Simvastatin Increases Endothelial Nitric Oxide Synthase and Ameliorates Cerebral Vasospasm Resulting From Subarachnoid Hemorrhage

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Background and Purpose—Endothelial nitric oxide synthase (eNOS) activity is decreased after subarachnoid hemorrhage (SAH). Simvastatin increases eNOS activity. We hypothesized that simvastatin would increase eNOS protein and ameliorate SAH-induced cerebral vasospasm.

Methods—Mice were treated with subcutaneous simvastatin or vehicle for 14 days and then subjected to endovascular perforation of the right anterior cerebral artery or sham surgery. Three days later, neurological deficits were scored (5 to 27; 27=normal), and middle cerebral artery diameter and eNOS protein were measured. The study was repeated, but simvastatin treatment was started after SAH or sham surgery.

Results—In SAH mice, simvastatin pretreatment increased middle cerebral artery diameter (SAH-simvastatin=74±22 μm, SAH-vehicle=52±18 μm, P=0.03; sham-simvastatin=102±8 μm, sham-vehicle=105±6 μm). Pretreatment reduced neurological deficits (SAH-simvastatin=25±2, SAH-vehicle=20±2, P=0.005; sham-simvastatin and sham-vehicle=27±0). Simvastatin pretreatment also increased eNOS protein. Simvastatin posttreatment caused a modest increase in middle cerebral artery diameter in SAH mice (SAH-simvastatin=56±12 μm, SAH-vehicle=45±4 μm, P=0.03; sham-simvastatin=92±13 μm, sham-vehicle=99±10 μm) and reduced neurological deficits (SAH-simvastatin=21±1, SAH-vehicle=19±2, P=0.009). Simvastatin posttreatment did not significantly increase eNOS protein.

Conclusions—Simvastatin treatment before or after SAH attenuated cerebral vasospasm and neurological deficits in mice. The mechanism may be attributable in part to eNOS upregulation. (Stroke. 2002;33:2950-2956.)

Key Words: HMG-CoA reductase inhibitors • simvastatin • subarachnoid hemorrhage • vasospasm • mice

Delayed cerebral vasospasm is a major cause of morbidity and mortality in patients with aneurysmal subarachnoid hemorrhage (SAH). Current medical treatments fail to consistently prevent or reverse vasospasm.1 The etiology of vasospasm may result from an imbalance of vasodilatation and vasoconstriction.2 Vascular endothelium regulates smooth muscle tone by generating nitric oxide (NO)3 and endothelial-derived constriction factors.4 Disruption of endothelium or its relaxing factors may alter this balance, initiating vasospasm.5 SAH has been associated with histopathological damage to cerebrovascular endothelium and impaired endothelium-dependent relaxation responses to pharmacological challenge.5,6 NO depletion accompanies these changes, suggesting that SAH-induced endothelial dysfunction contributes to loss of NO. Furthermore, NO replacement reverses cerebral vasospasm in animal studies.7 However, it is not clear whether decreased endogenous NO production or increased NO breakdown underlies these changes in NO bioavailability. NO synthase, the primary source of NO in vascular tone regulation, metabolizes L-arginine to NO and citrulline. The endothelial isoform (eNOS) is constitutively expressed in cerebrovascular endothelium. Immunoreactivity for eNOS mRNA and protein has been shown to decrease after SAH.8 Although the mechanism of eNOS reduction is not known, it likely causes reduction of NO production and disruption of endothelial-dependent vasodilation after SAH.

The 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, also referred to as statins, are potent inhibitors of cholesterol biosynthesis.9 Treatment with statins improves endothelial function without changes in serum cholesterol.10 Furthermore, statins directly upregulate eNOS expression under cholesterol-controlled conditions.11 An increase in eNOS mRNA, protein, and enzymatic activity has been demonstrated after statin treatment, resulting in increased cerebral blood flow.12

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Mean arterial blood pressure, pH, PaCO$_2$, and PaO$_2$ were measured above. In addition, a catheter was placed in the left femoral artery. Underwent anesthesia and surgical preparation for SAH as described and vehicle injections were continued. Mice were observed until recovery of the righting reflex and were returned to their cages for 3 days. Daily simvastatin was extubated. Mice were observed until recovery of the righting reflex, thereby avoiding arterial perforation. After SAH. In sham-operated mice the suture was advanced only until the right ACA. The suture was withdrawn, allowing reperfusion and was encountered, and then advanced 3 mm further to perforate the (ACA) – middle cerebral artery (MCA) bifurcation, where resistance was encountered, and then advanced 3 mm further to perforate the right ACA. The suture was withdrawn, allowing reperfusion and SAH. In sham-operated mice the suture was advanced only until the point of resistance, thereby avoiding arterial perforation. After removal of the filament, the skin was sutured, and halothane was discontinued. On recovery of spontaneous ventilation, the trachea was extubated. Mice were observed until recovery of the righting reflex and were returned to their cages for 3 days. Daily simvastatin and vehicle injections were continued.

Additional vehicle- (n=5) and simvastatin-treated (n=5) mice underwent anesthesia and surgical preparation for SAH as described above. In addition, a catheter was placed in the left femoral artery. Mean arterial blood pressure, pH, PaCO$_2$, and PaO$_2$ were measured immediately before SAH.

A neurobehavioral examination (scoring scale 5 to 27) was performed 72 hours after SAH or sham surgery by an examiner blinded to group assignment. A motor score (0 to 12) was derived from spontaneous activity, symmetry of limb movements, climbing, balance, and coordination, with each scored 0 to 3. A sensory score (5 to 15) was derived from examination of body proprioception and vibrissae, visual, olfactory, and tactile responses to stimulus, with each parameter scored on a scale of 1 to 3.

After neurological examination, mice underwent either cerebral vascular perfusion or eNOS Western blot analysis. Cerebral vascular perfusion was performed, by an observer blinded to group assignment, 72 hours after surgery, when vasospasm has been reported to peak in this model. Mice were anesthetized with halothane. The chest was opened, and the aorta was cannulated with a blunted 20-gauge needle. Flexible plastic tubing (3.2-mm internal diameter) connected to the needle was used to deliver infusion solutions by manual pulsatile syringe pressure. The tubing was connected to a 30-ml syringe, the cannulated aorta, and a mercury manometer, establishing a closed circuit to monitor perfusion pressure. An incision was made in the right atrium to allow for outflow of perfusion solutions. Twenty milliliters of 0.9% NaCl was infused followed by 15 minutes of 10% formalin and 10 minutes of gelatin–india ink solution. All perfusates were passed through a 0.2-μm pore size filter and delivered at 60 to 80 mm Hg. The mouse was refrigerated for 24 hours. The brains were harvested and stored in 4% neutral buffered formaldehyde (Figure 1).

Brains were analyzed under light microscopy (×6 magnification) to determine the magnitude of SAH by an observer blinded to treatment group. Hemorrhage size was graded by 2 characteristics: area of hemorrhage distribution and density of clot formation. Hemorrhage size was scored as follows: 1 = SAH extends < 1.0 mm from MCA-ACA bifurcation; 2 = SAH extends > 1.0 mm from bifurcation; and 3 = SAH extends > 1.0 mm from bifurcation with extension to the contralateral internal carotid artery. Hemorrhage density was scored as follows: 1 = brain parenchyma visualized through clot; 2 = brain parenchyma not visualized through clot. Hemorrhage grade (2 to 5) was defined as the sum of size and density scores. No hemorrhage was scored as 0.

Arteries were imaged with a dissecting microscope (×6 magnification) integrated with an image analyzer. MCA images were divided into proximal and distal 1.0-mm segments beginning at the ACA-MCA bifurcation. The proximal MCA was selected to determine magnitude of vasospasm. The smallest proximal MCA diameter was recorded by digital measurement. The basilar artery diameter was also measured in SAH mice. Mice were excluded from vascular diameter analysis when air emboli or particulate emboli
were observed or when extravasation of ink into the subarachnoid space prevented visualization of the MCA. A single observer blinded to group performed all measurements.

Recovery mice not subjected to cerebrovascular perfusion underwent eNOS Western blot analysis after the day 3 neurological examination. Brain tissue ipsilateral to SAH or sham surgery, within the MCA distribution, was harvested rapidly and stored at −80°C.

For immunoblot analysis, tissue was placed into 0.3 to 0.4 mL of extraction buffer (45 mmol/L Tris-HCl; 0.4% SDS; 0.2 mmol/L phenylmethylsulfonyl fluoride; 1 mmol/L sodium vanadate; 0.5% Sigma protease inhibitor cocktail), sonicated, and centrifuged for 5 minutes at 12,000 rpm. A portion of the supernatant was used for total protein analysis, and the remainder was combined with a Western loading buffer. Ten micrograms of protein (diluted in buffer) was loaded onto each lane of the gel (7.5% SDS-PAGE gel with 5% stacking gel and Tris-glycine running buffer). Each gel was presented with 2 samples from each group, along with a standard (human umbilical vein endothelial cell extract). To confirm that each lane contained equivalent amounts of protein, 1 duplicate gel was run in parallel and stained for total protein with Gelcode Blue. The original gel was wet-transferred to a PVDF membrane with the use of CAPS/methanol buffer. The membranes were blocked with 5% nonfat dry milk in PBS/Tween 20. This was followed by overnight incubation at 4°C with mouse monoclonal anti-eNOS antibody (Transduction Laboratories). After they were rinsed with PBS/Tween, the specimens underwent a 2-hour incubation with the secondary antibody (goat anti-mouse IgG: horseradish peroxidase) and were rinsed again in PBS/Tween. To measure eNOS expression, bands were visualized by incubation in enhanced chemiluminescence reagents and exposure to x-ray film. Quantitative assessment of band intensities (integrated optical densities) was performed by an observer blinded to group using an Alpha Innotech Imaging 2000 system. Background intensities (determined from an equal-sized area of the film adjacent to the band of interest) were subtracted. To confirm that differences in eNOS expression, when observed, were specific, additional gels were run, in which both eNOS and β-tubulin (as an internal control) expressions were detected. For these analyses, 20 μg total protein was loaded onto each lane, and both anti-eNOS (see above) and anti-β-tubulin (monoclonal, from Transduction Laboratories, 1:3500 dilution) were added. Subsequent processing was done as described earlier.

The entire experiment was repeated to determine whether simvastatin could attenuate cerebral vasospasm when given only after SAH (posttreatment). Subcutaneous injection with simvastatin (20 mg/kg per day; n = 37) or vehicle (n = 38) was performed 30 minutes after SAH or sham surgery and repeated at 24 and 48 hours after SAH. All mice underwent neurological examination 72 hours after SAH or sham surgery following either cerebrovascular perfusion or eNOS Western blot analysis.

One-way ANOVA was used to compare continuous variables. Between-group differences were compared with the Scheffé test. The α error was set at 0.05. Neurological scores were compared by the Kruskal-Wallis test. Between-group differences were compared with the Mann-Whitney test. Association between neurological score and MCA diameter/SAH grade was tested with the Spearman rank correlation. Parametric values are given as mean±SD. Nonparametric values are given as median (interquartile range).

Results

There were no differences in arterial pH (vehicle = 7.41±0.04; simvastatin = 7.45±0.04), PaCO₂ (vehicle = 42±2 mm Hg; simvastatin = 45±2 mm Hg), PaO₂ (vehicle = 187±9; simvastatin = 181±17), temperature (vehicle = 37.0±0.1°C; simvastatin = 37.0±0.1°C), or mean arterial blood pressure (vehicle = 74±2 mm Hg; simvastatin = 79±3 mm Hg) among surrogate 14-day simvastatin- and vehicle-treated mice (n = 5 per group). Body weight did not differ between simvastatin groups.

Thirty-four mice were treated with simvastatin and 36 mice were treated with vehicle before randomization to SAH or sham groups. Six simvastatin- and 4 vehicle-treated mice subjected to SAH died within 72 hours of surgery and were not included in the study. In all of these mice, severe SAH extending bilaterally was evident at necropsy.

Three mice treated with vehicle were excluded from vascular diameter measurement because of perfusion artifact. Two demonstrated vascular air emboli, while ink extravasation into the subarachnoid space prevented MCA measurement in the third.

SAH was distributed in the basal cistern of all SAH mice. In mice used for MCA diameter analysis, SAH grade was not different between groups (vehicle = 3±1, n = 10; simvastatin = 3±2, n = 9; P = 0.64).

A main effect was present for vascular diameter 72 hours after SAH or sham surgery (P < 0.0001). In vehicle-treated mice, SAH caused a 50% reduction in MCA diameter (SAH = 52±18 μm, n = 10; sham = 105±6 μm, n = 9; P < 0.001). In simvastatin-treated mice, SAH caused a 27% reduction in MCA diameter (SAH = 74±22 μm, n = 9; sham = 102±8 μm, n = 9; P = 0.003). In mice subjected to SAH, MCA lumen diameter was greater in the simvastatin group than in the vehicle group (P = 0.03) (Figure 2A). There was no difference between vehicle and simvastatin sham groups (P = 0.98). There also was no difference in basilar artery diameter between simvastatin-SAH (150±15 μm, n = 9) and vehicle-SAH groups (155±8 μm, n = 10; P = 0.53).

SAH grades in vehicle- (3±0, n = 15) and simvastatin-treated mice (3±1, n = 14) were similar (P = 0.898). SAH grades in the sham groups were 0.

In vehicle-treated mice, neurological function was worsened 3 days after SAH (20±4.5, n = 15) compared with the vehicle-sham group (27±0, n = 14; P < 0.001). In the simvastatin-SAH group, neurological function was also worsened (25±4, n = 14) compared with the simvastatin-sham group.

Figure 2. A, MCA lumen diameter 72 hours after SAH or sham surgery in mice treated with vehicle or simvastatin 14 days before SAH or sham surgery and 3 days after surgery. Bars indicate mean values. Circles indicate individual mice. B, Neurological examination scores 72 hours after SAH or sham surgery (27 = no deficit). Bars indicate median values.
stated, n=5; vehicle, n=5) and SAH groups (statin, n=5; vehicle, n=5). eNOS expression was unchanged by SAH in vehicle-treated mice (Figures 3 and 4). No variations in β-tubulin expression were noted (Figure 3).

For the posttreatment-only study, a total of 37 mice were treated with simvastatin, and 38 mice were treated with vehicle after SAH or sham surgery. Body weight did not differ between groups. SAH was distributed in the basal cistern of all SAH mice. Six simvastatin-treated and 6 vehicle-treated mice subjected to SAH died and were excluded from analysis. In each of these mice, severe SAH extending bilaterally was evident at necropsy. In mice used for MCA diameter analysis, SAH grade in vehicle-treated (3±1, n=9) and simvastatin-treated mice (3±1, n=9) was similar between groups (P=0.83).

In vehicle-treated mice, SAH reduced MCA diameter (SAH=45±4 μm, n=9; sham=99±10 μm, n=9; P<0.001). In simvastatin-treated mice, SAH also reduced MCA diameter (SAH=56±12 μm, n=9; sham=92±13 μm, n=10; P<0.001). In mice subjected to SAH, MCA diameter was increased in the simvastatin compared with the vehicle group (P=0.03) (Figure 5A). There was no difference in MCA diameter between sham groups (P=0.37). There also was no difference in basilar artery diameter between mice in the simvastatin-SAH (153±12 μm) and vehicle-SAH (153±11 μm; P=0.94) groups. SAH grades in vehicle- (3±0, n=18) and simvastatin-treated mice (3±0, n=17) were similar (P=0.839). SAH grades in the sham groups were 0.

In vehicle-treated mice, neurological function was worsened 3 days after SAH (19±5, n=18) compared with shams (27±0, n=14; P<0.001). In simvastatin-treated mice, neu-

Figure 3. Ipsilateral eNOS protein (percentage of background optical density) 72 hours after SAH or sham surgery in mice treated with vehicle or simvastatin. Pre- and post- indicates treatment for 14 days before surgery and 3 days after surgery, respectively; Post-, treatment begun after SAH or sham surgery and repeated twice at 24-hour intervals. *P<0.05 relative to vehicle-treated cohorts. Values are mean±SD.

Figure 4. Immunoblots showing eNOS in the hemisphere ipsilateral to SAH or sham surgery in mice treated with vehicle or simvastatin. Pre- and post- indicates treatment for 14 days before surgery and 3 days after surgery; Post-, treatment begun after SAH or sham surgery and repeated at 24-hour intervals. At 72 hours after surgery, simvastatin increased eNOS expression only in the "Pre- and post-" groups. The relative constancy of β-tubulin expression indicates an eNOS-specific effect of simvastatin.
Discussion

Subcutaneous simvastatin given either before or after SAH reduced morphological vasospasm and neurological deficits resulting from SAH. After SAH, reduced vascular diameter was observed in mice receiving 14-day vehicle compared with 14-day simvastatin pretreatment. A smaller effect was observed when simvastatin treatment was initiated after SAH. Similarities of basilar artery diameters between vehicle- and simvastatin-treated mice after SAH indicate that the india ink–gelatin perfusion technique did not contribute to these effects. Neurological deficits were most likely attributable to vasospasm on the basis of both a correlation with MCA diameter and localization of behavioral deficits to the MCA vascular distribution. Neurological deficits were reduced by simvastatin pretreatment. In fact, many simvastatin-treated mice had minimal neurological deficit after SAH. Although less potent, initiation of simvastatin treatment after SAH also reduced neurological deficit. Simvastatin pretreatment increased cerebrovascular eNOS expression 2- to 3-fold in both sham and SAH mice. SAH did not alter eNOS expression in vehicle mice. Simvastatin posttreatment resulted in a statistically insignificant pattern of increased cerebral eNOS expression in SAH mice only. The similarity of vascular diameter and neurological function after sham surgery, as well as similar physiological responses to SAH surgery in vehicle and simvastatin groups, suggested no other baseline differences between groups.

Endovascular perforation of cerebral arteries in rats and mice produces SAH without craniotomy or other invasive techniques that may confound in vivo responses. However, SAH magnitude cannot be controlled precisely. Severity of vasospasm is dependent on SAH magnitude. We used a SAH grading scale to account for variability in hemorrhage magnitude. Similar SAH grades between simvastatin and vehicle groups indicate that variation in SAH size did not confound conclusions drawn from these end points.

Although pharmacological agents have been shown to partially resolve vasospasm after SAH, the importance of their effect on vasospasm-induced neurological injury has not been measured. In this study motor and sensory deficits were correlated with the magnitude of MCA vasospasm and were localized predominantly to the MCA distribution. These findings suggest that the measured neurological deficits specifically resulted from MCA vasospasm and resultant hypoperfusion of its vascular bed.

Neurological deficit after SAH was substantively decreased by simvastatin pretreatment and partially reduced when simvastatin treatment was initiated after SAH. It is unclear whether this improvement was a result of a macrovascular effect (attenuation of MCA vasospasm), a microvascular effect, or both. Previous studies have demonstrated reduction in infarct size with administration of HMG-CoA reductase inhibitors, corresponding to an increase in peri-infarct blood flow. Blood flow was increased after statin treatment in nonpathological conditions, suggesting that both macrovasculature and microvasculature are affected. In the setting of SAH vasospasm, both macrovascular and microvascular dysfunction contribute to clinical deterioration. The marked neurological effect observed with simvastatin pretreatment in this study may have resulted from both ischemic protection and attenuation of vasospasm.

It has been controversial whether cerebral eNOS expression is altered by SAH. eNOS protein expression was unchanged in spastic canine basilar arteries 7 days after SAH. The reduction in eNOS protein observed by Park et al occurred only 20 minutes after SAH, while the 56% reduction in eNOS mRNA reported by Hino et al was not accompanied by a decrease in ipsilateral brain eNOS protein 7 days after SAH. Similarly, our study did not find an effect of SAH on eNOS protein 3 days after SAH. It is possible that eNOS depletion occurs early and contributes to the acute phase of cerebral vasospasm. However, our eNOS analysis included both MCA and peri-MCA parenchymal tissue. Different results may be found if eNOS activity is measured only in vessels excised from the site of...
hemorrhage. Such a study was prohibitive in our experiments because of the mouse MCA size. Regardless, the increase in cerebrovascular eNOS expression observed ipsilateral to SAH with simvastatin treatment is consistent with the attenuation of delayed cerebral vasospasm. A study of the effects of simvastatin in eNOS-depleted mice subjected to SAH would be of interest to further characterize the vascular specific effects of this intervention. Regardless, other evidence has recently been provided that upregulation of eNOS expression can ameliorate cerebral vasospasm. Khurana et al.\(^\text{21}\) using an established canine model of 7-day recovery from SAH, provided angiographic evidence that vasospasm can be ameliorated by intrathecal viral transfection of the perivascularu with eNOS mRNA.

A 2-fold increase in eNOS expression in noncerebral endothelial cells has been shown with chronic HMG-CoA reductase inhibitor treatment in focal ischemia models.\(^\text{16,22}\) Similarly, we observed a 2.6-fold increase in eNOS expression with prolonged simvastatin administration before SAH. The absence of a significant increase in eNOS expression observed with only 3 days of simvastatin treatment after SAH is consistent with observations made by others,\(^\text{12,16,22}\) although eNOS protein was numerically increased by 26%. Because beneficial effects were still observed with simvastatin with respect to MCA diameter and neurological function, it is plausible that only small changes in eNOS expression are required. Alternatively, other effects of simvastatin may have played a role. Because neither eNOS activity nor NO bioavailability was measured, whether the observed efficacy was a direct result of increased NO production remains unclear.

In contrast to the mouse, in which vasospasm has been reported to peak within 72 hours after SAH,\(^\text{13}\) human vasospasm peaks within 4 to 12 days. This would allow a longer therapeutic window for statin therapy to induce eNOS upregulation. Confirmation in a canine or primate model may better assess simvastatin as a clinical therapy. Further work is also required to define dose-response effects for simvastatin. Our mice were given simvastatin 20 mg/kg per day. The maximum recommended dose of simvastatin for humans is 80 mg/d to treat hyperlipidemia. Whether more clinically relevant simvastatin doses will be beneficial for simvastatin is 80 mg/d to treat hyperlipidemia. Further work is also required to define dose-response effects for simvastatin. Our mice were given simvastatin 20 mg/kg per day. The maximum recommended dose of simvastatin for humans is 80 mg/d to treat hyperlipidemia. Whether more clinically relevant simvastatin doses will be beneficial for simvastatin is numerically increased by 26%. Because beneficial effects were still observed with simvastatin with respect to MCA diameter and neurological function, it is plausible that only small changes in eNOS expression are required. Additionally, other effects of simvastatin may have played a role. Because neither eNOS activity nor NO bioavailability was measured, whether the observed efficacy was a direct result of increased NO production remains unclear.

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In conclusion, treatment with simvastatin attenuated cerebral vasospasm and improved neurological outcome after SAH in mice. This effect was associated with an increase in cerebrovascular eNOS protein, suggesting that augmentation of the endogenous NO synthase pathway mediated the observed benefit. HMG-CoA reductase inhibitors, such as simvastatin, may potentially serve as agents in the prevention of cerebral vasospasm after SAH.

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