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ORIGINAL RESEARCH

Sirtuin 1 Mediates Protection Against Delayed Cerebral Ischemia in Subarachnoid Hemorrhage in Response to Hypoxic Postconditioning

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BACKGROUND: Many therapies designed to prevent delayed cerebral ischemia (DCI) and improve neurological outcome in aneurysmal subarachnoid hemorrhage (SAH) have failed, likely because of targeting only one element of what has proven to be a multifactorial disease. We previously demonstrated that initiating hypoxic conditioning before SAH (hypoxic preconditioning) provides powerful protection against DCI. Here, we expanded upon these findings to determine whether hypoxic conditioning delivered at clinically relevant time points after SAH (hypoxic postconditioning) provides similarly robust DCI protection.

METHODS AND RESULTS: In this study, we found that hypoxic postconditioning (8% O_2 for 2 hours) initiated 3 hours after SAH provides strong protection against cerebral vasospasm, microvessel thrombi, and neurological deficits. By pharmacologic and genetic inhibition of SIRT1 (sirtuin 1) using EX527 and global $Sirt1^{-/-}$ mice, respectively, we demonstrated that this multifaceted DCI protection is SIRT1 mediated. Moreover, genetic overexpression of SIRT1 using $Sirt1\text{-}Tg$ mice, mimicked the DCI protection afforded by hypoxic postconditioning. Finally, we found that post-SAH administration of resveratrol attenuated cerebral vasospasm, microvessel thrombi, and neurological deficits, and did so in a SIRT1-dependent fashion.

CONCLUSIONS: The present study indicates that hypoxic postconditioning provides powerful DCI protection when initiated at clinically relevant time points, and that pharmacologic augmentation of SIRT1 activity after SAH can mimic this beneficial effect. We conclude that conditioning-based therapies administered after SAH hold translational promise for patients with SAH and warrant further investigation.

Key Words: delayed cerebral ischemia ■ microvessel thrombi ■ postconditioning ■ resveratrol ■ SIRT1 ■ subarachnoid hemorrhage ■ vasospasm

A neurysmal subarachnoid hemorrhage (SAH) is a devastating cerebrovascular disease with significant morbidity and mortality.[1] Despite effective aneurysm treatment, ≈30% of patients develop long-term cognitive and neurological deficits that significantly affect their capacity to return to work or daily life unassisted.[2] Much of this stems from a secondary ischemic phenomenon referred to as delayed cerebral ischemia (DCI), which typically occurs 4 to 12 days after the initial hemorrhage.[3] Though primarily attributed for many years to large-artery vasospasm, DCI is now understood to be a multifactorial process driven by additional pathophysiological events including microvessel thrombosis,[4] microvascular autoregulatory dysfunction,[5] and cortical spreading depolarization.[6] Given this improved understanding of

CLINICAL PERSPECTIVE

What Is New?

- Hypoxic conditioning initiated after subarachnoid hemorrhage (hypoxic postconditioning) provides robust protection against delayed cerebral ischemia (DCI) including alleviating large-artery vasospasm, microvessel thrombosis, and sensory-motor neurological deficits.
- Genetic and pharmacologic inhibition of the nicotinamide adenine dinucleotide–dependent deacetylase, SIRT1 (sirtuin 1), blocks the DCI protection afforded by hypoxic postconditioning.
- Genetic overexpression of SIRT1 mimics the DCI protection afforded by hypoxic postconditioning in subarachnoid hemorrhage; post–subarachnoid hemorrhage administration of the SIRT1 activator, resveratrol, reduces DCI and improves neurological outcome in a SIRT1-mediated fashion.

What Are the Clinical Implications?

- Our results indicate that conditioning-based strategies, in particular SIRT1 activation, carry promise as a novel therapeutic approach to reducing DCI and improving neurological outcome in patients suffering from a ruptured brain aneurysm.

Nonstandard Abbreviations and Acronyms

DCI	delayed cerebral ischemia
EBI	early brain injury
eNOS	endothelial nitric oxide synthase
HPostC	hypoxic postconditioning
HPreC	hypoxic preconditioning
MCA	middle cerebral artery
SIRT1	sirtuin 1

underlying mechanisms, it is logical to conclude that efficacious future therapeutic strategies for reducing DCI and improving functional outcome in patients with SAH must elicit protection against not only large-artery vasospasm, but also other pathophysiological events including those disrupting the microcirculation. Given its well-described powerful and pleiotropic protective effects against ischemic brain injury shown by us[7,8] and others,[9,10] we began studies to assess the benefits of a therapeutic approach known as conditioning in experimental models of SAH.

Conditioning describes the phenomenon wherein endogenous injury resilience in brain and other tissues is augmented by exposure to a stressful, but sublethal, stimulus.[11] A variety of conditioning agents and strategies have been examined in ischemic stroke and are found to enhance endothelial-dependent vasodilation,[12] reduce blood–brain barrier breakdown[13] and vasogenic edema,[14] and increase vascular patency,[15] thereby supporting a vascular contribution to conditioning-induced protection. Given the central role vascular deficits play in DCI pathophysiology (large-artery vasospasm and microcirculatory deficits including microvessel thrombosis and autoregulatory dysfunction), the wide therapeutic window between SAH onset, and development of DCI, we have leveraged conditioning-based approaches to improve outcome after experimental SAH. In our initial proof-of-principle study, we showed that a brief period of hypoxia initiated before SAH (hypoxic preconditioning [HPreC]) markedly reduces large-artery vasospasm and improves neurological outcome, and that this protection is critically dependent on endothelial NO synthase (eNOS).[16] In a follow-up study also examining HPreC, we discovered that SIRT1 (sirtuin 1) is an additional mediator of hypoxia-induced protection against vasospasm and neurological dysfunction.[17] In the present study, we turned our attention toward translating these initial proof-of-principle discoveries to strategies that can ultimately be applied to patients in the clinic (ie, a conditioning-based therapy initiated after aneurysmal SAH).

SIRT1 is a well-known nicotinamide adenine dinucleotide–dependent epigenetic modulator that regulates gene transcription and thereby impacts numerous functions including stress, inflammation, vessel function, and thrombosis.[18] Although SIRT1 is known to have protective effects in central nervous system diseases including Alzheimer's, Parkinson's,[19] ischemic stroke,[20] and traumatic brain injury,[21] it has only recently been explored in the setting of SAH. Zhang and colleagues provided evidence that SIRT1 plays a role in SAH-induced early brain injury (EBI).[22] This finding has subsequently been validated in follow-up studies.[23] Our laboratory showed SIRT1 is a key mediator of HPreC-induced protection against large-artery vasospasm and neurological deficits after experimental SAH.[17] Hence, we undertook the present study to build upon these initial findings and determine whether hypoxic conditioning initiated after SAH (hypoxic postconditioning [HPostC]) provides similarly robust protection against large-artery vasospasm and neurological deficits, whether it also provides protection at the level of microcirculatory deficits, and whether this multifaceted endogenous protective phenotype is mediated by SIRT1.

METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request and approval of the appropriate institutional review board.

Experimental Animals

All experimental procedures performed were approved by the Institutional Animal Care and Use Committee at Washington University and conformed to the National Institutes of Health *Guidelines for the Care and Use of Animals in Research*. We obtained 12- to 14-week-old male C57BL/6J mice from Jackson Laboratories (Bar Harbor, ME). Whole body adult-inducible SIRT1-knockout and SIRT1-overexpressing mice were generated as described previously.[24] In brief, mice with a targeted Sirt1 floxed mutant allele (B6;129-Sirt1tm1Ygu/J) were obtained from Jackson Laboratories and bred with mice expressing whole-body tamoxifen-inducible Cre (B6.129-Gt[ROSA]26Sortm1[cre/ERT2]Tyj/J) obtained from Jackson Laboratories to generate tamoxifen-inducible whole-body SIRT1-knockout mice and wild-type littermate controls. Hemizygous constitutive whole-body SIRT1-overexpressing mice (B6. CgTg[Sirt1]ASrn/J) and noncarriers on the same background were also obtained from Jackson Laboratories and bred to generate constitutive whole-body SIRT1-overexpressing mice (*Sirt1-Tg*) and littermate controls. All animals were acclimated under a 12-hour light/dark cycle controlled environment with free access to water and a standard mouse chow diet.

Experimental SAH Model

Experimental SAH was induced in mice by the endovascular perforation technique as described in our earlier publications.[16,25,26] Briefly, mice were anesthetized with isoflurane (2% induction, 1.5% maintenance) in room air with core body temperature maintained at 37 °C by a thermo-regulated heating pad. A midline incision made in the neck and the external carotid artery was exposed. A 5-0 nylon suture was introduced into the external carotid artery and advanced distally through the internal carotid artery until reaching the internal carotid artery bifurcation. The suture was then advanced further to induce SAH. The suture was removed, and the external carotid artery was ligated. Mice were allowed to recover from anesthesia in an incubator and then returned to their cages. Mice undergoing sham surgery underwent the same procedure except for suture perforation of the internal carotid artery.

Neurological deficits were assessed daily until the time the animal was euthanized, and DCI-related pathology (vasospasm and microvessel thrombus) was assessed on post-SAH day 2. This time point was chosen based on the established time course of DCI-related pathology in the endovascular perforation mouse model of SAH, a time course that is considerably faster than that seen in humans (for review, see LeClerc et al[27]). To evaluate distribution of blood along subarachnoid vessels, mice were transcardially perfused with heparinized PBS, and the brain was carefully removed. In sham-operated animals, there is no visible blood, whereas after SAH, extravasated blood consistently covers the middle cerebral artery (MCA) and is distributed along the larger arteries that branch from the MCA (Figure S1). Animals that died during or soon after the surgical procedure, those from the SAH group with absence of subarachnoid blood, those from the sham group with presence of subarachnoid blood, and those with the presence of dense hemiparesis within 6 hours of surgery (indicative of inadvertent MCA occlusion) were excluded. Otherwise, all SAH and sham-operated animals that survived and were able to complete neurobehavioral assessment were included in our findings.

Hypoxic Postconditioning

Figure S1 demonstrates the schematic representation of our experimental design and protocols. In the initial dose-finding experiment (Figure S2), mice were placed in a hypoxic chamber 3 hours after SAH and exposed to air containing 8% O_2/92% N_2 for 2 hours or 11% O_2/89% N_2 for 1 hour with access to food and water ad libitum; animals were then returned to their cages. For subsequent experiments, exposure to air containing 8% O_2/92% N_2 for 2 hours was used as the conditioning stimulus and administered beginning 3 hours after SAH. This stimulus was repeated daily at roughly 24-hour intervals following SAH until the day the animal was euthanized. Normoxic controls were placed in chambers containing room air.

SIRT1 Deacetylase Activity

SIRT1 deacetylase activity in the cerebral cortex was determined using an SIRT1 Activity Assay Kit (ab156065; Abcam, Cambridge, MA). In brief, fluoro-substrate peptide, fluoro-deacetylated peptide, and nicotinamide adenine dinucleotide were successively added to the SIRT1 assay buffer followed by incubation with the tissue sample, as well as the developer buffer and recombinant SIRT1 (5 mg) at 37 °C for 60 minutes. After excitation at 340 nm, emitted light was detected at 440 nm using a microtiter plate fluorometer. The fluorescence intensity of the assay buffer was subtracted from each experimental sample.

Drug Administration

A selective SIRT1 inhibitor EX-527 (10 mg/kg; Tocris Bioscience, Bristol, UK), or vehicle (8% dimethylsulfoxide

in β-cyclodextrin:PBS) was administered to mice via intraperitoneal injection beginning 2 hours after surgery and 1 hour before HPostC and continued once daily thereafter. Resveratrol (6 mg/kg IP, BID; Sigma, St. Louis, MO) or vehicle (7.5% dimethylsulfoxide in corn oil) was administered starting 3 hours after SAH or sham surgery and repeated every 12 hours until the morning of post-SAH day 2. Vehicle-treated animals were administered equal volumes of dimethylsulfoxide. We (Figure S3) and others have shown that both EX-527 and resveratrol are potent regulators of SIRT1 deacetylase activity.[28] A whole-body Cre-inducible SIRT1-knockout mouse was generated by intraperitoneal administration of tamoxifen (75 mg/kg) for 5 consecutive days and subjected to SAH 9 to 10 days after the last injection. The resultant deletion of the catalytic region of SIRT1 in SIRT1-knockout mice was evaluated 1 week after the last tamoxifen injection by protein expression analysis.

Cerebral Vasospasm Assessment

Vasospasm was assessed in the ipsilateral MCA on postsurgery day 2 via cerebrovascular casting, as previously described.[16,25,26,29] Briefly, mice were anesthetized with isoflurane and transcardially perfused at a constant pressure of 80±5 mm Hg with 15 mL of 10 mmol/L glucose PBS–heparin (1000 USP U/mL), followed by 20 mL of 4% paraformaldehyde and 20 mL of the 20 µmol/L 5-(and-6)-carboxy-X-rhodamine, succinimidyl ester (5(6)-ROX, SE) working solution. A 10-mmol/L stock solution of 5(6)-ROX, SE (AS-81113; AnaSpec, Freemont, CA) in dimethylsulfoxide was diluted into 10 mmol/L glucose–PBS for a final working solution of 20 µmol/L 5(6)-ROX, SE in 10 mmol/L glucose–PBS. After perfusion fixation, brains were extracted under a dissecting microscope (SMZ 800; Nikon) and stored in 4% paraformaldehyde at 4 °C for 48 hours and then transferred into a 30% sucrose solution. Blood vessels imaged using a charge-coupled device (CCD) camera (CoolSNAP 3s; Roper Scientific, Tucson, AZ). Spasm of the ipsilateral proximal MCA was assessed by recording 3 independent measurements of the smallest lumen diameter within the first 1000 µm of the MCA outside the lateral sulcus. All vascular measurements were performed by a single observer blinded to experimental groups.

Microvessel Thrombus Immunohistochemistry

The extent of microvessel thrombi formation was assessed by immunohistochemistry in a blinded fashion by an independent investigator for the presence of fibrinogen deposition, as previously described,[26] on post-SAH day 2. Briefly, mice were transcardially perfused with heparinized PBS, brains were then extracted and fixed in 4% paraformaldehyde for 48 hours, and 50-µm thick, free floating, coronal brain sections were then blocked in PBS containing 1% bovine serum albumin, 0.2% dry milk, and 0.1% Triton-X100 for 1 hour, followed by overnight incubation at 4 °C with with rabbit anti-fibrinogen antibody (1:1000 dilution; Abcam). The free-floating sections were then incubated with biotinylated goat-anti rabbit antibody (Bio-Rad, Hercules, CA) at room temperature for 2 hours, followed by incubation with VECTASTAIN Elite ABC Kit solution (Vector Laboratories, Burlingame, CA), and 3,3′-diaminobenzidine tetrachloride solution. Stained sections of the left cerebral cortex were imaged using a Nikon E-600ME microscope. Microvessel thrombi formation was quantified by measuring at 20× magnification, and the percent coverage of fibrinogen-immunoreactive areas of parietal cortex (4–5 sections per brain) ipsilateral to SAH injury was determined using the threshold function in ImageJ software (National Institutes of Health, Bethesda, MD).

Neurological Deficit Testing

Neurobehavioral outcome in mice was examined daily using our Neuroscore test, as previously described.[26] Briefly, neurological function was graded based on the combination of a motor score (0–12) that evaluated spontaneous activity, symmetry of limb movements, climbing, balance and coordination, and a sensory score (4–12) that evaluated body proprioception and vibrissae, visual, and tactile responses.

Western Immunoblotting

All the extraction operations were performed on ice. Briefly, the tissues of the cerebral cortex were purified using radioimmunoprecipitation assay lysis buffer. Protein concentration was quantified by bicinchoninic protein assay kit (DC protein assay kit; Bio-Rad). The equivalent extracted protein lysates (40–60 µg) were diluted with 2× Laemmli buffer, denatured for 5 minutes at 95 °C, loaded onto sodium dodecyl sulphate polyacrylamide gels, and blotted as previously described.[30] After transferring onto immobilon-P/polyvinylidene difluoride membranes (Millipore, Billerica, MA), the membranes were blocked in TBST buffer (0.05 M Tris pH 7.4, 0.15 M NaCl, 0.05% Tween 20) containing 5% (w/v) albumin (Sigma) at room temperature for 1 hour. Subsequently, membranes were incubated at 4 °C overnight with the following primary antibodies, which were dissolved in 5% albumin in TBST solution: mouse anti-mouse SIRT1 (Abcam) and anti-α tubulin antibody (ab18251; rabbit 1:3000). Blots were developed using an HRP (horseradish peroxidase)-conjugated

immunoglobulin G secondary antibody for 1 hour at room temperature. The relative expression of protein bands was visualized using the GeneSys Software (Alpha Metrix Biotec, Rödermark, Germany) with the enhanced chemiluminescence substrate (Millipore). Densitometric quantification of the bands was performed using Image J software.

Statistical Analysis

We estimated sample sizes using effect sizes based on our previous publications as well as pilot experiments, using a significance level of 0.05 and a power of 0.80. Therefore, our studies were appropriately powered for each end point to obtain statistical significance. Following testing for the homoscedasticity and the normal distribution by Shapiro-Wilk test, 1-way ANOVA followed by Tukey multiple comparisons post hoc tests were performed for vasospasm and microvessel thrombosis. Neuroscore data were analyzed using repeated-measures ANOVA followed by Tukey post hoc test for multiple comparisons. Nonparametric data with 2 groups were analyzed using the Mann-Whitney U test. Data are presented as mean with SD. $P<0.05$ was considered as statistically significant.

RESULTS

HPostC Attenuates SAH-Induced Vasospasm, Microvessel Thrombosis, and Neurological Deficits

First, we assessed whether HPostC provides macro- and microvascular protection. Mice were subjected to SAH and then exposed 3 hours later to an episode of severe hypoxia (8% O_2 for 4 hours) with the intention of evaluating large-artery vasospasm and neurological deficits for protective effects; however, mortality with this severe dose of HPostC was ≈60% (data not shown). We then subjected mice that underwent SAH to mild (11% O_2 for 1 hour) or moderate (8% O_2 for 2 hours) hypoxia, both initiated 3 hours after SAH, and then repeated daily until the animal was euthanized. Both doses were well tolerated in control and SAH mice, with mortality rates of 2% and 10%, respectively. Although mild HPostC produced no significant neurovascular protection, moderate HPostC led to a significant reduction in large-artery vasospasm, improved neurological deficits, and provided marked protection against SAH-induced cortical microvessel thrombosis compared with untreated control mice (Figure S2). Given the robust neurovascular protection provided by moderate HPostC initiated 3 hours after SAH, this treatment paradigm was used for all subsequent experiments.

HPostC Increases SIRT1 Enzymatic Activity

Because SIRT1 is upregulated by hypoxia[31] and is a known regulator of eNOS,[32] we hypothesized that SIRT1 directly participates in HPostC-induced protection against SAH. To examine this hypothesis, we assessed the impact of HPostC on SIRT1 deacetylase activity and protein expression in mice subjected to SAH. We found that SIRT1 activity, but not protein expression levels (data not shown), was significantly upregulated by HPostC in SAH group, remaining elevated for 48 hours after SAH (Figure 1A). In contrast, SIRT1 activity in nonpostconditioned mice was only significantly elevated at 12 hours after SAH, perhaps reflecting an endogenous response to help protect the brain (Figure 1B).

SIRT1 Contributes to the DCI Protection Afforded by HPostC: Validation by Pharmacologic and Genetic Inhibition

We next used a pharmacologic and genetic approach to obtain causal evidence for the involvement of SIRT1 in HPostC-induced DCI protection. One cohort of mice was treated with the SIRT1-specific pharmacologic inhibitor, EX527, before HPostC. We found that the strong protection against DCI-associated large-artery vasospasm (Figure 2A), microvessel thrombosis (Figure 2B), and neurological deficits (Figure 2C) afforded by HPostC was blocked by EX527. We also examined the role of SIRT1 in HPostC-induced DCI protection using tamoxifen-inducible global SIRT1-knockout (Sirt1$^{-/-}$) mice of either sex, the latter of which we showed by immunoblotting to exhibit an absence of SIRT1 protein in the brain (Figure 3A). As with the pharmacologic study, the robust protection against large-artery vasospasm (Figure 3B), microvessel thrombosis (Figure 3C), and neurological deficits (Figure 3D) in Sirt1$^{+/+}$ mice was lost in their littermate Sirt1$^{-/-}$ mice. Taken together, these pharmacologic and genetic studies indicate HPostC-induced protection against SAH-induced DCI and accompanying neurological deficits is critically dependent on SIRT1.

SIRT1 Contributes to the DCI Protection Afforded by HPostC: Validation by Pharmacologic and Genetic Potentiation

Additional genetic and pharmacologic studies were undertaken to support SIRT1 as a mediator of DCI protection. Constitutive global SIRT1-overexpressing mice of either sex were used to assess the efficacy of SIRT1 augmentation as a strategy to attenuate SAH-induced DCI and neurological deficits. We found a significant reduction in SAH-induced large-artery
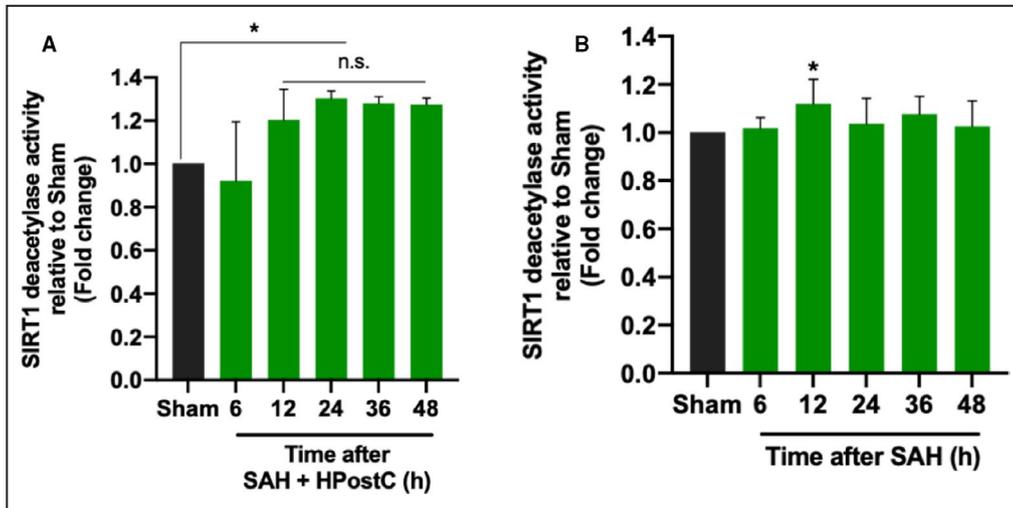
Figure 1. SIRT1 (sirtuin 1) deacetylase activity after subarachnoid hemorrhage (SAH) and hypoxic postconditioning (HPostC).
SIRT1 deacetylase activity in brain lysates was quantified 6 to 48 hours after SAH plus HPostC (**A**) and 6 to 48 hours after SAH or sham surgery (**B**) (n=5 per group). *$P<0.05$ vs sham and n.s. $P>0.05$ by 1-way ANOVA with Tukey multiple comparisons test. Data represent the mean with SD. n.s. indicates not significant.

vasospasm (Figure 4A), microvessel thrombosis (Figure 4B), and neurological deficits (Figure 4C) in SIRT1-transgenic mice (*Sirt1-Tg*), compared with wild-type littermate controls, in the absence of HPostC. Notably, the degree of neurovascular protection in

SIRT1-transgenic mice was comparable to that seen in wild-type mice treated with HPostC. We then assessed the ability of the SIRT1 activator resveratrol to pharmacologically mimic the DCI-protective effects of HPostC. Resveratrol, administered 3 hours after
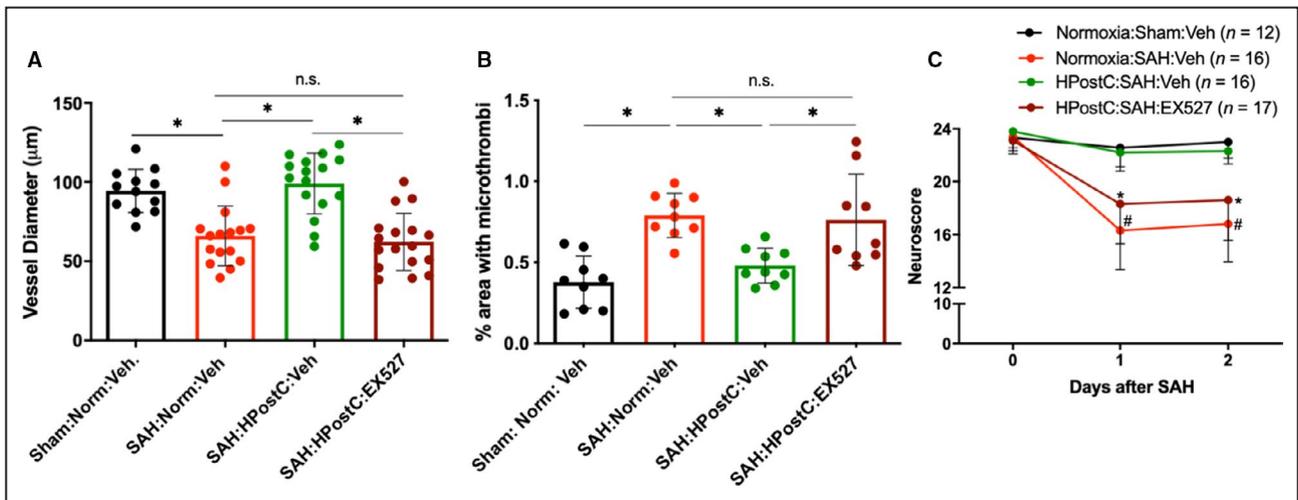

Figure 2. Hypoxic postconditioning (HPostC)-induced delayed cerebral ischemia protection against subarachnoid hemorrhage (SAH) is lost following pharmacological inhibition of SIRT1 (sirtuin 1).
HPostC-induced protection against vasospasm (**A**), microvascular thrombi (**B**), and neurological deficits (**C**) induced by SAH is abrogated by the SIRT1 inhibitor EX527. Mice were subjected to SAH or sham surgery followed 2 hours later by treatment with the SIRT1 inhibitor, EX527 (10 mg/kg, IP) or vehicle (Veh), and then underwent HPostC (8% $O_2 \times 2$ hours, repeated daily) or normoxia (Norm; room air×2 hours, repeated daily) starting at 3 hours after SAH/sham surgery. **A**, Vasospasm in the left middle cerebral artery was assessed on postsurgery day 2 (n=12–17 mice). *$P<0.05$ and n.s. $P>0.05$ by 1-way ANOVA with Tukey multiple comparisons test. **B**, Cortical microthrombosis was quantified as the percent of fibrinogen-positive microvessels in the ipsilateral parietal cortex microcirculation (n=9). *$P<0.05$ and n.s. $P>0.05$ by 1-way ANOVA with Tukey multiple comparisons test. **C**, Neuroscore was assessed on days 0 to 2 (n=12–17 mice). *$P<0.05$ and #$P<0.05$ vs Normoxia:Sham:Veh and HPostC:SAH:Veh. Statistical significance was analyzed by repeated-measures ANOVA followed by Tukey multiple comparisons test. Data represent the mean with SD. Excluded=1 mouse from each SAH:Norm:Veh., SAH:HPostC:Veh., and SAH:HPostC:EX527 groups. n.s. indicates not significant.
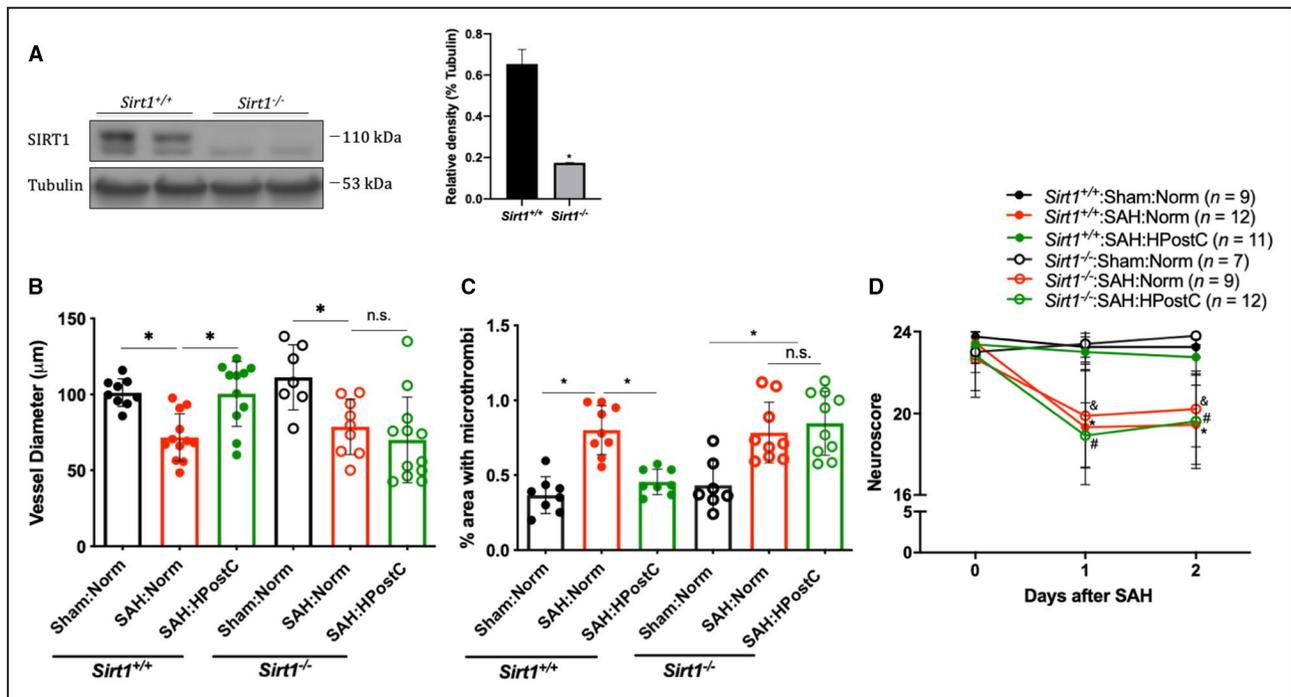
Figure 3. Hypoxic postconditioning (HPostC)-induced protection against subarachnoid hemorrhage (SAH) is lost in inducible SIRT1 (sirtuin 1)-null mice.
HPostC-induced protection against SAH-induced vasospasm (**B**), microvascular thrombi (**C**), and neurological deficits (**D**) seen in wild-type (*Sirt1$^{+/+}$*) mice is lost in tamoxifen-induced SIRT1-null (*Sirt1$^{-/-}$*) littermates. *Sirt1$^{+/+}$* and *Sirt1$^{-/-}$* mice were subjected to SAH or sham surgery, and then underwent HPostC (8% O_2×2 hours, repeated daily) or normoxia (Norm; room air×2 hours, repeated daily) beginning 3 hours after surgery. **A**, Representative immunoblot for SIRT1 in brain cortex of 2 wild-type (*Sirt1$^{+/+}$*) and 2 SIRT1-null (*Sirt1$^{-/-}$*) mice, with β-tubulin as a loading control. **B**, Vasospasm in the left middle cerebral artery was assessed on postsurgery day 2 (n=7–12). *$P<0.05$ and n.s. $P>0.05$ by 1-way ANOVA with Tukey multiple comparisons test. **C**, Cortical microthrombosis, quantified as the percent of fibrinogen-positive microvessels in the ipsilateral parietal cortex microcirculation (n=8–10). *$P<0.05$ and n.s. $P>0.05$ by 1-way ANOVA with Tukey multiple comparisons test. **D**, Neuroscore was assessed on days 0 to 2 (n=7–12). *$P<0.05$ vs *Sirt1$^{+/+}$*:Sham:Norm and *Sirt1$^{+/+}$*:SAH:HPostC. #$P<0.05$ and &$P<0.05$ vs *Sirt1$^{-/-}$*:Sham:Norm. Statistical significance was analyzed by repeated-measures ANOVA followed by Tukey multiple comparisons test. Data represent the mean with SD. Excluded=1 mouse from SAH:Norm: (*Sirt1$^{+/+}$*), SAH:HPostC: (*Sirt1$^{+/+}$*) and (*Sirt1$^{-/-}$*), and 2 mice in SAH: Norm: (*Sirt1$^{-/-}$*) groups. n.s. indicates not significant.

SAH in mice, significantly attenuated large-artery vasospasm (Figure 5A), microvessel thrombosis (Figure 5B), and neurological deficits (Figure 5C) as compared with vehicle-treated control mice. Given that resveratrol not only activates SIRT1 but also has antioxidant and neuroprotective properties,[33] we sought to verify that the resveratrol-induced DCI protection was SIRT1 mediated by cotreating a subgroup of mice with the SIRT1 inhibitor, EX527. We found that the DCI protection afforded by resveratrol was lost in EX527-treated mice (Figure 5A through 5C). Moreover, we demonstrated that resveratrol-induced increases in cerebral SIRT1 activity in mice with SAH were abrogated by EX527 treatment (Figure S3), also supporting our contention that resveratrol-mediated DCI protection was largely the result of increased SIRT1 activity secondary to resveratrol administration. Taken together, these results indicate that resveratrol provides robust SIRT1-mediated protection against DCI and neurological deficits.

DISCUSSION

In the present study, several important insights were advanced: (1) Hypoxic conditioning initiated at a therapeutically relevant time (3 hours) after SAH (HPostC) provides robust protection against large-artery vasospasm and neurological deficits. (2) HPostC not only protects against macrovessel dysfunction, but also protects at the level of the microcirculation by reducing thrombosis. (3) Multifaceted HPostC-induced DCI protection is critically dependent on SIRT1, given that our neurovascular protection metrics were lost in SIRT1-null mice as well as in EX527-treated mice. (4) Genetic overexpression of SIRT1 mimics the DCI protection afforded by HPostC. (5) Administration of the SIRT1 activator, resveratrol, provides strong protection against DCI and improves neurological outcome in an SIRT1-dependent manner. Collectively, our findings demonstrate that conditioning strategies designed to activate powerful endogenous protective mechanisms can
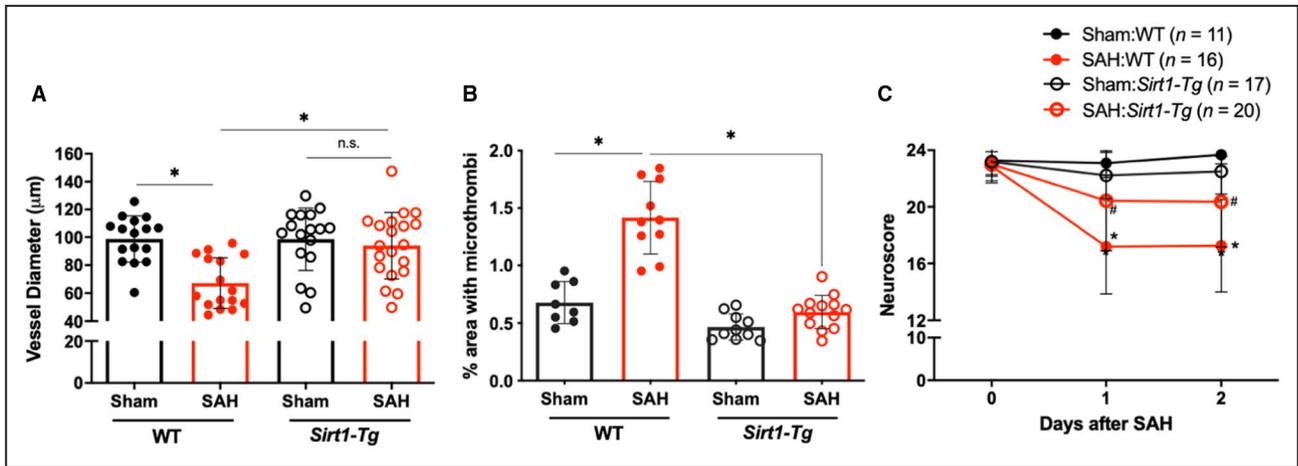
Figure 4. Constitutive overexpression of SIRT1 (sirtuin1) provides strong protection against subarachnoid hemorrhage (SAH).

Extent of SAH-induced vasospasm (**A**), microvascular thrombosis (**B**), and neurological deficits (**C**) are all significantly reduced in constitutive SIRT1-overexpressing (*Sirt1-Tg*) relative to wild-type (WT) littermate controls. WT and *Sirt1-Tg* mice underwent sham or SAH surgery. **A**, Vasospasm in the left middle cerebral artery was assessed on day 2 (n=11–20). *$P<0.05$ and n.s. $P>0.05$ by 1-way ANOVA with Tukey multiple comparisons test. **B**, Cortical microthrombosis was quantified as the percent of fibrinogen-positive microvessels in the ipsilateral parietal cortex microcirculation (*n*=8–13).; *$P<0.05$ by 1-way ANOVA with Tukey multiple comparisons test. **C**, Neuroscore was assessed on days 0 to 2 (n=11–20). *$P<0.05$ vs Sham:WT. #$P<0.05$ vs SAH:WT. Statistical significance was analyzed by repeated-measures ANOVA followed by Tukey multiple comparisons test. Data represent the mean with SD. Excluded=1 mouse from SAH:WT and SAH:*Sirt1-Tg* groups. n.s. indicates not significant.

provide strong neurovascular protection even when initiated after induction of SAH. As such, we contend that postconditioning with SIRT1-promoting therapeutics represents a clinically viable treatment strategy for

reducing secondary brain injury and improving overall outcome in patients presenting with SAH.

In recent years, we and others have documented that a variety of conditioning agents including hypoxia[15,16]
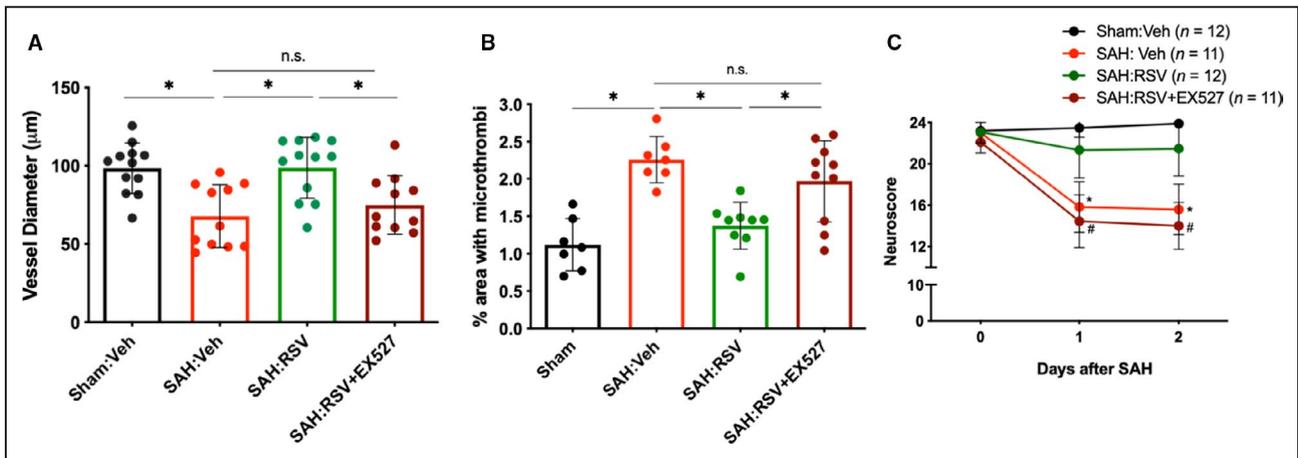

Figure 5. SIRT1 (Sirtuin 1) activation by post-subarachnoid hemorrhage (SAH) resveratrol (RSV) provides strong protection against SAH.

Extent of SAH-induced vasospasm (**A**), microvascular thrombus (**B**), and neurological deficits (**C**) are all significantly reduced by RSV treatment initiated 3 hours after SAH. Administration of the SIRT1 inhibitor EX527 concomitant with RSV abrogates the protective effect of RSV on SAH-induced vasospasm (**A**), microvascular thrombosis (**B**), and neurological deficits (**C**). Wild-type mice underwent sham or SAH surgery and were treated with RSV or RSV+EX527 starting 2 hours after SAH. (**A**) Vasospasm in the left middle cerebral artery was assessed on postsurgery day 2 (n=11–12). *$P<0.05$ and n.s. $P>0.05$ by 1-way ANOVA with Tukey multiple comparisons test. **B**, Cortical microvessel thrombosis was quantified as the percent of fibrinogen-positive microvessels in the ipsilateral parietal cortex microcirculation (*n*=7–10). *$P<0.05$ and n.s. $P>0.05$ by 1-way ANOVA with Tukey multiple comparisons test. **C**, Neuroscore was assessed on days 0 to 2 (n=11–12). *$P<0.05$. #$P<0.05$ vs Sham:Veh and SAH:RSV. Statistical significance was analyzed by repeated-measures ANOVA followed by Tukey multiple comparisons test. Data represent the mean with SD. Excluded=1 mouse from SAH:Veh., SAH:RSV, and SAH:RSV+EX527 groups. n.s. indicates not significant; and Veh, vehicle.

anesthetics[34] and lipopolysaccharide[35] provide powerful protection in the setting of brain ischemia and have also shown evidence that conditioning approaches such as anesthetics and remote limb ischemia may similarly mitigate SAH-induced neurovascular dysfunction in patients.[34] Although the suitability of conditioning-based therapy for SAH had been proposed for years,[9,36] the first application of a conditioning-based strategy to SAH came from our group in 2011,[16] in which we demonstrated that a one-time exposure to hypoxia before the induction of SAH provides robust protection against subsequent large-artery vasospasm and neurological deficits. We also provided mechanistic evidence that this DCI protection involved eNOS-derived NO, a molecule critically involved in endothelial cell and cerebral vessel physiology, strongly implicated in vasospasm pathophysiology and regulated by multiple mechanisms at transcriptional and posttranslational levels.[37] One well-described regulator of eNOS is SIRT1, which is hypoxia responsive and important in regulating endothelium-dependent, eNOS-mediated vascular homeostasis.[38] Given this regulatory connection between eNOS and SIRT1, we recently extended findings in our 2011 study to show that SIRT1 is likely involved in HPreC protection against SAH-induced macro- and microvascular injury.[17] In the present study, we extend these proof-of-principle findings in 3 key ways: (1) We show hypoxic conditioning initiated after SAH (HPostC) produces DCI protection at a magnitude similar to HPreC[17]. (2) We demonstrate that DCI protection occurs not only by attenuating vasospasm in large cerebral arteries, but also reduces the extent of SAH-induced thrombosis in the microcirculation. (3) We provide cross-validating genetic and pharmacologic evidence that SIRT1 contributes significantly to HPostC-induced DCI protection. These findings support postconditioning with SIRT1-activating agents as a novel, clinically viable therapeutic approach to attenuating SAH-induced secondary brain injury.

Importantly, SIRT1 has previously been explored as a therapeutic target for another important, although less common, pathophysiological event following SAH, EBI. This form of secondary brain injury involves a complex set of pathological processes including blood–brain barrier disruption, neuroinflammation, neuronal apoptosis, and cerebral edema.[39] Studies have demonstrated that administering resveratrol soon after[40,41] experimental SAH provides significant protection against a variety of histologic markers of EBI as well as early neurological deficits (24 hours after SAH). More direct evidence supporting the possibility that SIRT1 mediates EBI-protective effects was provided by Zhang and colleagues, showing that a SIRT1 activator (activator 3) reduces and inhibitor (sirtinol) exacerbates

EBI in the rat prechiasmatic cistern model of SAH.[22] Taken together with our pharmacologic and genetic studies reported herein implicating SIRT1 in mediating powerful protection against the more common and more morbid condition of DCI, a promising therapeutic approach for SAH emerges. Moreover, as opposed to the majority of therapeutic approaches designed to target one pathophysiological process in SAH-induced brain injury (eg, large-artery vasospasm), hypoxic conditioning, and more specifically SIRT1-directed therapies, have the benefit of targeting multiple pathophysiological processes implicated in poor outcomes after SAH (eg, EBI and DCI, macro- and microvessel dysfunction, and neuronal cell death).

There are several limitations to our study. First, although we provide evidence indicating hypoxia and resveratrol increase SIRT1 activity in a temporally relevant fashion, the mechanisms by which this occurs were not explored. Second, although we rigorously examined 2 key components of SAH-induced DCI (ie, large-artery vasospasm and microvessel thrombosis), other contributors were not assessed including autoregulatory dysfunction and cortical spreading depression. Third, we did not perform long-term assessment of SAH-induced neurobehavioral deficits, which will be important to pursue given the morbidity associated with SAH-induced cognitive dysfunction in patients. Fourth, we were unable to test SIRT1 activators with higher potency and specificity given that these agents are largely proprietary and unavailable. Fifth, we recognize that it is possible that the protective effect of SIRT1 activation on DCI may have resulted, at least in part, from a beneficial impact on EBI, because some have suggested a causal connection between onset of EBI and subsequent development of DCI.[22,42,43]

In summary, results from the present study indicate that HPostC elicits a powerful protective response against DCI, that this protection is multifaceted, acting at both macro- and microvascular levels, and that SIRT1 is a critical downstream inducer of these DCI-protective actions. Thus, SIRT1-directed pharmacologic strategies implemented after SAH carry great promise for combating SAH-induced DCI and improving patient outcomes. Additional studies are warranted to further elucidate the molecular cascade leading to HPostC-induced DCI protection, to determine whether HPostC also provides significant protection against EBI and if so whether this is also SIRT1 mediated, and to explore the therapeutic potential of novel approaches for augmenting and/or activating SIRT1 in patients presenting with SAH.

ARTICLE INFORMATION

Supplementary Material

Figures S1–S3

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SUPPLEMENTAL MATERIAL

Figure S1. Experimental protocol.
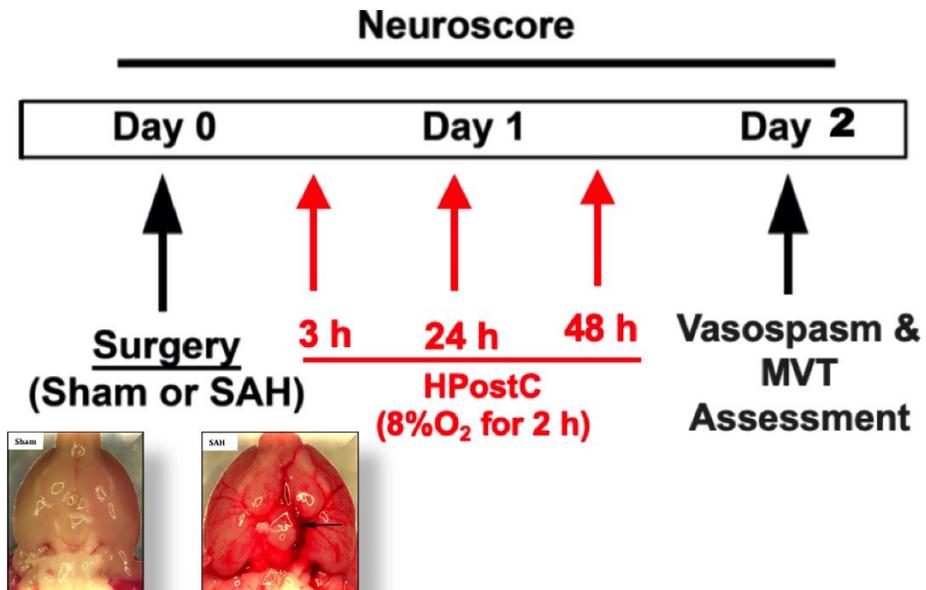
Red and black arrows indicate time points for surgical procedures (sham or SAH surgery), hypoxic postconditioning (HPostC) treatments, and assessment of vasospasm, microvessel thrombi (MVT), and neurological deficit testing (Neuroscore).

Figure S2. Hypoxic Postconditioning (HPostC) protects against SAH-induced vasospasm, neurological deficits, and microvascular thrombosis.
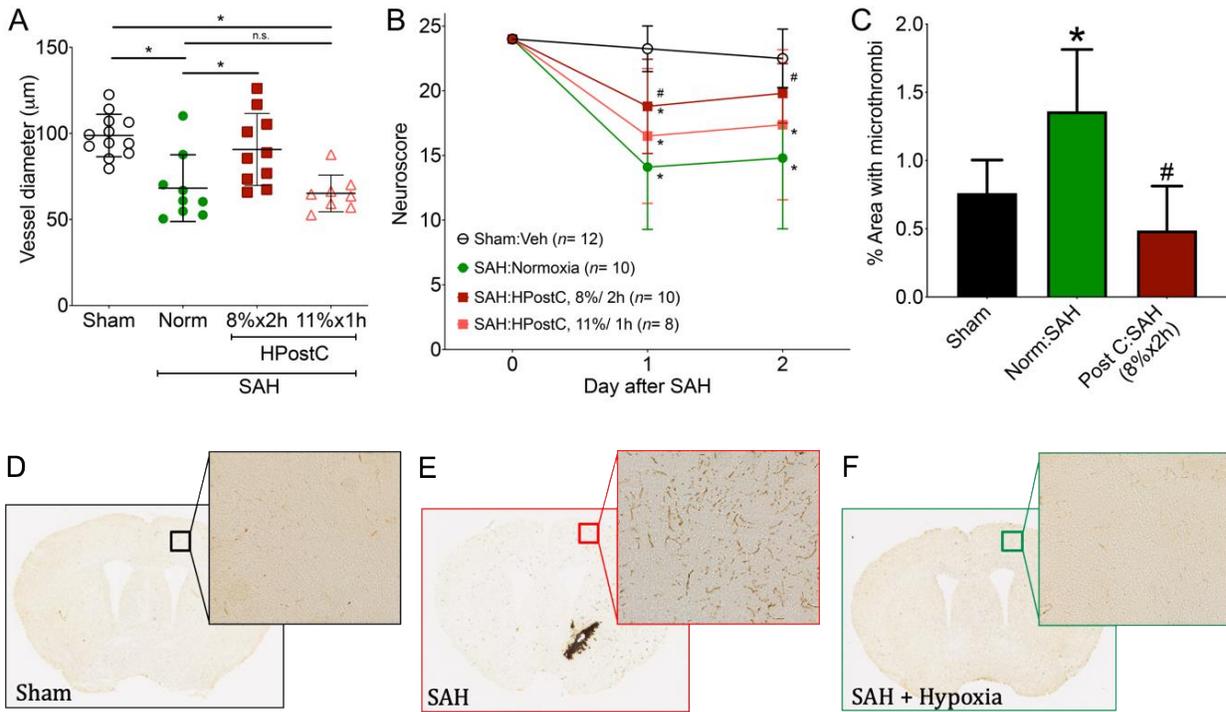
Mice underwent SAH or sham surgery followed 3 h later by HPostC (8% O_2 for 2h, or 11% O_2 for 1h) or Normoxia (Norm; room air x 2 h, repeated daily). HPostC was then repeated daily until animal sacrifice. (A) Vasospasm was assessed in the left middle cerebral artery (MCA) on Day 3; *$p<0.05$, n.s. $p>0.05$ by one-way ANOVA with Tukey multiple comparisons test. (B) Neuroscore was assessed daily until animal sacrifice; *$p<0.05$ vs. Sham:Norm; #$p<0.05$ vs. SAH:Normoxia by repeated-measures ANOVA followed by Tukey's multiple comparisons test. (C) Cortical microthrombosis was determined as percent coverage of ipsilateral parietal cortex; *$p<0.05$ by one-way ANOVA with Tukey multiple comparisons test. Data represent the mean with SD. n.s. indicates not significant. Representative images of mice that underwent (D) Sham and (E) SAH surgery without HPostC and (F) with HPostC demonstrates a marked reduction in microthrombosis.

Figure S3. Effects of treatment with either resveratrol (RSV) or resveratrol + EX527 on Sirtuin 1 (SIRT1) enzymatic activity.
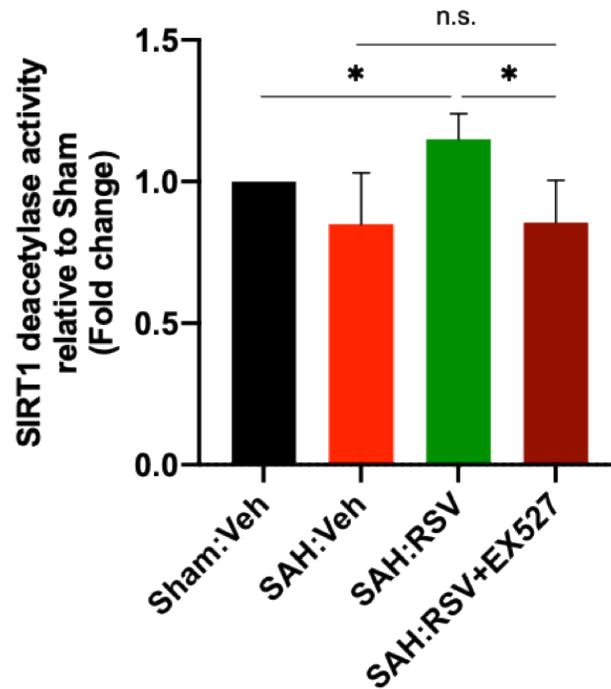
Wild-type mice underwent sham or SAH surgery and were treated with resveratrol or resveratrol plus the SIRT1 inhibitor EX527 starting 2 h after SAH and continued daily until animal sacrifice. SIRT1 deacetylase activity in brain lysates was quantified 48 h after surgery. (n=5-7) *p<0.05 vs Sham and n.s. *p*>0.05 by one-way ANOVA with Tukey multiple comparisons test. Data represents the mean with SD. n.s. indicates not significant.