7-18-2023

Isoflurane conditioning-induced delayed cerebral ischemia protection in subarachnoid hemorrhage-Role of inducible nitric oxide synthase

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Journal of the American Heart Association

ORIGINAL RESEARCH

Isoflurane Conditioning-Induced Delayed Cerebral Ischemia Protection in Subarachnoid Hemorrhage—Role of Inducible Nitric Oxide Synthase

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BACKGROUND: Recent evidence implicates inflammation as a key driver in delayed cerebral ischemia after aneurysmal subarachnoid hemorrhage (SAH). Inducible nitric oxide synthase (iNOS) is one of the known major mediators of inflammation. We previously showed that an inhalational anesthetic, isoflurane, provides strong protection against delayed cerebral ischemia after SAH. Our current study aims to define the role of iNOS in isoflurane conditioning-induced protection against delayed cerebral ischemia in a mouse model of SAH.

METHODS AND RESULTS: The experiments used 10- to 14-week-old male wild-type (C57BL/6) and iNOS global knockout mice. Anesthetic conditioning was initiated 1 hour after SAH with isoflurane 2% for 1 hour. Isoflurane-induced changes in iNOS expression were measured. N-(3-(aminomethyl) benzyl) acetamidine, a highly selective iNOS inhibitor, was injected intraperitoneally immediately after SAH and then daily. Vasospasm, microvessel thrombosis, and neurological assessment was performed. Data were analyzed by 1-way ANOVA and 2-way repeated measures ANOVA followed by Student Newman Keuls comparison test. Statistical significance was set at $P<0.05$. Isoflurane conditioning downregulated iNOS expression in naïve and SAH mice. N-(3-(aminomethyl) benzyl) acetamidine attenuated large artery vasospasm and microvessel thrombosis and improved neurological deficits in wild-type animals. iNOS knockout mice were significantly resistant to vasospasm, microvessel thrombosis, and neurological deficits induced by SAH. Combining isoflurane with N-(3-(aminomethyl) benzyl) acetamidine did not offer extra protection, nor did treating iNOS knockout mice with isoflurane.

CONCLUSIONS: Isoflurane conditioning-induced delayed cerebral ischemia protection appears to be mediated by downregulating iNOS. iNOS is a potential therapeutic target to improve outcomes after SAH.

Key Words: DCI ■ iNOS ■ isoflurane ■ neurovascular protection ■ subarachnoid hemorrhage

Delayed cerebral ischemia (DCI), a form of secondary brain injury after aneurysmal subarachnoid hemorrhage (SAH), is a critical contributor for poor outcomes in patients with SAH.[1] DCI occurs in 30% of patients, 4 to 12 days after ictus, and it is characterized by large artery vasospasm, microvessel thrombosis, autoregulatory dysfunction, and cortical spreading depolarizations.[2] Many strategies to prevent DCI in patients with SAH have failed so far, probably due to targeting a single element of what is known to be a multifactorial process. We previously demonstrated both in mouse SAH and in patients with SAH that volatile anesthetics can significantly mitigate DCI leading to improved neurologic outcomes.[3–10] Elucidating the

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This article was sent to Neel S. Singhal, MD, PhD, Associate Editor, for review by expert referees, editorial decision, and final disposition.

For Sources of Funding and Disclosures, see page 8.

JAHA is available at: www.ahajournals.org/journal/jaha

RESEARCH PERSPECTIVE

What Is New?

- We examined the role of isoflurane conditioning and inducible nitric oxide synthase, a major mediator of inflammation in delayed cerebral ischemia after aneurysmal subarachnoid hemorrhage.
- Our results show that isoflurane conditioning provides robust protection against subarachnoid hemorrhage-induced delayed cerebral ischemia by downregulating inducible nitric oxide synthase.

What Question Should Be Addressed Next?

- In this study, we showed that either isoflurane conditioning or inhibition of inducible nitric oxide synthase (pharmacologic or genetic deletion) provides powerful protection against subarachnoid hemorrhage-induced delayed cerebral ischemia and short-term neurologic deficits.
- In future studies, we will investigate whether isoflurane conditioning and inducible nitric oxide synthase inhibition provides sustained protection against long-term neurobehavioral deficits after subarachnoid hemorrhage.

Nonstandard Abbreviations and Acronyms

DCI	delayed cerebral ischemia
eNOS	endothelial nitric oxide synthase
iNOS	inducible nitric oxide synthase
MCA	middle cerebral artery
1400W	N-(3-(aminomethyl) benzyl) acetamidine

molecular underpinnings of anesthetic conditioning-based neurovascular protection is an essential next step in translating these results into practical therapies to treat patients with SAH and improve outcomes.

Recent evidence suggests that neuroinflammation is closely associated with DCI after SAH.[11] Inducible nitric oxide synthase (iNOS) has been implicated in the pathogenesis of inflammation.[12] Interestingly, iNOS is shown to be activated after SAH in animal models and in the brain tissues of patients with SAH.[13–16] In addition, these studies[14,16] show that pharmacologic inhibition of iNOS via aminoguanidine, a relatively selective iNOS inhibitor, attenuates vasospasm in a rat endovascular perforation and rabbit cisterna magna injection SAH models. However, the impact of iNOS on other critical components of DCI such as microvessel thrombi, autoregulatory dysfunction, and neurological outcome is not known. Also, additional studies are needed to definitely implicate iNOS inhibition in the DCI protection afforded by isoflurane conditioning. So, the aim of our current study is to examine the role of iNOS in isoflurane conditioning-induced DCI protection after SAH. Our hypothesis is that isoflurane conditioning provides strong protection against SAH-induced DCI, through downregulating iNOS.

METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request. Washington University in Saint Louis animal care and use committee approved all the experiments conducted in this study. For the experiments 10- to 14-week-old C57BL/6J wild-type and iNOS knockout male mice (B6.129P2-Nos2tm1Lau/J, strain #:002609, C57BL/6J genetic background) were obtained from Jackson Laboratories (Bar Harbor, ME). Mice were acclimatized in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International under a 12-hour light–dark cycle with free access to food and water. All the experiments were conducted in a blinded and randomized manner. For the first experiment assessing DCI and neurobehavioral assessment in wild-type mice, mice were divided into the following groups: sham (n=18), SAH (n=15), SAH+N-(3-(aminomethyl) benzyl) acetamidine (1400W) (n=16), SAH+isoflurane conditioning (n=9), and SAH+ isoflurane conditioning+1400W (n=17). For iNOS knockout experiments, mice were divided into sham (n=14), SAH (n=19), and SAH+ isoflurane conditioning (n=20). The sample size was based on our previous studies.[3,6] A separate cohort of wild-type mice was used for Western blot experiments (n=22). A total of 162 mice were used for the experiments. Out of 162, 102 wild-type and 60 iNOS knockout mice were used. A total of 7 mice died in the SAH group, and middle cerebral artery vessel (MCA) measurement was not possible in 5 mice. These mice were excluded from the analysis and rest of the animals in sham and SAH group were included in the analysis. The overall experimental design of the study is represented in Figure 1. We used Animal Research: Reporting of In Vivo Experiments guidelines for this study.[17]

Subarachnoid Hemorrhage Mouse Model

SAH was induced through endovascular perforation as per our published methods.[3–6] Briefly, mice were anesthetized with isoflurane (4% induction, 1.5% maintenance) in room air and a midline incision was made in the neck to expose the external carotid artery. Then, a 5–0 nylon suture was introduced through it and advanced distally through the internal carotid

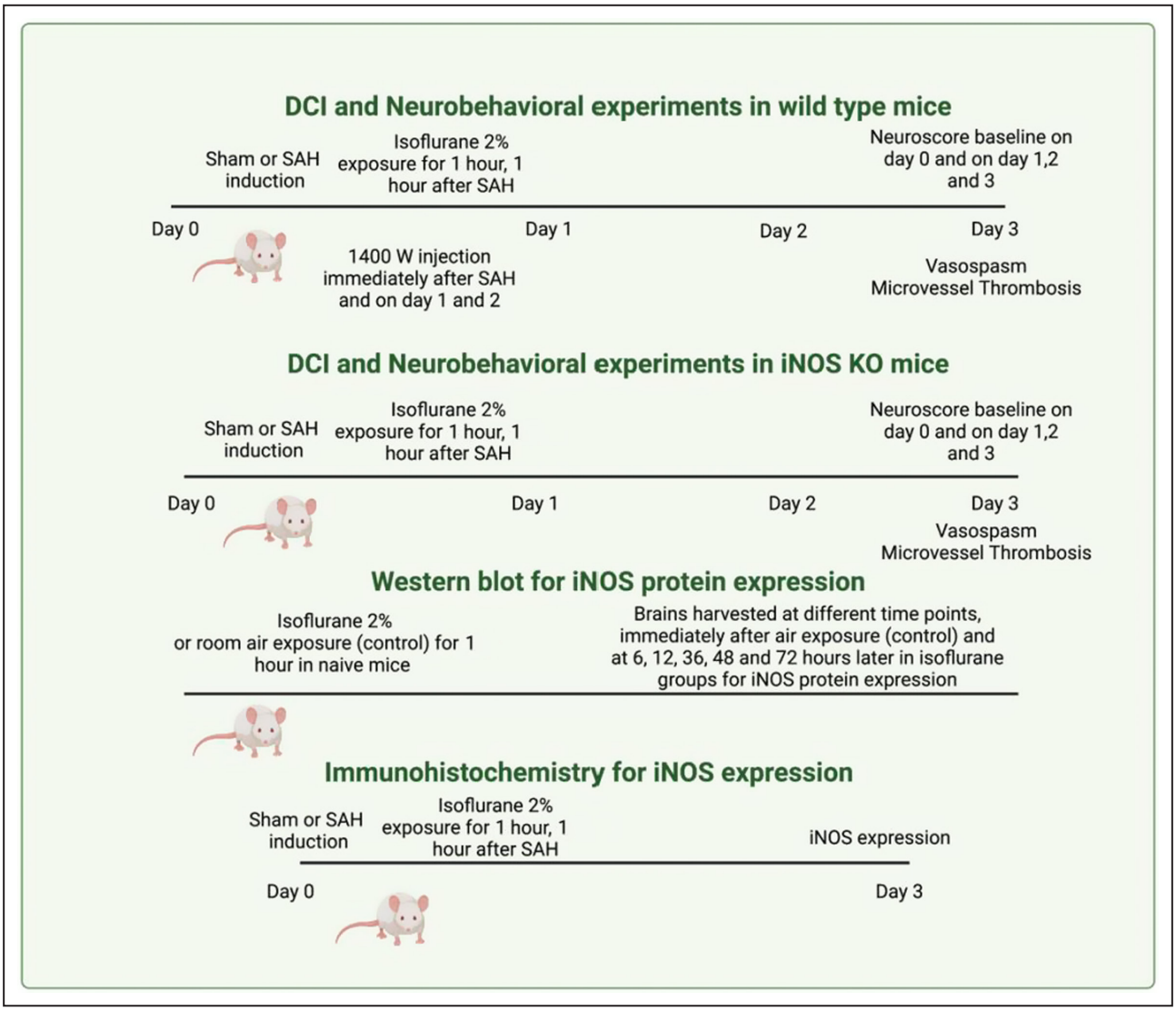

Figure 1. Experimental design of the study.
DCI indicates delayed cerebral ischemia; iNOS, inducible nitric oxide synthase; SAH, subarachnoid hemorrhage; and 1400W, N-(3-(aminomethyl)benzyl) acetamidine.

artery until the bifurcation is reached. The suture was advanced further to induce SAH, followed by external carotid artery ligation. Sham mice underwent the same operation except for perforation. Normothermia at 37 °C (±0.2 °C) was maintained throughout the procedure by a thermoregulated heating pad (mTCII micro-Temperature Controller by Cell MicroControls, Norfolk, VA).

Anesthetic Conditioning

Anesthetic conditioning was established by exposing the mice to 2% isoflurane for 1 hour. This dose was chosen based on our previous experiments.[3–5] To examine the impact of isoflurane on DCI and neurologic outcomes, a single time 2% isoflurane was exposed to mice for 1 hour, beginning 1 hour after SAH surgery. A gas analyzer (Datex Ohmeda, Capnomac Ultima, Louisville, KY) was used to measure isoflurane concentration in the mice chamber. Normal temperature was maintained during the anesthetic exposure using a heat therapy pump connected to a warming blanket (HTP-1500 heat therapy pump, Kent Scientific Corporation, Torrington, CT).

1400W Treatment

N-(3-(aminomethyl) benzyl) acetamidine (1400W) (10 mg/kg) was diluted with saline and injected intraperitoneally in mice. A total of 3 doses were administered, 1 dose immediately after SAH and 2 doses were injected on day 1 and 2 after SAH. 1400W was diluted fresh every day for all injections. Other groups received same volume of saline.

Neurobehavioral Assessment

Neurological function was examined as explained in the literature.[3–6] Briefly, neuroscore was done at baseline before SAH and repeated for next 3 days until the animals were euthanized. Briefly, neurological function was graded based on a motor score (0 to 12) that evaluates spontaneous activity, symmetry of limb movements, climbing, balance, and coordination and a sensory score (4 to 12) that evaluates body proprioception and vibrissae, visual, and tactile responses.

Vasospasm Assessment

Cerebral vasospasm was assessed in the MCA vessel as previously described.[3–6] Briefly, a pressure-controlled cerebrovascular casting of mice was performed with ROX SE, 72 hours after sham or SAH surgery. The brains were extracted and stored in 4% paraformaldehyde at 4 °C for 48 hours and then transferred into 30% sucrose solution. Blood vessels in the circle of Willis were imaged under a fluorescent microscope using a charged-coupled device camera (CoolSNAP EZ, Photometrics, Tucson, AZ) and MetaMorph software (Universal Imaging, West Chester, PA). Vasospasm measurement for each brain sample was obtained by recording the narrowest diameter within the first-1000 μm segment of the left (ipsilateral) MCA.

Microvessel Thrombosis Assessment

Ipsilateral (left) cortical microvessel thrombosis was measured for fibrinogen on day 3 after SAH by immunofluorescence staining.[5,6] Briefly, free floating, fixed brain sections with 50-μm thickness were incubated with rabbit antifibrinogen antibody (1:3000, Abcam, Cambridge, MA) overnight at 4 °C, followed by incubation with donkey antirabbit secondary antibody (1:2000, Invitrogen Waltham, MA), overnight at 4 °C. Sections were then analyzed for fibrinogen using NanoZoomer 2.0-HT digital slide scanner (Hamamatsu Corporation, Bridgewater, NJ), followed by estimation of microvessel thrombosis density by ImageJ.

Western Blot

A separate group of mice were exposed to either air (control) or 2% isoflurane for 1 hour and were subjected to Western blot experiment. The cortical tissue was collected at different time points, immediately after the exposure in the control group and at 6, 12, 36, 48 and 72 hours after isoflurane exposure. According to our previously published reports,[3] extracted and separated protein from brain tissue was transferred to polyvinylidene difluoride membrane. The polyvinylidene difluoride membrane containing protein was blocked by 5% milk in 1× Tris-buffered saline, 0.1% Tween detergent for 1 hour at room temperature and was incubated overnight at 4 °C with rabbit anti-iNOS primary antibody (1:1000, Abcam, #3523) and rabbit anti-B actin primary antibody (1:1000, Cell Signaling Tech, #8457). Next, the blot was incubated with anti-rabbit horseradish peroxidase-conjugated secondary antibody (1:1000, Cell Signaling Tech, #7074) at room temperature for 1 hour followed by the visualization of the protein bands using an enhanced chemiluminescence kit (BioRad). It was then quantified by ImageJ.

Immunostaining

On day 3 after SAH, mice were perfused and the brains were stored in 4% paraformaldehyde. Fixed brains were sectioned into 50-μm coronal sections and were incubated with a blocking buffer (0.4% Triton X-100 TBS-T with 5% donkey serum). Next, the sections were incubated with rabbit anti-iNOS antibody (1:100, Abcam, #3523) in 0.1% Triton X-100 TBS-T overnight at 4 °C, followed by incubation with donkey anti-rabbit fluor antibody (1:1000, Thermofisher, #A-21208) in TBS-T for 45 minutes. After rinse, the brain sections were mounted on the slide. The images were taken by Nikon confocal microscopy and the signal of iNOS was analyzed by ImageJ.

Statistical Analysis

GraphPad prism (version 9.0.1) was used for the statistical analysis. Data are presented as mean±SEM. Shapiro–Wilk test was used to examine the normality of the data. Cerebral vasospasm, microvessel thrombosis, Western blot, and immunostaining data were analyzed using 1-way ANOVA followed by Student Newman Keuls multiple comparison test. Neuroscore was evaluated using 2-way repeated measures ANOVA followed by Student Newman Keuls multiple comparison test. Statistical significance was set at $P<0.05$.

RESULTS

Isoflurane Conditioning Reduces iNOS Expression in Naïve and SAH Animals

A separate cohort of naïve mice were exposed to either air for 1 hour (control) or isoflurane 2% for 1 hour. Cortical tissue from naïve mice were harvested at different time points, immediately after 1-hour air exposure in control group and at 6, 12, 36, 48 and 72 hours after isoflurane exposure, which then was subjected to Western blot. In addition, iNOS expression was examined after sham or SAH surgery on day 3 via immunofluorescence staining. Isoflurane conditioning significantly attenuated iNOS expression both in naïve and SAH mice ($P<0.05$; Figure 2A through 2D).

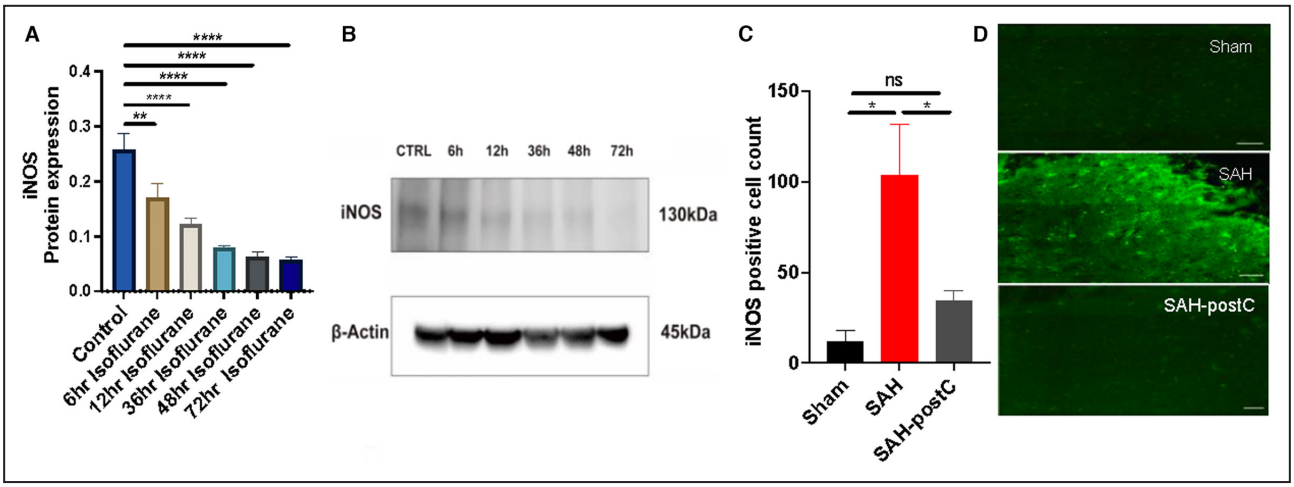

Figure 2. Isoflurane conditioning reduces iNOS expression in naïve and SAH mice.
A and **B**, Wild-type male mice were exposed to air (control) or 2% isoflurane for 1 hour, and cortical tissue harvested at 6, 12, 36, 48, and 72 hours after isoflurane exposure in naïve animals was subjected to Western blot. Data indicate mean±SEM. **A**, *P<0.05 vs control (n=3–4), by ANOVA followed by Newman–Keuls multiple comparisons test. **B**, Representative Western blot images. Beta-actin was used as protein loading control. **C** and **D**, Wild-type male mice underwent SAH or sham surgery followed 1 hour later by exposure to 2% isoflurane (postC) or room air for 1 hour. iNOS expression assessment was performed by immunofluorescence staining in brain around the vascular perforation area. Data represent mean±SEM. **C**, *P<0.05 sham vs SAH; *P<0.05 SAH vs SAH-postC by ANOVA with Newman–Keuls multiple comparisons test (n=3). **D,** Representative immunofluorescence staining images for iNOS expression. Scale bar=50 μm. CTRL indicates control; iNOS, inducible nitric oxide synthase; postC, isoflurane post conditioning; and SAH, subarachnoid hemorrhage.

Isoflurane Conditioning or 1400W Separately Provides DCI Protection After SAH, and Isoflurane Coadministration With 1400W Did Not Afford Additional Protection

Four out of 80 wild-type mice died in the SAH group, and MCA was not visible in 1 mouse. Significant MCA vasospasm, microvessel thrombosis, and neurological deficits were noted in the SAH group (MCA diameter, 76.66±4.32) compared with the sham group (106.50±7.43) (P<0.05; Figure 3A through 3E). Isoflurane conditioning (103.95±7.01) and the highly selective iNOS inhibitor, 1400W (101.51±6.05), independently provided significant protection against SAH-induced vasospasm, microvessel thrombosis, and neurological deficits (P<0.05; Figure 3A through 3E). Combining isoflurane with 1400W (106.97±4.24) did not offer extra protection compared with the isoflurane conditioning or the 1400W group alone (P>0.05; Figure 3A through 3E).

iNOS Deletion Affords DCI Protection After SAH, and Isoflurane Coadministration Did Not Afford Additional Protection

Three out of 60 iNOS knockout mice died in the SAH group, and MCA was not visible in 4 mice. iNOS knockout mice subjected to SAH (MCA diameter, 98.63±5.93) did not have a noticeable vasospasm, microvessel thrombosis, or the neurologic deficits compared with the sham group (104.14±6.60) (P>0.05; Figure 4A through 4E). Also, isoflurane conditioning in iNOS knockout mice (92.17±5.84) did not appear to provide any additional protection related to vasospasm, microvessel thrombosis, or the neurologic deficits compared with the sham or SAH groups (P>0.05; Figure 4A through 4E).

DISCUSSION

The main findings of our study are (1) iNOS expression after SAH is downregulated by isoflurane conditioning; (2) isoflurane conditioning provides strong protection against DCI after SAH, which is consistent with our previous results[3–6]; (3) pharmacological inhibition of iNOS via 1400W or genetic abrogation of iNOS provides robust protection against SAH-induced DCI; and (4) combining isoflurane conditioning with either 1400W or iNOS knockout mice did not offer additional protection. These findings indicate that isoflurane conditioning-induced DCI protection after SAH is, at least in part, mediated via downregulating iNOS.

Nitric oxide an endothelium derived relaxing factor has been shown to play a critical role in the pathophysiology of SAH.[18] Nitric oxide is produced by three isoforms of NOS, (1) NOS-1 (neuronal NOS), (2) NOS-2 (iNOS), and (3) NOS-3 (endothelial NOS, or

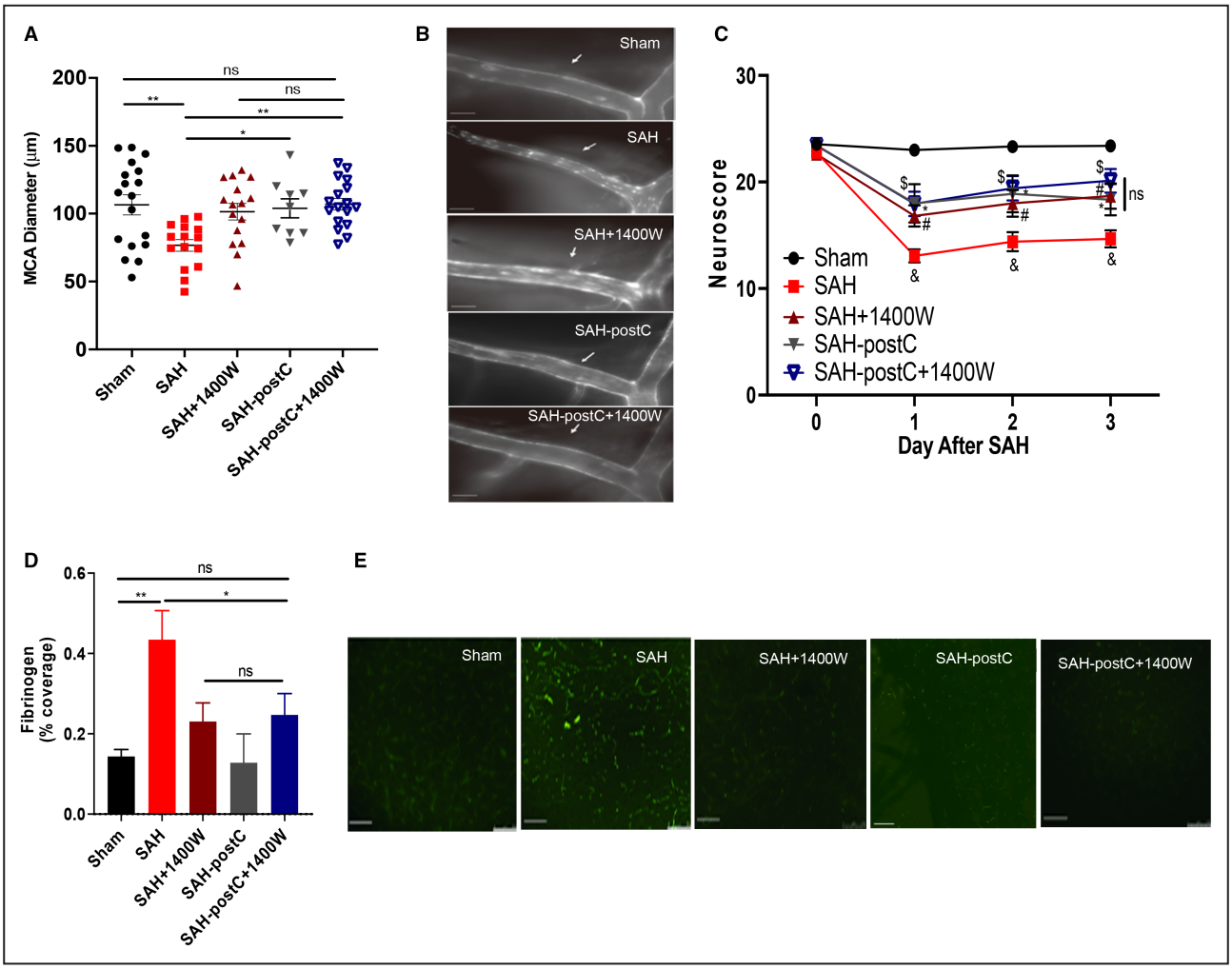

Figure 3. Isoflurane conditioning and 1400W provides DCI and neurological protection after SAH.
Wild-type male mice were subjected to sham surgery or endovascular perforation SAH and treated with vehicle (saline) or 3 doses of 1400W, 10 mg/kg with one 1400W group exposed to 2% isoflurane for 1 hour, 1 hour after SAH. A separate group of mice were exposed to isoflurane 2% alone for 1 hour, beginning 1 hour after SAH. **A**, Vasospasm was assessed on day 3. Data represent mean±SEM. *P<0.05 sham vs SAH, SAH vs SAH+1400W, SAH-postC, and SAH-postC+1400W, (ns) P>0.05 SAH+1400W vs SAH-postC vs SAH-postC+1400W, by 1-way ANOVA Newman–Keuls multiple comparisons test. **B,** Representative images for vasospasm. Arrow mark points to ipsilateral left middle cerebral artery. **C**, Neuroscore was assessed baseline and daily for 3 days. Data represent mean±SEM. &P<0.05 sham vs SAH, *#$ P<0.05 SAH vs SAH+1400W, SAH-postC, and SAH-postC+1400W, (ns)ns P>0.05 SAH+1400W vs SAH-postC vs SAH-postC+1400W, by 2-way repeated measures ANOVA with Newman–Keuls multiple comparisons test. **D**, Microvessel thrombosis was assessed on day 3 (n=3–5). Data represent mean±SEM. *P<0.05 sham vs SAH, SAH vs SAH+1400W, SAH-postC, and SAH-postC+1400W, (ns) ns P>0.05 SAH+1400W vs SAH-postC vs SAH-postC+1400W, by one-way ANOVA Newman–Keuls multiple comparisons test. **E**, Representative images for microvessel thrombosis. Scale corresponds to 100 μm. MCA indicates middle cerebral artery; ns, nonsignificant; postC, isoflurane post conditioning; SAH, subarachnoid hemorrhage; and 1400W, N-(3-(aminomethyl)benzyl) acetamidine.

eNOS). eNOS is a constitutive isoform and its activity is controlled by intracellular calcium. On the other hand, iNOS is an inducible isoform and its activity is controlled only by the degradation of the enzyme itself. Although eNOS is primarily expressed on vascular endothelial cells regulating vascular tone and blood flow, iNOS is mainly expressed in peripheral and central immune cells, playing a significant role in inflammation.[12] We previously showed that isoflurane conditioning upregulated eNOS protein expression after SAH and through pharmacologic and genetic interventions, we causally related eNOS as one of the critical mediators of isoflurane conditioning-induced DCI protection.[3] However, the role of iNOS and isoflurane conditioning in SAH-induced DCI has not yet been explored.

Emerging evidence points to the major role of neuroinflammation in DCI, and neurologic outcomes in patients with SAH.[4] iNOS, one of the isoforms of NOS, when excessively stimulated, generates a disproportionate quantity of nitric oxide, explaining its critical role in neuroinflammation and as a mediator of cytotoxicity. Multiple studies have indicated a key role

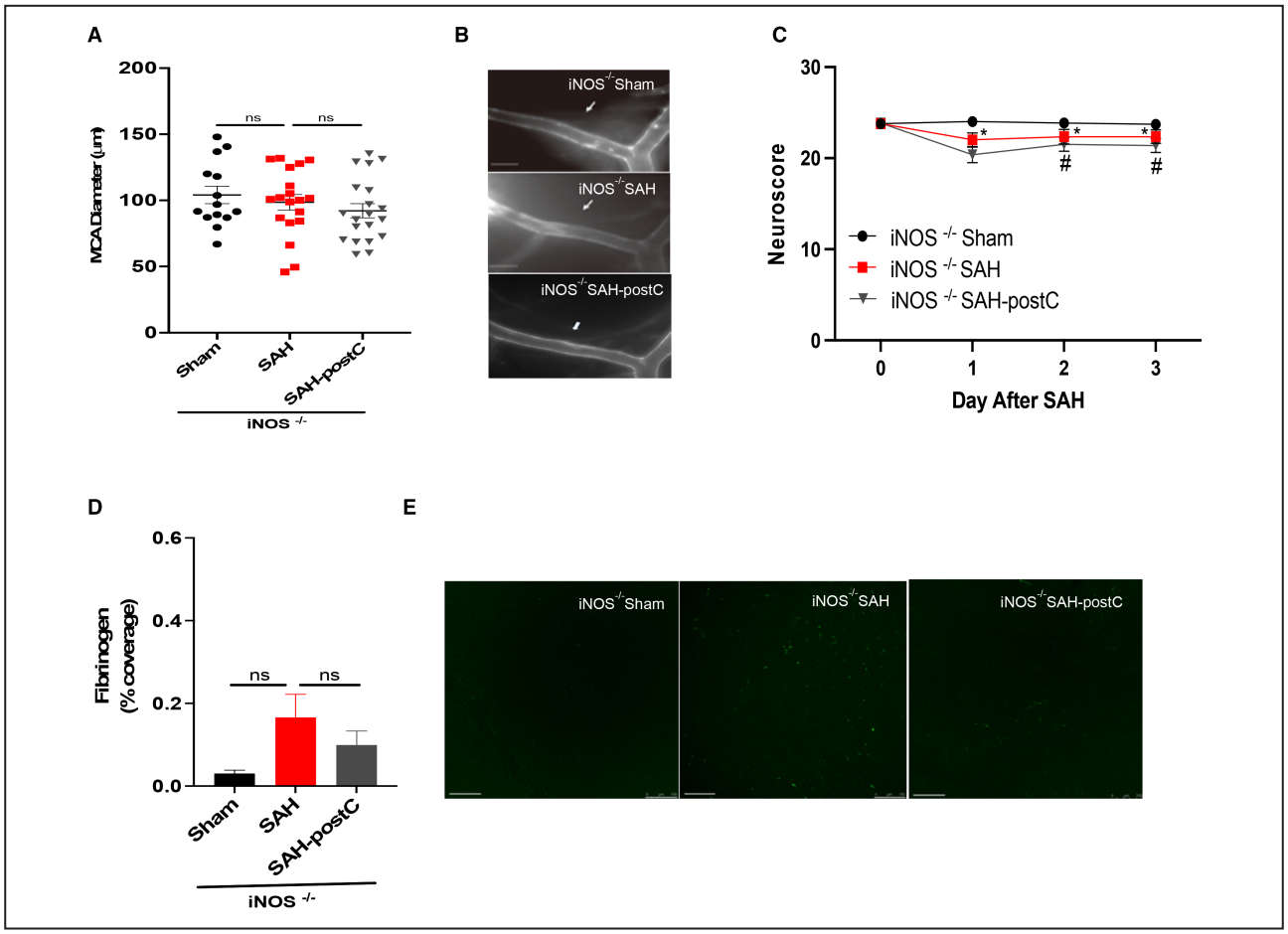

Figure 4. iNOS deletion provides DCI and neurological protection after SAH.
iNOS global knockout male mice were subjected to sham surgery or endovascular perforation SAH followed by exposure to 2% isoflurane for 1 hour, 1 hour after SAH, or room air for 1 hour. **A**, Vasospasm was assessed on day 3. Data represent mean±SEM. *P*>0.05, sham vs SAH vs SAH-postC, by 1-way ANOVA with Newman–Keuls multiple comparison test. **B**, Representative images for vasospasm. Arrow mark points to ipsilateral left middle cerebral artery. **C**, Neuroscore was assessed baseline and daily for 3 days. Data represent mean±SEM. *#*P*>0.05, sham vs SAH vs SAH-postC, by 2-way repeated measures ANOVA with Newman–Keuls multiple comparison test. **D**, Microvessel thrombosis was assessed on day 3 (n=4–5). Data represent mean±SEM. *P*>0.05, sham vs SAH vs SAH-postC, by one-way ANOVA with Newman–Keuls multiple comparison test. **E**, Representative images for microvessel thrombosis. Scale corresponds to 100 μm. DCI indicates delayed cerebral ischemia; iNOS, inducible nitric oxide synthase; ns, nonsignificant; postC, isoflurane post conditioning; SAH, subarachnoid hemorrhage.

of iNOS in initiating or exacerbating several neurodegenerative or inflammatory conditions, including ischemic stroke.[12,19] Intriguingly, iNOS expression has been shown to be upregulated after SAH, and early studies have shown that a relatively selective iNOS inhibitor, aminoguanidine, reduces SAH-induced large artery vasospasm.[15,16] Literature related to iNOS and SAH-induced DCI is otherwise limited, and studies exploring the role of iNOS against other components of DCI (eg, microvessel thrombosis, autoregulatory dysfunction) and neurological deficits have not been reported yet.

In consistent with the previous reports showing that volatile anesthetics, specifically isoflurane strongly reduces iNOS expression after an inflammatory stimulus,[20,21] our data show that isoflurane conditioning significantly downregulated iNOS expression in the naïve and SAH mice. Next, we examined the impact of a highly selective iNOS inhibitor 1400W[22] and isoflurane conditioning on DCI after SAH. In addition to replicating our previous results showing that isoflurane conditioning provided robust protection against DCI, we also show that pharmacological inhibition of iNOS via 1400W provides protection against multiple components of DCI leading to improved neurological outcomes after SAH. Interestingly, coadministration of isoflurane with 1400W did not offer any additional protection, compared with either the isoflurane or the 1400W group alone. Subsequently, we used a global genetic iNOS knockout mice to examine its impact on the DCI after SAH. We noticed that the SAH-induced iNOS knockout mice were refractory to vasospasm and microvessel thrombosis and also showed very

minimal neurologic deficits compared with the sham iNOS knockout mice. Adding isoflurane conditioning to the SAH-induced iNOS knockout mice did not offer any additional protection, implying that isoflurane conditioning-induced DCI protection is possibly mediated through iNOS downregulation.

LIMITATIONS

Our study has several limitations. The current study used a more common mouse SAH model, the endovascular perforation model. Though this model more closely recapitulates the features of human SAH, it is necessary to validate our findings in other SAH animal models. Our study used only male mice for the experiments. Given the fact that SAH occurs more frequently in women compared with men, future studies should include female animals to address sex as a relevant biological variable. Long-term neurological deficits induced by SAH were not examined in the current study. Future studies should be designed to elucidate the effects of isoflurane conditioning and iNOS inhibition on long-term neurobehavioral deficits after SAH.

CONCLUSIONS

Our study is important for several reasons: (1) using pharmacologic (1400W) and genetic (iNOS knockout mice) means to inhibit iNOS, we cross-validated 2 previous studies that suggested iNOS inhibition (aminoguanidine) attenuates vasospasm after SAH[14,16]; (2) we extended these findings by showing that iNOS inhibition protects not only against large artery vasospasm but also microvessel dysfunction and that these vascular benefits lead to improved neurological outcome after SAH; and (3) we provide compelling evidence that isoflurane conditioning-induced DCI protection is mediated via downregulation of iNOS. Based on these results, we conclude that isoflurane conditioning has the potential to improve SAH outcome, that this protection is likely iNOS-mediated, and that iNOS has promise as a therapeutic target to prevent/attenuate DCI after SAH.

ARTICLE INFORMATION

Received February 23, 2023; accepted June 20, 2023.

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Acknowledgments

We thank Ernie Gonzales for performing the SAH surgeries. Figure 1 was created with BioRender.com.

Sources of Funding

This work was supported by a K08 grant (K08NS125038) awarded to Dr Athiraman, Brain Aneurysm Foundation grant (GR0026849) awarded to Dr Athiraman, and a R01 grant (NS091603) awarded to Dr Zipfel.

Disclosures

None.

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