Basic Sciences

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Pial Collateral Reactivity During Hypertension and Aging
Understanding the Function of Collaterals for Stroke Therapy

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Background and Purpose—We investigated vasoactive properties of leptomeningeal arterioles (LMAs) under normotensive conditions and during hypertension and aging that are known to have poor collateral flow and little salvageable tissue.

Methods—LMAs, identified as distal anastomotic arterioles connecting middle and anterior cerebral arteries, were studied isolated and pressurized from young (18 weeks) or aged (48 weeks) normotensive Wistar Kyoto (WKY18, n=14; WKY48, n=6) rats and spontaneously hypertensive rats (SHR18, n=16; SHR48, n=6). Myogenic tone and vasoactive responses to pressure as well as endothelial function and ion channel activity were measured.

Results—LMAs from WKY18 had little myogenic tone at 40 mm Hg (8±3%) that increased in aged WKY48 (30±6%). However, LMAs from both WKY groups dilated to increased pressure and demonstrated little myogenic reactivity, a response that would be conducive to collateral flow. In contrast, LMAs from both SHR18 and SHR48 displayed considerable myogenic tone (56±8% and 43±7%; $P<0.01$ versus WKY) and constricted to increased pressure. LMAs from both WKY and SHR groups had similar basal endothelial nitric oxide and IK channel activity that opposed tone. However, dilation to sodium nitroprusside, diltiazem and 15 mmol/L KCl was impaired in LMAs from SHR18.

Conclusions—This study shows for the first time that LMAs from young and aged SHR are vasoconstricted and have impaired vasodilatory responses that may contribute to greater perfusion deficit and little penumbral tissue. These results also suggest that therapeutic opening of pial collaterals is possible during middle cerebral artery occlusion to create penumbral tissue and prevent infarct expansion. (***Stroke***. 2016;47:1618-1625. DOI: 10.1161/STROKEAHA.116.013392.)

Key Words: cerebrovascular circulation ■ hypertension ■ ion channel ■ nitric oxide ■ nitroprusside ■ potassium channels ■ stroke

Collateral perfusion is emerging as a key variable in outcome from acute ischemic stroke.[1–3] Collateral status at the time of occlusion has been shown to be the strongest predictor for therapeutic recanalization and is being considered for use in clinical decision making in stroke treatment.[1–3] Importantly, patients with good collateral flow or a favorable pattern of leptomeningeal collateral vessels on computed tomographic angiography have better reperfusion, smaller infarcts, and less hemorrhagic transformation,[1,2] whereas patients with poor collateral status do poorly even if recanalization is achieved.[3]

Although several collateral systems exist within the brain vasculature, it is generally recognized that the leptomeningeal arterioles (LMAs) are particularly important for stroke outcome.[4–6] LMAs are secondary collaterals that connect distal branches of major cerebral artery territories.[4–6] During middle cerebral artery occlusion (MCAO), LMA anastomoses promote flow from the anterior cerebral artery (ACA) territory to sustain flow to the penumbra, a region of constrained blood supply that is potentially salvageable if reperfusion occurs rapidly or neuroprotective agents are present to arrest cell death.[7,8] In fact, the primary goal of neuroprotection for acute stroke is to salvage penumbral tissue at risk of infarction, further highlighting a critical role of LMAs in acute stroke therapy.

The ability to sustain or promote penumbral flow has been attributed to passive increases in bidirectional flow during an occlusion (eg, from ACA to MCA territories) that varies depending on the size and number of LMAs.[6,9,10] In addition, expansion of infarct to incorporate the penumbra has been attributed to collateral failure, but factors that contribute to this process are largely unknown because of our limited understanding of the vascular biology of LMAs.[5,8] In contrast to nonanastomotic pial arterioles that have been more extensively studied, LMAs experience unique hemodynamic forces, including low shear stress and bidirectional flow.[11] The influence of this unique environment may be associated with distinct functional properties of LMAs that could significantly influence collateral perfusion, penumbral flow, and

stroke outcome. Furthermore, understanding the functional properties of LMAs under normal and pathological conditions goes beyond identifying patients that will benefit from reperfusion therapies, but may provide for targeted therapy to extend the time for treatment in those patients that have poor outcome even with rapid recanalization with endovascular approaches.[12]

In this study, we investigated the vasoactive properties of LMAs that could actively influence vascular resistance and collateral flow. We developed methodology to study LMAs isolated and pressurized, as we have previously done with brain parenchymal arterioles.[13] We compared myogenic tone and vasoactive responses to changes in pressure as well as endothelial function and ion channel function in LMAs from normotensive Wistar Kyoto (WKY) and spontaneously hypertensive rats (SHR) that were young (18 weeks) or aged (48 weeks). The importance of understanding LMA function during hypertension and aging is that these factors are associated with poor collateral status and small penumbral tissue and more closely mimic the comorbid demographic of patients with stroke.[2,14–18] We hypothesized that compared with LMAs from normotensive animals, LMAs from hypertensive and aged animals are acutely vasoconstricted, a response that contributes to increased perfusion deficit and collateral failure during MCAO.[14–18]

Materials and Methods

Animals
Male SHR that were 18- or 48-weeks old (SHR18 and SHR48) and age-matched normotensive control WKY (WKY18 and WKY48) rats (Charles River) were used in this study. Animals were housed in the Animal Care Facility at the University of Vermont, an Association for Assessment and Accreditation of Laboratory Animal Care-accredited facility. Rats were maintained on a 12-hour light/dark cycle and allowed food and water ad libitum. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Vermont and complied with the National Institutes of Health guidelines for care and use of laboratory animals.

Identification and Isolation of LMA and Non-LMAs
Animals were decapitated under deep isoflurane anesthesia (3% in oxygen). The brain was removed and placed in cold, oxygenated physiological saline solution. LMAs were identified on the surface of the brain within the leptomeninges that connected the MCA and ACA branches (Figure 1A). A vascular segment was then carefully dissected that included an MCA branch, an LMA, and an ACA branch. LMAs were identified as the smallest segment between the 2 larger MCA and ACA branches at both the ends. LMAs were then mounted onto glass

cannulas in an arteriograph chamber (Living Systems Instrumentation, Burlington, VT; Figure 1B). Non-LMAs were identified as pial arterioles that did not connect the MCA and ACA branches and were taken from the same animals and studied similarly as LMAs.

Experimental Protocols
LMAs and non-LMAs were equilibrated at 20 mm Hg for 1 hour to allow spontaneous development of myogenic tone, after which intravascular pressure was increased stepwise to 80 mm Hg to measure myogenic reactivity. Lumen diameter was recorded at each pressure. Intravascular pressure was returned to 60 mm Hg for the remainder of the experiment. In separate sets of experiments, reactivity to various pharmacological agents were measured: NS309, a small- and intermediate-conductance calcium-activated potassium channel (SK/IK) agonist; NG-nitro-L-arginine methyl ester (10^{-3} M), a nitric oxide (NO) synthase inhibitor; sodium nitroprusside (SNP), norepinephrine, diltiazem; KCl in the absence and presence of the inward rectifier potassium (K_{ir}) channel blocker $BaCl_2$; and apamin and 1-[(2-chlorophenyl) diphenylmethyl]-1H-pyrazole (TRAM-34), inhibitors of SK and IK channels, respectively. Passive structural measurements were obtained in fully relaxed arterioles at the end of each experiment. Please see online-only Data Supplement for detailed protocols.

Determination of Perivascular Innervation
To determine whether LMAs are innervated, immunohistochemical staining of perivascular nerves of LMAs with the pan neuronal marker protein gene product 9.5 was performed, as previously described with modifications.[19] Please see online-only Data Supplement for details.

Drugs and Solutions
Details on Drugs and Solutions are available in the online-only Data Supplement.

Data Calculations and Statistical Analysis
All results are presented as mean±SEM. Differences between ≥3 groups were determined by 1-way ANOVA with a post hoc Tukey or Dunnett multiple comparison test, where appropriate. Differences between 2 groups were determined by *t* test. Differences were considered significant at $P<0.05$. Please see online-only Data Supplement for all data calculations.

Results

Effect of Hypertension and Aging on LMA Myogenic Reactivity and Tone
To determine if LMAs respond myogenically, diameter changes in response to pressure were measured. Figure 2A shows that LMAs from WKY18 animals increased diameter with increasing pressure suggesting little to no myogenic
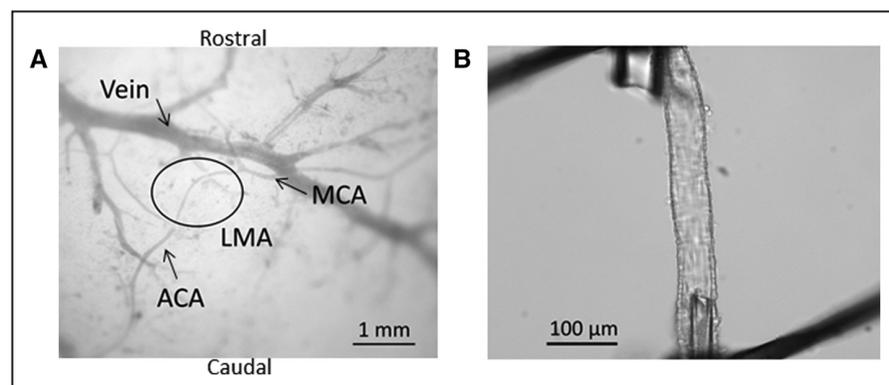

Figure 1. Photomicrographs showing leptomeningeal arterioles (LMA) taken for study. **A**, LMA were identified as connecting distal branches of middle cerebral artery (MCA) and anterior cerebral artery (ACA). **B**, LMA isolated, mounted on glass cannulas within an arteriograph chamber and pressurized.
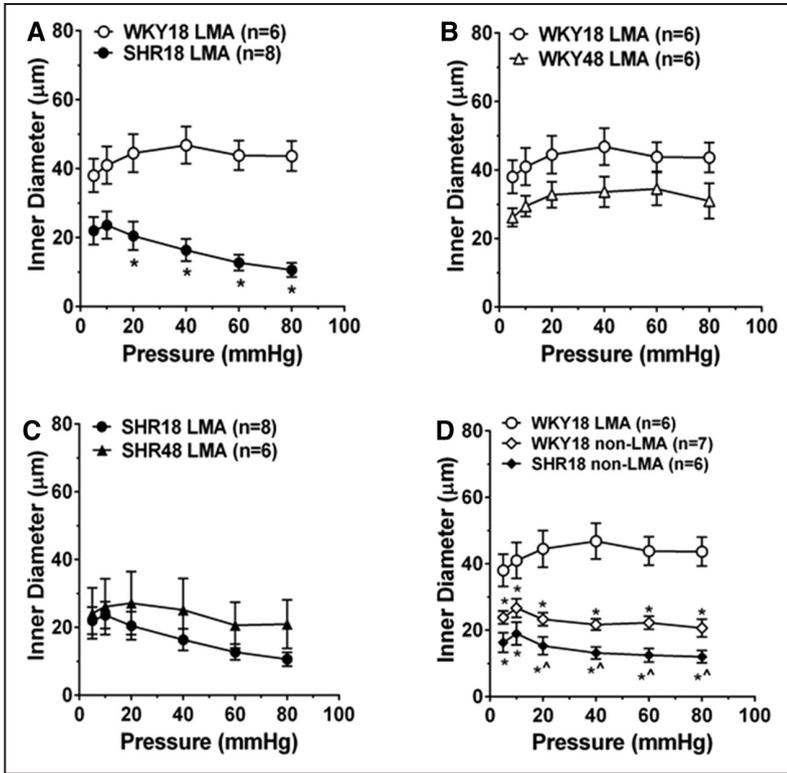
Figure 2. Active myogenic vasoconstriction of leptomeningeal arterioles (LMA) and non-LMA. **A**, Changes in inner diameter in response to pressure of LMAs from Wistar Kyoto (WKY) 18 and spontaneously hypertensive (SHR) 18 animals. LMAs from WKY18 animals had little myogenic reactivity and responded with dilation to increased pressure, whereas LMAs from SHR18 animals significantly constricted to pressure and displayed considerable myogenic reactivity. **B**, There was a little difference in the amount of myogenic reactivity in LMAs from aged WKY48 vs young WKY18 animals. **C**, LMAs from aged SHR48 animals displayed similar myogenic reactivity as young SHR18 animals. **D**, Comparison of LMA vs non-LMAs shows that the lack of myogenic reactivity in WKY18 was unique to LMAs. *$P<0.05$ vs WKY18 LMA; ^$P<0.05$ vs WKY18 non-LMA.

reactivity. In contrast, LMAs from SHR18 animals constricted significantly to pressure ≥20 mm Hg, demonstrating considerable myogenic reactivity. Representative tracings of diameter changes in response to pressure steps are shown in Results section in the online-only Data Supplement (Figure I in the online-only Data Supplement). Aging alone did not seem to have an influence on myogenic reactivity as LMAs from aged WKY48 responded similarly to pressure as from young WKY18, with little to no myogenic reactivity (Figure 2B). However, LMAs from aged hypertensive SHR48 responded similarly as young SHR18, with significant myogenic reactivity (Figure 2C). To determine if the lack of myogenic reactivity was unique to LMAs, we compared the response to pressure in pial arterioles that did not anastomose. Figure 2D shows that non-LMAs had considerable myogenic reactivity compared with LMAs, a response that was similar between WKY18 and SHR18.

LMAs from both groups of hypertensive animals had smaller diameters compared with WKY. This was partially because of structural remodeling as shown in Table I in the online-only Data Supplement. Please see online-only Data Supplement for all structural data. Passive inner diameters of LMAs were not different at 60 mm Hg from WKY18, SHR18, WKY48, and SHR48 (µm): 54±8, 37±4, 49±2, and 42±10; $P>0.05$. However, aging and hypertension caused wall hypertrophy in LMAs from SHR48 only. Wall thickness was (µm): 4.5±0.2, 4.8±0.4, 4.3±0.3, and 6.0±0.2; $P<0.01$. The smaller lumen diameters of LMAs from SHR animals were mostly because of considerable myogenic tone that caused vasoconstriction. Figure 3A shows that LMAs from WKY18 animals had little tone at all pressures studied that increased nonsignificantly in aged WKY48 animals. However, tone in LMAs from SHR18 and SHR48 was considerable being >50% at most pressures. The low level of tone in LMAs from WKY18 animals was unique to these arterioles as tone in non-LMAs
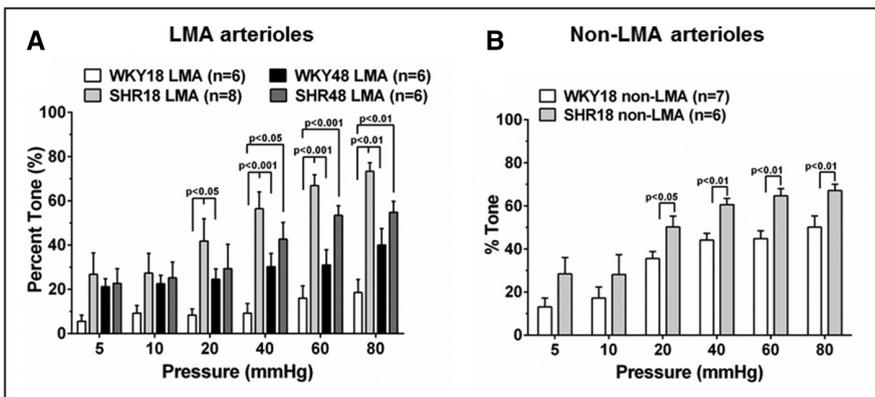
Figure 3. Myogenic tone in leptomeningeal arteriole (LMA) and non-LMA. **A**, Comparison of percent myogenic tone at each pressure studied of LMAs from all groups of animals. LMAs from normotensive young Wistar Kyoto (WKY) 18 animals had little tone at all pressures that was nonsignificantly increased in aged WKY48 animals. Hypertension increased tone in LMAs from both young and aged spontaneously hypertensive (SHR) compared with WKY animals. **B**, Percent myogenic tone in non-LMA from WKY18 and SHR18 animals. Non-LMA had considerable tone at pressures >20 mm Hg that was significantly increased in SHR18 animals.

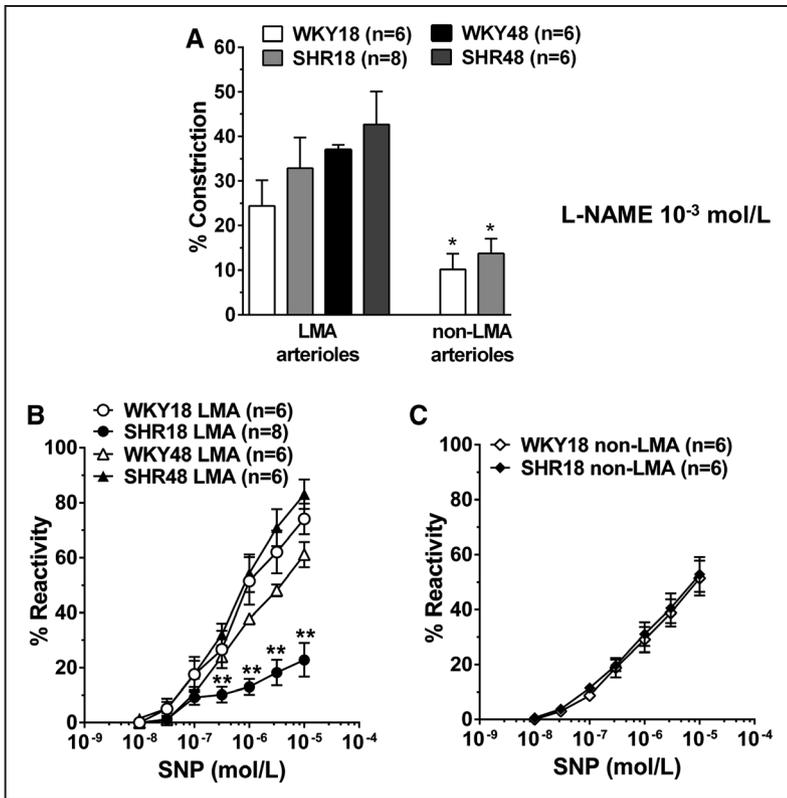**Figure 4.** Reactivity of leptomeningeal arteriole (LMA) and non-LMA to nitric oxide (NO). **A**, Percent constriction to the NO synthase (NOS) inhibitor ʟ-arginine methyl ester (L-NAME). Vessels from all groups constricted in response to NOS inhibition, a response that was greater in LMA than in non-LMA. **B**, Reactivity to the NO donor sodium nitroprusside (SNP). Arterioles from all groups of animals dilated to SNP; however, reactivity was significantly impaired in LMAs from spontaneously hypertensive (SHR) 18 animals. **C**, Reactivity to SNP in non-LMA from Wistar Kyoto (WKY) 18 and SHR18 animals. Arterioles from both groups dilated similarly to SNP. *$P<0.05$ vs LMA; **$P<0.01$ vs all.

was greater than in LMAs. In addition, tone in non-LMAs was increased in SHR18 (Figure 3B).

Effect of Hypertension and Aging on LMA Response to NO

To understand the dilatory influence of NO on LMA tone and if this varied with hypertension or aging, constriction in response to a single high concentration of the NO synthase inhibitor ʟ-arginine methyl ester was measured. Figure 4A shows that LMAs from all groups of animals constricted considerably to NO synthase inhibition that was not different between groups. The constriction of LMAs was greater than that of non-LMAs, demonstrating a greater influence of NO that inhibits tone in LMAs. Figure 4B shows the dilatory response to the NO donor SNP. LMAs from all groups except SHR18 dilated considerably to SNP. Dilation of LMAs to SNP from SHR18 animals was severely impaired compared with the other groups. There was no difference in dilation to SNP in non-LMAs with hypertension (Figure 4C).

Ion Channel Function in LMAs

To better understand LMAs, we investigated the functional effects of ion channel activity on diameter regulation and myogenic responses using pharmacological approaches. Vascular reactivity of LMAs to various ion channel activators and inhibitors were compared from WKY18 and SHR18 animals only. Figure 5A shows the diameter response of LMAs to increasing concentrations of KCl from 3 to 20 mmol/L. KCl can cause dilation through activation of K_{ir} channels at concentrations <15 mmol/L, after which it causes constriction because of depolarization and activation of voltage-dependent

L-type calcium channels.[20] LMAs from WKY18 animals dilated to KCl ≤15 mmol/L then constricted to 20 mmol/L. The response to KCl was inhibited by $BaCl_2$, suggesting the dilation was specific to K_{ir} channels. In contrast, LMAs from SHR18 did not dilate to KCl and there was no effect of $BaCl_2$, suggesting impaired K_{ir} channel function in these vessels. Dilation to KCl was unique to LMAs as this response did not occur in non-LMAs, as shown in Figure II in the online-only Data Supplement. Figure 5B shows the reactivity of LMAs to the L-type calcium channel inhibitor diltiazem. Diltiazem caused dilation in both groups of LMAs that was significantly diminished in LMAs from SHR18 animals. Non-LMAs also dilated significantly to diltiazem that was diminished to a lesser extent in arterioles from SHR18 animals (Figure III in the online-only Data Supplement).

SK/IK channels are expressed in cerebral endothelium whose activation can cause endothelium-derived hyperpolarization of vascular smooth muscle (VSM) and potent vasodilation.[13] In addition, we have shown SK/IK channels to be basally active and inhibit tone only in brain parenchymal arterioles, not MCAs.[13] Thus, we determined if LMAs had SK/IK channels and if their inhibition caused constriction, indicating basal activity. Figure 5C shows that LMAs from both groups of animals dilated to increasing concentrations of the SK/IK activator NS309, demonstrating the presence of these channels in LMAs. Non-LMAs also dilated to NS309 that was greater at the maximum concentration than LMAs (Figure IV in the online-only Data Supplement). Figure 5D shows the percent change in diameter of LMAs in response to SK and IK channel inhibition with apamin and TRAM-34, respectively. Inhibition of IK channels with TRAM-34, but
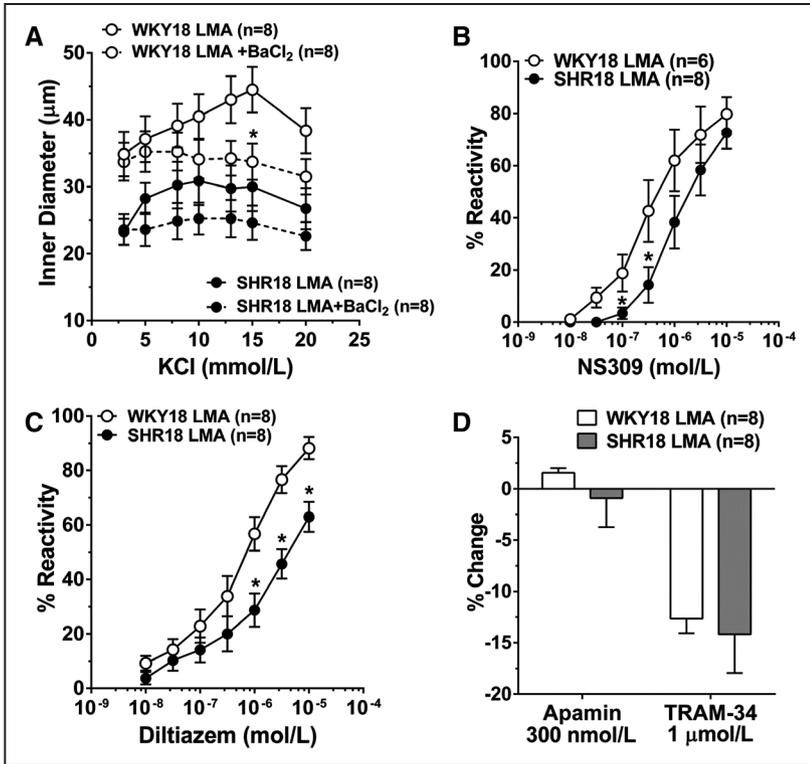
Figure 5. Ion channel activity and the contribution to tone in leptomeningeal arterioles (LMAs). **A**, Diameter changes of LMAs in response to increases in KCl from 3 to 20 mmol/L in the absence and presence of the K_{ir} channel blocker $BaCl_2$. LMAs from Wistar Kyoto (WKY) 18 animals dilated to KCl at concentrations ≤15 mmol/L, after which they constricted. The dilation to KCl was blocked by $BaCl_2$ demonstrating the presence of K_{ir} channels. LMAs from spontaneously hypertensive (SHR) 18 animals dilated less to KCl, suggesting impaired K_{ir} channel function. **B**, Percent reactivity to the L-type calcium channel inhibitor diltiazem. Diltiazem caused dilation of LMAs from both WKY18 and SHR18 animals; however, reactivity was significantly impaired in arterioles from SHR18. **C**, Percent reactivity to the small- and intermediate-conductance calcium-activated potassium channel (SK/IK) channel activator NS309 in LMAs from WKY18 and SHR18 animals. Arterioles from both groups of animals dilated considerably to NS309, demonstrating the presence of SK/IK channels. Dilation to NS309 was diminished in LMAs from SHR18. **D**, Percent change in diameter in response to inhibition of SK and IK channel with apamin and 1-[(2-chlorophenyl)diphenylmethyl]-1H-pyrazole (TRAM-34), respectively. LMAs from both groups reacted little to SK channel inhibition with apamin, whereas both constricted in response to IK channel inhibition with TRAM-34, suggesting that these arterioles have active IK channels that inhibit tone under basal conditions. *$P<0.05$ vs WKY18.

not SK with apamin, caused constriction of LMAs from both WKY18 and SHR18 animals, suggesting only IK channels are basally active and inhibiting tone. Non-LMAs constricted to both apamin and to a greater extent to TRAM-34 (Figure V in the online-only Data Supplement).

Extrinsic Innervation of LMAs

Perivascular innervation of pial arteries and arterioles has been shown to have a role in limiting autoregulatory breakthrough and hypertrophic responses during hypertension.[21] Of 15 LMAs total that were assessed, we found no perivascular nerves on LMAs from WKY18 or SHR18 animals (Figure 6A and 6B). This was in contrast to MCAs in which there were considerable varicose nerve fibers present (Figure 6C). To confirm a lack of influence of perivascular innervation on LMA function, reactivity to norepinephrine was measured. There was no response of LMAs from any of the groups to norepinephrine (Figure 6D).

Discussion

The goal of acute stroke therapy with thrombolysis or endovascular treatment is to prevent salvageable tissue from progressing to infarction with reperfusion. The pial collaterals that connect distal branches of major cerebral artery territories have long been thought to be the major determinant of flow to the ischemic penumbra, a region which is salvageable during acute stroke if reperfusion occurs rapidly.[8,18,22,23] The capacity of LMAs to sustain salvageable tissue requires perfusion from the unobstructed to the obstructed arterial territory, which has been shown to relate to luminal size and number of LMAs.[5,6] Importantly, the ability of LMAs to sustain flow during an occlusion can limit infarct growth and is associated with better

outcome.[8,23] Although therapies to increase collateral flow have been shown to reduce infarct expansion in experimental animals,[9,24,25] the use of this approach in humans is limited by our lack of understanding of the LMA function, especially in conditions such as hypertension and aging that are known to have poor collateral status and limited salvageable tissue, yet comprise the majority of patients with stroke.[14–16]

In this study, we found that LMAs from young normotensive animals were larger than non-LMAs from the same animals that did not anastomose and were relatively unresponsive to pressure. The diminished myogenic reactivity and tone and larger lumens of LMAs from WKY18 animals would be conducive to bidirectional flow during an occlusion. Aging increased myogenic tone of LMAs from WKY48 animals; however, these vessels also had little to no myogenic reactivity and behaved similarly to increases in pressure as LMAs from WKY18 animals. In contrast, LMAs from both groups of hypertensive animals were myogenic and constricted significantly in response to pressure that resulted in considerable tone. In fact, tone in LMAs from SHR18 and SHR48 animals was similar to non-LMAs and suggest these arterioles become high resistance vessels during hypertension that would impede collateral flow and possibly contribute to collateral failure. Although the smaller lumen of LMAs from both groups of SHR was partial because of structural remodeling, the majority of size decrease was because of vasoconstriction. This finding fits with previous studies showing hypertension is associated with poor collateral status and little salvageable tissue.[17,18,26] We speculate that the limited penumbra in animals and humans with hypertension is at least partly because of vasoconstriction of LMAs that restricts collateral flow. These findings also importantly suggest that therapeutic opening of LMAs is possible; however,
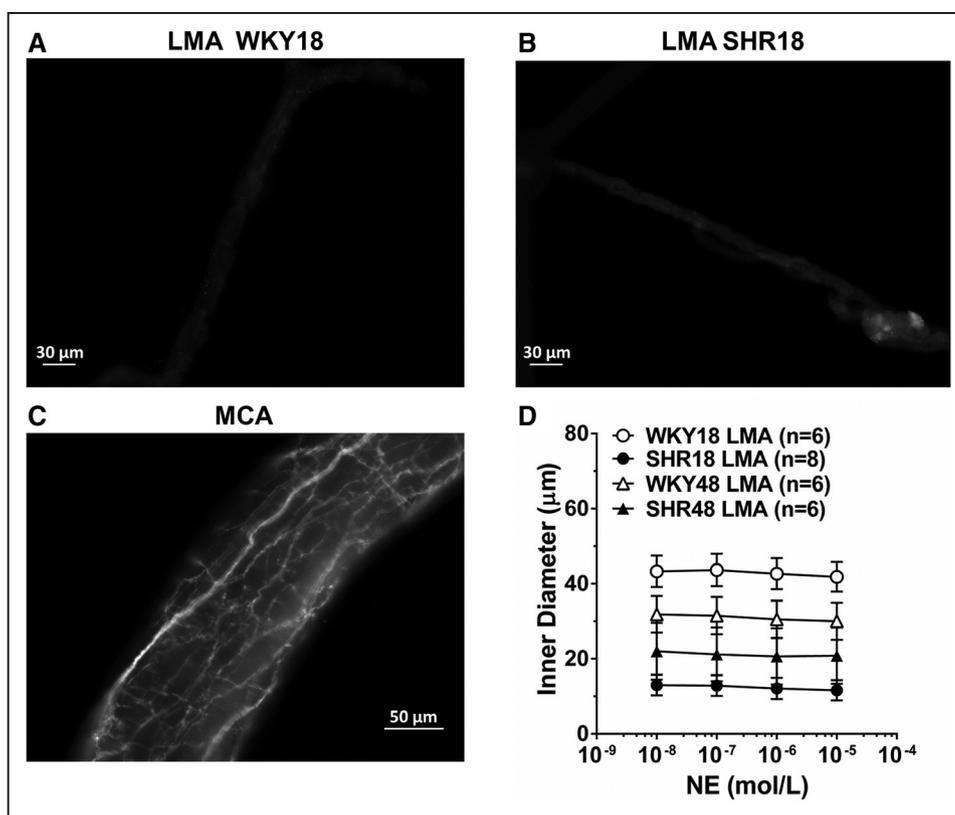
Figure 6. Perivascular innervation of leptomeningeal arterioles (LMAs) and reactivity to norepinephrine. **A–C**, Representative photomicrographs of vessels stained with the pan neuronal marker protein gene product 9.5. No perivascular innervation was found in LMAs from Wistar Kyoto (WKY) 18 or spontaneously hypertensive (SHR) 18 animals. In contrast, middle cerebral arteries (MCAs) contained numerous protein gene product 9.5 positive nerve fibers. **D**, Changes in inner diameter of LMAs from all groups of animals in response to increasing concentrations of norepinephrine (NE). Consistent with no perivascular innervation, LMAs did not response to NE.

understanding of the mechanisms by which they are vasoconstricted is needed for effective treatment.

Several approaches have been used to increase collateral flow during MCAO. Mild-induced hypertension was shown in rats and mice to increase cerebral blood flow and cerebral metabolic rate of oxygen in the core and penumbra, and prevented the expansion of infarct.[9,25] The efficacy of this approach was likely because of the passive nature of LMAs in normotensive animals, but may not have succeeded in hypertension in which LMAs are myogenic and constrict in response to increased pressure. Similarly, inhaled NO was shown to dilate cerebral pial arterioles, improve collateral flow during MCAO, and salvage penumbral tissue in mice.[24] However, our results here show that LMAs from SHR18 animals were significantly less sensitive to SNP, an NO donor, suggesting this approach may not work in hypertension either.

The mechanism by which hypertension increased myogenic reactivity, and tone of LMAs is not clear. Myogenic reactivity and tone are dependent on pressure-induced depolarization of VSM that opens voltage-dependent L-type calcium channels to cause vasoconstriction.[27] The increased tone, together with decreased sensitivity to diltiazem and NS309 of LMAs from SHR18 animals suggest that these vessels have VSM that is more depolarized. Hypertension is well-known to cause alterations in L-type calcium channels in VSM that promote vasoconstriction of cerebral arteries and arterioles.[28]

Oxidative stress is a primary mechanism by which ion channel activity is affected to increase vasoconstriction of cerebral vessels[29] and a similar mechanism may be affecting LMAs to have increased tone. It is also possible that LMAs from normotensive animals have increased vasodilator influence that is inhibited during hypertension, including K^{+} channels in both VSM and endothelium. For example, SK/IK channels, expressed only in cerebral endothelium, are basally active in parenchymal arterioles and serve a vasodilatory role that opposes tone.[13] In this study, LMAs constricted to TRAM-34, but not to apamin, suggesting an important role for IK but not SK channels in opposing myogenic vasoconstriction. There was no difference in the constriction to TRAM-34 of LMAs between WKY18 and SHR18, suggesting IK activation may be a means to open collaterals during hypertension.

Previous studies on LMAs and the effect on stroke outcome have focused on structural changes under conditions, such as hypertension or the influence of genetics. Using stroke-prone SHR as a model of hypertension compared with WKY, Coyle and Heistad[30] found that vascular resistance in stroke-prone SHR increased and cerebral blood flow decreased in the MCA territory both during MCAO and seizure that presumably caused full vasodilation. In a related study, the number of LMAs in stroke-prone SHR were not found to be appreciably different than WKY, but their lumen diameters were smaller under fully relaxed conditions.[4]

Another study using different genetic strains of mice found a negative correlation with number of LMAs and infarction such that the fewer LMAs, the greater infarction.[6] Although these studies only measured diameter or number of LMAs under fully relaxed conditions, this study importantly found that LMAs from SHR had smaller lumens mostly because of vasoconstriction and to a lesser extent remodeling.

Aging is associated with increased collateral vascular resistance and rarefaction that is thought to contribute to worse outcome from stroke.[15,26,31] In this study, we found that aged WKY48 animals had smaller lumens structurally and functionally than those from young WKY18 animals, although this was not statistically significant. However, LMAs from aged SHR48 animals had increased tone and inward remodeling that was associated with vessel wall hypertrophy. Thus, it seems that the combination of hypertension and aging, a common comorbid state of patients with stroke, had a combined negative impact on LMA structure that may lead to rarefaction and ultimately decreased collateral flow.

In summary, LMAs from normotensive animals were larger and did not respond to pressure myogenically compared with non-LMAs from the same animals, an effect that would be conducive to collateral flow during occlusion. In contrast, LMAs from hypertensive animals responded with significant myogenic vasoconstriction to increased pressure and had impaired vasodilation. It is likely that the significant vasoreactivity of LMAs during hypertension contributes to greater perfusion deficit and less salvageable tissue. Importantly, vasoconstriction of LMAs from hypertensive animals suggest that therapeutic opening of these collaterals is possible during MCAO to create penumbral tissue and prevent infarct expansion.

Sources of Funding

Disclosures

None.

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