteries and the cerebral microcirculation, as well as on other important CNS cell types including neurons and glia.

As a follow-up to our proof-of-concept study examining pre-SAH delivery of a conditioning stimulus (hypoxia), we next turned our attention towards translating this concept to a post-SAH conditioning paradigm. Preliminarily, we noted that transient focal ischemia initiated soon after experimental SAH afforded strong neurovascular protection; however, we also found that isoflurane alone (included as an anesthetic control for ischemic postconditioning) provided similarly strong neurovascular protection. Given this volatile anesthetic’s much greater clinical translatability as a conditioning stimulus (as compared to potentially deleterious approaches such as hypoxia or ischemia), we sought to characterize the breadth and extent of isoflurane-induced neurovascular protection in SAH, and to define its underlying mechanism.

One molecule that has been identified as a mediator of isoflurane-induced conditioning, at least in the context of cerebral ischemia (a fundamentally different form of experimental brain injury), is hypoxia-inducible factor 1 (HIF-1). The central function of this molecule relates to maintenance of cellular homeostasis during periods of low oxygen tension – a function that has been strongly conserved across evolutionary lines. Under hypoxic conditions, the alpha subunit (HIF-1α) accumulates, associates with the beta subunit, and translocates to the nucleus, where it modulates transcription of over 100 genes. Whereas HIF-1α has been strongly implicated in protecting neurons from experimental ischemia (both *in vivo* and *in vitro*), its protective role in the cerebrovasculature has thus far only been examined in *in vitro* studies with cultured vascular endothelial cells. As such, whether HIF-1α-mediated ischemic tolerance is
driven (at least in part) by improved cerebral vessel function (e.g., microvessel function, cerebral blood flow, or vessel patency) remains unknown. Moreover, while HIF-1α has been examined in several reports employing experimental models of SAH, none has assessed HIF-1α in the context of conditioning.

We thus sought to determine whether a clinically relevant conditioning paradigm (i.e., isoflurane postconditioning) protects against experimental SAH–induced DCI and neurological deficits, and whether HIF-1α contributes to this protective phenotype. To obtain direct, causal data, we employed complementary HIF-1α-directed interventions including pharmacologic inhibition of HIF-1α via 2-methoxyestradiol (2ME2) administration and genetic inhibition of vascular endothelial HIF-1α knockout mice via a Cre-Lox approach.

** MATERIALS AND METHODS **

**Ethical Statement:** All experimental protocols were approved by the Animals Studies Committee at Washington University in St. Louis and complied with the NIH Guide for the Care and Use of Laboratory Animals and with Washington University Department of Comparative Medicine guidelines.

**Study Design:** Allocation of animals to a given experiment and experimental subgroup was performed randomly prior to each experiment: one experimenter numbered tails and another experimenter assigned mice according to these numbers. All data were collected by experimenters blinded to experimental group. Each experiment included a minimum of three
independent replications (i.e., cohorts subjected to surgery on separate days, with every experimental group represented in each cohort).

Experimental animals were housed in an AAALAC-accredited facility in temperature- and humidity-controlled rooms with a 12-hour light-dark cycle. Mice were housed 5 to a cage and had *ad libitum* access to lab chow and tap water. A total of 276 mice were used at 12-14 weeks of age (24-30 g): 203 male C57BL/6 mice (Jackson Labs, Bar Harbor, ME) and 67 male endothelial cell HIF-1α null (EC HIF-1α−/−); the latter were bred by crossing *Tie2-Cre* and HIF-1α^{fl/fl} transgenic mice lines (both on C57BL/6 background; both Jax) as described: \(^{139}\) *Tie2-Cre*-positive, HIF-1α^{fl/fl} mice were used. In addition, a total of 6 male mice derived from crossing *Tie2-Cre* and ROSA26 reporter mice (background: 129X1/SvJ; Jax) were used to assess cell-specific expression of *Tie2-Cre*: tomato-red fluorescence is changed to green fluorescence with Cre expression. Only male mice were used due to known neuroprotective effects of estrogen. \(^{140}\)

**Sample sizes:** When comparing vasospasm, cortical microthrombosis, and neurological outcome, based on our previous studies\(^{1,141}\) we estimated 80% power to detect a 20% difference between groups with N=14 per group based on a one-way analysis of variance (ANOVA) model at a significance of 5%. When comparing microvascular reactivity and qPCR, based on previous studies\(^{142}\) and the literature\(^{44,143}\) we estimated 80% power to detect a 25% difference between groups with N=5-8 per group based on a one-way ANOVA model at a significance level of 5%.

**Endovascular perforation SAH** was performed per established protocol.\(^{1,141}\) Briefly, a 5-0 blunted nylon suture was advanced from the left external carotid into the internal carotid artery and advanced distally to the point of feeling resistance at its bifurcation into the anterior and middle cerebral arteries (MCA). For SAH, the suture was advanced further to cause perforation. For sham, the suture was removed without advancement. Mice were allowed to recover in a
heated incubator and then returned to their home cages. Surgeries were performed in the late morning and early afternoon in the Animal Surgery Core at Washington University.

Isoflurane postconditioning was performed in an anesthetic induction chamber, as described with modification. Briefly, mice were placed in the chamber perfused with 2% isoflurane in room air for 1 h; temperature was maintained via a homeothermic blanket. Controls were placed in the same chamber perfused only by room air. In experiments assessing isoflurane-induced transcriptional changes, these same parameters were used. In a subset of mice, physiological parameters were examined 2 h after surgery in three groups: sham surgery, SAH surgery, SAH surgery + isoflurane postconditioning (1 h of 2% isoflurane in room air beginning 1 h after SAH surgery). Arterial pH, pCO₂, pO₂, hematocrit, and hemoglobin were assessed via a femoral artery catheterization.

Gross neurological outcome was assessed in the morning prior to surgery and daily thereafter via sensorimotor scoring per established protocol. Briefly, a motor score (0–12; comprising spontaneous activity, symmetry of limb movement, climbing, and balance and coordination) and a sensory score (4–12; comprising proprioception plus vibrissae, visual, and tactile responses) were added together.

SAH-induced DCI was assessed 3 d after surgery according to three components: cerebral vasospasm was assessed per established protocol via pressure-controlled casting with gelatin–India ink solution and measurement of the proximal MCA. Second, microvessel reactivity was assessed per established protocol. Briefly, a closed cranial window was made to allow visualization of leptomeningeal arterioles; vasodilation to three stimuli was examined: physiological hypercapnea; superfusion of the endothelium-dependent vasodilator acetylcholine (ACh, 100 µM); and superfusion of the endothelium-independent vasodilator S-Nitroso-N-
acetylpencillamine (SNAP; 500 µM, both Sigma-Aldrich, St. Louis, MO). Third, cortical microthrombosis was assessed via 3,3'-diaminobenzidine (DAB) staining for fibrinogen as described with modification. Briefly, following transcardial perfusion with heparinized PBS, brains were removed and sliced coronally at 50 µm. Floating sections were incubated with 0.3% H₂O₂ in PBS for 10 min, washed three times in PBS for 5 min, blocked (blocking buffer: 0.1% Triton-X100, 0.2% dry mild, and 1% BSA in PBS) on a shaker for 1 h, then incubated with rabbit anti-fibrinogen antibody (1:1000 in blocking buffer; abcam, Cambridge, MA) at 4°C overnight. After three further washes, sections were incubated with goat–anti-rabbit biotinylated secondary antibody (1:1000; BioRad, Hercules, CA) for 1 h, washed three times, and incubated with VECTASTAIN Elite ABC Kit solution (Vector Laboratories, Inc., Burlingame, CA), washed three times, then transferred to peroxidase substrate solution (1 tab DAB, 40µL H₂O₂, and 10µL NiSO₄ in 40mL ddH₂O). After ~2.5 min, tissues were transferred to PBS to stop the peroxidase reaction and mounted onto glasses slides, dried, and cover-slipped. Percent coverage of ipsilateral parietal cortex was determined using the threshold function in ImageJ (NIH, Bethesda, MD).

The HIF inhibitor 2-methoxyestradiol (2ME2, Sigma) was administered at a dose of 15 mg/kg IP once daily (vehicle: normal saline), with the first dose given prior to isoflurane exposure or surgery. This dose was chosen based on a previous report showing its efficacy in preventing HIF-mediated transcriptional effects in adult rodent brain. 2ME2 is known to inhibit both HIF-1α and HIF-2α.

Quantitative real-time PCR (qPCR) was performed as described. Briefly, following transcardial perfusion with heparinized PBS, cortex was rapidly frozen on dry ice followed by extraction of messenger RNA using TRIzol (Life Technologies, Grand Island, NY) and reverse
transcription (of 2 µg mRNA) with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster, CA). qPCR of HIF-1α and HIF-2α transcriptional targets was performed using the ABI 7500 in default mode with SYBR Green Master Mix (Applied Biosystems) using the following primers (Integrated DNA Technologies, Coralville, IA): HIF-1α: forward GAA CAT CAA GTC AGC AAC GTG, reverse TTT GAC GGA TGA GGA ATG GG; erythropoietin (EPO): forward GAG GTA CAT CTT AGA GGC CAA G, reverse TCT TCC ACC TCC ATT CTT TTC C; glucose transporter 1 (GLUT1): forward GAT TGG TTC CTT CTC TGT CGG, reverse CCC AGG ATC AGC ATC TCA AAG; BCL2/adenovirus E1B 19 kd-interacting protein (BNIP3): forward ACC ACA AGA TAC CAA CAG AGC, reverse CGA CTT GAC CAA TCC CAT ATC C; and glyceraldehyde 3-phosphate dehydrogenase (GAPDH): forward CTT TGT CAA GCT CAT TTC CTG G, reverse TCT TGC TCA GTG TCC TTG C. Messenger RNA levels were calculated relative to GAPDH via the ΔCt method and are expressed relative to naïve controls.

Cre-mediated expression was assessed by crosses of Tie2-Cre mice with floxed ROSA26 reporter mice per established protocols. Briefly, following transcardial perfusion with heparinized PBS, brains were removed and sliced coronally at 50 µm. Sections were counterstained with DAPI then mounted on glass slides and cover-slipped with VectaShield (Vector Laboratories, Burlingame, CA).

Statistical Analysis: Data represent individual animals and are expressed as means±SEM. Following testing for normality, vasospasm, microthrombosis, microvessel reactivity, and qPCR were analyzed by ANOVA followed by Tukey’s HSD test. Following testing for normality, Neuroscore was analyzed by repeated measures ANOVA followed by Newman-Keuls multiple comparison. Statistical significance was set at p < 0.05.
**RESULTS**

Postconditioning Attenuates SAH-Induced Cerebrovascular Dysfunction

To determine the temporal window for postconditioning-induced protection, we exposed mice to isoflurane at various times after SAH surgery. Mortality was not significantly different between groups (6.1% in non-postconditioned SAH mice *versus* 6.9% in postconditioned SAH mice). However, substantial DCI protection was noted: postconditioning beginning at 15 min, 1 h, or 3 h after SAH eliminated SAH-induced vasospasm; this protection was lost when postconditioning began at 6 h (Fig 6A and B; *p*<0.05, omnibus ANOVA). Given the robust protection seen with postconditioning starting at 1 h, this time point was used for subsequent experiments. Other vascular contributors to DCI were also significantly improved by postconditioning. Extensive cortical microthrombosis was noted in MCA-territory cerebral cortex after SAH, which was significantly reduced by postconditioning (Fig 7A and B; *p*<0.05, omnibus ANOVA). SAH-induced microvessel function was also restored by postconditioning (Fig 8). Cerebrovascular function was significantly impaired after SAH as assessed by responses to physiologic hypercapnia, as well as to local application of the endothelium-dependent and endothelium-independent dilators ACh and SNAP, respectively, compared to sham animals. Postconditioning fully restored the vasodilatory responses to hypercapnia and SNAP (Fig 8; *p*<0.05, omnibus ANOVA) and produced a strong trend towards improving ACh-induced vasodilation, though this did not reach our predetermined threshold for statistical significance (Fig 8; *p*=0.062, omnibus ANOVA). Together, these results show that postconditioning after mouse SAH can positively impact multiple contributors to cerebrovascular-mediated DCI, establishing it as a novel therapeutic approach for this devastating secondary brain injury.
Postconditioning Improves Neurological Outcome After SAH

To determine whether the breadth of protection afforded by postconditioning extends from the cerebrovasculature to functional outcomes, neurological status was assessed before SAH and daily thereafter via sensorimotor scoring. SAH caused significant neurological deficits, which were markedly attenuated by isoflurane postconditioning beginning at 15 min, 1 h, or 3 h, but not at 6 h, after ictus (Fig 9; \( p<0.05 \), omnibus repeated measures ANOVA). The neurovascular protection afforded by isoflurane postconditioning was not related to isoflurane-induced physiological changes, as no significant differences in arterial pH, pCO2, pO2, O2 saturation, hematocrit, and hemoglobin were noted across experimental groups (Table 1).

Pharmacologic Inhibition of HIF Prevents Isoflurane’s Transcriptional Effect and Postconditioning’s Neurovascular Protection

Next, we sought to determine whether this SAH-tolerant phenotype is dependent on HIF. In naïve WT mice, isoflurane exposure significantly modulated the HIF transcriptional targets EPO, GLUT1, and BNIP3 in a time-dependent manner. Administration of 2ME2 (a pharmacologic inhibitor of HIF-1\( \alpha \) and HIF-2\( \alpha \)) significantly attenuated these effects for each gene (Fig 10A; \( p<0.05 \), omnibus ANOVA). The same dose of 2ME2 eliminated the protection afforded by isoflurane postconditioning against SAH-induced vasospasm (Fig 10B; \( p<0.05 \), omnibus ANOVA) and neurological deficits (Fig 10C; \( p<0.05 \), omnibus repeated measures ANOVA). Together, these results show that isoflurane modulates HIF-driven transcription, and that pharmacologic inhibition of this transcriptional response abolishes the protection against SAH-induced neurovascular dysfunction afforded by isoflurane postconditioning.
Genetic Deletion of Endothelial HIF-1α Inhibits Isoflurane’s Transcriptional Effect and Postconditioning’s Neurovascular Protection

To test our hypothesis that HIF-1α-driven transcriptional activation in endothelial cells in response to isoflurane postconditioning drives the observed vasculoprotective phenotype, we generated endothelial cell–specific HIF-1α knockout mice. Endothelial cell expression of Cre in our Tie2-Cre mice was verified by crossing them with ROSA26 reporter mice and examining cerebral microvascular fluorescence in the brains of their progeny. As shown in Figure 11, green fluorescence – indicative of Cre expression – was seen throughout the cerebrovascular endothelium of Cre-positive mice (Fig 11Av to viii), but the endothelium of Cre-negative mice fluoresced red (Fig 11Ai to iv). In naïve EC HIF-1α−/− mice, isoflurane exposure did not significantly affect transcription of the HIF-1α targets GLUT1 and BNIP3; by contrast, transcription of EPO was significantly increased (Fig 11B; p<0.05, omnibus ANOVA), which is consistent with a known role of vascular HIF-2α (retained in these mice) in regulating EPO.147 Genetic deletion of endothelial HIF-1α eliminated the protection afforded by isoflurane postconditioning against SAH-induced vasospasm (Fig 11C; p<0.05, omnibus ANOVA) and neurological deficits (Fig 11D; p<0.05, omnibus repeated measures ANOVA). Collectively, these results provide causal evidence that vascular endothelium–derived HIF-1α is critical for isoflurane’s transcriptional effect and the neurovascular protection afforded by its use as a postconditioning treatment.


**DISCUSSION**

DCI is the most common and potentially treatable cause of secondary neurological injury following SAH;\(^1\) among patients affected by DCI, up to one-third experience poor outcome or death.\(^4\) We\(^1\) and others\(^9\) have hypothesized that DCI may represent an ideal clinical scenario for a conditioning-based therapy due to several factors: 1) the predictability of ischemia – after a stereotypical delay of many days – in a significant fraction of this clinical population; 2) the severity of DCI and its contribution to poor patient outcome; and 3) the multifactorial nature of DCI that could be positively affected by a conditioning stimulus. Whereas the terms DCI and cerebral vasospasm have traditionally been used interchangeably, the last decade has seen an expanded appreciation for the contribution to DCI of other pathophysiological processes including cortical microthrombosis, microvessel dysfunction, and cortical spreading depression (for review, see Macdonald, 2014\(^4\)).

We thus sought to determine whether postconditioning – with a clinically relevant stimulus and at a clinically relevant time point – could mitigate the deleterious effects of DCI following experimental SAH. Our main findings are as follows: First, we demonstrated that a brief “dose” of isoflurane, administered 15 min, 1 h, or 3 h after SAH (but not 6 h later) strikingly attenuated the SAH-induced vasospasm and neurological deficits observed days later. This shows that a clinically relevant paradigm of isoflurane postconditioning provides strong neurovascular protection in SAH. Notably, the afforded protection is dependent on timing of the conditioning stimulus, similar to findings in experimental ischemic stroke.\(^14\) Second, we found that isoflurane postconditioning markedly attenuated two additional contributors to DCI, cortical microthrombosis and microvessel dysfunction. This shows that isoflurane provides broad vascular protection, at not only the macro-vessel level (vasospasm) but also at the
microcirculatory level; this breadth enhances its translational potential. Third, we documented that isoflurane exposure modified HIF target gene expression and that this transcriptional modulation was prevented by pharmacologic and genetic inhibition of HIF in an internally consistent manner. Specifically, pharmacologic inhibition of HIF-1α and HIF-2α with 2ME2 prevented isoflurane-induced modulation of all HIF target genes (GLUT1, BNIP3, and EPO), while selective genetic deletion of HIF-1α in vascular endothelial cells prevented isoflurane-induced modulation of HIF-1α target genes (GLUT1 and BNIP3) but not HIF-2α target genes (EPO).147 Fourth, we demonstrated that pharmacologic and genetic inhibition of HIF reversed the protective phenotypes we identified in postconditioned mice. Specifically, pharmacologic inhibition of HIF-1 and HIF-2 with 2ME2 administration blocked isoflurane-induced neurovascular protection in SAH, as did selective genetic deletion of HIF-1α in vascular endothelial cells. Taken together, these data indicate that a clinically relevant paradigm of isoflurane postconditioning strongly inhibits macro- and microvascular contributors to DCI, that this multifaceted vascular protection is mediated via endothelial-cell-derived HIF-1α, and that the robust vascular protection afforded by isoflurane postconditioning leads to markedly improved neurological outcome after SAH.

Our group was the first to show that SAH may be amenable to a conditioning strategy: we showed that hypoxic preconditioning significantly attenuated SAH-induced neurovascular dysfunction in the mouse, and that this protection was critically dependent on eNOS-derived nitric oxide.1 Subsequently, Altay and colleagues showed that early brain injury after mouse SAH can be impacted by isoflurane postconditioning, as cerebral edema,95 neuronal cell death,96 and neurological deficits were all reduced at 24 h post-SAH. However, in contrast to our finding of sustained neurovascular protection at 72h post-SAH, these investigators noted that isoflurane-
induced protection in SAH appeared transient, as reductions in cerebral edema and neurological deficits were lost at 72 h post-SAH (neuronal cell death was not examined beyond 24 h). This discrepancy between the two studies in the sustainability of isoflurane-induced protection has a variety of potential explanations including variations between our respective endovascular perforation techniques (4-0 versus 5-0 suture used herein), our neurological assessment scales (6-point versus 8-point sensorimotor scoring used herein), our vascular endpoints (BBB disruption versus vasospasm, microthrombosis, and microvessel dysfunction used herein), or a combination. Regardless, the present study significantly extends upon these initial findings in several important ways: 1) we demonstrated isoflurane postconditioning produced sustained neurological protection in SAH; 2) we found that it protected against three separate contributors to DCI that act at both the macro-vessel level (vasospasm) and micro-vessel level (microthrombosis and microvessel dysfunction); 3) we identified an extended therapeutic window of opportunity (3 h post-SAH); and 4) we identified vascular endothelium–derived HIF-1α as an essential factor in the neurovascular protection afforded by isoflurane postconditioning.

Isoflurane conditioning has been shown to reduce neuronal cell injury and neurological deficits in a variety of acute cerebrovascular conditions including neonatal hypoxia-ischemia, ischemic brain injury, and intracerebral hemorrhage. Notably, several groups have linked the brain protection afforded by isoflurane to HIF-1α. Isoflurane increases HIF-1α and HIF target genes; isoflurane protects against OGD-induced neuronal cell death in a HIF-1α-dependent fashion; and administration of pharmacologic agents that augment HIF-1α provide protection against experimental cerebral ischemia (e.g., DMOG, a prolyl hydroxylase inhibitor that augments HIF-1α but also inhibits numerous other enzymes, and deferoxamine, an iron chelator that indirectly inhibits prolyl hydroxylase and augments HIF-
HIF-1α but also inhibits other iron-dependent enzymes\textsuperscript{159} and stabilizes the mitochondrial permeability transition pore\textsuperscript{160}). Importantly, none of these studies established a causal role of HIF-1α in the protection afforded by isoflurane in animal models of acute cerebrovascular injury, nor have they linked HIF-1α to isoflurane-induced \textit{vascular} protection. In the present study, we provide such evidence. First, we establish that isoflurane-induced protection extends to SAH, a fundamentally different form of acute cerebrovascular injury. Second, utilizing complementary pharmacologic and genetic techniques we show that vascular endothelium–derived HIF-1α is a key mediator of this protection – the first to directly implicate HIF-1α in the brain protection afforded by isoflurane in any \textit{in vivo} paradigm of acute cerebrovascular injury. Third, we show that HIF-1α mediates not only isoflurane-induced neural protection but also isoflurane-induced vascular protection – the first study to our knowledge to make this important link.

Our preclinical results are substantiated by recent studies examining the issue of conditioning-induced protection in patients with cerebrovascular disease: first, patients with transient ischemic attacks who later suffered ischemic strokes have smaller infarctions\textsuperscript{97} and lower rates of in-hospital mortality\textsuperscript{98} than patients without prior history of such attacks. More recently, in collaboration with Hoh and colleagues, we showed that SAH patients with pre-existing steno-occlusive cerebrovascular disease are less likely to develop angiographic vasospasm than those without such disease:\textsuperscript{100} the first evidence in humans of a potential preconditioning effect on SAH-induced vascular dysfunction. Although these reports do not establish causation, they intriguingly suggest that the human cerebrovasculature may in fact benefit from non-injurious stressors in ways similar to what has been documented in animal models.
Our study raises several questions that warrant further investigation. First, by what mechanism(s) does isoflurane activate HIF-1α in the context of postconditioning in SAH? Here, several possibilities exist including isoflurane up-regulation of HIF-1α via phosphorylation by phosphatidyl inositol phosphate 3-kinase, S-nitrosylation by nitric oxide, and reduced hydroxylation by prolyl hydroxylases (for review, see Hieber et al.\textsuperscript{161}) Second, by what downstream molecular effector(s) does HIF-1α reduce the brain’s susceptibility to SAH? One possibility is that the HIF-1α-mediated protection afforded by isoflurane is the consequence of HIF-1α’s effect on gene expression and protein synthesis in its cell of origin, the vascular endothelium. If so, several observations suggest that eNOS could be the downstream mediator: 1) eNOS is expressed in vascular endothelial cells and can be modulated by HIF-1α,\textsuperscript{154, 162} 2) eNOS has been linked to vasospasm,\textsuperscript{138} cortical microthrombosis,\textsuperscript{102} and microvessel dysfunction;\textsuperscript{44} and 3) neurovascular protection afforded by a different conditioning paradigm (brief hypoxia 24hr prior to SAH) is critically dependent on eNOS. Another possibility (though not mutually exclusive from HIF-1α’s transcriptional effects in vascular endothelial cells) is that one or more HIF-1α-driven secreted proteins (e.g., vascular endothelial growth factor, which has been linked to vasospasm protection in at least one study\textsuperscript{163}) exerts paracrine protective effects on neighboring cells. Third, is the neurovascular protection afforded by isoflurane postconditioning present across SAH model systems (endovascular perforation, cisterna magna injection, and pre-chiasmatic injection), species (lissencephalic \textit{versus} gyrencephalic), gender (male \textit{versus} female), age (young \textit{versus} old), comorbidities (hypertension, diabetes, etc.), and outcome measures (short-term \textit{versus} long-term)? These cross-validation experiments are critical if isoflurane and/or HIF-1α-based therapies are to be considered for translational studies, and are strongly advocated by the Stroke Therapy Academic Industry Roundtable.\textsuperscript{81}
Another important issue to be examined is the impact of HIF-1α up-regulation by SAH itself, which several groups have documented in various animal models of SAH, versus that induced by isoflurane postconditioning as we documented in the present study. In this instance, timing, severity, and sustainability of HIF-1α up-regulation and its downstream transcriptional effects may matter greatly since pharmacologic inhibition of HIF-1α activation has been shown to be protective or deleterious depending on the specific experimental conditions, in a variety of acute cerebrovascular injury paradigms including ischemic stroke (for review, see Singh et al., 2012) and SAH. This “double-edged sword” impact of HIF-1α must be fully understood in the setting of SAH if HIF-1α-based therapies are to be pursued in translational studies. Alternatively, it may be that the therapeutic index for pharmacologic manipulation of HIF-1α is too narrow to be a viable target for SAH therapeutics, in which case isoflurane itself (or potentially other agents including different inhalational anesthetics or alternate classes of anesthetics) may prove a more promising intervention against SAH-induced DCI and the resultant morbidity and mortality.

In conclusion, this study demonstrates that isoflurane has beneficial vasculoprotective effects on experimental SAH–induced DCI, and that this protection is critically dependent on endothelial cell HIF-1α-driven gene transcription. This is a novel and exciting finding given that isoflurane is already FDA-approved for use in this patient population and that administration of isoflurane at a clinically applicable dose and at a clinical relevant time point provided robust protection against several contributors to DCI. Moreover, the stereotypical delay between SAH and DCI provides a meaningful therapeutic window of opportunity for an isoflurane-based postconditioning strategy.
Figure 6: Postconditioning eliminates SAH-induced vasospasm.

Mice underwent sham surgery; subarachnoid hemorrhage (SAH) surgery; or SAH surgery followed by isoflurane postconditioning (2% for 1 h, SAH-postC) starting 15 minutes, 1h, 3h, or 6 h after surgery. On post-surgery day 3, pressure-controlled cerebrovascular casting was performed with gelatin–India ink. A. Representative images of the middle cerebral artery (MCA) ipsilateral to endovascular perforation. B. Vessel diameter of the ipsilateral MCA was assessed. N=6 sham, N=10 SAH, N=16 SAH-postC-15', N=12 SAH-postC-1h, N=14 SAH-postC-3h, N=15 SAH-postC-6h. Data represent mean±SEM. *p<0.05 by ANOVA.
Figure 7: Postconditioning attenuates SAH-induced cortical microthrombosis.

Mice underwent sham surgery, subarachnoid hemorrhage (SAH) surgery, or SAH surgery followed 1 h later by isoflurane postconditioning (2% for 1 h, SAH-postC). On post-surgery day 3, brains were subjected to fibrinogen immunohistochemistry. A. Representative images of parietal cortex ipsilateral to endovascular perforation. B. Cortical microthrombosis was determined as percent coverage of ipsilateral parietal cortex. N=15 sham, N=16 SAH, N=12 SAH-postC. Data represent mean±SEM. *p<0.05, **p<0.01 by ANOVA.
Figure 8: Postconditioning reverses SAH-induced microvessel dysfunction.

Mice underwent sham surgery, subarachnoid hemorrhage (SAH) surgery, or SAH surgery followed 1 h later by isoflurane postconditioning (2% for 1 h, SAH-postC). On post-surgery day 3, microvessel function was examined through an open cranial window. Pial arteriolar vasodilatory responses to hypercapnia (CO₂), the endothelium-dependent vasodilator acetylcholine (ACh), and the endothelium-independent vasodilator S-nitroso-N-acetylpenicillamine (SNAP) were assessed. N=7 per group. Data represent mean±SEM. *p<0.05 by ANOVA.
Figure 9: Postconditioning improves neurological outcome after SAH.

Mice underwent sham surgery; subarachnoid hemorrhage (SAH) surgery; or SAH surgery followed by isoflurane postconditioning (2% for 1 h, SAH-postC) starting 15 minutes, 1h, 3h, or 6 h after surgery. Neurobehavioral assessment was performed on post-surgery days 0-3 via sensorimotor scoring. N=6 sham, N=10 SAH, N=16 SAH-postC-15', N=12 SAH-postC-1h, N=14 SAH-postC-3h, N=15 SAH-postC-6h (the same animals as were assessed for vasospasm in Figure 6). Data represent mean±SEM. *p<0.05 vs. sham, #p<0.05 vs. SAH, by repeated measures ANOVA and Newman-Keuls multiple comparison test.
Table 1: Physiological Parameters in Arterial Blood.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>pH</th>
<th>pCO₂ (mmHg)</th>
<th>pO₂ (mmHg)</th>
<th>Hematocrit (%)</th>
<th>Hemoglobin (g/dL)</th>
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</thead>
<tbody>
<tr>
<td>Sham</td>
<td>4</td>
<td>7.40 ± 0.001</td>
<td>41.5 ± 1.1</td>
<td>90.7 ± 3.4</td>
<td>43.6 ± 0.5</td>
<td>14.2 ± 0.3</td>
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<tr>
<td>SAH</td>
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<td>7.37 ± 0.001</td>
<td>42.9 ± 1.9</td>
<td>87.9 ± 0.9</td>
<td>442. ± 0.5</td>
<td>14.3 ± 0.3</td>
</tr>
<tr>
<td>SAH-postC</td>
<td>5</td>
<td>7.39 ± 0.001</td>
<td>41.1 ± 1.9</td>
<td>90.7 ± 4.2</td>
<td>44.1 ± 0.6</td>
<td>14.7 ± 0.2</td>
</tr>
</tbody>
</table>
Figure 10: HIF-1 mediates isoflurane-induced transcription and postconditioning-induced neurovascular protection after SAH.

A. Mice were administered vehicle or the HIF-1 inhibitor 2-methoxyestradiol (2ME2), exposed to isoflurane (2% for 1 h), sacked at 3 h, 24 h, or 72 h, and cortical tissue was subjected to quantitative real-time PCR. Data represent mean±SEM. *p<0.05 vs. naïve, #p<0.05 vs. time-
matched isoflurane-only by ANOVA. N=6 mice per group. **B-C.** Mice were administered vehicle and subjected to sham surgery; were administered vehicle and subjected to subarachnoid hemorrhage (SAH) surgery followed 1 h later by isoflurane postconditioning (2% for 1 h, SAH-postC); or administered 2ME2 and subjected to SAH-postC. On post-surgery day 3, pressure-controlled cerebrovascular casting was performed with gelatin–India ink (**B**). Data represent mean±SEM. *p<0.05 by ANOVA. Neurobehavioral assessment was performed on post-surgery days 0-3 via Neuroscore (**C**). N=8 sham, N=10 SAH, N=10 SAH-postC, N=9 SAH-postC-2ME2. Data represent mean±SEM. *p<0.05 vs. sham, #p<0.05 vs. SAH, ‡p<0.05 vs. SAH-postC-veh by repeated measures ANOVA and Newman-Keuls multiple comparison test.
Figure 11: Endothelial HIF-1 mediates isoflurane-induced transcription and postconditioning-induced neurovascular protection after SAH.

Endothelial cell hypoxia-inducible factor 1 null (EC HIF-1/−) mice were bred using a Cre-lox system. A. Tie2-Cre mice were bred to ROSA26 reporter mice. Note green fluorescence in cerebrocortical endothelial cells (indicating Tie2-Cre expression) but not in other cell types (red) in the brains of the offspring. B. EC HIF-1/− mice were subjected to normoxia (naïve) or isoflurane (2% for 1 h), sacked at 3 h or 24 h, and cortical tissue was subjected to quantitative real-time PCR. N=5 mice per group. Data represent mean±SEM. *p<0.05 vs. naïve by ANOVA. C-D. EC HIF-1/− mice underwent sham surgery, subarachnoid hemorrhage (SAH) surgery, or SAH surgery followed 1 h later by isoflurane postconditioning (2% for 1 h, SAH-postC). On post-surgery day 3, pressure-controlled cerebrovascular casting was performed with gelatin-
India ink. C. Vessel diameter of the ipsilateral middle cerebral artery was assessed. N=21 sham, N=20 SAH, N=11 SAH-postC. Data represent mean±SEM. *p<0.05 by ANOVA. C. Neurobehavioral assessment was performed on post-surgery days 0-3 via Neuroscore. Data represent mean±SEM. *p<0.05 vs. sham by repeated measures ANOVA and Newman-Keuls multiple comparison test.

CHAPTER 3: Endovascular Perforation Subarachnoid Hemorrhage Fails to Cause Morris Water Maze Deficits in the Mouse

INTRODUCTION

Aneurysmal subarachnoid hemorrhage (SAH) is a neurologically devastating disease that affects 6-7 people per 100,000 each year. Mortality remains near 50%;\textsuperscript{11} while relatively few SAH survivors have significant focal neurological deficits of the sort that are common following ischemic stroke, long-term cognitive dysfunction is seen in 50–60%, allowing only 33% to return to their previous level of employment despite good neurological outcome.\textsuperscript{66} The long-term cognitive deficits after SAH cross numerous cognitive domains, including memory, executive function, and language (for review, see Al-Khindi, 2010\textsuperscript{67}). Of the memory deficits, most\textsuperscript{51, 68, 69} but not all\textsuperscript{70} have documented visuospatial memory as being particularly affected. Strikingly, no treatment has been shown to improve cognitive outcome after SAH. It is therefore critical to devise experimental methods to elucidate underlying mechanism and develop novel therapeutics for these deficits, as they are a principal driver of the long-term loss of quality of life in SAH survivors.

Experimental models of SAH vary in species used and in method of induction; each has its unique advantages and disadvantages.\textsuperscript{79} A key benefit of mouse models is the ability to incorporate myriad transgenic lines to query mechanism precisely. This is directly relevant to SAH, as apolipoprotein E (ApoE) and haptoglobin genotypes have been identified as genetic risk factors for poor patient outcome, the former being independently associated with poor cognitive outcome after SAH.\textsuperscript{71, 72}
The importance of including long-term outcomes in preclinical studies has been substantiated by disappointing results from recent therapeutic trials. Despite successfully reducing angiographic vasospasm, long-term patient outcome was not affected by administration of the non-glucocorticoid 21-aminosteroid tirilazad or the endothelin receptor antagonist clazosentan. It may be that, beyond examining short-term preclinical outcomes (such as early brain injury and cerebral vasospasm), the successful translation of putative therapies would be predicted by evaluation of longer-term neurobehavioral outcomes in preclinical models, as recommended by the Stroke Therapy Academic Industry Roundtable’s criteria.

Experimental SAH research has thus sought to model long-term neurobehavioral outcomes and elucidate the mechanisms responsible. Morris water maze (MWM) deficits have been documented in all three principal rat models 3-5 weeks after SAH, along with T-maze deficits in endovascular perforation SAH; moreover, treatment with statins and minocycline has decreased SAH-induced cognitive deficits. Notably, a strong correlation was seen in rat SAH between MWM performance and neuronal cell counts in hippocampus at 5 weeks.

Despite these reports in rat models of SAH, to date there has been no characterization of longer-term neurobehavioral deficits in mouse models of SAH. We thus undertook to characterize cognitive deficits in the most commonly used mouse model of SAH, endovascular perforation, using the gold standard of spatial learning, the MWM.

**Materials and Methods**

**Animals:** All experimental protocols were approved by the Animals Studies Committee at Washington University in St. Louis and complied with the Guide for the Care and Use of
Laboratory Animals; the Public Health Service Policy on Humane Care and Use of Laboratory Animals; and Washington University Department of Comparative Medicine guidelines. Mice were housed in an AAALAC-accredited facility in temperature- and humidity-controlled rooms with a 12-hour light-dark cycle. Mice were allowed *ad libitum* access to standard chow and autoclaved tap water. A total of 250 male wild-type C57BL/6 mice (Jackson Labs, Bar Harbor, ME) were used starting at 12–14 weeks of age (24–30 g). Allocation to experimental group was performed prior to the beginning of each experiment: tails were numbered by one experimenter and another experimenter assigned mice randomly according to these numbers. All data were collected by experimenters blinded to experimental group. The study included 5 independent experiments (summarized in Table 2): in Experiment 1, mice were subjected to SAH, given 3 days of fluid support, and assessed for cerebral vasospasm on post-SAH day 3. In Experiment 2, mice were subjected to SAH, given 3 days of fluid support, and assessed via Place MWM. Significant long-term mortality was seen, and so attempts were made to improve survival, first by extending fluid support to post-operative day 7 (Experiment 3) and second by not only extending fluid support to 7 days but also administering ampicillin\(^{165}\) (Experiments 4 and 5). A more sensitive MWM protocol, the Learning Set task, was employed in a subset of studies (Experiment 4, cohort 2, and Experiment 5). TBI was used as a positive control (Experiment 5).

**Endovascular Perforation SAH:** For Experiments 1-4, endovascular perforation SAH was performed as described.\(^1,^{141}\) Briefly, mice were anesthetized with isoflurane (4% induction, 1.5% maintenance) and a midline neck incision was made. The external carotid artery (ECA) was isolated, and through it a 5-0 blunted nylon suture was introduced into the internal carotid artery (ICA). The suture was advanced distally until resistance was felt at the bifurcation of the ICA into the anterior and middle cerebral arteries. Advancing the suture a further 5 mm caused
perforation and SAH. The suture was immediately removed and the ECA was ligated. Sham-operated mice were subjected to all surgical procedures except that, upon feeling resistance at the ICA bifurcation, the suture was removed without advancement. Cerebral vasospasm was assessed on post-SAH day 3 via pressure-controlled casting with gelatin–India ink solution.

**Controlled Cortical Impact Traumatic Brain Injury:** In Experiment 5, electromagnetically-controlled cortical impact model of TBI was performed as previously described. Briefly, mice were anesthetized with isoflurane (4% induction, 2% maintenance) and placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA). A 1-cm midline scalp incision was made and a left craniotomy was performed over the parietotemporal cortex using a 5-mm trephine (Meisinger, Neuss, Germany) attached to an electric drill (Foredom, Bethel, CT). The impact device was mounted on the stereotaxic frame at an angle of 15° and moved 3.0 mm anterior to lambda and 2.7 mm left of midline, inside the craniotomy. Zero depth was determined, the tip was cocked, and the device was lowered using the stereotaxic arm to set a depth of impact of 2 mm. TBI was triggered via Matlab (The Mathworks, Natick, MA). A 6-mm plastic disc was glued to the skull over the craniotomy and the incision was sutured. Sham-operated mice were subjected to all surgical procedures except for TBI.

**Fluid Support:** In all cases, mice were administered 0.5 ml of 10% dextrose in normal saline IP twice daily. In Experiments 1 and 2, support was provided on post-operative days 0-3, as previously described. In Experiment 3, this regimen was extended to post-operative day 7. For Experiments 4 and 5, mice were administered 50 mg/kg ampicillin in dextrose in normal saline twice daily on post-operative days 0-3, followed on post-operative days 4-7 by dextrose in normal saline.
Neuroscore: For all experiments, gross neurologic function was assessed on post-operative days 0-3, 7, and 12 via sensorimotor scoring as previously described. Briefly, neurological function was graded based on a motor score (0 to 12) that evaluates spontaneous activity, symmetry of limb movements, climbing, and balance and coordination; and a sensory score (4 to 12) that evaluates body proprioception and vibrissae, visual, and tactile responses. For Experiments 2-4, data presented include sham-operated mice; SAH mice that survived long term and were able to complete MWM testing (survival group); and mice that survived initially (i.e., at least 3 d after SAH), but either died before MWM testing or were excluded owing to being unable to complete MWM testing (mortality group, SAH-mort).

Morris Water Maze:

For all MWM experiments, a 100-cm pool was filled with water rendered opaque with non-toxic white paint. A 10-cm escape platform was made to either protrude 1 cm above the surface of the water (Cued trials) or was submerged 1 cm (Place and Learning Set trials). Mice were released from one of four cardinal drop points. If the mouse found the platform, it was allowed to sit for 15 seconds before removal; if the mouse failed to find the platform, it was placed on the platform for 15 seconds. An overhead camera recorded swim paths. SMART software (San Diego Instruments, San Diego, CA, USA) analyzed escape latency, swim distances, and swim speeds for all trials. All experiments were conducted by a single investigator (EM) blinded to experimental groups.

Two different protocols were used. For the Place MWM, mice were given 4 trials per days, one from each drop point. On post-SAH days 13-15, mice were subjected to 4 trials of the Cued task (visible platform) with a single platform location. On days 18-22, mice were
subjected to the Place task with a single (hidden) platform location. On day 23, a 30-second Probe trial was performed.

For the Learning Set MWM (modified from Hartman et al., 2005167), each day mice consisted of 8 blocks of 2 consecutive trials. On post-SAH day 13, mice were subjected to the Cued task: a new (visible) platform location was used for each block, and mice were dropped from diametrically opposite the platform. On days 14-20, mice were subjected to the Learning Set task with a new (hidden) platform position each day. For block 1, the same distant drop point was used; for blocks 2-8, drop points were counter-balanced for near-far. On days 15-21, mice were subjected to a 30-second Probe trial (i.e., a probe trial ~16 hours after completing the previous day’s task).

Mice were disqualified from MWM testing if they failed to swim to the visible platform during Cued training. This was due to either an inability to learn or to complete the procedural aspects involved, including staying above water for 4 or 16 daily trials (Place and Learning Set, respectively) and lifting onto the elevated platform; or due to deficits in vision or motivation to escape the water. Sensorimotor data from these mice are included along with mice that died prior to MWM testing (mortality group; see Neuroscore section above).

Neuronal Cell Counting: After completing the final Probe trial, mice were sacrificed and transcardially perfused with heparinized PBS. Brains were post-fixed overnight in 4% paraformaldehyde in PBS, equilibrated in 30% sucrose, sectioned coronally at 50 µm, and stained with cresyl violet. Unbiased stereology was performed using Stereo Investigator (MBF Bioscience, Williston, VT). A single investigator blinded to treatment assessed 3-5 hippocampal sections taken at 600-µm intervals; morphologically intact neurons were counted in hippocampal CA1 from midline to the lateral extent of the superior limb of the angular gyrus.
Statistical Analysis: Data are expressed as means ± SEM. Mortality was analyzed by Kaplan-Meier estimator. Following testing for normality, Neuroscore and MWM performance were analyzed by repeated measures analysis of variance followed by Newman-Keuls multiple comparison. Probe performance, CA1 neuronal cell count, and CA1 volume were first assessed for normality and then analyzed by unpaired $t$-test. Correlation between neuronal cell counts and MWM performance was analyzed by least-squares linear regression. Statistical significance was set at $p < 0.05$.

**RESULTS**

Short-term SAH outcome

Acute SAH mortality (0-3 days) was similar in Experiments 1-4 ($p=0.80$, Kaplan-Meier estimator). Marked cerebral vasospasm was noted 3 days after SAH (arrows) in Experiment 1 (Figure 12A-B; $p<0.05$, $t$-test); cerebral vasospasm was not assessed in Experiments 2-4 due to the post-mortem nature of this assessment in the mouse. Significant sensorimotor neurological deficits were noted 1-3 days after SAH in Experiments 1-4 (Figures 12C, 13A, 14A, 15A), and were similar across SAH groups. Overall, these data are in line with our previous reports$^{1,141}$ and demonstrate the short-term reproducibility of our endovascular perforation SAH mouse model.
Long-term SAH outcome

Mortality

Delayed SAH mortality (after day 3) was noted in all long-term experiments (Figure 12D; \( p=0.0010, p=0.0152, p=0.0053 \) for Experiments 2-4, respectively, Kaplan-Meier estimator). A significant reduction in delayed SAH mortality was noted in Experiment 4 versus Experiment 2 (Figure 12D; \( p=0.048 \), Kaplan-Meier estimator). Together, these data indicate that long-term SAH survival can be increased by extending fluid support from 3 to 7 days and adding antibiotic prophylaxis, but nonetheless long-term SAH survival remains suboptimal.

Neurological deficits

Mild but statistically significant neurological deficits were noted in long-term SAH survivors (Figure 13A, 14A, 15A; \( p<0.001, p<0.05, p<0.01 \) for Experiments 2-4, respectively, omnibus repeated measures ANOVA). Mice that survived to day 3 (the typical point of assessment for vasospasm-driven delayed cerebral ischemia) but did not complete the MWM (due to either delayed mortality or disqualification) evinced dramatically more severe neurological deficits than mice that survived and were able to complete the MWM. In total, these data demonstrate that SAH survivors had mild sensorimotor deficits, whereas mice that were excluded from long-term assessments (MWM and unbiased stereology) had severe sensorimotor deficits.

Morris Water Maze

Performance in the Place MWM was similar between SAH and sham groups (Figure 13B-C, 14B-C, 15B-C). No significant differences were seen in the Cued task (visible platform)
No significant differences were seen in the Place task (hidden platform) \((p=0.77, p=0.11, p=0.85, \text{Experiments 2-4, respectively, omnibus repeated measures ANOVA})\). No significant differences were seen in Probe trial performance: Time spent in the target quadrant was similar \((p=0.15, p=0.64, p=0.65 \text{ for Experiments 2-4, respectively, } t\text{-test})\) and time spent in the target area was similar \((p=0.61, p=10, p=0.17 \text{ for Experiments 2-4, respectively, } t\text{-test})\). In all cases, Probe performance exceeded chance levels (i.e., 25% for target quadrant, 1% for target area).

Performance in the more sensitive Learning Set MWM was also similar between SAH and sham groups (Figure 15D-E). No significant differences were seen in the Learning Set task \((p=0.89, \text{omnibus repeated measures ANOVA})\). No significant differences were seen in Probe trial performance: time spent in the target quadrant and in the target area was similar \((p=0.26, p=0.52, \text{respectively, } t\text{-test})\). Again, Probe performance exceeded chance levels in both groups.

A total of four SAH mice were disqualified from MWM testing due to an inability to complete the MWM: one in Experiment 2 (unable to learn procedural aspects of Cued training), two in Experiment 3 (one unable to learn procedural aspects of Cued training; one unable to swim due to dense hemiparesis), and one in Experiment 4 (unable to stay above water for the 16 daily trials).

In summary, these data demonstrate that, despite reducing SAH mortality and utilizing multiple MWM protocols, endovascular perforation SAH causes consistent (albeit mild) sensorimotor deficits – but no visuospatial learning or memory deficits – in mice surviving the acute post-SAH period.
Unbiased stereology

Brain sections from mice that completed the MWM were examined for cortical cavitation consistent with chronic infarction: no clear example was found. In these mice, cortical volume of the ipsilateral hemisphere was not significantly reduced (96.9±2.8% of contralateral side, versus 97.9±3.3% in sham). In the four mice that survived but were excluded from MWM testing, cavitation consistent with chronic infarction was seen in only one (data not shown).

Morphologically intact hippocampal CA1 neurons (ipsilateral to side of SAH) were counted via unbiased stereology in mice subjected to the Learning Set MWM in Experiment 4. Minimal injury was noted (Figure 17A): neuronal cell counts and spared CA1 volume were similar in SAH and sham groups (Figure 17B-C; \( p=0.88, \ p=0.90 \), respectively, \( t \)-test). No correlation was noted between CA1 neuronal cell counts and MWM performance (Figure 17D; \( p=0.83 \), Pearson correlation). In the four mice disqualified from MWM testing, mean neuronal cell counts were 199±47, similar to those completing the MWM. In total, these data demonstrate that endovascular perforation SAH fails to cause significant damage to hippocampal CA1.

Long-term TBI outcome

In Experiment 5, included as a positive control, mice were subjected to controlled cortical impact–TBI or sham surgery. All mice survived (\( N=10 \) in each group). TBI mice evinced significant neurological deficits (Figure 16A; \( p<0.001 \), omnibus repeated measures ANOVA). TBI caused significant deficits in the Learning Set MWM (Figure 16B; \( p=0.0027 \), omnibus repeated measures ANOVA). Probe performance exceeded chance levels in sham mice; by contrast, TBI mice performed at or below chance levels (Figure 16C; \( p<0.00001 \) versus sham, \( t \)-test, for both target quadrant and target area). TBI caused severe injury in ipsilateral CA1 (Figure
A), ranging from reduction in CA1 thickness (arrow) to near-complete obliteration (arrowhead): significantly fewer neuronal cells were counted in CA1 in TBI than in TBI mice (Figure 17B; \(p<0.001\), \(t\)-test), and CA1 volume was strikingly reduced in TBI compared to sham mice (Figure 17C; \(p<0.001\), \(t\)-test). A significant correlation was noted between CA1 neuronal cell counts and MWM performance (Figure 17E; \(p<0.001\), Pearson correlation).

**Discussion**

Results of the present study provide strong evidence that, in mice, the endovascular perforation SAH model fails to cause long-term learning or memory deficits as assessed by the MWM. This is a major experimental limitation, as it is by far the most commonly utilized mouse model of SAH owing to its relative ease in development, close recapitulation of the physiological events involved with aneurysmal SAH (e.g., arterial perforation, localization of blood in the basal cisterns, acute rise in intracranial pressure, and transient global ischemia), and applicability to the use of powerful, targeted genetic manipulations. We rigorously examined possible reasons for our finding, including high mortality (albeit comparable to the 20–33% reported in rat studies\(^{74, 76-78}\)), lack of sensitivity of the MWM protocol, and extent of neuronal cell loss in hippocampal CA1. We increased survival by modifying post-operative support; moreover, we employed different MWM protocols, the standard Place task and the more sensitive Learning Set task. Neither manipulation identified MWM deficits. We examined the extent of neuronal cell loss in hippocampal CA1, finding that it was inconsistent and did not correlate to MWM deficits. As a positive control, we examined controlled cortical impact–TBI, demonstrating reliable CA1 neuronal cell loss and significant MWM deficits, which are consistent with and extend our past
results with this model. In total, our results indicate that endovascular perforation SAH in mice
does not produce demonstrable long-term learning or memory deficits as assessed by the MWM
and that the cause of this is likely inconsistent neuronal cell loss in CA1. The latter may have
resulted from significant delayed mortality that preferentially affected mice having the most
severe neurological deficits and thus most likely to have had significant CA1 neuronal cell loss
and cognitive deficits. As far as we are aware, this is the first report examining long-term
neurobehavioral outcome in a mouse model of SAH.

Cognitive deficits afflict half of SAH survivors and are discernable as long as 6 years
after ictus. Such deficits are seen even in patients with “good outcome”: those with a Glasgow
Outcome Scale of 5 nonetheless have demonstrable deficits in multiple assessments of executive
function at 6 months. These cognitive deficits have major implications with regard to patient-
relevant outcome as they strongly predict long-term morbidity, including poor self-reported
quality of life, reduced ADLs, impaired instrumental ADLs, and the inability to return to work in
at least 50% of patients.

Though the frequency and gravity of long-term cognitive deficits in SAH patients are
well documented, their underlying neurophysiological processes have only recently begun to be
elucidated. Multiple lines of evidence implicate hippocampal impairment as playing a central
role. First, hippocampal volume is reduced in SAH patients at 1 year, and this reduction
correlates to performance on visual memory tests – a particularly compelling result given the
well-known central involvement of the hippocampi and other medial temporal lobe structures in
many forms of declarative memory. Second, multiple molecular alterations in the hippocampi
occur following rat SAH, including loss of synaptic function and long-term potentiation.
Third, cognitive deficits have been strongly correlated to hippocampal CA1 neuronal cell
number after experimental SAH. To be sure, other structural areas may also play a role, including left hemispheric lesions, reductions in total gray and white matter volume (in patients), and cortical neuronal cell loss (in rats). In total, these data provide substantial evidence that hippocampal damage plays a key role in SAH-induced cognitive deficits, but that alterations in other brain areas also contribute.

We thus examined hippocampus-dependent long-term neurobehavioral outcome following endovascular perforation SAH in the mouse. Despite attenuating mortality and employing two separate protocols of the MWM – the most commonly used metric for cognitive outcome in rodents – no significant deficit by any measure was seen after SAH. Most likely, this was because of insufficient CA1 neuronal cell loss in long-term SAH survivors. Neuronal cell loss following endovascular perforation SAH in mice has only been examined in two previous reports, both of which assessed cell death at acute time points and only in cerebral cortex (and not in CA1). We documented significant neuronal cell death (as assessed by terminal deoxynucleotidyl transferase dUTP nick end labeling, TUNEL) in the ipsilateral parietal cortex 72 hours post-SAH, and Altay and colleagues reported significant TUNEL-positive neuronal cell death in the ipsilateral basal cortex 24 hours post-SAH. When considered in the context of our present results – showing no chronic SAH-induced decrease in morphologically intact CA1 neurons – these results suggest that some combination of severity or location of injury (hippocampus versus cortex, ipsilateral versus bilateral) and timing or method of assessment (acute versus chronic, actively dying versus intact neurons) may account for the lack of MWM deficits in the endovascular perforation mouse model.

Another possible explanation for our negative findings is the long-term mortality associated with this model. While we succeeded in mitigating delayed mortality by increasing
post-SAH support, it nonetheless remained substantial (~20%). It is possible that significant neuronal cell loss occurred in the mice that died, a contention substantiated by the strikingly greater sensorimotor deficits seen in such mice compared to SAH survivors. If this were the case, by assessing neuronal cell survival approximately one month after SAH (as opposed to acutely as in previous reports with this model), we would have inadvertently selected against mice with quantifiable neuronal cell loss and attendant MWM deficits.

Others have examined neuronal cell death in alternate mouse models of SAH. Using the prechiasmatic injection model, Sabri et al. reported significant TUNEL-positive neuronal cell death in ipsilateral middle cerebral artery–territory cortex and hippocampus 48 hours post-SAH. Interestingly, far less neuronal cell death was seen in the hippocampus than in the cortex, but it was not specifically quantified in CA1. Following cisterna magna injection of oxyhemoglobin, Huang et al. documented TUNEL-positive neuronal cell death in cortex at 24 and 72 hours, but did not specify what area of cortex was assessed and provided no quantification. Given the paucity of data regarding the extent and especially the location of neuronal cell injury – particularly in CA1, the brain region most implicated in MWM deficits in rat SAH and rodent TBI – it is difficult to predict whether either of these alternative mouse models might better recapitulate the long-term cognitive deficits seen in SAH patients.

In contrast, neuronal cell death and long-term outcomes in rat models of SAH have been more thoroughly characterized. Takata and colleagues were the first to report that rats displayed significant deficits in the Learning Set MWM following cisterna magna double-injection SAH, and that MWM performance correlated with hippocampal and cortical neuronal cell counts. Multiple subsequent reports have documented cognitive deficits in the three most commonly used rat models of SAH: cisterna magna injection, endovascular perforation, and
prechiasmatic cistern injection.\textsuperscript{76, 78} Each of these studies employed the MWM, widely considered the gold standard for spatial learning in rodent models of CNS injury;\textsuperscript{179, 180} Sherchan et al. also showed deficits in T maze performance following rat endovascular perforation SAH.\textsuperscript{77} Two reports also show treatment effects, of simvastatin\textsuperscript{75} and minocycline,\textsuperscript{77} establishing the suitability of the MWM as an outcome measure for therapeutic studies in rat SAH.

Our study has several limitations. First, we assessed only the endovascular perforation mouse model of SAH. We chose to examine this model as it is by far the most commonly used mouse model of SAH and it faithfully recapitulates the arterial damage, basal subarachnoid blood localization, increased intracranial pressure, and transient global ischemia associated with aneurysmal rupture in patients.\textsuperscript{79} However, as delineated above, other mouse models of SAH exist, and one may cause MWM deficits. Second, we examined long-term neurobehavioral deficits using only the MWM, albeit with two distinct protocols. The MWM is considered to be the gold standard for examining spatial deficits after rodent CNS injury, and has been reported to show dysfunction in numerous CNS disease models, including vascular disease, TBI, developmental disorders, Alzheimer’s disease, AIDS dementia complex, and more. However, there are other tests of visuospatial memory, and so we cannot exclude the possibility that deficits in our model may be revealed by another measure. Third, we examined a single mouse strain. C57BL/6 is one of the most commonly used strains in mouse SAH and has been shown by multiple groups to recapitulate numerous short-term sequelae; however, it is possible that, while neuronal cell loss and long-term neurobehavioral deficits were absent in this line, other strains would demonstrate them. Fourth, we examined morphologically intact neurons rather than markers of cell death. This was deliberate, as the processes examined by cell death assays would have ended by the time of our assessment. Nonetheless, it is possible that despite not affecting
the number of surviving hippocampal neurons, SAH could have had more subtle molecular effects. This possibility is distinctly unlikely given the absence of MWM deficits (an assessment critically dependent on hippocampal function) documented herein. Finally, SAH-related morality remained at 18% despite significant post-SAH support. It therefore remains possible that significant neuronal cell loss and MWM deficits would have occurred in the mice that died.

**Conclusions**

Our results strongly indicate that the endovascular perforation mouse model of SAH does not cause reliable CA1 neuronal cell death and does not produce demonstrable long-term neurobehavioral deficits as assessed by the MWM. This is a major experimental limitation, given that long-term cognitive deficits in SAH patients have such a profound effect on quality of life to the individual and have a similarly significant effect to society with regard to the cost of care and loss of productivity related to these patients. Development of an experimental model of SAH-induced cognitive dysfunction that permits use of genetically modified mice to determine the influence of certain genotypes on SAH outcome (e.g., ApoE, haptoglobin, and others), and allow targeted mechanistic inquiry, would represent a major advance forward for the field. Until that time, however, translational studies employing long-term assessments of outcome – including cognitive measures – should likely employ rat models of SAH, aided by the increasing availability of genetically modified lines.
Table 2: Summary of Experimental Protocols.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Surgery</th>
<th>Fluid Support</th>
<th>MWM Protocol</th>
<th>Duration of Experiment</th>
<th>Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SAH</td>
<td>Days 0–3: dex-NS</td>
<td>N/A</td>
<td>3 days</td>
<td>7%</td>
</tr>
<tr>
<td>2</td>
<td>SAH</td>
<td>Days 0–3: dex-NS</td>
<td>Place</td>
<td>23 days</td>
<td>34%</td>
</tr>
<tr>
<td>3</td>
<td>SAH</td>
<td>Days 0–7: dex-NS</td>
<td>Place</td>
<td>23 days</td>
<td>25%</td>
</tr>
<tr>
<td>4</td>
<td>SAH</td>
<td>Days 0–3: amp in dex-NS Days 4–7: dex-NS</td>
<td>Place (cohort 1) Learning Set (cohort 2)</td>
<td>23 days 21 days</td>
<td>18%</td>
</tr>
<tr>
<td>5</td>
<td>TBI</td>
<td>Days 0–3: amp in dex-NS Days 4–7: dex-NS</td>
<td>Learning Set</td>
<td>21 days</td>
<td>0%</td>
</tr>
</tbody>
</table>
Figure 12: SAH causes short-term neurological deficits and delayed cerebral vasospasm; long-term survival after SAH is increased by extended fluid support and antibiotic administration.

Mice underwent subarachnoid hemorrhage (SAH) or sham surgery and received fluid support for 3 days (0.5 ml 10% dextrose in normal saline IP twice daily). Mice were casted with gelatin–India ink on post-surgery day 3; representative images of casted vessels are shown (A). B. Cerebral vasospasm was assessed via measurement of the proximal middle cerebral artery. Neurobehavioral assessment was performed on post-surgery days 1-3 via sensorimotor scoring (C). Data indicate mean±SEM. *p<0.05 vs. sham by t-test (B) or repeated measures ANOVA and
Newman-Keuls multiple comparison test (C) D. SAH was induced and mortality was recorded with three regimens of post-surgical support: post-surgical injections of dextrose-saline solution for 3 days (dex-NS–3d) or 7 days (dex-NS–7d), or ampicillin in dextrose-saline for 3 days post-surgery followed by another four days of dextrose-saline (amp + dex-NS–7d). No sham-operated control (sham) died.
Figure 13: SAH followed by 3 days of fluid support causes deficits in neurological outcome but not in the Place Morris water maze.

Mice underwent subarachnoid hemorrhage (SAH) or sham surgery and received fluid support for 3 days (0.5 ml 10% dextrose in normal saline IP twice daily). Neurobehavioral assessment was performed on post-surgery days 1-3, 7, and 12 via sensorimotor scoring (A). Long-term neurocognitive outcome was performed on post-surgical days 13-22 by the Place Morris water maze (B). Data indicate mean±SEM. *p<0.05 vs. sham, #p<0.05 vs. SAH (surv) by repeated measures ANOVA and Newman-Keuls multiple comparison test. C. A 30-second Probe trial was performed 24 hours after the Place task; time spent in the target quadrant and in the previous location were determined. The dashed line denotes chance performance; data represent 95% confidence interval of the mean. NS P > 0.05 vs. sham by t-test. SAH-mort, SAH mortality group.
Figure 14: SAH followed by 7 days of fluid support causes deficits in neurological outcome but not in the Place Morris water maze.

Mice underwent subarachnoid hemorrhage (SAH) or sham surgery and received fluid support for 7 days (0.5 ml 10% dextrose in normal saline IP twice daily). Neurobehavioral assessment was performed on post-surgery days 1-3, 7, and 12 via sensorimotor scoring (A). Long-term neurocognitive outcome was performed on post-surgical days 13-22 by the Place Morris water maze (B). Data indicate mean±SEM. *p<0.05 vs. sham, #p<0.05 vs. SAH (surv) by repeated measures ANOVA and Newman-Keuls multiple comparison test. C. A 30-second Probe trial was performed 24 hours after the Place task; time spent in the target quadrant and in its previous location were determined. The dashed line denotes chance performance; data represent 95% confidence interval of the mean. NS P > 0.05 vs. sham by t-test. SAH-mort, SAH mortality group.
Figure 15: SAH followed by 7 days of fluid support plus antibiotic prophylaxis causes deficits in neurological outcome but not in the Place or Learning Set Morris water maze.

Mice underwent subarachnoid hemorrhage (SAH) or sham surgery and received antibiotics for 3 days (50 mg/kg ampicillin IP once daily, first dose prior to surgery) followed by fluid support on days 4-7 (0.5 ml 10% dextrose in normal saline IP twice daily). Neurobehavioral assessment was
performed on post-surgery days 1-3, 7, and 12 via sensorimotor scoring (A). Long-term neurocognitive outcome was performed on post-surgical days 13-22 by the Place Morris water maze (B). A 30-second Probe trial was performed 24 hours after the Place task; time spent in the target quadrant and in the previous location were determined (C). A separate cohort of mice was subjected to the Learning Set Morris water maze on post-surgical days 13-21 (D). A 30-second Probe trial was performed 16 hours after the completion of the previous day’s task; time spent in the target quadrant and in the previous location were determined (E). \( A,B,E \): Data indicate mean±SEM. \(*p<0.05\) vs. sham, \(#p<0.05\) vs. SAH (surv) by repeated measures ANOVA and Newman-Keuls multiple comparison test. \( C,E \): The dashed line denotes chance performance; data represent 95% confidence interval of the mean. NS \( P > 0.05\) vs. sham by \( t\)-test. SAH-mort, SAH mortality group.
Figure 16: Experimental TBI causes significant deficits in neurological outcome and in the Learning Set Morris water maze.

Mice underwent traumatic brain injury (TBI) or sham surgery and received antibiotics for 3 days (50 mg/kg ampicillin IP once daily, first dose prior to surgery) followed by fluid support on days 4-7 (0.5 ml 10% dextrose in normal saline IP twice daily). Neurobehavioral assessment was performed on post-surgery days 1-3, 7, and 12 via sensorimotor scoring (A). Long-term neurocognitive outcome was performed on post-surgical days 13-21 by the Learning Set Morris water maze (B). A 30-second Probe trial was performed 16 hours after the completion of the previous day’s task; time spent in the target quadrant and in the previous location were determined (C). A,B: Data indicate mean±SEM. *p<0.05 vs. sham by repeated measures ANOVA and Newman-Keuls multiple comparison test. C: The dashed line represents chance performance; data represent 95% confidence interval of the mean. *p<0.05 vs. sham by t-test.
Figure 17: Histological damage in CA1 correlates to performance in the Learning Set task following experimental TBI but not SAH.

Following completion of the Learning Set task, mice from Experiments 4 and 5 were sacrificed and their brains were sliced. Representative histological images of cresyl violet–stained coronal sections are shown (A). Scale bar = 500 µm. Traumatic brain injury (TBI) – but not subarachnoid
hemorrhage (SAH) – caused significant loss of morphologically intact neurons in hippocampal CA1 (B) and in ipsilateral CA1 volume (C). Data represent mean±SEM. *p<0.05 vs. sham TBI by t-test. NS P > 0.05 vs. sham by t-test. Scatter plots relating neuronal counts to mean distance swum in the Morris water maze following SAH (D) and TBI (E). Dashed lines represent 95% confidence bands. Pearson correlations are shown, NS P > 0.05.
FUTURE DIRECTIONS AND CONCLUSIONS

Delayed cerebral ischemia (DCI) remains a major source of morbidity in patients with aneurysmal subarachnoid hemorrhage (SAH). Moreover, cognitive dysfunction is the primary driver of poor long-term outcome in SAH survivors; modeling such deficits preclinically is thus key for mechanistic and translational investigation. We thus sought to determine whether activating innate neurovascular protective mechanisms by conditioning could reduce SAH-induced dysfunction and, if so, by which molecular mediators. We found that endogenous protective mechanisms against contributors to DCI exist and are powerful; can be induced by conditioning stimuli, including with clinically relevant parameters; and depend critically on endothelial nitric oxide synthase (eNOS) and hypoxia-inducible factor 1α (HIF-1α). As such, a conditioning strategy may represent a novel therapeutic approach to mitigate SAH-induced DCI and its resultant morbidity and mortality. To further assess the translational potential of such strategies, we sought to model long-term neurobehavioral outcomes in our preclinical model. Unfortunately, we were unable to identify robust or consistent SAH-induced deficits in the most commonly used mouse model of SAH, endovascular perforation, using the gold standard of spatial learning, the Morris water maze (MWM). As such, preclinical studies examining the efficacy of interventions on long-term outcomes should employ rat models, as multiple groups have documented significant SAH-induced neurobehavioral deficits in them.

Our results raise several questions that warrant further investigation. Experiment 1, a proof-of-concept study demonstrating that hypoxic PC protects against SAH-induced DCI, strongly supports a causal role for eNOS-derived nitric oxide (NO) in the phenotype. However, we did not directly assess whether the other isoforms of NOS (iNOS and nNOS) are also necessary for the protection afforded. This is unlikely given that PC had no significant effect on
the expression of either, in contrast to the increase in eNOS expression we documented. Nonetheless, the possibility remains that the neurovascular protective phenotype would be reversed in mice deficient for iNOS or nNOS similar to what we documented in eNOS-null mice.

Second, while we demonstrated that hypoxic PC significantly reduced SAH-induced DCI and neurological deficits, we did not assess its effect on early brain injury (EBI). Given the multifactorial nature of PC-induced protection across experimental modalities, it is likely that the neurovascular effects extends to BBB disruption and neuronal cell death, the principal contributors to EBI. Similarly, we did not assess whether hypoxic PC attenuates SAH-induced inflammation. Such studies would support the notion that PC, by impacting multiple injurious processes, might succeed in improving SAH outcomes where other, more selective interventions have failed.

Finally, the paradigm used in Experiment 1 is not clinically relevant for two reasons. First, PC was administered prior to SAH. This was by design, as we were proving the concept that SAH-induced vasospasm was amenable to a strategy to boost endogenous protection. Nonetheless, given the inherent unpredictability of aneurysm rupture, it is unrealistic to expect to employ PC in the SAH population. Similarly, the use of hypoxia as the PC stimulus is highly unlikely to be used clinically (though it is possible that pharmacologic agents that mimic its effects could be employed). Again, though, choosing it was by design due to our collaborator Dr. Gidday’s experience with the paradigm.

Experiment 2 was conceived in part to address the translational shortcomings of the proof-of-concept study: the conditioning stimulus was delivered after SAH rather than before, and the stimulus employed is a standard-of-care anesthetic in SAH patients. Nonetheless, these studies raise interesting questions that warrant further investigation. Broadly, these fall under
three categories: (1) further elaborating the breadth of the protection afforded by isoflurane postconditioning; (2) further delineation of its underlying mechanism, in particular the cause of HIF-1α up-regulation by isoflurane and the critical downstream effectors; and (3) cross-validation as recommended by the Stroke Therapy Academic Industry Roundtable’s criteria for translational studies.\textsuperscript{81}

Regarding the breadth of the protective phenotype: though we expanded our consideration of SAH-induced DCI relative to Experiment 1, there are nonetheless several other SAH-induced injurious processes that we did not assess. Regarding DCI, we assessed the effect of isoflurane postC not just on cerebral vasospasm (as in Experiment 1) but also cortical microthrombosis and microvessel dysfunction. We did not, however, assess its effect on cortical spreading depression. To our knowledge there are no published reports documenting cortical spreading depression (CSD) in mouse SAH, though one prominent CSD researcher has attempted to assess it with minimal success (Dr. Cenk Ayata, personal communication to GJZ). Nonetheless, CSD could be assessed as part of our cross-validation studies in other models in which it has been documented, specifically in the cat\textsuperscript{181} or more likely the rat\textsuperscript{182,183}.

Future investigation of the breadth of postC-induced protection should also examine processes other than DCI. Altay and colleagues showed that two contributors to EBI (cerebral edema\textsuperscript{95} and neuronal cell death\textsuperscript{96}) are attenuated by isoflurane postconditioning. However, other SAH-induced deleterious processes include damage to axonal projections, glial cells, and even extra-cerebral organs,\textsuperscript{184} have not been examined in the context of endogenous protection.

The prospect of a conditioning-based intervention to attenuate damage to glial cells and white matter is particularly intriguing, for several reasons: (1) multiple groups have documented such injury, both clinically and in animal models of SAH. Petzold and colleagues documented
increased CSF levels of neurofilament in SAH patients. In preclinical studies, Sabri et al. found astrocyte apoptosis in the hippocampus, cerebral cortex and brain stem following cisterna magna–injection SAH in the dog. We recently documented that axonal injury is widespread following endovascular perforation SAH in the mouse. (2) Glial cell death and dysfunction have been implicated in the pathogenesis of DCI (for review, see Mutch, 2010). Astrocytes are connected via gap junctions and thereby form a functional syncytium that is critical for buffering extracellular potassium and glutamate. Ischemia-induced acidosis uncouples astrocytes, decreasing their buffering capacity and thus the ability of nearby neurons to repolarize, thereby contributing to CSD. Moreover, increased intracellular calcium is seen in astrocytic end-foot processes due to increased 20-HETE and decreased NO, which can result in increased vasomotor tone and thus contribute to microvessel dysfunction. (3) Glial cells and white matter have been shown to be amenable to conditioning-based intervention. Our collaborator Dr. Gidday has demonstrated that retinal ganglion cell axonal injury in a mouse model of glaucoma was decreased by repetitive hypoxic PC. Other groups have shown that heat-stress PC improved MWM performance and maintained LTP following diffuse axonal injury and that hyperbaric oxygen PC attenuated axonal injury following spinal cord injury in the rat. Given this greater appreciation for the role of the neurovascular unit in SAH-induced pathology, recognizing the pleiotropic effects of conditioning-based therapies raises the exciting possibility that such interventions could have wide-ranging salubrious effects.

Second, beyond examining the pleiotropic effects of isoflurane postconditioning, future studies should also consider its underlying molecular and cellular mechanisms. Experiment 2 established HIF-1α as critical mediator of the vasculoprotective phenotype we documented. However, we did not examine the mechanism by which isoflurane up-regulated HIF-1α, nor did
we establish which downstream effectors were responsible for the observed resistance of the brain to SAH-induced dysfunction (see Chapter 2 Discussion). Moreover, cell-specific analysis will be valuable given the varying pathophysiological processes responsible for large-artery vasospasm, cortical microthrombosis and microvessel dysfunction.

Third, cross-validation studies will be critical to translating our preclinical findings to the SAH patient population. Our studies have employed rigorous experimental design, including calculation of power and sample sizes, \textit{a priori} determination of inclusion/exclusion criteria, randomized allocation of animals, blinded assessment, and disclosure of potential conflicts of interest – a standard of rigor that puts them, disappointingly, in the minority of animal studies.\textsuperscript{192}

To date, we have utilized young, healthy male mice. As recommended by the Stroke Therapy Academic Industry Roundtable,\textsuperscript{81} follow-up studies should cross-validate these initial findings in several other experimental cohorts, including: females; aged animals; animals with commonly comorbid conditions (in SAH, hypertension, diabetes, and cigarette smoking); employing other experimental models, both in terms of method of induction as well as animal, particularly in gyrencephalic species; and assessing the additive effects of the intervention in animals given standard-of-care drugs (e.g. the calcium-channel blocker nimodipine). Performing such experiments would substantiate the translational potential of isoflurane postconditioning into a patient population.

Fourth, collaborating with independent laboratories to replicate certain key findings would further substantiate our conclusions to date. Though most isoflurane postconditioning papers report a protective effect, regardless of the disease being modeled, this almost certainly represents publishing bias. Even when negative results are published, synthesizing them with positive papers is typically complicated by key methodological differences among groups. For
example, in the context of experimental intracerebral hemorrhage (ICH), Esposito and colleagues found no beneficial effect of post-ICH isoflurane,\textsuperscript{193} in contrast to Khatibi et al.\textsuperscript{155} Reconciling these broadly different results is challenging in light of differences in species employed and method of induction (respectively rat \textit{versus} mouse, injection of collagenase \textit{versus} of autologous blood). Such problems to interpretation could be minimized through direct collaboration with other groups: replicating key results would increase confidence in their veracity and strengthen the case for further translational studies.

Finally, future studies should assess delayed outcomes (at least 2-3 weeks after SAH), both behavioral and histologic. This emphasis on long-term outcome led us to perform Experiment 3. Unfortunately, despite reducing SAH mortality and employing two distinct versions of the gold-standard assessment for rodent learning and memory, we concluded that the endovascular perforation SAH in the mouse fails to cause demonstrable long-term MWM deficits, likely due to unreliable CA1 neuronal cell death. Given the frequency and severity of long-term cognitive deficits in SAH patients, further efforts are warranted. One possibility is to examine whether experimental parameters employing the mouse, which has myriad genetically modified lines, can be altered to detect neurobehavioral deficits. As discussed (see Chapter 3 Discussion), other mouse strains or methods of SAH induction could be employed, or other outcomes measures assessed. However, given that MWM deficits are seen in the rat in the three most commonly employed SAH models (endovascular perforation, cisterna magna injection and prechiasmatic injection), future studies should assess the effect of conditioning strategies on long-term neurobehavioral and histologic outcomes in the rat.

In summary, we demonstrate that conditioning strategies can reduce SAH-induced DCI. Significantly, protection was seen not just in proof-of-concept studies (employing pre-SAH
hypoxia) but also with a clinically relevant stimulus (isoflurane) delivered at a clinically relevant dose and time point. Moreover, HIF-1α and eNOS were identified as critical mediators of the neurovascular protection. Regarding cognitive outcomes after SAH, a significant source of long-term morbidity, we found that the endovascular perforation mouse model of SAH does not cause reliable CA1 neuronal cell death and does not produce demonstrable long-term neurobehavioral deficits as assessed by the MWM.

We thus conclude that conditioning strategies represent a promising therapeutic approach to combat SAH-induced DCI. Future studies should further delineate the molecular pathways responsible for this protection, as well as the impact on long-term neurobehavioral deficits employing rat models.
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