Endothelial Nitric Oxide Synthase Mediates Endogenous Protection Against Subarachnoid Hemorrhage-Induced Cerebral Vasospasm

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Background and Purpose—Vasospasm-induced delayed cerebral ischemia remains a major source of morbidity in patients with aneurysmal subarachnoid hemorrhage (SAH). We hypothesized that activating innate neurovascular protective mechanisms by preconditioning (PC) may represent a novel therapeutic approach against SAH-induced vasospasm and neurological deficits and, secondarily, that the neurovascular protection it provides is mediated by endothelial nitric oxide synthase (eNOS).

Methods—Wild-type mice were subjected to hypoxic PC or normoxia followed 24 hours later by SAH. Neurological function was analyzed daily; vasospasm was assessed on post-surgery Day 2. Nitric oxide availability, eNOS expression, and eNOS activity were also assessed. In a separate experiment, wild-type and eNOS-null mice were subjected to hypoxic PC or normoxia followed by SAH and assessed for vasospasm and neurological deficits.

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

Conclusions—Endogenous protective mechanisms against vasospasm exist, are powerful, and can be induced by PC. eNOS-derived nitric oxide is a critical mediator of PC-induced neurovascular protection. These data provide strong “proof-of-principle” evidence that PC represents a promising new strategy to reduce vasospasm and delayed cerebral ischemia after SAH. (Stroke. 2011;42:776-782.)

Key Words: cerebral vasospasm ■ hypoxia ■ nitric oxide synthase ■ preconditioning ■ subarachnoid hemorrhage

Preconditioning (PC) refers to a strategy whereby exposure to a sublethal quantity of a normally injurious stimulus can be used to augment the brain’s inherent evolutionarily conserved protective mechanisms to attenuate subsequent injury. Although the contribution of neurons to the observed protection is well documented, the role of the cerebrovasculature (vascular PC) has only recently been appreciated (for review, see Gidday).

Most studies examining PC have used physiological stimuli such as hypoxia, ischemia, or lipopolysaccharide administration in the setting of brain ischemia. Although these studies have provided critical insight into the underlying mechanisms of endogenous brain protection, it appears that clinical application of a PC strategy may be limited to select patient populations with especially high risk for ischemic events, including those undergoing carotid endarterectomy, cerebral aneurysm surgery, and coronary artery bypass grafting. Another potential population is aneurysmal subarachnoid hemorrhage (SAH), although preclinical studies examining this possibility are currently lacking.

SAH is a disease associated with high morbidity and mortality, a significant portion of which is due to the development of delayed cerebral ischemia. This condition may be ideal for a PC strategy because (1) there is a wide therapeutic window between SAH and delayed cerebral ischemia (typically 4 to 12 days); (2) vasospasm is a key factor responsible for delayed cerebral ischemia, and PC stimuli have known vasculoprotective effects; and (3) endothelial nitric oxide synthase (eNOS)-derived nitric oxide (NO), a known mediator of vascular PC, is strongly linked to SAH-induced vasospasm.

Therefore, in this “proof-of-principle” study, we sought to determine whether PC results in protection against SAH-induced vasospasm and neurological dysfunction and

Received November 29, 2010; final revision received December 22, 2010; accepted December 28, 2010.
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© 2011 American Heart Association, Inc.
Stroke is available at http://stroke.ahajournals.org DOI: 10.1161/STROKEAHA.110.607200
whether eNOS-derived NO contributes to the mechanism underlying this protective effect.

## Materials and Methods

### Animals

All experimental protocols were approved by the Animal Studies Committee at Washington University in St Louis. Three to four months old male wild-type mice (C57BL/6J) and homozygous eNOS<sup>−/−</sup> mice (B6.129P2-Nos3<sup>tm1Unc</sup>/J with a C57BL/6 genetic background) from Jackson Laboratories (Bar Harbor, ME) were used.

### Hypoxic PC

Mice were placed in a hypoxic chamber and exposed to air containing 8% O<sub>2</sub>-92% N<sub>2</sub> for 4 hours with access to food and water ad libitum<sup>3,4</sup>; mice were then returned to their cages. Normoxic controls were placed in chambers containing room air.

### Pharmacological Inhibition of NO Synthase

To examine the effect of NO synthase inhibition on PC-induced neurovascular protection in SAH, wild-type mice were administered the pan-NO synthase inhibitor L-NG-nitroarginine methyl ester (L-NAME; 20 mg/kg intraperitoneally) or vehicle (normal saline) starting 1 hour before PC and continued once daily thereafter.

### Experimental Protocols and Groups

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

### Experimental SAH

Endovascular perforation SAH was performed as described.<sup>13</sup> Briefly, mice were anesthetized with isoflurane (4% induction, 1.5% maintenance), and a 5-0 nylon suture was introduced into the external carotid artery and advanced through the internal carotid artery until the internal carotid artery bifurcation. The suture was then advanced to induce SAH, then removed and the external carotid artery ligated. Mice in the sham surgery groups underwent these procedures except for suture perforation.

### Neurobehavioral Tests

Neurobehavioral outcome was examined daily using Neuroscore and Rotarod tests as described.<sup>15,16</sup> Briefly, neurofunctional judgment was graded based on a motor score (0 to 12) that evaluated spontaneous activity, symmetry of limb movements, climbing, balance, and coordination and a sensory score (4 to 12) that evaluated body proprioception and vibrissae, visual, and tactile responses. Balance and coordination were assessed by performance on the Rotarod (Rotarod-5; Columbus Instruments, Columbus, OH). Mice were pretrained on the Rotarod 1 day before surgery. Latency on 3 trials of 180 seconds was averaged daily.

### Vasospasm Assessment

Vasospasm assessment was performed on post-surgery Day 2 through cerebrovascular casting, as described.<sup>15</sup> Briefly, mice were anesthetized and transcardially perfused with phosphate-buffered saline, 10% formalin, and 3% gelatin–India ink solution. Brains were removed, SAH was graded as described,<sup>16</sup> and blood vessels imaged under a microscope using a charge-coupled device camera. The narrowest diameter within the first 1000 μm of the middle cerebral artery was measured to assess vasospasm.

### NO Availability Assay

NO availability was determined using DAF-2DA (fluorophore 4,5-diamino-2-[3-(4,6-dimethoxy-2-methylphenyl)diazenyl]-2H-1,2-dichloroquinoline) as described.<sup>17</sup> Briefly, brain lysates were incubated with 50 μmol/L DAF-2DA for 30 minutes at 22°C in the dark. The reaction mixture was excited at 495 nm and emission read at 515 nm in a spectrophotometer (Biotek, Winooski, VT).

### Western Blot

Western blots were performed as described.<sup>18</sup> Briefly, brains were harvested 48 hours after sham or SAH surgery. Tissue from the hemisphere ipsilateral to endovascular perfusion was lysed in a buffer containing 10 mmol/L 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid, 5 mmol/L MgCl₂, 1 mmol/L dithiothreitol, 2 mmol/L ethylenediaminetetra-acetic acid, 2 mmol/L ethylene glycol bis(β-aminoethyl ether)-N,N,N’,N’-tetra-acetic acid, 1 mmol/L phenylmethanesulfonyl fluoride, 1% Triton X-100, 0.5 mmol/L sodium orthovanadate, 0.1 mmol/L okadaic acid, 25 mmol/L β-glycerophosphate, and protease inhibitor cocktail...
bronchial arteries. The following primary antibodies were used: mouse anti-eNOS (BD Biosciences), rabbit anti-inducible nitric oxide (iNOS; Santa Cruz Biotechnology), mouse anti-neuronal nitric oxide synthase (nNOS; Santa Cruz Biotechnology), and mouse anti-α-tubulin (Sigma). Blots were subsequently incubated with anti-mouse or anti-rabbit horseradish peroxidase-conjugated IgG and visualized using an enhanced chemiluminescence kit (BioRad).

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

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

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

**PC Improves Neurological Outcome After SAH**  
SAH led to significant neurobehavioral deficits by Neuroscore and Rotarod latency test (*P<0.05*) in nonpreconditioned mice (Figure 3). These neurobehavioral deficits were significantly attenuated in preconditioned mice (*P<0.05*; Figure 3).

**PC Attenuates SAH-Induced Reduction in NO Availability**  
NO availability was assessed 72 hours after PC (48 hours after sham or SAH surgery). SAH reduced NO availability by 42%±20% (*P<0.05*) in nonpreconditioned mice (Figure 4). However, in preconditioned mice, NO availability was unchanged after SAH (ie, SAH-induced reduction in NO availability was prevented; Figure 4). PC also increased NO availability in sham-operated mice by 69%±34% (*P<0.05*).

**NO Synthase Inhibition Blocks PC-Induced Neurovascular Protection in SAH**  
NO synthase inhibition with L-NAME was found to (1) reduce baseline middle cerebral artery diameter by 12%±4% (*P=0.03*); (2) have no impact on the degree of vasospasm after SAH (29%±5% vessel constriction in vehicle-treated versus 32%±8% vessel constriction in l-NAME-treated nonpreconditioned mice, *P=0.7*); and (3) completely block PC-induced protection against vasospasm (66%±15% vasospasm protection in vehicle-treated versus −25%±23% vasospasm protection in l-NAME-
treated mice, *P = 0.003) and neurological deficits by Neuroscore and Rotarod (*P > 0.05) after SAH (Figure 4). Hemorrhage grade was not significantly different between L-NAME-treated SAH groups (nonpreconditioned = 3.5; preconditioned = 4.1); however, mortality was different (nonpreconditioned = 31%; preconditioned = 0%, *P < 0.05 by Fisher exact test).

PC Selectively Increases eNOS Expression After SAH
eNOS, nNOS, and iNOS expression was assessed 72 hours after PC (48 hours after sham or SAH surgery). PC increased eNOS expression by 2.3-fold (*P < 0.05) in sham-operated mice and by 1.6-fold (*P < 0.05) in SAH-operated mice (Figure 5); SAH alone did not alter eNOS activity (Figure 5).

eNOS Contributes to PC-Induced Protection Against Vasospasm and Neurological Deficits After SAH
To directly assess the role of eNOS in PC-induced neurovascular protection, a separate group of wild-type (eNOS+/+) and eNOS-null (eNOS−/−) mice was examined. SAH caused significant (*P < 0.05) vasospasm in both eNOS+/+ (21%±5% constriction) and eNOS−/− (24%±5% constriction) mice (Figure 6A). As expected, SAH-induced vasospasm was nearly completely prevented (94%±19% vasospasm reduction; *P < 0.05) in preconditioned eNOS+/+ mice (Figure 6A–B). However, vasospasm protection by preconditioning was largely absent (24%±20% vasospasm reduction) in eNOS−/− mice (Figure 6A–B). A commensurate loss in PC-induced neurobehavioral protection (by Neuroscore) was also found in eNOS−/− mice (Figure 6C). Rotarod testing was not performed because naïve eNOS−/− mice had substantial Rotarod deficits at baseline (data not shown). Hemorrhage grade (eNOS+/+ nonpreconditioned mice = 3.2; eNOS+/+ preconditioned mice = 3.7; eNOS−/− nonpreconditioned mice = 3.1; and eNOS−/− preconditioned mice = 3.7) and mortality (eNOS+/+ nonpreconditioned mice = 6.7%; eNOS+/+ preconditioned mice = 10.3%; eNOS−/− nonpreconditioned mice = 5.0%; and eNOS−/− preconditioned mice = 4.7%) were not significantly different between SAH groups.
result is similar to that reported in studies examining PC in the setting of cerebral ischemia\textsuperscript{7,12,21} and neonatal hypoxia-ischemia.\textsuperscript{9,20} Collectively, these data strongly suggest that NO synthase-derived NO is a critical mediator of PC-induced neurovascular protection in SAH.

To identify the NO synthase isoform that contributes to the observed protective effect of PC, we examined the effect of PC on the expression of all 3 NO synthase isoforms (eNOS, nNOS, and iNOS) in the setting of SAH. Given that eNOS expression was selectively upregulated after PC and that eNOS is the primary enzymatic source of NO in cerebral vessels\textsuperscript{9} and the most strongly implicated NO synthase isoform in vasospasm pathophysiology\textsuperscript{13} and vascular PC,\textsuperscript{7,9,12} we next examined the effect of PC on eNOS activity. Our finding that PC increased eNOS activity in both sham and SAH mice, when coupled with the aforementioned observation that NO synthase-derived NO is critical for PC-induced neurovascular protection, suggests that the vasculoprotective effects of PC likely occur through increased NO secondary to a persistent upregulation in eNOS.

Our findings in eNOS-null mice provided further support for this hypothesis. That PC-induced abrogation of vasospasm and neurobehavioral deficits in wild-type mice was lost in eNOS-null mice (although the degree of vasospasm and neurological deficits after SAH was similar between the 2 groups when not preconditioned) indicates that the innate protective mechanisms by which PC reduces SAH-induced neurovascular deficits are dependent, to a significant extent, on eNOS. More broadly for the field of PC, our results lend additional support to the notion that eNOS is a key contributor to PC-induced neuroprotection. Other studies of PC-induced vasculoprotection in focal ischemic stroke have advanced similar conclusions regarding the participation of eNOS.\textsuperscript{7,22}

The effect of SAH on eNOS expression and/or activity is controversial. We found no effect of SAH on either metric, but previous studies report no change,\textsuperscript{23–25} a reduction,\textsuperscript{26} or an increase\textsuperscript{17} in eNOS expression after SAH. Similarly, SAH was found to either have no effect\textsuperscript{24,27} on phosphorylated eNOS (at Ser1177, one of many active forms of eNOS\textsuperscript{77}) or an increase.\textsuperscript{17,25,26} In addition, our experiments identified what could be considered a discrepancy between eNOS activity (no change) and NO availability (decreased) in response to SAH. Several possibilities could explain this observation. First, it is possible that a SAH-induced decrease in eNOS activity actually occurred in vivo but was undetected by our ex vivo assay. This could occur if availability of one or more substrates (e.g., L-arginine) or cofactors (e.g., reduced nicotinamide adenine dinucleotide phosphate), which were artificially provided in our ex vivo assay, became limited; or if the endogenous NO synthase inhibitor asymmetrical dimethyl L-arginine was upregulated by SAH.\textsuperscript{13} Second, it is possible that NO availability was decreased after SAH despite unchanged eNOS activity. For example, this could occur through NO scavenging by oxyhemoglobin\textsuperscript{28} and/or leukocytes\textsuperscript{29} present in the subarachnoid space. In either case, PC-induced changes appear significant enough to overwhelm these factors and restore NO production to pre-SAH levels.

**Discussion**

In the present study, we established the existence of endogenous protection against SAH-induced neurovascular deficits by demonstrating that preconditioned mice develop almost no vasospasm and exhibit reduced neurological deficits after SAH. This finding demonstrates, for the first time, that PC is efficacious in a fundamentally different form of stroke, SAH, and provides support to the evolving concept of vascular PC for brain ischemia.\textsuperscript{1,2} Moreover, we demonstrated that the neurological protection afforded by PC in the setting of SAH is due, in part, to decreased vasospasm, a well-recognized risk factor for poor outcome after SAH.

With respect to the molecular mechanism underlying PC-induced vasospasm protection, we examined NO given its influence on vascular function\textsuperscript{13} and its role in both vasospasm pathophysiology\textsuperscript{13,17} and PC-induced neuroprotection.\textsuperscript{5,12,20} We found that NO availability decreased after SAH and that PC increased NO levels in both sham- and SAH-operated mice. Both results are consistent with past studies,\textsuperscript{1,2,13} although the latter has not been previously reported in the setting of SAH. We also demonstrated that inhibition of NO synthesis abolished the protective effect of PC on SAH-induced vasospasm and neurological dysfunction. This
Some limitations of our study should be noted. First, PC was administered before SAH. This was by design, because our study was conceived as a “proof-of-concept” one to establish whether endogenous protective mechanisms against vasospasm are present and thus permit their exploration. Studies examining the efficacy of post-SAH conditioning paradigms have already been initiated in our laboratory. Second, although the clinical applicability of hypoxia as a preconditioning stimulus may be difficult to envision, several pharmacological agents with known PC effects are available for future testing. Third, because no animal model fully recapitulates human SAH, it is essential to validate our findings in complementary SAH models. Fourth, although vasospasm is a major contributor to delayed cerebral ischemia, other factors may also contribute (for review, see MacDonald et al11); whether PC also positively affects these factors will require future study. Fifth, our study does not exclude a contribution from nNOS or iNOS; however, it is very unlikely that either plays a major role given that neither was upregulated after PC in the setting of SAH. Finally, we recognize that the neurological deficits documented in our SAH model are likely multifactorial and that the observed neuroprotection could be explained in part by beneficial effects of PC on reducing neuronal apoptosis,32 inflammation,33 and/or blood–brain barrier breakdown. In fact, the robustness of the PC-induced protection noted in our study suggests a multifaceted underlying mechanism; therapeutic strategies for SAH that target several deleterious cascades would likely have great potential for clinical efficacy.

In conclusion, results from this “proof-of-principle” study indicate that powerful endogenous protective mechanisms against vasospasm exist, that they can be activated by a preconditioning stimulus, and that their underlying mechanism is at least partly dependent on eNOS-derived NO. Further studies to more fully elucidate the innate molecular mechanisms underpinning these endogenous protective mechanisms against vasospasm are required. We thank Ernesto Gonzales and for performing subarachnoid hemorrhage surgery and The Hope Center for Neurological Disorders at Washington University School of Medicine.

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Disclosures

None.

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

Source of Funding

This work was supported by grants from the McDonnell Center for Cellular and Molecular Neurobiology (B.H.H.), American Heart Association (G.J.Z.), Brain Aneurysm Foundation (G.J.Z. and B.H.H.), Neurosurgery Research and Education Foundation (G.J.Z.), McDonnell Center for Higher Brain Function (G.J.Z.), and Howard Hughes Medical Institute (E.M.).


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Stroke. published online February 11, 2011; Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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