

RESEARCH ARTICLE

HIF-1 α Mediates Isoflurane-Induced Vascular Protection in Subarachnoid Hemorrhage

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Abstract

Objective: Outcome after aneurysmal subarachnoid hemorrhage (SAH) depends critically on delayed cerebral ischemia (DCI) – a process driven primarily by vascular events including cerebral vasospasm, microvessel thrombosis, and microvascular dysfunction. This study sought to determine the impact of postconditioning – the phenomenon whereby endogenous protection against severe injury is enhanced by subsequent exposure to a mild stressor – on SAH-induced DCI. **Methods:** Adult male C57BL/6 mice were subjected to sham, SAH, or SAH plus isoflurane postconditioning. Neurological outcome was assessed daily via sensorimotor scoring. Contributors to DCI including cerebral vasospasm, microvessel thrombosis, and microvascular dysfunction were measured 3 days later. Isoflurane-induced changes in hypoxia-inducible factor 1 α (HIF-1 α)-dependent genes were assessed via quantitative polymerase chain reaction. HIF-1 α was inhibited pharmacologically via 2-methoxyestradiol (2ME2) or genetically via endothelial cell HIF-1 α -null mice (EC-HIF-1 α -null). All experiments were performed in a randomized and blinded fashion. **Results:** Isoflurane postconditioning initiated at clinically relevant time points after SAH significantly reduced cerebral vasospasm, microvessel thrombosis, microvascular dysfunction, and neurological deficits in wild-type (WT) mice. Isoflurane modulated HIF-1 α -dependent genes – changes that were abolished in 2ME2-treated WT mice and EC-HIF-1 α -null mice. Isoflurane-induced DCI protection was attenuated in 2ME2-treated WT mice and EC-HIF-1 α -null mice. **Interpretation:** Isoflurane postconditioning provides strong HIF-1 α -mediated macro- and microvascular protection in SAH, leading to improved neurological outcome. These results implicate cerebral vessels as a key target for the brain protection afforded by isoflurane postconditioning, and HIF-1 α as a critical mediator of this vascular protection. They also identify isoflurane postconditioning as a promising novel therapeutic for SAH.

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.¹ The primary pathophysiological events implicated in DCI involve the cerebrovasculature. Cerebral vasospasm, felt by many to be a principal driver of DCI, is characterized by delayed and severe narrowing of large cerebral arteries.² This striking vascular pathology has been repeatedly identified as an independent risk factor for both brain infarction

and poor outcome after SAH.^{2–4} Several additional vascular processes are also linked to DCI, including microvascular autoregulatory dysfunction and microvessel thrombosis.² In fact, many believe a combination of these pathological vascular events are required to ultimately produce DCI (for review, see Macdonald²).

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.^{5,6} 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.⁷ Given that the pathophysiological events that underlie DCI are primarily vascular (vasospasm, microvascular dysfunction, and microvessel thrombosis), a conditioning-based strategy capitalizing on endogenous protective cascades that robustly protect the cerebrovasculature (as well as neurons and glia) would represent a powerful, novel intervention for SAH-induced DCI.

We previously applied such a strategy to SAH,⁸ showing that hypoxic preconditioning (i.e., exposure to hypoxia prior to SAH) prevented vasospasm and markedly improved neurological outcome, and that this protection depended critically on endothelial nitric oxide synthase (eNOS), a molecule whose dysregulation after SAH is known to contribute to vasospasm,⁹ microvascular dysfunction,¹⁰ and microvessel thrombosis.¹¹ As a follow-up to this proof-of-concept study, we next turned our attention toward translating this concept to a post-SAH conditioning paradigm. Given the strong experimental evidence that volatile anesthetics when delivered not only as a preconditioning stimulus^{12–16} but also as a postconditioning therapeutic agent^{17,18} provide robust protection against acute brain injury, we began our translational studies in SAH by examining the neurovascular protection afforded by isoflurane postconditioning.

Herein, we characterize the breadth and extent of the protection afforded by isoflurane postconditioning in SAH – a unique acute cerebrovascular condition where delayed vascular pathological events play a dominant role in determining long-term patient outcome.² We also begin to elucidate the molecular mediator(s) of this neurovascular protective response. In particular, we critically examined vascular endothelium-derived hypoxia-inducible factor 1alpha (HIF-1 α) in the protection afforded by isoflurane postconditioning in SAH, given that this molecule has been frequently implicated in the protection afforded by conditioning-based strategies for ischemic brain injury^{19–27} and has recently been linked (though not causally established) to the protection afforded by isoflurane postconditioning in experimental focal cerebral ischemia.²⁸ To obtain direct,

causal data, we employed complementary HIF-1 α -directed interventions including pharmacologic inhibition of HIF-1 α via 2-methoxyestradiol (2ME2) administration and genetic inhibition of vascular endothelial HIF-1 α 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

Experimental animals were housed in an AAALAC-accredited facility in temperature- and humidity-controlled rooms with a 12-h light–dark cycle. Mice were housed five to a cage and had ad libitum access to laboratory chow and tap water. A total of 261 mice were used at 12–14 weeks of age (24–30 g): 188 male C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) and 67 male endothelial cell HIF-1 α null (EC HIF-1 α ^{−/−}); the latter were bred by crossing *Tie2*-Cre and HIF-1 α ^{fl/fl} transgenic mice lines (both on C57BL/6 background; both purchased from Jackson Laboratory) as described²⁹: *Tie2*-Cre-positive, HIF-1 α ^{fl/fl} mice were used. In addition, a total of six male mice derived from crossing *Tie2*-Cre and ROSA26 reporter mice (background: 129X1/SvJ; Jackson Laboratory) 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.³⁰

Sample sizes

When comparing vasospasm, cortical microthrombosis, and neurological outcome, based on our previous studies⁸ 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 quantitative real-time polymerase chain reaction (qPCR), based on previous studies³¹ and the literature^{32,33} 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%.

Experimental procedures

Endovascular perforation SAH

Endovascular perforation SAH was performed per established protocol.⁸ 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

Isoflurane postconditioning was performed in an anesthetic induction chamber, as described with modification.³⁴ Briefly, mice were placed in the chamber perfused with 2% isoflurane in room air for 1 h; temperature was continuously 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 including arterial pH, pCO₂, pO₂, hematocrit, and hemoglobin were assessed via a femoral artery catheter in three experimental groups – sham surgery, SAH surgery, SAH surgery + isoflurane postconditioning (1 h of 2% isoflurane in room air beginning 1 h after SAH surgery). These parameters were assessed 2 h after sham or SAH surgery in all groups.

Gross neurological outcome

Gross neurological outcome was assessed in the morning prior to surgery and daily thereafter via sensorimotor scoring per established protocol.⁸ 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

SAH-induced DCI was assessed 3 days after surgery according to three components: *cerebral vasospasm* was assessed per established protocol⁸ via pressure-controlled casting with gelatin–India ink solution and measurement of the proximal MCA. Second, *microvascular reactivity* was assessed as per established protocol.³¹ Briefly, a closed cranial window was made to allow visualization of leptomeningeal arterioles; vasodilation to three stimuli was examined: physiological hypercapnia; superfusion of the endothelium-dependent vasodilator acetylcholine (ACh, 100 μ mol/L); and superfusion of the endothelium-independent vasodilator S-Nitroso-N-acetylpenicillamine (SNAP; 500 μ mol/L, both Sigma-Aldrich, St. Louis, MO). Third, *cortical microvessel thrombosis* was assessed via 3,3'-diaminobenzidine (DAB) staining for fibrinogen as described with modification.³⁵ Briefly, following transcardial perfusion with heparinized Phosphate buffered saline (PBS), brains were removed fixed in 4% paraformaldehyde, and sliced coronally at 50 μ m. Six coronal sections from the genu of the corpus callosum to the end of the dorsal hippocampus were incubated with blocking buffer (0.1% Triton-X100, 0.2% dry mild, and 1% bovine serum albumin [BSA] in PBS) on a shaker for 1 h, then incubated with rabbit antifibrinogen antibody (1:1000; Abcam, Cambridge, MA) at 4°C overnight. After wash with PBS, sections were incubated with goat-anti-rabbit biotinylated secondary antibody (1:1000; BioRad, Hercules, CA) for 1 h, incubated with VECTASTAIN Elite ABC Kit solution (Vector Laboratories, Inc., Burlingame, CA), and DAB solution. Sections were then mounted on a slide glass and coverslipped. Photographic images of fibrinogen immunostaining were taken using a Nikon Eclipse 600ME digital video microscopy system and MetaMorph imaging software (Molecular Devices, Sunnyvale, CA). Percent coverage of fibrinogen-immunoreactive areas of parietal cortex (3 fields/section; 6 sections/mouse) ipsilateral to SAH injury was determined using the threshold function in ImageJ software (NIH, Bethesda, MD).

The HIF inhibitor 2ME2

The HIF inhibitor 2ME2 (Sigma St. Louis, MO, USA) 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 report³⁶ showing its efficacy in preventing HIF-mediated transcriptional effects in adult rodent brain. 2ME2 is known to inhibit both HIF-1 α and HIF-2 α .³⁷

Quantitative real-time PCR

qPCR was performed as described.³² 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 μ g mRNA) with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster, CA). qPCR of HIF-1 α and HIF-2 α 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-1 α – forward GAACATCAAGTCAGCAACGTG, reverse TTTGACGGATGAGGAATGGG; erythropoietin (EPO) – forward GAGGTACATCTTAGAGGCCAAG, reverse TCTTCCACCTCCATTCTTTTCC; glucose transporter 1 (GLUT1) – forward GATTGGTTCCTTCTCTGTCGG, reverse CCCAGGATCAGCATCTCAAAG; BCL2/adenovirus E1B 19 kd-interacting protein (BNIP3) – forward ACCACAAGATACCAACAGAGC, reverse CGACTTGACCAATCCCATATCC; and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) – forward CTTTGTCAAGCTCATTTCCTGG, reverse TCTTGCTCAGTGCCTTGC. mRNA levels were calculated relative to GAPDH via the ΔC_t method and are expressed relative to naïve controls.

Cre-mediated expression

Cre-mediated expression was assessed by crosses of *Tie2*-Cre mice with floxed ROSA26 reporter mice per established protocols.³⁸ Briefly, following transcatheter perfusion with heparinized PBS, brains were removed and sliced coronally at 50 μ m. Sections were counterstained with 4',6-DiAmidino-2-PhenylIndole (DAPI) then mounted on glass slides and coverslipped with VectaShield (Vector Laboratories, Burlingame, CA).

Statistical analysis

Data represent individual animals and are expressed as means \pm 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

Isoflurane postconditioning attenuates SAH-induced vasospasm, microvessel thrombosis, and microvascular dysfunction

To determine the temporal window for postconditioning-induced protection, we exposed mice to isoflurane at various times after SAH surgery. Nonpostconditioned mice served as controls. All mice subjected to SAH

surgery were found to have SAH at the time of animal sacrifice; no mice subjected to sham surgery were found to have SAH at the time of animal sacrifice. Mortality was not significantly different between groups (nonpostconditioned = 6.1%; postconditioned = 6.9%). Substantial DCI protection, however, was noted between postconditioned and nonpostconditioned mice. Specifically, SAH-induced vasospasm was eliminated when postconditioning was initiated 15 min, 1 h, or 3 h after SAH; this protection was lost when postconditioning began 6 h after SAH (Fig. 1A and B; $P < 0.05$, 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 microvessel thrombosis was noted in MCA-territory of the cerebral cortex after SAH, which was significantly reduced by postconditioning (Fig. 2A and B; $P < 0.05$, ANOVA). SAH-induced microvascular dysfunction was also attenuated by postconditioning (Fig. 3). Cerebral microvascular 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. 3; $P < 0.05$, ANOVA). Together, these results show that isoflurane postconditioning after experimental SAH positively impacts multiple vascular contributors to DCI. This is direct evidence that isoflurane postconditioning induces strong protection of the cerebrovasculature and indicates this strategy is a novel and promising therapeutic approach toward ameliorating the devastating effects of SAH-induced DCI.

Isoflurane 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. 4; $P < 0.05$, 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).

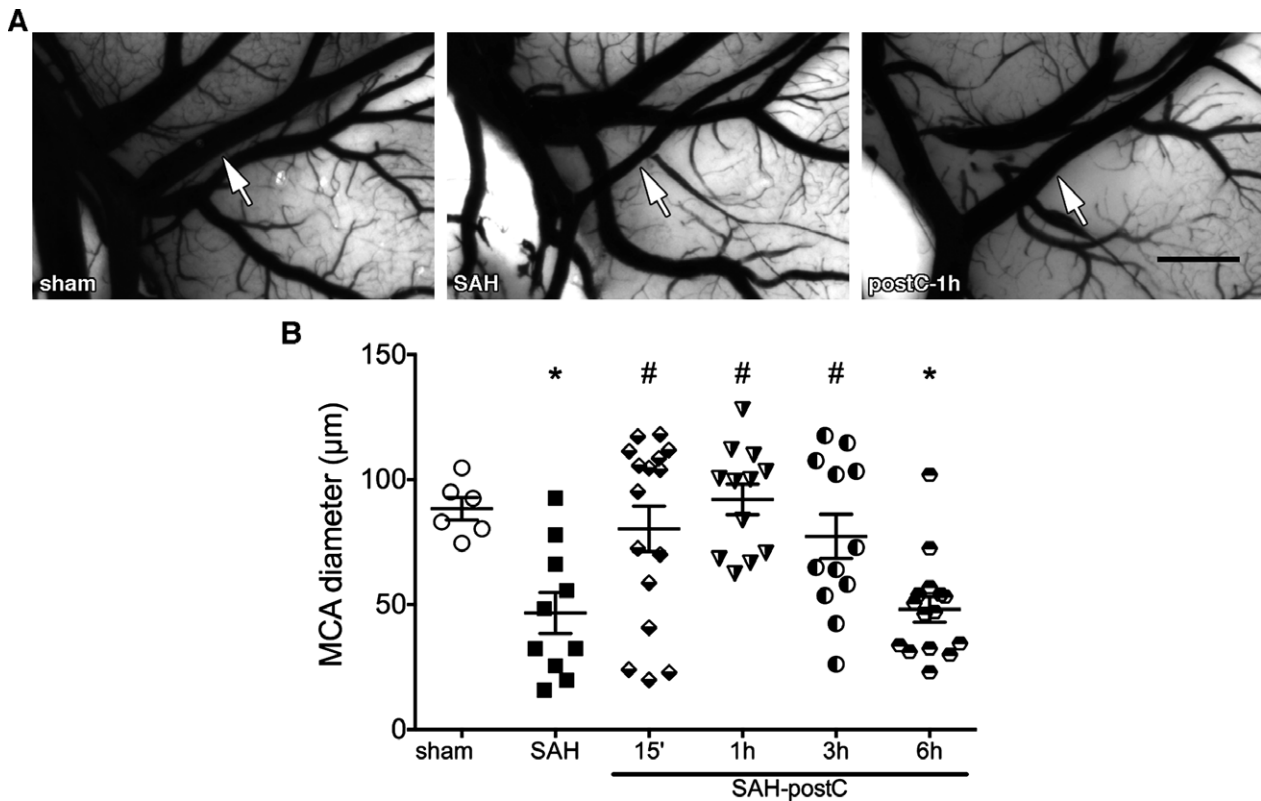


Figure 1. Postconditioning eliminates SAH-induced vasospasm. Mice underwent sham surgery; subarachnoid hemorrhage (SAH) surgery; or SAH surgery followed by isoflurane postconditioning (2% for 1 h, SAH-postC) starting 15 min, 1 h, 3 h, or 6 h after surgery. On post surgery day 3, pressure-controlled cerebrovascular casting was performed with gelatin–India ink. (A) Representative images of the middle cerebral artery (MCA) ipsilateral to endovascular perforation. Scale bar = 500 μm . (B) Vessel diameter of the ipsilateral MCA was assessed. $N = 6$ sham, $N = 10$ SAH, $N = 16$ SAH-postC-15', $N = 12$ SAH-postC-1 h, $N = 14$ SAH-postC-3 h, $N = 15$ SAH-postC-6 h. Data represent mean \pm SEM. * $P < 0.05$ versus sham, # $P < 0.05$ versus SAH, by repeated measures ANOVA.

Pharmacologic inhibition of HIF prevents isoflurane's transcriptional effect and isoflurane postconditioning's neurovascular protection

Next, we sought to determine whether this SAH-tolerant phenotype is dependent on HIF. Because HIF-1 α regulation does not entail an increase in gene transcription (activation of HIF-1 α by hypoxia occurs via inhibition of HIF-1 α degradation by the proteasome; activation of HIF-1 α by nonhypoxic stimuli occurs via increase in HIF-1 α protein translation³⁹), we assessed isoflurane-induced activation of HIF-1 α by quantitating changes to several of its transcriptional targets via qPCR. In naïve wild-type (WT) mice, isoflurane exposure did not impact HIF-1 α mRNA expression (as expected); however, it significantly modulated HIF-1 α transcriptional targets GLUT1 and BNIP3 as well as HIF-2 α transcriptional target EPO in a time-dependent manner (Fig. 5A). Administration of 2ME2 (a pharmacologic inhibitor of both HIF-1 α and

HIF-2 α) significantly attenuated these effects for each gene (Fig. 5A; $P < 0.05$, ANOVA). The same dose of 2ME2 eliminated the protection afforded by isoflurane postconditioning against SAH-induced vasospasm (Fig. 5B; $P < 0.05$, ANOVA) and neurological deficits (Fig. 5C; $P < 0.05$, repeated measures ANOVA). Together, these results show that isoflurane modulates HIF-driven gene 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-1 α inhibits isoflurane's transcriptional effect and isoflurane postconditioning's neurovascular protection

To test our hypothesis that HIF-1 α -driven transcriptional activation in ECs in response to isoflurane postconditioning drives the observed vasculoprotective phenotype, we

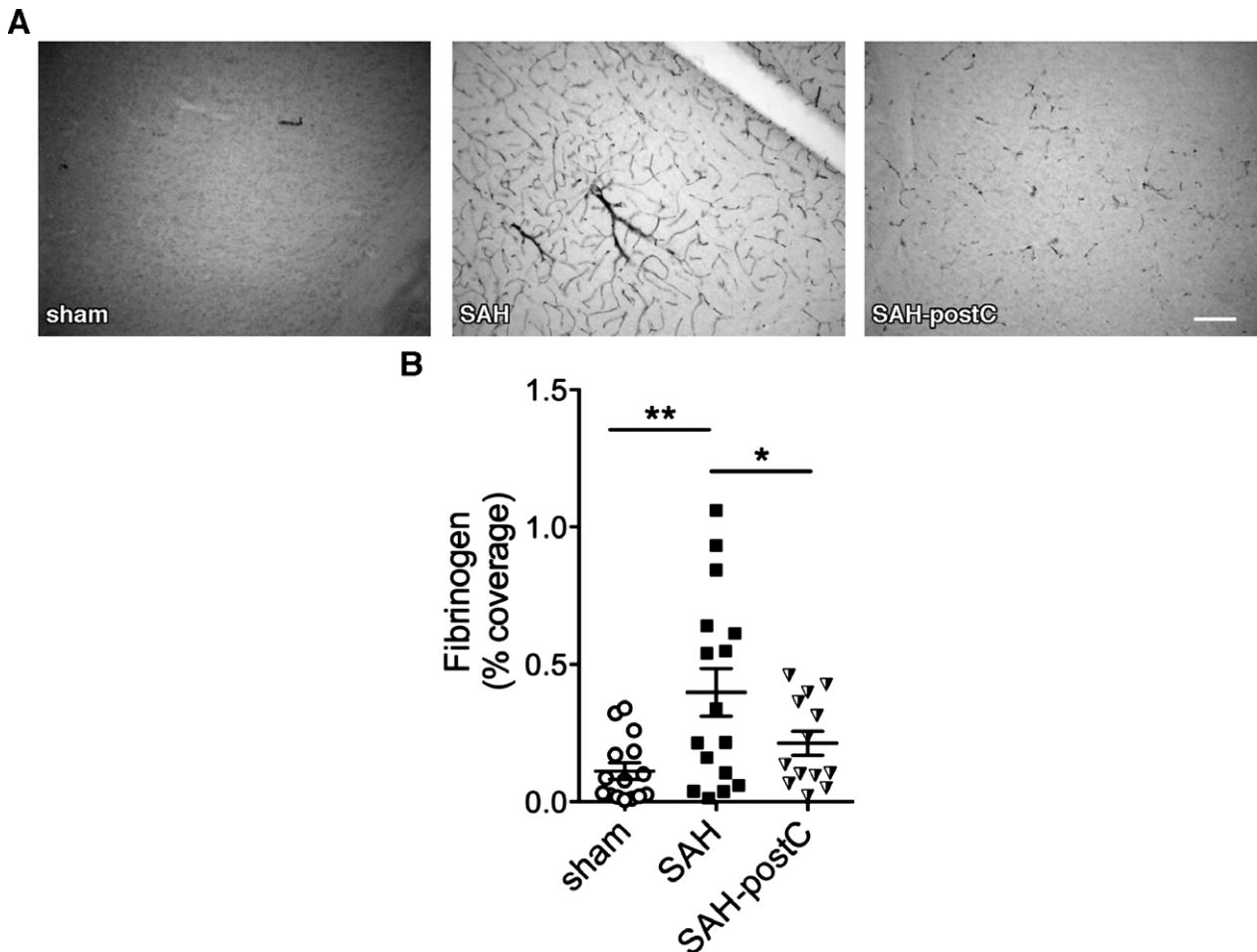


Figure 2. Postconditioning attenuates SAH-induced cortical microvessel thrombosis. Mice underwent sham surgery, subarachnoid hemorrhage (SAH) surgery, or SAH surgery followed 1 h later by isoflurane postconditioning (2% for 1 h, SAH-postC). On post surgery day 3, fixed brain sections were subjected to fibrinogen immunohistochemistry. (A) Representative images of parietal cortex ipsilateral to endovascular perforation. Scale bar = 100 μ m. (B) Cortical microvessel thrombosis was determined as percent coverage of ipsilateral parietal cortex. $N = 15$ sham, $N = 16$ SAH, $N = 12$ SAH-postC. Data represent mean \pm SEM. * $P < 0.05$, ** $P < 0.01$ by ANOVA.

generated EC-specific HIF-1 α -null mice. EC 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 6, green fluorescence – indicative of Cre expression – was seen throughout the cerebrovascular endothelium of Cre-positive mice (Fig. 6Av–viii), but the endothelium of Cre-negative mice fluoresced red (Fig. 6Ai–iv). In naïve EC HIF-1 α ^{-/-} mice, isoflurane exposure did not significantly affect transcription of the HIF-1 α targets GLUT1 and BNIP3; in contrast, transcription of EPO was significantly increased (Fig. 6B; $P < 0.05$, ANOVA), which is consistent with a known role of vascular HIF-2 α (retained in these mice) in regulating EPO.⁴⁰ Genetic deletion of endothelial HIF-1 α eliminated the protection afforded by isoflurane postcon-

ditioning against SAH-induced vasospasm (Fig. 6C; $P < 0.05$, ANOVA) and neurological deficits (Fig. 6D; $P < 0.05$, repeated measures ANOVA). Collectively, these results provide causal evidence that vascular endothelium-derived HIF-1 α is critical for isoflurane's transcriptional effect and the neurovascular protection afforded by its use as a postconditioning treatment in SAH.

Discussion

DCI is the most common and potentially treatable cause of secondary neurological injury following SAH¹; among patients affected by DCI, up to one-third experience poor outcome or death.² A central role of cerebral vasospasm in DCI is supported by the observations that vasospasm and DCI coincide temporally³; that DCI-related

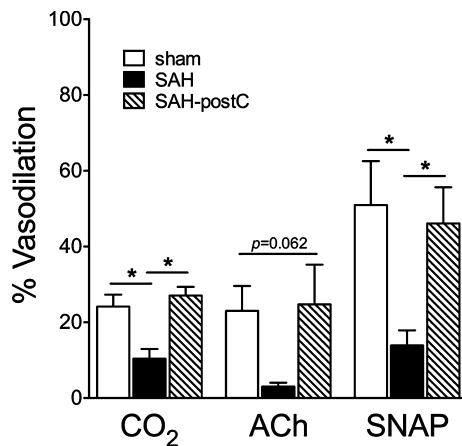


Figure 3. Postconditioning reverses SAH-induced microvascular dysfunction. Mice underwent sham surgery, subarachnoid hemorrhage (SAH) surgery, or SAH surgery followed 1 h later by isoflurane postconditioning (2% for 1 h, SAH-postC). On post surgery day 3, microvessel reactivity of the distal middle cerebral artery (MCA) 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*-acetyl-penicillamine (SNAP) were assessed. $N = 7$ per group. Data represent mean \pm SEM. * $P < 0.05$ by ANOVA.

symptoms occur within the territory of spastic arteries in many patients with vasospasm⁴; and that targeted endovascular treatment of vasospasm often improves patients' neurological status.⁴¹ Whereas the terms DCI and vasospasm were previously used interchangeably, the last decade has seen an expanded appreciation for the contribution of other pathophysiological processes including cortical microvessel thrombosis and microvascular dysfunction to DCI (for review, see Macdonald²). We⁸ and others⁵ have hypothesized that SAH 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 SAH patients; (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.

We thus sought to determine whether postconditioning – with a clinically relevant stimulus and at clinically relevant time points – could mitigate the deleterious effects of DCI. 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 SAH-induced vasospasm and neurological deficits. This shows that a clinically relevant paradigm of isoflurane postconditioning provides strong vascular protection in SAH and that this protection leads to a substantial improvement in neurological outcome. Second,

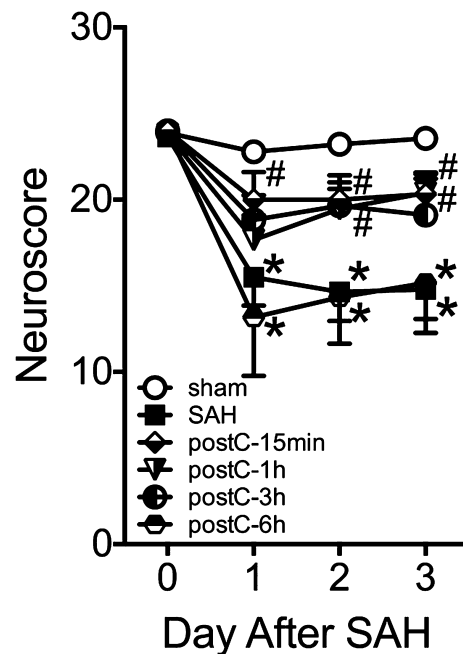


Figure 4. Postconditioning improves neurological outcome after SAH. Mice underwent sham surgery; subarachnoid hemorrhage (SAH) surgery; or SAH surgery followed by isoflurane postconditioning (2% for 1 h, SAH-postC) starting 15 min, 1 h, 3 h, or 6 h after surgery. Neurobehavioral assessment was performed on post surgery days 0–3 via sensorimotor scoring. $N = 6$ sham, $N = 10$ SAH, $N = 16$ SAH-postC-15', $N = 12$ SAH-postC-1 h, $N = 14$ SAH-postC-3 h, $N = 15$ SAH-postC-6 h (the same animals as were assessed for vasospasm in Fig. 1). Data represent mean \pm SEM. * $P < 0.05$ versus sham, # $P < 0.05$ versus SAH, by repeated measures ANOVA and Newman-Keuls multiple comparison test.

we found that isoflurane postconditioning markedly attenuated two additional vascular contributors to DCI, cortical microvessel thrombosis and microvascular dysfunction. This shows that isoflurane provides broad vascular protection – both at the macrovessel level (vasospasm) and at the microcirculatory level. This breadth of vascular protection enhances the translational potential of an isoflurane-based conditioning approach for SAH. 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-1 α and HIF-2 α with 2ME2 prevented isoflurane-induced modulation of all HIF target genes (GLUT1, BNIP3, and EPO), while selective genetic deletion of HIF-1 α in ECs prevented isoflurane-induced modulation of HIF-1 α target genes (GLUT1 and BNIP3) but not HIF-2 α target genes (EPO).⁴⁰ Fourth, we demonstrated that both pharmacologic (2ME2 administration) and genetic inhibition (selective deletion of HIF-1 α in ECs) of HIF-1 α reversed

Table 1. Physiological parameters in arterial blood.

Group	n	pH	pCO ₂ (mmHg)	pO ₂ (mmHg)	Hematocrit (%)	Hemoglobin (g/dL)
Sham	4	7.40 ± 0.01	41.5 ± 1.1	90.7 ± 3.4	43.6 ± 0.5	14.2 ± 0.3
SAH	6	7.37 ± 0.01	42.9 ± 1.0	87.9 ± 0.9	44.2 ± 0.5	14.3 ± 0.3
SAH:postC	5	7.39 ± 0.01	41.1 ± 1.9	90.7 ± 4.2	44.1 ± 0.6	14.7 ± 0.2

SAH, subarachnoid hemorrhage.

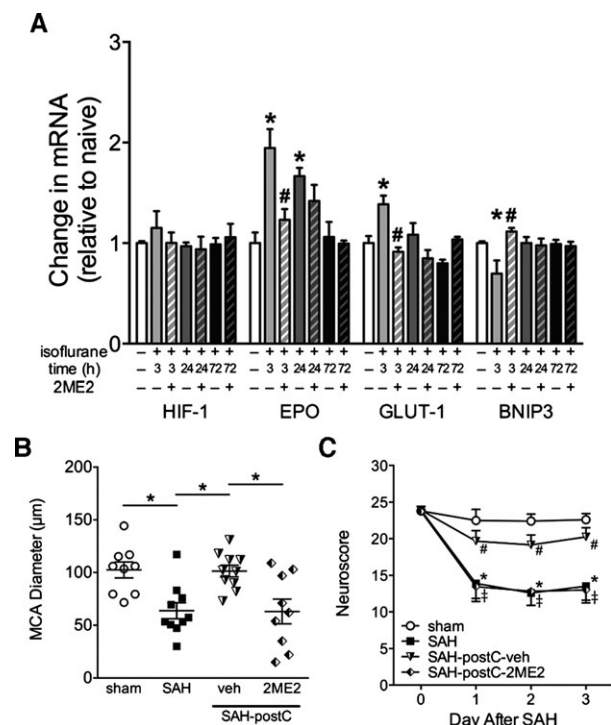


Figure 5. Hypoxia-inducible factor (HIF)-1 mediates isoflurane-induced transcription and postconditioning-induced neurovascular protection after subarachnoid hemorrhage (SAH). (A) Mice were administered vehicle or the HIF-1 inhibitor 2-methoxyestradiol (2ME2), exposed to isoflurane (2% for 1 h), sacked 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 versus naïve, #*P* < 0.05 versus time-matched isoflurane only by ANOVA. *N* = 6 mice per group. (B and C) Mice were administered vehicle and subjected to sham surgery; were administered vehicle and subjected to 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 versus sham, #*P* < 0.05 versus SAH, ‡*P* < 0.05 versus SAH-postC-veh by repeated measures ANOVA and Newman–Keuls multiple comparison test.

the neurovascular protective phenotype we identified in isoflurane postconditioned mice. These data shed new light on the mechanism by which isoflurane produces

vascular protection against acute cerebrovascular injury – that is, vascular endothelium-derived HIF-1 α is a key mediator of the robust vascular protection afforded by isoflurane postconditioning. 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 EC-derived HIF-1 α , and that the robust vascular protection afforded by isoflurane postconditioning leads to markedly improved neurological outcome after SAH.

Previously, we reported that SAH is amenable to a conditioning strategy: we showed that hypoxic preconditioning attenuated SAH-induced neurovascular dysfunction in mice, and that this protection was critically dependent on eNOS-derived nitric oxide.⁸ Subsequently, Altay and colleagues showed that early brain injury after mouse SAH can be impacted by isoflurane postconditioning, as cerebral edema, neuronal cell death, and neurological deficits were all reduced at 24 h post-SAH.^{34,42} However, in contrast to our finding of sustained isoflurane-induced neurovascular protection at 72 h post-SAH, these investigators noted that the protection was 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 in the sustainability of isoflurane-induced protection has a variety of potential explanations including variations between our respective endovascular perforation techniques (4–0 vs. 5–0 suture), our neurological assessment scales (6-point vs. 8-point sensorimotor scoring), our vascular endpoints (blood-brain barrier [BBB] disruption vs. vasospasm, microvessel thrombosis, and microvascular dysfunction), or a combination of the above. Regardless, this 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 vascular contributors to DCI that act at both the macrovessel (vasospasm) and microvessel (microvessel thrombosis and microvascular dysfunction) levels; (3) we show an extended therapeutic window of opportunity (3 h post-SAH); and (4) we identified vascular endothelium-derived HIF-1 α as an essential factor in the neurovascular protection afforded by isoflurane postconditioning.

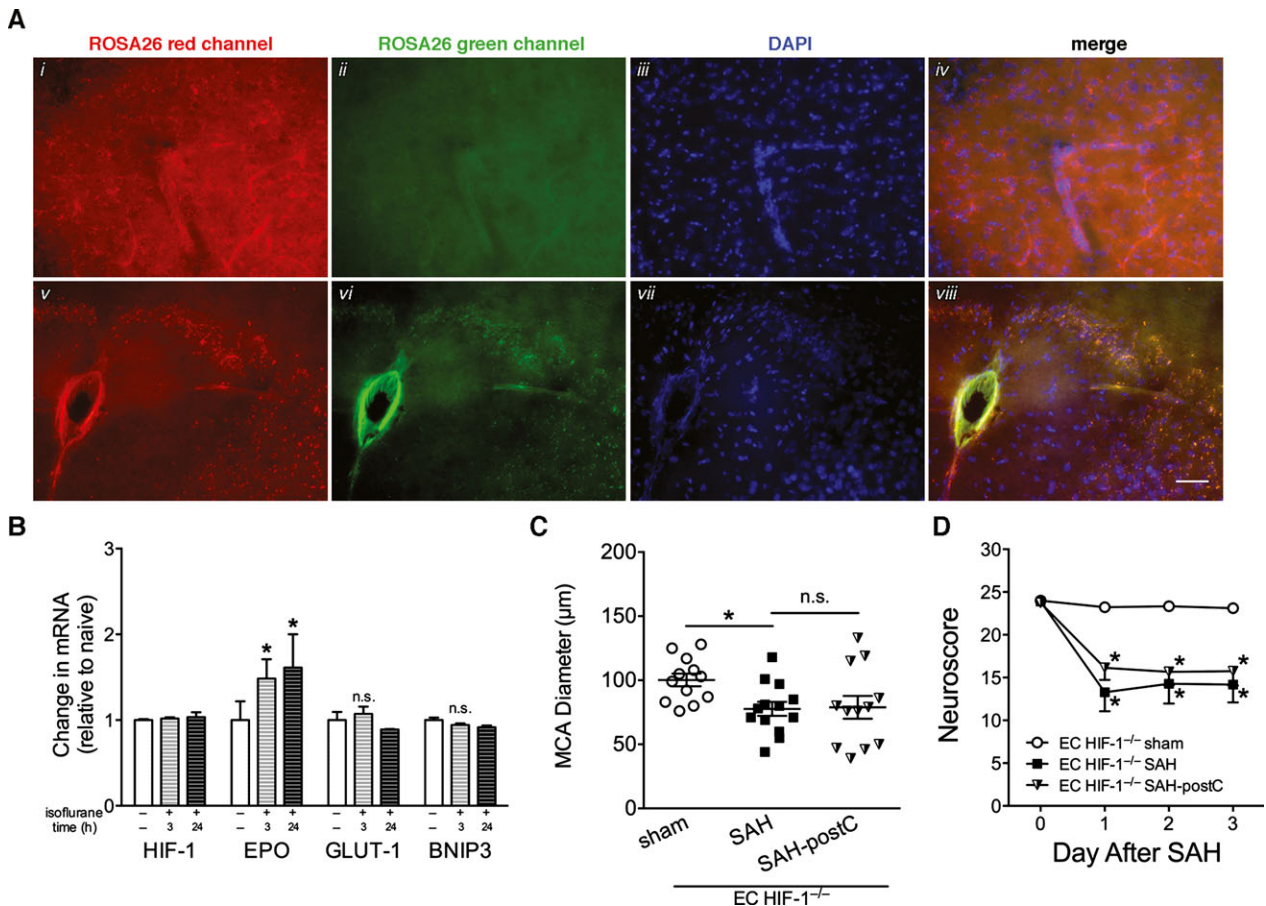


Figure 6. Endothelial hypoxia-inducible factor (HIF)-1 mediates isoflurane-induced transcription and postconditioning-induced neurovascular protection after subarachnoid hemorrhage (SAH). Endothelial cell HIF-1 null (EC HIF-1^{-/-}) mice were bred using a Cre-lox system. (A) *Tie2*-Cre mice were bred 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. Scale bar = 500 μm. (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 versus naïve by ANOVA. n.s. *P* > 0.05. (C–D) EC HIF-1^{-/-} mice underwent sham surgery, 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. n.s. *P* > 0.05. (D) Neurobehavioral assessment was performed on post surgery days 0–3 via Neuroscore. Data represent mean ± SEM. **P* < 0.05 versus sham by repeated measures ANOVA and Newman–Keuls multiple comparison test.

Inhalational anesthetic-induced conditioning with agents such as isoflurane has been reported to produce robust neuroprotection in a variety of acute cerebrovascular conditions including cerebral ischemia,^{12–14} neonatal hypoxia–ischemia,^{43,44} and cerebral hemorrhage.^{15,16} The majority of these studies have focused on characterizing and mechanistically understanding the *neural* protection afforded by volatile anesthetics – that is, how anesthetics such as isoflurane protect against neuronal cell death, ischemic brain injury, and neurological deficits. Recently, however, Chi and colleagues⁴⁵ examined whether the brain protection afforded by isoflurane is, in part, due to vascular protection induced via specific vascular-related

molecular cascades. They found that rats subjected to isoflurane preconditioning had improved regional cerebral blood flow in the ischemic cortex 1 and 3 h after induction of focal cerebral ischemia. They also showed that this improved regional cerebral blood flow was abolished with pharmacologic inhibition of inducible nitric oxide synthase (iNOS). Whether similar vascular protection is afforded when isoflurane is administered in a postconditioning paradigm or when it is applied to other acute cerebrovascular conditions such as SAH, however, has yet to be explored; nor has the upstream molecular inducer (s) of isoflurane-induced vascular protection been identified.

As opposed to the limited understanding of the mechanism(s) by which inhalational anesthetics induce vascular protection, the cascades by which such agents induce neuronal protection (at least in the setting of cerebral ischemia) have been well studied, (for reviews, see Kitano et al.⁴⁶ and Wang et al.⁴⁷) with several lines of evidence indicating that HIF-1 α may play an essential role. Isoflurane increases HIF-1 α ^{28,48} and HIF target genes^{28,48}; isoflurane protects against oxygen–glucose deprivation-induced neuronal cell death in a HIF-1 α -dependent fashion²⁸; and several strategies for augmenting HIF-1 α have been shown to be reduce neuronal injury and improve neurological outcome after cerebral ischemia (for review, see Ratan et al.⁴⁹) as well as cerebral hemorrhage.⁵⁰ Importantly, however, a causal role of HIF-1 α in the brain protection afforded by isoflurane has yet to be proven with in vivo experiments; and the role of HIF-1 α in the vascular protection afforded by isoflurane has yet to be examined. In this study, we provide such evidence: (1) We establish that isoflurane-induced protection extends to SAH, a fundamentally different form of acute cerebrovascular injury; (2) We found that this protection is strongly dependent on the protective effects of isoflurane on the cerebrovasculature – both at the macro- and microlevels; and (3) We show through a combination of targeted pharmacologic and genetic approaches that vascular endothelium-derived HIF-1 α is an essential mediator of the vascular protection afforded by isoflurane postconditioning in SAH. These results have important implications. First, they demonstrate that isoflurane postconditioning – when initiated at a clinically relevant dose and at clinically relevant time points – is a promising therapeutic approach to combat SAH-induced DCI. Second, they provide new evidence that the mechanism by which isoflurane reduces brain injury after acute cerebrovascular insults is, in part, via induction of vascular protection. Third, they provide evidence that HIF-1 α is a key molecular inducer of the vascular protection afforded by isoflurane. When coupled with the finding of Chi and colleagues that iNOS is required for isoflurane-induced vascular protection in ischemic stroke⁴⁵ and evidence indicating that iNOS is a downstream genetic target of HIF-1 α ,⁵¹ these results suggest that HIF-1 α -iNOS may be the molecular cascade by which isoflurane promotes vascular protection in acute brain injury.

Our experimental results are substantiated by recent studies examining the beneficial effect of cerebral conditioning in patients with acute cerebrovascular conditions. The first evidence of such a phenomena in cerebrovascular patients came from Zsuga and colleagues⁵² who demonstrated that patients with a history of transient ischemic attacks who later suffer an ischemic stroke have smaller infarctions and lower rates of in-hospital mortal-

ity than patients without a history of such attacks.⁵² More recently, in collaboration with Hoh and colleagues, we showed that a preconditioning effect may also exist in SAH patients.⁵³ In this study, we found that SAH patients with preexisting steno-occlusive cerebrovascular disease develop significantly less angiographic vasospasm than those without such disease. 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.

Results from our study raise several questions that warrant further investigation. First, by what mechanism (s) does isoflurane activate HIF-1 α in the context of postconditioning in SAH? Here, several possibilities exist including isoflurane upregulation of HIF-1 α 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.⁵⁴). Second, by what downstream molecular effector(s) does HIF-1 α reduce the brain's susceptibility to SAH? One possibility is that the protection afforded by isoflurane is the consequence of HIF-1 α '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 ECs and can be modulated by HIF-1 α ⁵⁵; (2) eNOS has been linked to vasospasm,⁹ microvascular dysfunction,¹⁰ and cortical microvessel thrombosis¹¹; and (3) the neurovascular protection afforded by a different conditioning paradigm (hypoxia 24 h prior to SAH) is critically dependent on eNOS.⁸ Another possibility – though not mutually exclusive from HIF-1 α 's effects on vascular ECs – is that one or more HIF-1 α -driven secreted proteins exert paracrine protective effects on neighboring cells. Potential target molecules here include iNOS (which, as mentioned above, has been implicated in the vascular protection afforded by isoflurane in cerebral ischemia⁴⁵) and vascular endothelial growth factor (which has been previously linked to vasospasm protection⁵⁶). Third is the neurovascular protection afforded by postconditioning present across SAH model systems (endovascular perforation, cisterna magna injection, prechiasmatic injection), species (lissencephalic vs. gyrencephalic), gender (male vs. female), age (young vs. old), comorbidities (hypertension, diabetes, etc.), and outcome measures (short-term vs. long-term)? These cross-validation experiments are critical if isoflurane therapy is to be considered for translational studies, and are strongly advocated by the Stroke Therapy Academic Industry Roundtable.⁵⁷

Another important issue to be examined is the impact of HIF-1 α upregulation by SAH itself (which several

groups have documented in various animal models of SAH^{58,59}) versus that induced by isoflurane postconditioning as documented in this study. In this instance, timing, severity, and sustainability of HIF-1 α upregulation and its downstream transcriptional effects may matter greatly since pharmacologic inhibition of HIF-1 α activation has been shown to be protective or deleterious depending on the specific experimental conditions in acute cerebrovascular injury paradigms including ischemic stroke (for review, see Singh *et al.*⁶⁰) and SAH.^{58,59,61,62} This “double-edged sword” impact of HIF-1 α must be fully understood in the setting of SAH if HIF-1 α -based therapies are to be pursued in translational studies. Alternatively, it may be that the therapeutic index for HIF-1 α -directed therapies is too narrow for HIF-1 α to be a viable druggable target, in which case isoflurane itself (or potentially other anesthetic agents with conditioning effects) may prove a more promising intervention against SAH-induced DCI.

In conclusion, this study demonstrates that isoflurane has strong vascular protective effects on SAH, that this protection produces robust improvement in neurological outcome after SAH, and that this vascular protective phenotype is critically dependent on vascular endothelial cell HIF-1 α -driven gene transcription. The latter raises the intriguing possibility that HIF-1 α is not only a mediator of vascular protection in SAH but may also be a previously unrecognized molecular target for the vascular protection provided by isoflurane postconditioning in other acute cerebrovascular conditions like ischemic stroke. These results are especially exciting given that isoflurane is already FDA approved for use in the SAH 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 SAH-induced DCI. Moreover, the stereotypical delay between SAH and DCI provides a meaningful therapeutic window of opportunity for an isoflurane-based postconditioning strategy. Such a window may also be exploited for HIF-1 α -directed therapies if future studies demonstrate that such approaches provide similar neurovascular protection in experimental SAH.

Acknowledgments

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Author Contributions

E. M. was involved in experimental conception and design, acquisition of data, analysis and interpretation of data, and writing the first draft of the manuscript and subsequent critical revisions. A. W. J. was involved in acquisition of data, analysis and interpretation of data, and critical revisions to the manuscript. J. W. N. and M. D. H. were involved in data acquisition and critical revisions to the manuscript. J. M. G., B. H. H., and G. J. Z. were involved in experimental conception and design, interpretation of data, and critical revisions to the manuscript.

Conflict of Interest

Dr. Milner reports grants from American Heart Association, during the conduct of the study. Dr. Zipfel reports grants from National Institutes of Health, American Heart Association, McDonnell Center for Higher Brain Function, and Neurosurgery Research and Education Foundation, during the conduct of the study.

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