Postischemic Reperfusion Causes Smooth Muscle Calcium Sensitization and Vasoconstriction of Parenchymal Arterioles

Marilyn J. Cipolla, PhD; Siu-Lung Chan, PhD; Julie Sweet, BS; Matthew J. Tavares, BS; Natalia Gokina, PhD; Joseph E. Brayden, PhD

Background and Purpose—Parenchymal arterioles (PAs) are high-resistance vessels in the brain that connect pial vessels to the microcirculation. We previously showed that PAs have increased vasoconstriction after ischemia and reperfusion that could increase perfusion deficit. Here, we investigated underlying mechanisms by which early postischemic reperfusion causes increased vasoconstriction of PAs.

Methods—Isolated and pressurized PAs from within the middle cerebral artery territory were studied in male Wistar rats that were either nonischemic control (n=34) or after exposure to transient middle cerebral artery occlusion (MCAO) by filament occlusion for 2 hours with 30 minutes of reperfusion (MCAO; n=38). The relationships among pressure-induced tone, smooth muscle calcium (using Fura 2), and membrane potential were determined. Sensitivity of the contractile apparatus to calcium was measured in permeabilized arterioles using Staphylococcus aureus α-toxin. Reactivity to inhibition of transient receptor potential melastatin receptor type 4 (9-phenanthrol), Rho kinase (Y27632), and protein kinase C (Gö6976) was also measured.

Results—After MCAO, PAs had increased myogenic tone compared with controls (47±2% versus 35±2% at 40 mm Hg; P<0.01), without an increase in smooth muscle calcium (177±21 versus 201±16 nmol/L; P>0.05) or membrane depolarization (−38±4 versus −36±1 mV; P>0.05). In α-toxin–permeabilized vessels, MCAO caused increased sensitivity of the contractile apparatus to calcium. MCAO did not affect dilation to transient receptor potential melastatin receptor type 4 or protein kinase C inhibition but diminished dilation to Rho kinase inhibition.

Conclusions—The increased vasoconstriction of PAs during early postischemic reperfusion seems to be due to calcium sensitization of smooth muscle and could contribute to infarct expansion and limit neuroprotective agents from reaching their target tissue. (Stroke. 2014;45:2425-2430.)

Key Words: arterioles ■ brain infarction ■ calcium ■ no-reflow phenomenon ■ smooth muscle

Restoration of blood flow to the ischemic brain is the most potent and efficacious means to improve outcome from ischemic stroke. Recanalization therapies, such as thrombolysis and mechanical removal or disruption of the clot, have shown clear benefit if reperfusion occurs within a narrow time window of 4.5 hours.1,2 However, recanalization after this time period can cause deleterious effects including hemorraghic transformation and edema that worsens outcome.1,3 Thus, early postischemic reperfusion to the brain parenchyma is one of the most important treatments for acute ischemic stroke.

Studies in animals and humans have shown that recanalization of an occluded artery does not necessarily lead to complete reperfusion or improvement of outcome.4-7 Incomplete microcirculatory reperfusion occurs in response to both global and focal ischemia in the brain and may be a primary factor that increases perfusion deficit, decreases efficacy of early thrombolysis, and limits neuroprotective agents from reaching their target.8-12 This no-reflow phenomenon occurs in other circumstances in response to postischemic reperfusion including the coronary circulation and is thought to be a major contributor to ischemic tissue injury.13 Importantly, a recent study in patients with stroke found that reperfusion was a more accurate predictor of final infarct volume than recanalization.4 The dissociation between recanalization and reperfusion is not entirely clear and may be because of distal embolization of thrombus or incomplete reperfusion of the brain parenchyma.14,15 Incomplete postischemic reperfusion has been mostly attributed to microcirculatory disturbances,14 including perivascular glial cell swelling that occludes capillaries5,16 and clogging of capillaries by microthrombi and immune cells.5,7,17,18 However, evidence from earlier studies also suggests that upstream vasoconstriction and increased cerebrovascular resistance occur in no-reflow zones.5,10,19 More recently, Shih et al20 used 2-photon microscopy to measure red blood cell flux and diameters of penetrating brain arterioles during early postischemic reperfusion and found both flux and diameters to be decreased below...
baseline, suggesting vasoconstriction occurs during reperfusion that may contribute to incomplete restoration of blood flow.

Our own studies using isolated and pressurized parenchymal arterioles have shown that these vessels have increased vasoconstriction in response to early postsischemic reperfusion. Although this previous study mainly focused on endothelial cell changes, we found that vascular smooth muscle was also more contractile after ischemia and reperfusion. In the present study, we investigated mechanisms by which vascular smooth muscle of parenchymal arterioles has increased vasoconstriction compared with nonischemic vessels, focusing on the relationship among myogenic tone, smooth muscle calcium, and membrane potential. Understanding this relationship may provide a therapeutic target to improve blood flow to the ischemic brain during reperfusion.

Materials and Methods

Animal Model of Transient Focal Ischemia

Experiments were performed using male Wistar rats (Harlan) that were 350 to 380 g. All procedures involving animals 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 the care and use of laboratory animals. Rats were housed in the Animal Care Facility at the University of Vermont, an Association for Assessment and Accreditation of Laboratory Animal Care–accredited facility, and were allowed food and water ad libitum. Proximal middle cerebral artery occlusion (MCAO) was performed using the filament technique, as previously described. Animals were anesthetized with isoflurane (1.5% in oxygen) and intubated and mechanically ventilated to maintain blood gases within a normal physiological range (Table 1 in the online-only Data Supplement). The MCA was occluded for 2 hours followed by suture removal for 30 minutes. Animals were randomized to sham, naive, or MCAO group, and individuals performing the experiments were blinded to group and outcome. Animals were excluded if the drop in cerebral blood flow was <60% from baseline.

Animals were decapitated under isoflurane anesthesia and the brain was removed and placed in cold physiological saline solution (PSS). Animals (n=5) and after MCAO (n=5), as previously described. Animals were randomized to sham, naive, or MCAO group, and individuals performing the experiments were blinded to group and outcome. Animals were excluded if the drop in cerebral blood flow was <60% from baseline. Sham control animals underwent anesthesia for 2.5 hours and received a midline neck incision, but no filament was inserted.

Preparation of Isolated Parenchymal Arterioles and the Relationship Between Myogenic Vasoconstriction and Smooth Muscle Calcium

Animals were decapitated under isoflurane anesthesia and the brain removed and placed in cold physiological saline solution (PSS). Parenchymal arterioles (PAs), branching off the MCA at right angles and penetrating into the brain tissue, were dissected and mounted in an arteriograph chamber, as previously described. Myogenic activity was measured in isolated middle cerebral artery (MCAO) and MCAO (n=12) arterioles by measuring diameter and tone at pressures from 40 to 80 mm Hg. To investigate the relationship between myogenic tone and calcium in PAs from sham control animals (n=6) and after MCAO (n=6), the calcium-sensitive dye Fura 2-acetoxyethyl ester, was used, as previously described and in the online-only Data Supplement. Arteriolar diameter and calcium were simultaneously recorded using IonWizard software (IonOptix). Briefly, PAs were pressurized to 40 mm Hg and equilibrated for 30 minutes to allow spontaneous development of myogenic tone. Changes in smooth muscle calcium and diameter were evaluated by stepwise increases in pressure from 40 to 80 mm Hg. At the conclusion of the experiment, diltiazem (10 μmol/L) in calcium-free PSS was added to obtain fully relaxed diameters.

Smooth Muscle Membrane Potential Measurement

Myogenic tone and smooth muscle membrane potential were simultaneously measured in isolated and pressurized PAs from sham control animals (n=5) and after MCAO (n=5), as previously described. Smooth muscle membrane potential was measured by insertion of a sharp glass microelectrode (≈100 mol/LΩ resistance) filled with 0.5 mol/L KCl into the vessel wall. Impalement was considered successful if there was an abrupt deflection to negative membrane potential on electrode entry, membrane potential was stable for ≥30 s, and the voltage returned abruptly to 0 mV on removal of the electrode. Membrane potential measurements were made with an electrometer (World Precision Instruments) and recorded via computer with Axotape and Dataq software.

Measurement of Calcium Sensitivity in Permeabilized Arterioles Using Staphylococcus aureus α-Toxin

The sensitivity of the contractile apparatus to calcium in arterioles from control animals (n=6) and after MCAO (n=7) was determined by permeabilizing the myocyte membrane with S aureus α-toxin and measuring the contractile response to addition of calcium, as previously described and in the online-only Data Supplement. S aureus α-toxin forms small (1–2 μm diameter) pores in the plasma membrane that allows ions but not proteins to pass. This technique is commonly used to study calcium sensitization because under these conditions intracellular calcium in smooth muscle can be tightly controlled. Briefly, PAs were carefully dissected and mounted in an arteriograph filled with HEPES-buffered PSS, pressurized to 40 mmHg, and equilibrated for 30 minutes. Vessels were permeabilized with S aureus α-toxin (800 U/mL) in relaxing solution at room temperature for 20 minutes. The S aureus α-toxin was then washed from the bath, and vessels were equilibrated in relaxing solution at 37°C for 30 minutes. The vasoactive response to calcium was determined by replacing relaxing solution with activating solution containing known concentrations of free ionic calcium (pCa or −log [Ca]: 7.0-6.0). For each concentration of calcium, the inner diameters were recorded once stable (5–7 minutes).

Reactivity of PAs to 9-Phenanthrol, Y27632, and Gö6976

In a separate set of PAs from sham control animals (n=6) or after MCAO (n=7), dilator responses to the transient receptor potential melastatin receptor type 4 (TRPM4) inhibitor, 9-phenanthrol, were determined. Arterioles were dissected and mounted in an arteriograph chamber, equilibrated at 40 mm Hg, and an air bubble passed through the lumen to remove the endothelium. Endothelial denudation was confirmed by lack of dilation to NS309. 9-Phenanthrol was cumulatively added to the bath and lumen diameters measured at each concentration, once stable. In a separate set of arterioles that were intact (not denuded of endothelium) from control animals (n=6) or after MCAO (n=6), reactivity to inhibitors of Rho kinase (Y27632) and protein kinase C (PKC; Gö6976) was determined by cumulative addition to the bath and measuring diameters at each concentration. Y27632 is a selective inhibitor of rho-associated protein kinase 1 (ROCK1) (IC50=140 nmol/L) that exhibits >200-fold selectivity over other kinases, including PKC and myosin light chain kinase. Gö6976 is a selective inhibitor of conventional PKCα (IC50=2.3 μmol/L) and PKCβI (IC50=6.2 μmol/L) and does not inhibit unconventional PKC isoforms (PKCδ, PKCγ, or PKCε).

Drugs and Solutions

All isolated vessel experiments, except calcium sensitivity measurements in S aureus α-toxin, were performed using a bicarbonate-based Ringer’s PSS, the ionic composition of which was (in mmol/L) NaCl 119.0, NaHCO3 24.0, KCl 4.7, KH2PO4 1.18, MgSO4·7H2O 1.17, CaCl2 1.6, EDTA 0.026, and glucose 5.5. PSS was made each week and stored without glucose at 4°C. Glucose was added to the PSS before each experiment. PSS was aerated with 5% CO2, 10% O2, and 85% N2 to maintain pH. S aureus α-toxin was purchased from Calbiochem (La Jolla, CA) and aliquoted in relaxing buffer and stored at −20°C until use. All other chemicals were purchased from Sigma (St. Louis, MO). Please see the online-only Data Supplement for details on Fura 2 measurements.
Data Calculations
Please see the online-only Data Supplement for all data calculations.

Statistical Analysis
All data are presented as mean±SEM. Unpaired \( t \) test with Welch correction was used to compare differences between control and MCAO arterioles.

Results
Effect of Early Postischemic Reperfusion on Myogenic Tone and Smooth Muscle Calcium
Figure 1 shows the relationship between myogenic tone and smooth muscle calcium in parenchymal arterioles. Arteriolar diameter in response to pressure. Arterioles displayed myogenic vasoconstriction and were smaller actively after middle cerebral artery occlusion (MCAO). Percent tone at 40 mm Hg. Arterioles had increased tone that contributed to their smaller diameter. Passive diameters were not different between groups (not shown). Smooth muscle calcium, measured using Fura 2 at 40 mm Hg, was not different in arterioles after MCAO. Relationship between percent tone and smooth muscle calcium. Arterioles were more sensitive to calcium after MCAO. *\( P<0.05 \) vs control (CTL); **\( P<0.01 \) vs CTL.

Effect of Early Postischemic Reperfusion on Smooth Muscle Membrane Potential and TRPM4 Activity
The increase in tone in parenchymal arterioles after MCAO could be because of greater pressure-induced depolarization and activation of voltage-dependent calcium channel activity. To test this possibility, we simultaneously measured smooth muscle membrane potential and tone of isolated arterioles pressurized to 40 mm Hg. Figure 2A shows the relationship between myogenic tone and smooth muscle membrane potential. PAs exposed to postischemic reperfusion had increased tone, but this was not a result of greater smooth muscle depolarization because membrane potential of arterioles from the 2 groups was similar and close to the values observed in these vessels previously under control conditions. We also tested the possibility that greater activation of TRPM4, a mechanosensitive ion channel that promotes depolarization in response to pressure, could account for the increased tone of PAs after MCAO. Figure 2B shows that both types of arterioles dilated to TRPM4 inhibition, but there was no difference in sensitivity to 9-phenanthrol, suggesting MCAO did not affect these channels.

Calcium Sensitization of Permeabilized Arterioles After Postischemic Reperfusion
To determine whether the contractile apparatus of smooth muscle was more sensitive to calcium after MCAO, which could explain the increased tone, a permeabilized vessel
preparation was used. The use of *S. aureus* α-toxin effectively eliminates the contribution of plasma membrane ion channels and measures the contractile response to addition of calcium. Figure 3A shows that arterioles after MCAO had increased tone at calcium concentrations ≥0.3 μmol/L. Figure 3A also shows that after MCAO, PAs were physically more constricted compared with controls at the highest level of calcium. When calcium sensitivity was compared between the 2 groups of arterioles (Figure 3B), there was an increase in calcium sensitivity after MCAO. The effective concentration that produced half-maximal constriction (EC50) was significantly less for arterioles after MCAO than controls.

**Effect of Early Postischemic Reperfusion on Reactivity to Rho Kinase and PKC Inhibition**

To begin to investigate the mechanism by which calcium sensitization occurs in arterioles after MCAO, the sensitivity to inhibition of ROCK and PKC was compared between groups. Figure 4A shows the sensitivity of PAs in response to ROCK inhibition with Y27632. Both types of arterioles dilated in response to Y27632; however, after MCAO arterioles were less sensitive to its effects. Figure 4A also shows that the IC50 value for Y27632 was significantly larger in arterioles after MCAO. We compared the IC50 values because at these concentrations Y27632 is not likely to be inhibiting other kinases that also promote vasodilation such as PKC or myosin light chain kinase because the IC50 for this compound is 140 nmol/L. In contrast, inhibition of PKCα and PKCβ with Gö6976 had little effect on either vessel type. Figure 4B shows there was almost no change in diameter in response to Gö6976, regardless of exposure to ischemia and reperfusion.

**Discussion**

In the present study, we show that after early postischemic reperfusion, PAs had increased pressure-induced tone that was not because of smooth muscle membrane depolarization or increased cell calcium but was related to an increase in calcium sensitivity. In fact, the capacity of PAs to constrict in response to calcium was also increased after MCAO, suggesting that other vasoconstrictor mechanisms (eg, endothelin-1) may also be enhanced. Increased vasoconstriction after ischemia and reperfusion has been shown previously in these arterioles,9,19–21 a response that is distinct from that of pial arteries that undergo vasodilation and decreased tone.22 However, this is the first study we are aware of to investigate the underlying mechanism by which increased vasoconstriction of PAs occurs with ischemia and reperfusion. Although capillary disturbances are well known to occur after ischemia and reperfusion that can limit reperfusion,5–7,17,18 upstream vasoconstriction of PAs may be another factor that contributes to heterogeneity of tissue recovery and incomplete perfusion.

This study focused on smooth muscle cell calcium and changes in membrane potential as underlying mechanisms by which myogenic tone could be increased in PAs after ischemia and reperfusion because it is well established that pressure induces membrane depolarization that opens voltage-dependent calcium channel to cause vasoconstriction.24 Thus, one means by which tone could be increased is through greater smooth muscle depolarization and increased cell calcium. However, we found that despite increased tone, smooth muscle calcium was not elevated compared with control vessels (177±21 nmol/L in MCAO versus 201±16 nmol/L in control; P>0.05). In addition, membrane potential of PAs pressurized to 40 mmHg was
not different (−38±4 mV in MCAO versus −36±1 mV in control; P=0.05). These values are in agreement with what has been published by our group previously in control arterioles pressurized to 40 mm Hg (−35±1 mV).23 In addition, sensitivity to 9-phenanthrol, a selective TRPM4 inhibitor, dilated both types of PA to a similar extent, suggesting that a difference in TRPM4 activity in response to pressure was not the underlying cause by which ischemia and reperfusion increased tone in PAs.

The findings described above suggest that the contractile apparatus of smooth muscle from PAs after ischemia and reperfusion may be more sensitive to calcium such that less calcium was needed to cause vasoconstriction at the same level of pressure. To measure calcium sensitivity directly, we used a permeabilized vessel preparation that eliminated the level of pressure. To measure calcium sensitivity directly, we used a permeabilized vessel preparation that eliminated the role of membrane potential and ionic fluxes and allowed direct and controlled access of calcium to the contractile apparatus. Calcium sensitization of smooth muscle occurs via several mechanisms, including phosphorylation of myosin light chain kinase by PKC, decreased myosin phosphatase activity, and increased Rho A–induced actin polymerization.27,28 We therefore compared the vasoactive response of arterioles with both ROCK and PKC inhibition. PKC inhibition with Go6976, a conventional PKC inhibitor, had little effect on basal tone of PAs, regardless of ischemia and reperfusion, suggesting PKC activation was not involved in calcium sensitization with MCAO. Conversely, ROCK inhibition with Y27632 caused significant vasodilation in both vessel types, confirming our previous study and others that the Rho A–ROCK pathway is involved in myogenic tone in these arterioles.22,29,30 However, sensitivity to Y27632 was decreased in arterioles after MCAO, suggesting ROCK was less activated and contributes less to tone in those arterioles and may not be the mechanism by which calcium sensitization occurs after ischemia and reperfusion either. Calcium sensitization by ROCK depends on its phosphorylation of myosin phosphatase target subunit 1 at Thr696 and Thr853 to suppress myosin light chain phosphatase activity.31 However, other kinases such as zipper-interacting protein kinase and integrin-linked kinase also have the ability to phosphorylate myosin phosphatase target subunit 1 at Thr696.32,33 The role of these kinases in calcium sensitization of PAs after MCAO clearly needs to be addressed with additional biochemical studies.

Conclusions

We show that PAs supplying the subcortical brain regions and striatum have increased vasoconstriction in response to early postischemic reperfusion, which could limit perfusion to these vulnerable brain regions including white matter. This seems to be because of ischemia and reperfusion–induced smooth muscle calcium sensitization. The main target of acute stroke treatment is the ischemic penumbra and peri-infarcted tissue—regions where the brain tissue is not dead and where reperfusion and neuroprotective agents can still provide benefit.

Understanding the mechanisms by which postischemic reperfusion preferentially promotes vasoconstriction of PAs is of interest to acute stroke treatment, because vasoconstriction in these vessels under these conditions would not seem to be beneficial. Therapeutic interventions opposing parenchymal arteriolar constriction during reperfusion should enhance local blood flow and aid recovery and delivery of neuroprotective agents to the penumbra.

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Disclosures

None.

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SUPPLEMENTAL MATERIAL

Post-ischemic Reperfusion Causes Smooth Muscle Calcium Sensitization and Vasoconstriction of Parenchymal Arterioles

Marilyn J. Cipolla, PhD1,2,3, Siu-Lung Chan, PhD1, Julie Sweet, BS1, Matthew J. Tavares, BS2, Natalia Gokina, PhD3 and Joseph E. Brayden, PhD2

1Department of Neurological Sciences, 2Department of Pharmacology, 3Department of Obstetrics, Gynecology & Reproductive Sciences, University of Vermont, Burlington, Vermont

Supplemental Methods

Measurement of Smooth Muscle Calcium using Fura 2

Fura 2-AM (1 mmol/L stock dissolved in anhydrous dimethylsulfoxide, DMSO) was premixed with an equal volume of 25% pluronic acid dissolved in DMSO and then diluted in aerated physiologic saline solution (PSS) to yield a final concentration of 5µmol/L. Each vessel segment was cannulated in an arteriograph, pressurized to 10 mm Hg, and equilibrated at 37 °C for 10–15 min. The arteriolar segments were then incubated in the Fura 2-AM/PSS loading solution at room temperature in the dark for 60 minutes. This solution was changed for freshly aerated loading solution after 30 minutes into the incubation period to maintain pH = 7.4. Fura 2-loaded arterioles were washed two to three times with PSS and then continuously superfused at 3 ml/min with oxygenated PSS (10% O2-5% CO2-balanced N2) at 37 °C. All experimental protocols were started after an additional 15-min equilibration period at 10 mm Hg to allow intracellular de-esterification of Fura 2-AM. Fura 2 fluorescence was measured using a photomultiplier system (IonOptix) in which background-corrected ratios of 510-nm emission were obtained at a sampling rate of 5 Hz from arteries alternately excited at 340 and 380 nm.

For the Fura 2 calibration procedure, the following solutions were used. Calcium-free calibration solution was composed of (in mmol/L): KCl 140.0, NaCl 20.0, HEPES 5.0, EGTA 5.0, MgCl2 1.0, 5 µmol/L nigericin, and 10 µmol/L ionomycin. Calcium-containing calibration solution was composed of (mmol/L): KCl 140.0, NaCl 20.0, HEPES 5.0, MgCl2 1.0, and CaCl2 10.0. Both solutions were adjusted to pH 7.15 at 37 °C with KOH. Ionomycin and nigericin were purchased from A.G. Scientific (San Diego, CA). Fura 2-AM and pluronic acid were purchased from Life Technologies (Grand Island, NY). Fura 2-AM was dissolved in dehydrated DMSO as a 1 mmol/L stock solution and frozen at -20 °C until use.

The concentration of intracellular calcium in vascular smooth muscle ([Ca2+]i) in intact (not permeabilized) arterioles was calculated using the following equation: [Ca2+]i = Kdβ(R - Rmin)/(Rmax - R), where Kd is the dissociation constant of Fura 2, R is experimentally measured ratio (340/380 nm) of fluorescence intensities, Rmin is the ratio in the absence of [Ca2+]i, Rmax is the ratio at Ca2+ saturation, and β is the ratio of the fluorescence intensities at 380-nm excitation wavelength at Rmin and Rmax. Rmin, Rmax, and β were determined using an in situ calibration procedure with nigericin (5 µmol/L) and ionomycin (10 µmol/L). These values were then used to convert the ratio values into a [Ca2+]i. The calibration procedure resulted in a β value of 4.43 ± 0.28 and Rmin and Rmax values of 0.55 ± 0.02 and 4.12 ± 0.26. The Kd was 282 nmol/L, as determined by using in situ titration of Ca2+ in Fura 2-loaded posterior cerebral arteries.1 Arterial diameter, pressure, and ratio values were recorded using an Ion Wizard data acquisition program.
Measurement of sensitivity of parenchymal arterioles to calcium using S. aureus α-toxin

**HEPES-buffered PSS** contained (in mmol/L): NaCl 142.0, KCl 4.7, MgSO₄ 1.17, EDTA 0.5, CaCl₂ 2.79, HEPES 10.0, KH₂PO₄ 1.2, glucose 5.5. pH was adjusted by 10 N NaOH to 7.4 at 37 °C. Relaxing solution contained (in mmol/L): potassium methanesulfonate 63.6, MgCl₂ 2.0, Mg-ATP 4.5, EGTA 2.0, phosphocreatine 10.0, and piperazine-N,N’-bis(2-ethanesulfonic acid) 30.0. Relaxing solution also contained 1.0 µmol/L carbonylcyanide p-trifluromethoxyphenyl-hydrazone, a mitochondrial blocker, and 1.0 µmol/L leupeptin, a protease inhibitor. pH was adjusted to 7.1 with 8 N KOH. The composition of the **activating solution** was similar to that of the relaxing solution, except it contained 10 mmol/L EGTA and 10 µmol/L GTP. The amount of CaCl₂ needed to yield the desired free ionic concentration of calcium in the activating solution was calculated by a web-based program Webmaxc Standard (http://www.stanford.edu/~cpatton/webmaxcS.htm). Ionic strength was kept at 200 mmol/L by adjusting the concentration of potassium methanesulfonate.

**Data Calculations**

Myogenic tone was calculated as a percent decrease in diameter from the fully relaxed diameter in calcium-free PSS with diltiazem or in calcium-free relaxing solution by the equation: \(1 - (\varphi_{\text{tone}} / \varphi_{\text{zero}}) \times 100\%\); where \(\varphi_{\text{tone}}\) = inner diameter of vessel with tone and \(\varphi_{\text{zero}}\) = inner diameter in calcium-free PSS with diltiazem. Percent sensitivity to calcium was calculated from the equation: \(((\varphi_{\text{calcium}} - \varphi_{\text{start}}) / (\varphi_{6.0} - \varphi_{\text{start}})) \times 100\%\) where \(\varphi_{7.00}\) is the inner diameter at \(-\log 7.0\) calcium, \(\varphi_{\text{start}}\) is diameter prior to giving the first concentration of calcium and \(\varphi_{6.00}\) is the inner diameter at \(-\log 6.0\) calcium which was the highest concentration of calcium. Percent dilation to 9-phenanthrol, Y27632 and Gö6976 was calculated from the equation: \((\varphi_{\text{drug}} - \varphi_{\text{zero Ca}}) / (\varphi_{\text{start}} - \varphi_{\text{zero Ca}})) \times 100\%\) where \(\varphi_{\text{drug}}\) is the inner diameter at a specific concentration of drug, \(\varphi_{\text{start}}\) is the inner diameter prior to giving the first concentration of drug and \(\varphi_{\text{zero Ca}}\) is the diameter fully relaxed in zero calcium PSS. Effective concentrations that produced half maximal dilation (EC₅₀) were determined from individual plots of concentration-response curves then averaged per group.

**Reference:**

**Supplemental Table I.** Physiological Parameters of Sham control and MCAO Animals.

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