Protein nitration impairs the myogenic tone of rat middle cerebral arteries in both ischemic and nonischemic hemispheres after ischemic stroke

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Coucha M, Li W, Johnson MH, Fagan SC, Ergul A. Protein nitration impairs the myogenic tone of rat middle cerebral arteries in both ischemic and nonischemic hemispheres after ischemic stroke. Am J Physiol Heart Circ Physiol 305: H1726–H1735, 2013. First published October 4, 2013; doi:10.1152/ajpheart.00535.2013.—The myogenic response is crucial for maintaining vascular resistance to achieve constant perfusion during pressure fluctuations. Reduced cerebral blood flow has been reported in ischemic and nonischemic hemispheres after stroke. Ischemia-reperfusion injury and the resulting oxidative stress impair myogenic responses in the ischemic hemisphere. Yet, the mechanism by which ischemia-reperfusion affects the nonischemic side is still undetermined. The goal of the present study was to determine the effect of ischemia-reperfusion injury on the myogenic reactivity of cerebral vessels from both hemispheres and whether protein nitration due to excess peroxynitrite production is the underlying mechanism of loss of tone. Male Wistar rats were subjected to sham operation or 30-min middle cerebral artery occlusion/45-min reperfusion. Rats were administered saline, the peroxynitrite decompositon catalyst 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrinato iron (III), or the nitration inhibitor epicatechin at reperfusion. Middle cerebral arteries isolated from another set of control rats were exposed to ex vivo oxygen-glucose deprivation with and without glycoprotein 91 tat (NADPH oxidase inhibitor) or N\(^{-}\)-nitro-L-arginine methyl ester. Myogenic tone and nitrotyrosine levels were determined. Ischemia-reperfusion injury impaired the myogenic tone of vessels in both hemispheres compared with the sham group (P < 0.001). Vessels exposed to ex vivo oxygen-glucose deprivation experienced a similar loss of myogenic tone. Inhibition of peroxynitrite parent radicals significantly improved the myogenic tone. Peroxynitrite scavenging or inhibition of nitration improved the myogenic tone of vessels from ischemic (P < 0.001 and P < 0.05, respectively) and nonischemic (P < 0.01 and P < 0.05, respectively) hemispheres. Nitration was significantly increased in both hemispheres versus the sham group and was normalized with epicatechin treatment. In conclusion, ischemia-reperfusion injury impairs vessel reactivity in both hemispheres via nitration. We suggest that sham operation rather than the nonischemic side should be used as a control in preclinical stroke studies.

contralateral hemisphere; ischemia-reperfusion injury; myogenic tone; nitration; peroxynitrite

ACUTE ISCHEMIC STROKE (AIS) is the fourth leading cause of death and disability in the United States (38). Currently, the only effective strategy for the management of AIS is the intravenous administration of recombinant tissue plasminogen activator to reestablish blood flow and salvage brain tissue. Vascular protection has become a promising strategy in the management of stroke. A well-functioning vasculature with a well-optimized cerebral blood flow (CBF) is essential to supply the necessary nutrients and even the requisite concentration of neuroprotectant to the jeopardized tissue while at the same time avoiding hemorrhage. Reduced CBF has been reported in the ischemic as well as nonischemic hemisphere after stroke; this phenomenon is known as cross-diaschisis (23). This persistent reduction in contralateral blood flow contributes to poor outcomes and recovery (36). However, less attention has been given to elucidating the underlying mechanisms of contralateral vascular dysfunction and how this can play a pivotal role in the salvage of the penumbra and subsequently improving stroke outcomes.

Cerebrovascular autoregulation is essential for maintaining constant CBF despite changes in cerebral perfusion pressure (29, 32). Large arteries, e.g., middle cerebral arteries (MCA), are major sites for vascular resistance, and they contribute significantly to the process of autoregulation in the brain (30). The myogenic response describes the intrinsic ability of smooth muscle cells to constrict in response to elevated pressure, and it contributes significantly to the autoregulation of CBF (6, 13, 15, 30). Therefore, it is essential to ensure a well-optimized myogenic tone to avoid detrimental consequences of the loss of myogenic tone that results in hemorrhage and edema after stroke. Several studies (2, 3) have investigated the impact of ischemia-reperfusion (I/R) injury on the myogenic tone of cerebral vessels isolated from the ischemic hemisphere, but, until now, the exact mechanism underlying the loss of tone is not fully understood. Moreover, the effect of I/R injury on the behavior of cerebral vessels from the nonischemic hemisphere has long been neglected; in addition, the contralateral hemisphere has long been used as a control in various previous studies.

Peroxynitrite is a powerful mediator of ischemic injury via acute and chronic posttranslational modifications of key factors. It has been shown that peroxynitrite can nitrosylate tyrosine residues on contractile proteins, leading to the depolymerization of F-actin and vasodilatation (25). However, the impact of peroxynitrite on vascular tone is complex and poorly understood, and the exact mechanism by which peroxynitrite leads to myogenic dysfunction remains undetermined. Therefore, mechanistic studies addressing this issue as well as identifying potential targets for peroxynitrite-induced myogenic dysfunction would be valuable in establishing new therapeutic strategies for AIS. In the present study, we tested the hypothesis that I/R injury impairs the myogenic tone in both ischemic and nonischemic hemispheres via increased peroxynitrite generation and protein nitration.

MATERIALS AND METHODS

Animals. All experiments were performed on weight-matched (250–350 g) male Wistar rats (Harlan, Indianapolis, ID). Animals were
housed at the animal care facility at Georgia Regents University, which is approved by the American Association for Accreditation of Laboratory Animal Care. All protocols were approved by the institutional Animal Care and Use Committee. Animals were fed standard rat chow and tap water ad libitum. All animals were euthanized by decapitation after being anesthetized with pentobarbital sodium ( Fatal-Plus, Vortech Pharmaceuticals, Dearborn, MI).

Model of ischemia and drug treatments. Focal cerebral ischemia was achieved using the monofilament suture MCA occlusion (MCAO) model previously described by our group (9). In brief, the middle cerebral artery (MCA) was isolated from both ischemic (IS) and nonischemic (NIS) hemispheres of the MCAO group and right (RT) and left (LT) sides of the sham group. Isolated vessels were pressurized using an arteriograph system.

**Fig. 1.** Ischemia-reperfusion (I/R) injury impairs the functional and mechanical properties of vessels in both ischemic (IS) and nonischemic (NIS) hemispheres. Wistar rats were exposed to 30-min middle cerebral artery (MCA) occlusion (MCAO)/45-min reperfusion or sham surgery (sham group). MCAs were isolated from both IS and NIS hemispheres of the MCAO group and right (RT) and left (LT) sides of the sham group. Isolated vessels were pressurized using an arteriograph system. A: myogenic tone (in %) of MCAs from both IS and NIS hemispheres were significantly decreased compared with sham RT and LT sides, respectively. B: myogenic tone (in %) in MCAs for each experimental group at 40 and 160 mmHg. C: passive vasodilation was significantly impaired in the IS hemisphere compared with the sham RT side but was similar in the NIS hemisphere and sham LT side. D: vessels from both IS and NIS hemispheres were stiffer than sham RT and sham LT sides, respectively. The $b$-coefficient ($b$-coefficient) is the slope of the stress-strain curve. E: cerebral blood flow (CBF; in %) in both hemispheres after 30-min MCAO. CBF was reduced in IS and NIS hemispheres compared with baseline, albeit to a different degree. $n = 8-9$. $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs. the sham RT side; $+P < 0.01$ and $^{***}P < 0.001$ vs. the sham LT side; $^#P < 0.001$ and $^{**}P < 0.01$ vs. baseline.

**Fig. 2.** Inhibition of peroxynitrite parent radicals nullified the effect of hypoxia on myogenic tone. MCAs isolated from control Wistar rats were exposed to 30 min (30') of oxygen-glucose deprivation (OGD) followed by 20 min of reoxygenation. During reoxygenation, glycoprotein (gp)91 tat, N$^\ominus$-nitro-L-arginine methyl ester (L-NAME), or catalase was added, and myogenic tones of the isolated vessels were determined across the pressure range. A: vessels exposed to ex vivo hypoxia experienced a loss of myogenic tone. The reduction of myogenic tone (in %) by OGD was similar to I/R injury. Treatment with gp91 tat restored the myogenic tone to normal. B: treatment with L-NAME significantly improved the myogenic tone. C: treatment with catalase could not restore tone to normal after exposure to 30'OGD. $n = 5–6$. $^{**}P < 0.01$ vs. sham RT side, 30'OGD and gp91 tat, and $^{***}P < 0.001$ vs. 30'OGD.
right MCA was occluded with 19- to 21-mm 3-0 surgical naylon filament. The suture was introduced from the external carotid artery into the internal carotid artery to block the origin of the MCA. Cipolla et al. (2, 3) previously reported that 30-min MCAO/45-min reperfusion is the threshold duration for the loss of myogenic tone. In our study, the same durations were used to evaluate the effect of I/R injury on the myogenic tone of vessels from both hemispheres. Assessment of MCAO and reperfusion was achieved by measuring blood flow using a scanning laser-Doppler (Perimed, North Royalton, OH). In sham-operated (sham) groups, animals were dissected in the neck region to locate the common carotid but were not subjected to MCAO and were euthanized similarly. The peroxynitrite decomposition catalyst 5,10,15,20-tetrakis(4-sulfonatophenyl)prophyrinato iron (III) (FeTPPs; 20 mg/kg ip, Calbiochem, San Diego, CA) (8, 22, 37), the selective nitration inhibitor (Epicatechin (30 mg/kg ip, Sigma-Aldrich, St. Louis, MO) (21), or the antioxidant N-acetyl-L-cysteine (NAC; 150 mg/kg ip, Sigma-Aldrich) (20) was administered in a single dose at reperfusion.

Pressurized arteriograph system. MCA segments from ischemic and nonischemic hemispheres were cannulated and pressurized in an arteriograph chamber, and pressure-diameter curves were obtained as previously described by our group (18). Briefly, MCA segments were pressurized at 15 mmHg for 1 h in HEPES bicarbonate buffer containing (in mM) 130 NaCl, 4 KCl, 1.2 MgSO4, 4 NaHCO3, 10 HEPES, 1.18 KH2PO4, 5.5 glucose, and 1.8 CaCl2 to develop spontaneous tone. Temperature was kept constant at 37 ± 0.5°C using a temperature controller. A video dimension analyzer connected to the arteriograph system was used to measure wall thickness and lumen diameter at pressures ranging from 0 to 180 mmHg in 20-mmHg increments. To determine the myogenic tone, pressure-diameter curves were obtained first in the presence of Ca2+ and then in Ca2+-free buffer with the addition of 0.2 mM papaverine hydrochloride.

Ex vivo hypoxia. MCAs isolated from control Wistar rats were exposed to ex vivo oxygen-glucose deprivation (OGD) for 30 min followed by 20 min of reoxygenation. To determine the contribution of peroxynitrite generation on myogenic tone, the NADPH oxidase inhibitor glycoprotein (gp)91 tat peptide (1 μM Anaspec), the nitric oxide synthase inhibitor N-nitro-L-arginine methyl ester (L-NAME; 0.3 mM, Sigma-Aldrich) (24), or catalase (1,000 U/ml, Sigma-Aldrich) (12) was added during reoxygenation. Pressure-diameter curves were obtained first in the presence of Ca2+ and then in Ca2+.
Ca\textsuperscript{2+}-free buffer with the addition of 0.2 mM papaverine hydrochloride, and myogenic tones across the pressure range were determined.

**Tissue markers of nitrosative stress.** Snap-frozen MCAs were homogenized as previously described by our group (10, 14). Total nitrotyrosine levels were determined via slot-blot analysis. In brief, equal amounts of the vessel lysate were immobilized onto a nitrocellulose membrane, and nitrotyrosine was detected using an anti-nitrotyrosine monoclonal antibody (Millipore, Lake Placid, NY). Relative levels of nitrotyrosine were quantified by densitometry software (Alpha Innotech). The total nitrotyrosine level in brain tissue was determined by immunohistochemistry. In brief, brains were processed in paraformaldehyde for 24 h and then in 30% sucrose in PBS. Brains were sectioned into 20-µm slices and stained for nitrotyrosine using a mouse anti-nitrotyrosine primary antibody at 1:500 and a goat anti-mouse secondary antibody at 1:1,000. Images were captured at ×10 magnification using an Axiovert 200 and processed using Zen 2008 software. ImageJ software (National Institutes of Health (NIH), Bethesda, MD) was used to quantify F/G-actin and nitrotyrosine staining.

**F/G-actin and nitrotyrosine staining and quantification.** MCA segments from ischemic and nonischemic hemispheres were pressure fixed in methanol-free 4% paraformaldehyde in PBS for 1 h at 15 mmHg. Whole mounted tissues were then stained for F-actin with an Oregon green 488-conjugated phalloidin fluorescent probe (Invitrogen, Carlsbad, CA), for G-actin with Alexa 594-conjugated DNase I (Invitrogen), and for nitrotyrosine with a mouse anti-nitrotyrosine primary antibody at 1:50 and a goat anti-mouse IgG conjugated to Cy5 (Molecular Probes) secondary antibody at 1:1,000. Z-stacks (5.5 µm, 0.3-µm intervals) of the vascular smooth muscle (VSM) layer were captured at ×63 magnification using an LSM 510 upright confocal microscope and processed using Zen 2008 software. ImageJ software (NIH) was used to quantify F/G-actin and nitrotyrosine volume intensity.

**Data calculations.** Using the wall thickness and lumen diameter measurements obtained in active (with Ca\textsuperscript{2+}) and passive (without Ca\textsuperscript{2+}) conditions, the following parameters could be measured: percent myogenic tone (%tone) = 1 − (active outer diameter/passive outer diameter) × 100; circumferential stress = (intraluminal pressure × passive lumen diameter)/2 × wall thickness; and circumferential strain = (passive lumen diameter − passive Lumen diameter at 0 mmHg)/passive lumen diameter at 0 mmHg. The β-coefficient is the slope of the stress-strain curve (a higher β-value is indicative of a stiffer vessel).

**Statistics.** The area under the curve was calculated across intraluminal pressures for vessels from each animal for myogenic tone (40–180 mmHg) and passive lumen diameter (0–180 mmHg) using NCSS 2007 (NCSS, Kaysville, UT) and was used in the analyses for these variables. Vessels from the ischemic side of the stroked brain were compared with vessels from the right side of a sham brain, and, likewise, vessels from the nonischemic side of the stroked brain were compared with vessels from the left side of a sham brain. A two-sample t-test was used to compare the myogenic tone, passive lumen diameter, and β-coefficient for sham and stroke animals (sham right side vs. ischemic side and sham left side vs. nonischemic side).

Fig. 4. The peroxynitrite-mediated loss of myogenic tone involves nitration. Wistar rats were subjected to 30-min MCAO/45-min reperfusion, and the selective nitration inhibitor epicatechin (EC; 30 mg/kg ip) was administered at reperfusion. MCAs were isolated from both hemispheres and pressurized in an arteriograph chamber. Treatment with EC restored the myogenic tone in IS (A) and NIS (B) hemispheres compared with the sham group at both pressure points. Sham rats treated with EC experienced a loss of myogenic tone in vessels isolated from the LT side (B), but EC had no effect on the RT side (A) compared with the untreated sham group. n = 6–9. *P < 0.05 and **P < 0.01 vs. the IS hemisphere; +P < 0.05 vs. the NIS hemisphere; #P < 0.05 vs. the sham LT side.
One-way ANOVA was used to compare the myogenic tone for sham, sham/H11001 NAC, and comparable vessels for stroke animals as well as passive lumen diameters for all comparisons. A series of 2 stroke (no vs. yes) treatment (no vs. yes) ANOVAs with interactions were used to determine the effect of FeTPPs and epicatechin on myogenic tone and nitrotyrosine levels of vessels from sham and stroke animals for the right and left sides of the brain. SAS 9.3 (SAS, Cary, NC) was used for all analyses. Statistical significance was determined at α < 0.05, and a Tukey’s post hoc test was used to compare means from significant ANOVAs. Results are presented as means ± SE.

RESULTS

Effect of I/R injury on vessel reactivity. MCAs isolated from sham right and left sides showed similar myogenic behavior in response to transmural pressure, where they developed myogenic tone at an early pressure point at 40 mmHg and were able to hold their percent myogenic tone at high pressure points (160 mmHg). Pressure-myogenic tone curves were significantly impaired in arteries isolated from ischemic and nonischemic hemispheres compared with the sham group (Fig. 1A). Arteries from ischemic hemispheres showed lower myogenic tone at both 40 and 160 mmHg, whereas vessels from nonischemic sides developed myogenic tone similar to sham at 40 mmHg but were unable to hold myogenic tone at 160 mmHg (Fig. 1B). The ability of vessels from the ischemic hemisphere to vasodilate under passive conditions was significantly impaired after I/R injury compared with vessels isolated from the sham right hemisphere (Fig. 1C). The β-coefficient was significantly increased in vessels from both ischemic and nonischemic hemispheres compared with the sham group, indicating that I/R increases the stiffness of vessels from both hemispheres compared with the sham group, indicating that I/R increases the stiffness of vessels from both hemispheres (Fig. 1D). CBF was reduced in both hemispheres after stroke, albeit to a different degree compared with baseline (Fig. 1E).

Effect of ex vivo hypoxia on vessel reactivity. Vessels exposed to 30-min OGD showed a similar reduction of myogenic tone as vessels isolated from the ischemic hemisphere, indicating that our OGD model is a valid ex vivo method to study...
the impact of hypoxia on the behavior of cerebral vessels (Fig. 2A). Inhibition of peroxynitrite parent radicals, superoxide using gp91 tat peptide or nitric oxide using l-NAME, significantly improved the myogenic tone of isolated vessels compared with the sham group (Fig. 2, A and B). To exclude the effect of the peptide on myogenic tone, scrambled gp91 tat peptide was added during reoxygenation. The addition of the scrambled peptide had no effect on myogenic tone compared with gp91 tat peptide (data not shown). Inhibition of hydrogen peroxide by catalase did not alter the myogenic tone (Fig. 2C).

**Effect of scavenging peroxynitrite on vessel reactivity.** To determine the effect of peroxynitrite on vessel reactivity, rats were treated with FeTPPs, a selective peroxynitrite scavenger, at reperfusion. Peroxynitrite scavenging with FeTPPs restored myogenic tone in the ischemic hemisphere at both pressure points, 40 and 160 mmHg, and improved passive vasodilation (Fig. 3, A and C). FeTPPs treatment normalized the percent myogenic tone in the nonischemic hemisphere at 160 mmHg and had no effect on passive vasodilation (Fig. 3, B and D). In sham animals treated with FeTPPs, levels of percent myogenic tone were decreased in vessels isolated from the right and left hemispheres at 40 and 160 mmHg, respectively (Fig. 3, A and B).

**Mechanism of the peroxynitrite-mediated loss of vessel reactivity.** To investigate whether the increased nitration by peroxynitrite is one of the mechanisms underlying the loss of tone and impaired vasodilation after I/R injury, rats were treated with epicatechin, a flavonoid and one of the green tea extracts, which selectively blocks the ability of peroxynitrite to

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**Fig. 7. Effects of I/R injury on vascular smooth muscle actin cytoskeleton and total protein nitration.** MCA segments from the ischemic hemisphere were pressure fixed and stained for F-actin with an Oregon green 488-conjugated phalloidin fluorescent probe, for G-actin with Alexa 594-conjugated DNase I, and for nitrotyrosine with an anti-nitrotyrosine primary antibody and an IgG conjugated to Cy5 secondary antibody. **A:** representative confocal microscopy images of MCA segments stained for F- and G-actin cytoskeletal filaments and the colocalization of actin filaments with nitrotyrosine. **B and C:** I/R injury mediated a decrease in the ratio of F-actin to G-actin (F/G actin; B) and increased vascular smooth muscle nitrotyrosine levels (C) in vessels isolated from the IS sides. Inhibition of nitration normalized this ratio. n = 3. *P < 0.05 vs. IS hemisphere + vehicle; **P < 0.01 vs. the sham RT side + vehicle; #P < 0.05, disease-treatment interaction.
nitrate tyrosine residues but has no effect on thiol oxidation (1, 7, 35). Prevention of nitrination with epicatechin was able to restore the myogenic tone in both hemispheres at both pressure points (Fig. 4, A and B). Interestingly, in sham animals treated with epicatechin, the levels of myogenic tone at 40 and 160 mmHg were significantly reduced in vessels isolated from the left hemisphere only (Fig. 4, A and B).

Tyrosine nitrination assessment in isolated MCAs. Nitrotyrosine levels in MCAs isolated from both ischemic and non-ischemic hemispheres were measured using slot blot. Nitrination was significantly increased in MCAs isolated from both hemispheres compared with the sham group. Treatment with epicatechin efficiently reduced the extent of nitrination back to normal (Fig. 5, A and B). Nitrotyrosine in brain tissue sections was significantly increased in both hemispheres compared with the sham group. Epicatechin treatment restored nitrotyrosine levels in both sides (Fig. 6, A–D).

F- and G-actin quantification. Using confocal imaging, we determined the effect of I/R on the ratio of F-actin to G-actin. VSM layers in MCAs isolated from both hemispheres were identified with their characteristic parallel arrangements of cells, and Z-stacks of these regions of interest were captured and processed (Figs. 7A and 8A). Ratios of F-actin to G-actin were reduced in both ischemic and nonischemic MCAs compared with the sham group, and inhibition of nitrination with epicatechin restored the ratios of F-actin to G-actin to normal levels (Figs. 7B and 8B). Nitrotyrosine staining was significantly pronounced in vessels isolated from ischemic hemispheres compared with vessels isolated from sham right hemispheres (Fig. 7C), and epicatechin treatment significantly reduced nitrination in vessels from both ischemic and nonischemic hemispheres (Figs. 7C and 8C).

Oxidation as a regulator of myogenic tone. To determine the role of oxidation in myogenic tone development under normal conditions, sham rats were treated with NAC. Inhibition of oxidation with NAC drastically reduced the myogenic tone in vessels isolated from the right hemisphere at 40 and 160 mmHg (Fig. 9A), whereas it had no effect on vessels from the left hemisphere (Fig. 9B).

DISCUSSION

Our results indicate that I/R injury significantly impairs myogenic tone in MCAs isolated from both ischemic and contralateral hemispheres. The loss of myogenic tone was restored by peroxynitrite scavenging or inhibition of nitrination, suggesting that increased peroxynitrite generation after I/R injury is involved in the loss of myogenic tone in both ischemic and contralateral hemispheres.

Autoregulation of CBF ensures the appropriate blood supply to the brain while complying with the limited space available in the cranium. Cerebrovascular autoregulation is essential for

![Image](http://ajpheart.physiology.org/)

**Fig. 8.** Effects of I/R injury on vascular smooth muscle actin cytoskeleton and total protein nitration. MCA segments from nonischemic hemisphere were pressure fixed and stained for F-actin with Oregon green 488-conjugated phalloidin fluorescent probe, for G actin with Alexa 594-conjugated DNsase I, and for nitrotyrosine with an anti-nitrotyrosine primary antibody and an IgG conjugated to Cy5 secondary antibody. A: representative confocal microscopy images of MCA segments stained for F- and G-actin cytoskeletal filaments and the colocalization of actin filaments with nitrotyrosine. White arrows indicate parts with increased nitrination. B: I/R injury mediated a decrease in the ratio of F-actin to G-actin ratio. C: inhibiting nitrination with EC normalized the ratio of F-actin to G-actin and reduced nitrotyrosine levels. n = 3. +P < 0.01 vs. NIS hemisphere + vehicle; #P < 0.05, disease-treatment interaction.
the protection of downstream microvessels from changes in perfusion pressure. The myogenic behavior of cerebral vessels significantly contributes to autoregulation (13, 30). They play a central role in preventing increases in blood flow during increases in perfusion pressure. Therefore, maintenance of the intrinsic myogenic tone is crucial for achieving optimum vascular resistance. It is well known in the literature that CBF is maintained relatively constant provided that cerebral perfusion pressure is between 60 and 160 mmHg (4, 28). We found that our vessels were able to develop a myogenic response at an early pressure in the range of 40–160 mmHg. Therefore, we were interested in determining the effect of I/R injury on myogenic tone at these pressure points. Cipolla et al. (2, 3) previously reported that I/R leads to loss of tone of cerebral vessels isolated from ischemic hemispheres with a threshold duration of ischemia between 15 and 30 min and 45 min of reperfusion. We used the same duration of 30-min MCAO/45-min reperfusion to study the impact of I/R injury on the myogenic reactivity of MCAs isolated from ischemic and contralateral hemispheres and compared with sham animals. We were interested in studying both hemispheres because clinical studies have shown that there is impairment of CBF in the nonischemic hemisphere, a phenomenon known as crossed cerebellar diaschisis, and that if this impairment is prolonged, it may impact stroke severity and recovery (16, 23). However, the underlying mechanisms contributing to altered CBF in the nonischemic hemisphere remain unknown. Experimental studies have provided us with this opportunity to address this question and to compare the vascular responses in the non-ischemic hemisphere with sham controls. Rasmussen et al. (33) showed that permanent distal occlusion of the MCA increased the contractile response to angiotensin, endothelin, and 5-HT in segments downstream of the occlusion. We (5) have previously shown that ACh-induced maximum relaxation was reduced in basilar arteries isolated from rats subjected to MCAO. These finding suggest that interruption of blood flow could alter the vascular reactivity of large vessels away from the site of injury. In this study, we demonstrated that I/R injury impairs the myogenic tone of vessels from the ischemic and contralateral hemisphere; importantly, we also showed that vessels from both hemispheres were stiffer compared with the sham group, which could exacerbate brain damage, leading to poor stroke outcomes. Our results are consistent with those of a previous study (2) that showed that isolated MCAs from the nonischemic hemisphere had lower tone compared with the sham group after 30-min MCAO/6-h reperfusion; Winters et al. (39) reported that transient focal cerebral ischemia for 1 h could induce long-term global cerebrovascular dysfunction. In our study, we propose that I/R injury also has a short-term global effect on the vascular reactivity of MCAs from both hemispheres.

There is growing evidence showing that I/R injury leads to increased production of ROS, including superoxide and nitric oxide; their pathological roles are intensified because of the generation of the extremely reactive peroxynitrite radical in the ischemic brain. Peroxynitrite is a powerful nitrating agent that triggers a cascade of molecular events that can cause detrimental alterations in brain functions and exacerbate brain tissue damage (17). We investigated whether excess production of peroxynitrite after stroke contributes to the loss of myogenic tone. We found that inhibition of the generation of the peroxynitrite parent radicals superoxide and nitric oxide nullified the effect of hypoxia and significantly improved the tone toward control levels. To further confirm the role of peroxynitrite, rats were treated with FeTPPs or epicatechin at reperfusion. FeTPPs a selective peroxynitrite scavenger is an iron porphyrin complex that catalytically isomerizes peroxynitrite into nitrate (27, 34), whereas epicatechin selectively blocks nitration reactions but not oxidation induced by peroxynitrite (1, 35). Either peroxynitrite scavenging or inhibition of nitrination enhanced the myogenic tone in ischemic and contralateral hemispheres. Additionally, the peroxynitrite-induced loss of tone was associated with a significant increase in nitrotyrosine levels in isolated MCAs from both hemispheres, and treatment with epicatechin restored those levels back to normal. The results of the present study expand on those of the previous studies, which reported that 1) FeTPPs given during reoxygenation reduces increased nitrotyrosine levels in MCAs after 20 min of OGD (19); 2) peroxynitrite diminishes myogenic activity, which is accompanied with increased nitrotyrosine levels in isolated cerebral arteries (25, 26); and 3) peroxynitrite decomposition ameliorates vascular dysfunction induced by perfusion of MCAs with plasma from rats subjected to hyperglycemic stroke, suggesting that circulating factors found in...
plasma in response to I/R injury can affect cerebrovascular function in the nonischemic MCA (31). In a preliminary study, we investigated whether I/R injury could modify vascular function in vascular beds away from the site of injury. I/R caused a significant leftward shift and enhanced relaxation after preconstriction with 5-HT in mesenteric arteries compared with sham arteries. ACh sensitivity and the maximum response to ACh were significantly augmented after I/R in mesenteric arteries ($P < 0.01$ and $P < 0.05$ vs. sham arteries, respectively). Taken together, these findings strongly suggest that the impact of I/R injury on vascular reactivity is global and mediated via increased peroxynitrite generation after stroke.

Previous studies have reported that peroxynitrite-induced myogenic dysfunction is associated with a shift in F/G-actin equilibrium for G-actin via nitrosylation of F-actin (26). Our laboratory (19) has previously demonstrated that OGD of vessels isolated from normal Wistar rats exhibit a loss of tone due to decreases in ratios of VSM F-actin to G-actin and increased nitration in the actin cytoskeleton. Immunohistochemical staining was conducted to see whether vessels isolated from both hemispheres would exhibit impaired myogenic tone because of the reduction in ratios of F-actin to G-actin. In agreement with the aforementioned studies, we provide evidence showing that ratios of F-actin to G-actin were reduced in vessels isolated from both ischemic and nonischemic hemispheres; in addition, our results show that F-actin depolymerization was accompanied by an increase in nitrotyrosine levels. Although different studies have provided important information showing on how peroxynitrite can alter myogenic tone, the exact mechanism remains largely unclear.

The present study also showed that physiological low levels of peroxynitrite are crucial for myogenic tone development in both right and left hemispheres, as peroxynitrite scavenging with FeTPPs in sham animal significantly decreased the myogenic tone. Our unique findings provide evidence showing that a balanced redox state is required for myogenic tone development under normal conditions. These findings are consistent with other studies that demonstrated that both contraction and relaxation of VSM cells can occur, but this depends on the concentration and potential targets of peroxynitrite (11, 26). Interestingly, we report that peroxynitrite could act through different mechanisms for the establishment of myogenic tone across both hemispheres under normal conditions in the absence of ischemia, as inhibition of nitration had no effect on vessels from the right hemisphere but significantly reduced the tone of vessels isolated from the left hemisphere in sham animals. To further test the differential effect of peroxynitrite and to determine the role of oxidation in the development of myogenic tone in both hemispheres, we treated sham rats with the thiol donor and glutathione precursor NAC. We found that blunting physiological oxidation with NAC drastically impaired the tone in the right hemisphere but had no effect on the left hemisphere. These results may be explained by the exquisite nature of the brain, in which CBF is highly regulated by multiple coordinated mechanisms to ensure optimum levels of perfusion and favorable nutrient delivery at all times. Finally, it is worth emphasizing that a full understanding of how peroxynitrite regulates myogenic tone will permit the development of novel strategies to prevent vascular dysfunction in diseased conditions such as AIS.

In summary, we believe that this study provides evidence showing that I/R injury has a global effect on the myogenic tone of cerebral arteries, and, as such, in preclinical stroke studies, changes in the ischemic hemisphere should not only be compared with the nonischemic hemisphere but also with sham animals. Previous studies have shown that a persistent drop in CBF in the contralateral hemisphere leads to poor stroke outcomes; therefore, the restoration of myogenic reactivity after AIS in the nonischemic as well as ischemic hemisphere is of therapeutic value.

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DISCLAIMER
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AUTHOR CONTRIBUTIONS
Author contributions: M.C. and A.E. conception and design of research; M.C. and W.L. performed experiments; M.C., M.J., and A.E. analyzed data; M.C., M.J., and A.E. interpreted results of experiments; M.C. prepared figures; M.C. drafted manuscript; M.C., M.J., S.C.F., and A.E. edited and revised manuscript; M.C., W.L., M.J., S.C.F., and A.E. approved final version of manuscript.

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