Soluble Guanylate Cyclase α1β1 Limits Stroke Size and Attenuates Neurological Injury

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Background and Purpose—Nitric oxide mediates endothelium-dependent vasodilation, modulates cerebral blood flow, and determines stroke outcome. Nitric oxide signals in part by stimulating soluble guanylate cyclase (sGC) to synthesize cGMP. To study the role of sGC in stroke injury, we compared the outcome of cerebral ischemia and reperfusion in mice deficient in the α1 subunit of sGC (sGCα1−/−) with that in wild-type mice.

Methods—Blood pressure, cerebrovascular anatomy, and vasoreactivity of pressurized carotid arteries were compared in both mouse genotypes. Cerebral blood flow was measured before and during middle cerebral artery occlusion and reperfusion. We then assessed neurological deficit and infarct volume after 1 hour of occlusion and 23 hours of reperfusion and after 24 hours of occlusion.

Results—Blood pressure and cerebrovascular anatomy were similar between genotypes. We found that vasodilation of carotid arteries in response to acetylcholine or sodium nitroprusside was diminished in sGCα1−/− compared with wild-type mice. Cerebral blood flow deficits did not differ between the genotypes during occlusion, but during reperfusion, cerebral blood flow was 45% less in sGCα1−/− mice. Infarct volumes and neurological deficits were similar after 24 hours of occlusion in both genotypes. After 1 hour of ischemia and 23 hours of reperfusion, infarct volumes were 2-fold larger and neurological deficits were worse in sGCα1−/− than in the wild-type mice.

Conclusion—sGCα1 deficiency impairs vascular reactivity to nitric oxide and is associated with incomplete reperfusion, larger infarct size, and worse neurological damage, suggesting that cGMP generated by sGCα1β1 is protective in ischemic stroke. (Stroke. 2010;41:1815-1819.)

Key Words: cerebral ischemia ■ gene knockout mice ■ mouse models

Nitrile oxide (NO) is synthesized by a family of enzymes referred to as NO synthases (NOS). Under physiological conditions, low levels of NO produced by the 2 constitutive Ca2+-dependent enzymes, NOS1 and NOS3, have diverse functions ranging from neuromodulation and vasodilation to inhibition of platelet adhesion and aggregation. We previously demonstrated that NO generated by NOS3 modulates vascular reactivity and cerebral blood flow (CBF) and limits stroke size in a mouse model of cerebral ischemia–reperfusion.1 In contrast, infarct volumes and neurological deficits are smaller in NOS1-deficient mice than in wild-type (WT) mice 24 and 72 hours after middle cerebral artery occlusion (MCAO),2 suggesting that NOS1-derived NO exacerbates acute ischemic injury. Importantly, administration of NO donor compounds reduces stroke lesion volume in both permanent and transient ischemia models of experimental stroke.3 Together, these findings highlight the complex role of NO in determining the injury associated with stroke.

NO reacts with a variety of intracellular and extracellular molecules, typically through thiol groups or transition metal centers. NO’s multiple targets contribute to the often-conflicting effects of this molecule. A major target of NO in the cardiovascular system is soluble guanylate cyclase (sGC), mediating many of the effects of NO on smooth muscle relaxation and blood pressure regulation. NO stimulates sGC; an obligate heterodimer composed of an α and a β subunit, to synthesize the intracellular second messenger cGMP. In the vasculature, the sGCα1β1 heterodimer appears to be the principal sGC isoform.4 However, recent studies suggest that low levels of cGMP generated by sGCα1β1 are sufficient to mediate many of NO’s cardiovascular effects.5-7

In brain homogenates, both sGCα1β1 and sGCα2β1 are abundant, and both are required to mediate hippocampal long-term potentiation.8 The expression of sGCβ1 is markedly reduced in the vascular wall of stroke-prone spontaneously hypertensive rats.9 However, whether sGC plays a role in the
physiological outcome of stroke remains to be determined. Therefore, we compared the impact of cerebral ischemia and reperfusion in WT and sGCα1−/− mice. sGCα1 deficiency was associated with larger infarct size and worse neurological damage, suggesting that cGMP generated by sGCα1,β1 is protective in ischemic stroke.

Materials and Methods

Animals
Mice deficient in the α1 subunit of sGC (sGCα1−/−) were generated as described previously.5,10 Male 8- to 12-week-old WT (Jackson laboratory) and sGCα1−/− mice on a C57BL/6 background were studied. All procedures were approved by the Massachusetts General Hospital Subcommittee on Research and Animal Care.

Anesthesia
Mice were anesthetized with 1.5% of isoflurane in 30% oxygen and 70% N2O. Body temperature was maintained at 36.5°C to 37.5°C.

Mean Arterial Blood Pressure
After anesthesia, the femoral artery was catheterized to monitor mean arterial blood pressure using a blood pressure transducer (ADInstruments).

Total Nitrate/Nitrite Concentration
Total nitrate/nitrite concentrations (NOx) were determined with a Nitric Oxide Colorimetric Assay Kit (Biovision).

Blood Glucose
Blood glucose measurements were performed in nonfasted mice before and 1 hour after MCAO using a Ascensia Breeze 2 Blood Glucose Meter (Bayer).

Cerebrovascular Anatomy
Cerebrovascular anatomy was studied by intracardiac carbon black line (ACh) and sodium nitroprusside (SNP) was studied with a pressure myograph as described.1 Carotid arteries were isolated, pressurized to 85 mm Hg, and constricted with 10−3 H11003. Passive diameters were determined by applying Ca2+−.free saline containing 2 mmol/L EGTA.

Cerebral Reperfusion Model
A fiberoptic probe (Perimed) was affixed to the skull over the middle cerebral artery (MCA) for measurement of relative CBF by laser Doppler flowmetry as described.12 Baseline values were measured before internal carotid artery ligation (considered to be 100% flow). For the transient MCAO model (1 hour of ischemia and 23 hours of reperfusion), the left common and internal carotid arteries were ligated and the external carotid artery was isolated and incised. Next, a silicon-covered nylon filament (Doccol) was introduced into the carotid artery and advanced to the MCA for 1 hour. After withdrawal of the filament after 1 hour of ischemia, reperfusion of the MCA through the circle of Willis was allowed for 30 minutes before opening of the ipsilateral carotid artery to allow complete cerebral reperfusion.

For the permanent MCAO model (24 hours of ischemia), the filament was introduced to the MCA for 24 hours.12 Neurological deficits and infarct volume were measured 24 hours after MCAO.

Neurological Scoring
Mice were examined using a 4-point scale as described previously.13 Briefly, flexion of the contralateral torso at the time of lifting the mouse was scored as 1, circling as 2, leaning to the contralateral side as 3, and no spontaneous motor activity as 4.

Infarct Volume
Brain coronal sections were stained with 2,3,5-triphenyltetrazolium chloride. Infarct sizes were determined by the indirect method (contralateral hemisphere volume minus ipsilateral hemisphere non-ischemic volume) as described previously.1

Distal MCAO
Mice were anesthetized, intubated, paralyzed (pancuronium bromide, 0.4 mg/kg intraperitoneally, given every 45 minutes), mechanically ventilated (SAR-830; CWE), and placed in a stereotaxic frame (Kopf) as described previously.1 Arterial blood pressure was continuously monitored, and arterial blood gases (Po2, and Pco2) and pH were measured every 30 minutes. A burr hole (2-mm diameter) was drilled over the MCA above the zygomatic arch, and the MCA was occluded using a microvascular clip (Zen clip; Oswa).

Laser Speckle Imaging
The areas of severe (0% to 20% residual blood flow) and moderate (21% to 30% residual blood flow) blood flow deficits were quantified using a thresholding paradigm as previously described.1

Statistics
Statistical analyses were performed using STATA 8.0. The Student t test was used to compare variables describing baseline characteristics. Cerebrovascular anatomy parameters were compared using the Mann-Whitney U or an unpaired t test. Two-way analysis of variance with Bonferroni post hoc testing was used when comparing infarct volumes. The Kruskal-Wallis test was implemented to assess differences in neurological score. CBF measurements and carotid artery relaxation were compared for the 2 genotypes using 2-way analysis of variance with repeated measures. All data are presented as mean±SD (or SEM, when indicated) with differences considered significant for P<0.05.

Results
Blood pressure was similar in WT and sGCα1−/− mice before and during ischemia (Table). Plasma glucose levels were similar in WT and sGCα1−/− mice both before (146±17 mg/dL, versus 133±15 mg/dL, respectively, n=10 for both) and 1 hour after ischemia (144±39 mg/dL, versus 135±78 mg/dL, respectively, n=10 for both, P=nonsignificant). Serum NOx levels at baseline did not differ between WT and sGCα1−/− mice (35±16 μmol/L, n=4 and 33±5 μmol/L, n=5, respectively). Similarly, brain NOx levels did not differ between WT and sGCα1−/− mice (13±6, n=4 and 8±4, n=5, respectively). Cerebrovascular anatomy did not differ between the groups (see Supplemental Figure I and Supplemental Table I, available online at http://stroke.ahajournals.org).

To evaluate the role of sGCα1,β1-derived cGMP in the response to cerebral ischemia and reperfusion, WT and sGCα1−/− mice were subjected to 1 hour of MCAO followed by 23 hours of reperfusion. Infarct volumes were larger in sGCα1−/− mice than in WT mice (104±31 versus 56±26 mm3, respectively; P<0.01; Figure 1). Neurological
scoring revealed more severe deficits in sGCα1−/− mice than in WT mice (Figure 2). Neurological scores were normal in sham-operated sGCα1−/− and WT mice (score=0, n=5 for both).

To determine whether the differences in stroke size and neurological outcome were associated with differences in blood flow during ischemia, we mapped the cortical CBF deficit with high spatial resolution using laser speckle flowmetry during distal MCAO. The areas of severe or moderate CBF reduction (≥20% or 21% to 30% residual CBF, respectively, compared with preischemic baseline) did not differ between WT and sGCα1−/− mice (Figure 3). Consistent with laser speckle flowmetry, laser Doppler flowmetry also did not detect any difference in cortical CBF between WT and sGCα1−/− mice during filament-induced MCAO (Figure 4). However, CBF was 45% less in sGCα1−/− than in WT mice during 1 hour of reperfusion (P<0.05; Figure 4). Together, these data suggest that sGC is a critical determinant of CBF recovery during reperfusion.

After permanent MCAO without reperfusion, infarct sizes (Figure 1) and neurological deficits (Figure 2) did not differ between WT and sGCα1−/− mice. These findings further support incomplete reperfusion as the likely mechanism of larger infarcts after transient ischemia in sGCα1−/− mice. Infarct volumes were smaller in WT mice subjected to 1 hour of MCAO followed by 23 hours of reperfusion than in WT mice subjected to 24 hours of MCAO without reperfusion. In contrast, infarct size was similar in sGCα1−/− mice subjected to 1 hour of MCAO followed by 23 hours of reperfusion and in sGCα1−/− mice subjected to 24 hours of MCAO without reperfusion.

To examine the possibility that altered vascular reactivity, previously suggested to modulate stroke-induced injury, affects the outcome after MCAO in WT and sGCα1−/− mice, vascular relaxation in response to ACh and SNP was compared in isolated
Figure 4. Effect of sGCα1 deficiency on CBF, measured by laser Doppler flowmetry (LDF), during 1 hour of MCAO, 30 minutes of MCA reperfusion, and 30 minutes of carotid artery reperfusion. N=10 and 9 for WT and sGCα1-deficient mice (sGCα1−/−), respectively. Data are shown as mean±SEM (*P<0.05 between WT and sGCα1−/− mice).

Discussion

In this study, mice deficient in sGCα1 were used to investigate the role of cGMP generated by sGCα1β1 in a murine model of brain injury induced by ischemia and reperfusion. Our results suggest that cGMP generated by sGCα1β1 attenuates the stroke damage associated with cerebral ischemia and reperfusion. Infarct size was larger and the neurological outcome was worse in sGCα1−/− mice than in WT mice subjected to 1 hour MCAO and 23 hours of reperfusion.

Male sGCα1−/− mice on a 129S6 background (sGCα1−/−S6 mice) develop systemic hypertension, whereas male sGCα1−/− mice on a C57BL6 background are normotensive. Because hypertension is a known risk factor for stroke, sGCα1−/− mice on a C57BL6 background were used in the current study. sGCα1−/− mice are normotensive, both before and during MCAO, indicating that increased blood pressure does not underlie the increased vulnerability of sGCα1−/− mice to cerebral ischemia-reperfusion.

Interestingly, passive diameters of carotid arteries were smaller in sGCα1−/− mice than in WT mice. Because vascular architecture is an important determinant of the infarct volume after MCAO and can differ among different mouse strains, we compared the anatomy of vessels in the brains of the WT and sGCα1−/− mice. The observation that the vascular architecture is similar in WT and sGCα1−/− mice suggests that blood flow alterations, correlating with structural modifications of blood vessels, do not underlie the different outcome between WT and sGCα1−/− mice after ischemia–reperfusion.

The ability of NO (produced by an NO donor compound or endogenously produced in response to ACh) to induce vasorelaxation was partially impaired in carotid arteries isolated from sGCα1−/− mice. We previously reported that both endothelium-dependent and endothelium-independent vascular relaxation were attenuated in aortic and femoral artery rings isolated from sGCα1−/−S6 mice, highlighting the importance of sGCα1β1-derived cGMP in the vascular smooth muscle cell layer for NO-induced relaxation. Impaired vascular relaxation may underlie the more marked reperfusion deficit, characterized by lower CBF, seen in sGCα1−/− mice than WT mice after MCA reperfusion. Although low levels of cGMP generated by sGCα1β1 are sufficient to mediate, at least in part, the effects of NO in the systemic cardiovasculature, the pulmonary vasculature of sGCα1−/− mice completely failed to dilate in response to NO, suggesting that pulmonary vessels critically rely on sGCα1 for vasodilation in response to NO. The observation that CBF is lower in sGCα1−/− mice than in WT mice during the first hour after MCA reperfusion suggests that vasorelaxation of the cerebral vasculature during reperfusion may rely on sGCα1 as well. Furthermore, more pronounced CBF deficits in sGCα1−/− mice than in WT mice during the first hour of reperfusion argue against the ability of endothelial-derived hyperpolarizing factor to preserve CBF during reperfusion in a setting of impaired NO-cGMP signaling.

Drugs that can improve endothelial function and cerebrovascular reactivity were previously shown to limit the infarct volume in rat models of cerebral ischemia. Furthermore, impaired vascular relaxation in NOS3-deficient mice was previously suggested to affect CBF during cerebral ischemia and to influence stroke size. Importantly, residual CBF during ischemia was less in NOS3-deficient mice than in WT mice, suggest-
ing that NOS3 activity is critical to sustain blood flow in focal cerebral ischemia.\(^1\) In contrast, residual CBF, measured by either laser Doppler flowmetry or laser speckle flowmetry, was similar during MCAO in WT and sGC\(\alpha_1\beta_1\) mice, suggesting that cGMP generated by sGC\(\alpha_1\beta_1\) is not an essential regulator of blood flow during MCAO. Importantly, the observation that infarct size and neurological outcome were similar in WT and sGC\(\alpha_1\beta_1\) mice in a model of permanent MCAO suggests that although sGC\(\alpha_1\) deficiency predisposes mice to reperfusion-injury induced injury, it does not affect perfusion through the collateral microvessels during MCAO.

CBF was lower in sGC\(\alpha_1\beta_1\) mice than in WT mice during reperfusion, suggesting that cGMP generated by sGC\(\alpha_1\beta_1\) regulates CBF during reperfusion. The finding that cerebral ischemia–reperfusion injury is exacerbated in sGC\(\alpha_1\beta_1\) mice, together with the observation that NOS3 modulates vascular reactivity and CBF and limits stroke size in a mouse model of cerebral ischemia–reperfusion,\(^1\) suggests that the protective effects of NO produced by NOS3 may be mediated by cGMP generated by sGC\(\alpha_1\beta_1\)-derived cGMP. Additional studies are required to determine whether the effects of NOS1 or NOS3-derived NO on the outcome of stroke are mediated by cGMP.

Although our results show that vascular reactivity at baseline and changes in blood flow after ischemia–reperfusion are affected by sGC\(\alpha_1\) deficiency, we cannot exclude the possibility of other mechanisms contributing to the adverse impact of sGC\(\alpha_1\) deficiency on the outcome of cerebral ischemia–reperfusion. For example, in platelets, sGC\(\alpha_1\beta_1\) mediates NO-induced inhibition of aggregation.\(^6\) Therefore, decreased reperfusion in sGC\(\alpha_1\beta_1\) mice may be, at least in part, secondary to the prothrombotic effect of sGC\(\alpha_1\) deficiency. Alternatively, the ability of cGMP to modulate delayed cerebral vasospasm\(^17\) or leukocyte-endothelial interactions,\(^18\) or a direct negative impact of sGC\(\alpha_1\) deficiency on the viability\(^19\) and function\(^20\) of neuronal cells may also underlie the increased stroke size and worse neurological outcome associated with cerebral ischemia and reperfusion in sGC\(\alpha_1\beta_1\) mice.

**Conclusion**

Impaired vascular reactivity and diminished CBF during reperfusion correlated with greater cerebral ischemic damage in sGC\(\alpha_1\beta_1\) mice than WT mice. Our findings indicate that sGC\(\alpha_1\beta_1\)-derived cGMP preserves CBF in ischemia–reperfusion injury. Accordingly, compounds that can enhance cGMP signaling, either by inhibiting catabolism of cGMP (eg, phosphodiesterase 5 inhibitors\(^16\)) or by increasing cGMP production (with sGC stimulators or activators\(^20\)), should be investigated for their therapeutic potential in stroke. Further elucidation of the exact role of sGC and its isoforms may provide important insights into the potential therapeutic benefit of existing sGC activating compounds and might promote the search for new, sGC isoform-specific compounds.

**Sources of Funding**

This study was supported by an American Heart Association Scientist Development Grant 0835344N (D.N.A.), National Institutes of Health grant T32-HL007208 (R.M.), grants from the Fonds Wetenschappelijk Onderzoek–Vlaanderen (FWO-Vlaanderen) and the Geconcerteerde Onderzoeksacties (P.B.), and National Institutes of Health grants P01-NS055104 (C.A.), P05-NS101828 (M.A.M.), and R01-NS033335 (P.L.H.).

**Disclosures**

None.

**References**


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Stroke. 2010;41:1815-1819; originally published online July 1, 2010; doi: 10.1161/STROKEAHA.109.577635
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2010 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

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