Threshold Duration of Ischemia for Myogenic Tone in Middle Cerebral Arteries
Effect on Vascular Smooth Muscle Actin

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Background and Purpose—We investigated the effect of different periods of ischemia on the myogenic tone of middle cerebral arteries (MCAs) and tested the hypothesis that ischemia disrupts the actin cytoskeleton in vascular smooth muscle.

Methods—The MCA occlusion model was used in male Wistar rats (n=27) to induce different periods of ischemia (15, 30, and 120 minutes) with 24 hours of reperfusion. Successful occlusion was determined by laser-Doppler flowmetry. MCAs were then studied in vitro with a specialized arteriograph system that allowed control of transmural pressure and measurement of lumen diameter. After equilibration for 1 hour at transmural pressure of 75 mm Hg, lumen diameter was measured, and the amount of spontaneous myogenic tone was determined. Arteries were then fixed with 10% formalin while still pressurized in the arteriograph bath and stained for filamentous (F-) actin with fluorescently labeled phalloidin, a specific probe for F-actin. The amount of F-actin was quantified by confocal microscopy.

Results—The amount of tone was similar between control and 15 minutes of ischemia (27.0±2.0% and 25.3±1.7%, respectively; P>0.05) but was significantly diminished after 30 and 120 minutes (11.7±2.0% and 8.5±2.0%, respectively; P<0.01 versus control). F-actin content also decreased at the longer ischemic periods and correlated significantly with vascular tone (P=0.04) such that the lesser the tone, the lesser was the F-actin content. Fluorescence intensity for control and 15, 30, and 120 minutes of ischemia was (×10^3) 3.21±0.25, 2.54±0.32 (P>0.05), 2.32±0.15 (P<0.01), and 2.22±0.16 (P<0.01), respectively.

Conclusions—These results demonstrate that ischemia disrupts the actin cytoskeleton in smooth muscle and diminishes vascular tone of MCAs in a threshold-dependent manner. This effect likely exacerbates brain tissue damage during stroke, including infarction and edema formation. (Stroke. 2001;32:1658-1664.)

Key Words: actins ■ cerebral arteries ■ cytoskeleton ■ reperfusion injury ■ stroke, acute ■ stroke, ischemic ■ rats

The cerebral circulation operates in a state of partial constriction or tone that provides a state from which an artery can increase or decrease diameter and hence cerebral blood flow (CBF) on demand.1 While there are several influences that determine vessel caliber (eg, neuronal, endothelial, metabolic), basal tone is primarily determined by the myogenic behavior of the cerebral vascular smooth muscle (VSM) that constrict in response to elevated pressure and dilate in response to decreased pressure.2 The innate myogenic tone of the cerebral VSM is crucial for establishment of an appropriate cerebrovascular resistance (CVR), which serves to protect downstream arterioles and capillaries in the face of changing perfusion pressures.3 Significant tissue damage occurs when autoregulation of CBF is lost. For example, reperfusion following transient cerebral ischemia causes autoregulatory loss and hyperperfusion.4 Cerebral hyperemia exacerbates neuronal injury and promotes brain edema due to a diminished CVR that exposes the microcirculation to excessive perfusion pressure.5,6 Although this period of posts ischemic hyperemia is due to vasodilatation of the cerebral vasculature, the mechanisms by which ischemia and reperfusion cause loss of myogenic tone are not clear. Accumulation of lactate and vasoactive ions is thought to have a role; however, this cannot completely explain the prolonged vasodilatation, particularly since it is demonstrated in vitro when these metabolites are not present and persists in humans for 2 to 3 weeks after stroke.5,7 In the present study we hypothesized that ischemia causes disruption of filamentous (F-) actin in cerebral artery VSM from occluded middle cerebral arteries (MCAs) that promotes the vasodilatation underlying posts ischemic hyperemia8 since this abundant protein is part of the contractile machinery and

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has been shown to be affected during ischemia.\(^9\) The MCA occlusion (MCAO) model was used in rats to induce different durations of ischemia, all with a constant reperfusion period of 24 hours.\(^{10}\) While this model is most widely used to study neuronal injury during stroke (i.e., infarct size), we utilized it as a way of exposing MCAs to different periods of ischemia (0, 15, 30, and 120 minutes) since the MCA is focally occluded. Arteries were then studied in vitro with a system that allowed measurement of lumen diameter, control of transmural pressure (TMP), and chemical fixation for determination of F-actin content. We found that the amount of intrinsic tone MCAs possessed decreased with increasing ischemic duration that correlated significantly with F-actin content. In addition, the threshold duration of ischemia for myogenic tone was between 15 and 30 minutes of ischemia. These results are similar to the threshold duration of ischemia for edema formation, suggesting that loss of myogenic tone may be an important contributor to posts ischemic reperfusion injury.

Materials and Methods

MCAO Model

Different periods of ischemia were produced by filament occlusion of the right MCA in male Wistar rats (weight, 280 to 300 g) with a modification of the technique developed by Koizumi et al.\(^9\) All procedures were approved by the Institutional Animal Care and Use Committee. The animals were anesthetized via inhalation mask with halothane and oxygen. With the aid of a dissecting microscope, the right carotid bifurcation was exposed, and the external carotid artery was coagulated distal to the bifurcation. After temporary ligation of the common carotid artery, a 5-0 nylon monofilament coated with silicone was inserted through the external carotid artery stump and gently advanced to occlude the origin of the MCA (Figure 1). Successful occlusion of the MCA was confirmed by laser-Doppler flowmetry. After initial anesthesia but before midline incision for placement of the monofilament, a skin incision in the right temporal area was made. The temporalis muscle was retracted, and the microtip of the laser-Doppler fiber-optic probe was glued to the skull with cyanoacrylate adhesive (Krazy Glue). The probe was left in place, and CBF was recorded during the experiment.

At 24 hours, before anesthesia was readministered, the animals were scored for neurological deficit by a 28-point scoring system that we developed.\(^{11}\) Two observers, blind to group, scored the animals independently, and the scores were averaged. After neurological deficit was scored and the MCA was removed for isolated artery studies, the brain was embedded in 1.5% agarose and sectioned with a Stoelting tissue slicer into 3-mm slices. The slices were placed in a well plate containing 2% 2,3,5-triphenyltetrazolium chloride (TTC) solution and allowed to develop for 30 minutes. The tissue was then placed in 10% formalin and scanned directly in the dish, and the images were placed in Adobe Photoshop. The National Institutes of Health image analysis program was used to measure the area of ischemia and total hemisphere volume. ANOVA was used to assess differences in lesion volume.

Preparation of MCAs and Pressurized Arteriograph System

After neurological deficit analysis, the animals were reanesthetized, and the brain was quickly removed and placed in oxygenated physiological saline solution (PSS). MCAs from the occluded side of the brain were quickly dissected and mounted on 2 glass microcannulas within the arteriograph chamber that was filled with PSS. The proximal cannula of the chamber was connected to an in-line pressure transducer and servomechanism that continually measured and adjusted TMP. The servo system consisted of a miniature peristaltic pump and controller that permitted TMP to be either maintained at a constant pressure (static) or increased at a variable rate. The distal cannula was closed off so that there was no flow within the arteries. The entire chamber was placed on an inverted microscope with an attached video camera and monitor. An optical window on the bottom of the chamber allowed lumen diameter to be measured by video dimensional analysis, as described previously.\(^{12}\)

Confocal Microscopy and Determination of F-Actin Content

Arterial segments were fixed and cannulated while pressurized at 75 mm Hg. Once diameter measurements were obtained, 1 mL of 37% formaldehyde was added to the 10 mL of PSS already present in the bath to obtain a final concentration of 3.7% (formalin). Arteries were fixed for 15 to 20 minutes, after which they were carefully removed from the cannulas, stained for F-actin with phalloidin, with standard staining techniques, and viewed with a BioRad MRC 1000 confocal scanning laser microscope. Rhodamine fluorescence was detected with an excitation of 568 nm and emission of 605 nm. Arteries were imaged with a ×20 objective, and z-lines representing approximately 1.5 μm thick and 1.0 μm apart in the z axis (into and out of plane) were obtained from optical sections of the arteries. To be consistent, each artery was focused so that the top of the artery was just out of the plane of focus (no image was present), and each artery was sectioned the same distance into the surrounding

Figure 1. MCAO in rat brain. The brain was removed after surgical placement of a silicone-coated nylon monofilament (green thread) into the ICA and advancement to occlude the MCA (arrows). For these studies, the monofilament was removed after different periods of ischemia to allow reperfusion, after which the MCA was dissected, cannulated, and pressurized for study of myogenic responses and ultrastructural studies with confocal microscopy.
VSM. Arteries with tone at 75 mm Hg were used to set the iris and gain optimally with the aid of image analysis software. A fluorescence intensity histogram was recorded with a set area that was appropriate for the size of the arteries. The gain and iris were set the same for all arteries, and the total intensity (gray scale value times the number of pixels that have that value) for a set number of pixels (determined by the set area) was compared. Differences in intensity were determined by ANOVA and considered significant at \( P<0.05 \).

**Experimental Protocol**

The MCAO procedure was used to induce ischemia of the MCA for different time periods: 15 minutes (\( n=6 \)), 30 minutes (\( n=7 \)), or 120 minutes (\( n=8 \)). Arteries subjected to ischemia and reperfusion were compared with a group of vessels from sham-operated control animals (\( n=6 \)) in which the animal underwent anesthesia and a midline incision, but without any impairment of CBF. After the MCAO procedure, arteries were dissected and mounted in the arteriograph bath. Arteries were equilibrated for 1 hour at TMP of 75 mm Hg, during which time spontaneous myogenic tone developed. Lumen diameter was recorded after initial mounting and again after the 1-hour equilibration period. Formalin was then added directly to the bath while the artery was still mounted in the chamber. The arteries were fixed for 20 minutes, after which they were rinsed with PSS, removed from the cannulas, and stored in PSS at 4°C. All arteries were stained at a later date for F-actin.

**Data Calculations and Statistical Analysis**

Percent tone was calculated as a percent decrease in diameter from the baseline diameter at 24°C (room temperature) before heating to 37°C. We and others\(^{13} \) have found this method to produce relaxed diameters within ±1% of relaxed diameters induced by pharmacological inhibition of smooth muscle contraction. No agents were given to relax smooth muscle since they could interfere with F-actin organization. Statistical significance between groups was determined by 1-way ANOVA with a post hoc Bonferroni test and considered significant at \( P<0.05 \).

**Drugs and Solutions**

PSS was composed of the following (mmol/L): NaCl 119.0, NaHCO\_3 24.0, KCl 4.7, KH\_2PO\_4 1.18, MgSO\_4 \cdot 7\_H\_2O 1.17, CaCl\_2 1.6, EDTA 0.026, and glucose 5.5. Phalloidin was obtained from Molecular Probes and mixed fresh before staining. TTC was purchased from Sigma and mixed fresh before each experiment.

**Results**

Figure 1 shows a rat brain in which a 5-0 nylon monofilament coated with silicone was surgically placed into the internal carotid artery (ICA), where it was advanced until it occluded the MCA. This MCAO procedure produces infarction similar to clinical stroke and is transient such that the suture can be removed to allow reperfusion. Because we were interested in how different periods of ischemia affected the myogenic tone of MCAs, we used the MCAO model to focally occlude the MCA and then recorded the change in lumen diameter of isolated and pressurized MCAs. The utility of this model is further strengthened because stroke outcome measures can be assessed, including infarct size and neurological deficit.

The Table shows several measurements of stroke outcome. CBF, measured by laser-Doppler flowmetry, decreased 56% to 72% for the experimental groups during the ischemic period. The reduction in CBF was accompanied by damage to brain tissue, producing infarction that was significant at 30 and 120 minutes of ischemia. Similarly, focal neurological deficit was found to increase with the longer periods of ischemia, as demonstrated by the increase in focal score.

**Discussion**

In the present study we demonstrated that spontaneous myogenic tone was diminished after longer periods of ische-
Cerebral ischemia, with the threshold duration between 15 and 30 minutes. In addition, the ischemia-induced loss of tone was associated with actin cytoskeletal disruption of the VSM. The loss of tone during ischemia and reperfusion could significantly affect stroke outcome since myogenic tone underlies autoregulation of CBF and CVR. Diminished CVR and autoregulatory failure are known to promote blood-brain barrier disruption and edema formation as a result of increased pressure on the microcirculation. In addition, ischemia-induced damage to the MCA may in and of itself negatively affect stroke outcome. For example, the increased wall tension due to dilation and loss of tone could increase vascular permeability of the MCA itself. Therefore, understanding how vascular structure and function are affected by ischemia and reperfusion, including the threshold durations for viability, is important in understanding the pathological process of stroke and for providing better stroke treatment.

It is well known that the depth and duration of ischemia affect the outcome of stroke, giving rise to the threshold concept of cerebral ischemia. Our previous studies demonstrated that longer periods of ischemia and reperfusion resulted in complete loss of myogenic tone in MCAs, whereas short periods produced normal tone. These results suggested that there may be a threshold duration of ischemia and reperfusion for viability of VSM and myogenic tone. Since viability thresholds tend to be flow dependent, changes in CBF were continuously measured transcranially with the use of a laser-Doppler flowmeter with a miniature fiber-optic probe. All animals that underwent the MCAO procedure experienced between 56% and 72% decrease in CBF on MCAO, rates below those required to maintain brain function, producing both infarction and neurological deficit (Table). The time dependence of ischemic injury of the brain is demonstrated in the Table, with longer durations giving rise to larger infarction and worse neurological deficit. While the size of infarct increased with increasing ischemic duration, as measured by TTC staining, these values were not statistically significant, likely because histological lesions require some time before they become visible. Focal neurological deficit, however, determined with a 28-point scoring system, progressively worsened with increasing ischemic period as well, becoming significantly different at 30 and 120 minutes of ischemia. While it is not surprising that these outcome measures of stroke worsened as ischemic duration lengthened, the results of the present study provide evidence that there is a threshold duration of ischemia for myogenic tone of MCAs that is between 15 and 30 minutes (Figure 2).

VSM contraction and myogenic tone have been shown to be dependent on an intact actin cytoskeleton. Disruption of the actin cytoskeleton by pharmacological agents that cause depolymerization of actin filaments or inhibit polymerization of monomeric G-actin into F-actin inhibits VSM contraction and causes vasodilation and loss of tone. Ischemia disrupts actin filaments in several cell types, which is considered a major contributor to ischemic damage. Given the dependence of myogenic tone on an intact actin cytoskeleton, it is possible that ischemia could be disrupting VSM actin and underlie the loss of tone in the MCAs. The effect of ischemia on F-actin was studied by chemically fixing pressurized MCAs with formalin subsequent to tone measurements. The arteries were then stained for F-actin with phalloidin and viewed with scanning laser confocal microscopy. Ischemia had a profound effect on the F-actin content of cerebral artery VSM such that the intensity of F-actin–stained arteries diminished with increasing ischemic duration (Figure 3A). The loss of F-actin in MCAs followed a pattern similar to that
for the loss of myogenic tone, strongly indicating an ischemia-induced effect on cytoskeletal organization in VSM (Figure 3B) as a mechanism underlying this loss of myogenic vasoconstriction. However, while these results demonstrate that longer periods of ischemia are associated with both diminished tone and loss of F-actin, we do not know whether the ischemia causes loss of tone, which in turn causes loss of F-actin, or vice versa. This is an important consideration; other studies have demonstrated that actin filament disruption causes loss of tone, whereas there have been no studies to our knowledge that have shown that loss of tone affects actin structure. However, the causality and sequence of these events are not known, and further studies are needed to fully understand the nature of ischemia-induced loss of tone and F-actin.

This study demonstrated that, similar to other cell types, the actin cytoskeleton of VSM is sensitive to ischemic damage, causing a loss of F-actin. The actin cytoskeleton is a complex and dynamic structure, suggesting that there may be several mechanisms of ischemia-induced damage that could be acting alone or in combination to affect its organization and myogenic tone (Figure 4). For example, actin polymerization is ATP dependent, and therefore ATP depletion during ischemia and its repletion during reperfusion likely have a profound effect on this process. There are many actin-binding proteins that control the dynamics and extent of polymerization, such as actin depolymerizing factor, which has been shown to be activated in kidney epithelial cells during ischemia and to cause actin filament disruption. Additionally, myogenic tone can be affected by factors released from the endothelium. For example, nitric oxide and superoxide anions are produced in large quantities from both the vascular endothelium and neurons during ischemia and can directly interact with actin and interfere with polymerization. Alteration in cytoskeletal structure and dynamics appears to be a common pathway for ischemia-induced damage in many cell types, including VSM. The subsequent loss of myogenic tone in cerebral arteries is a process that likely aggravates ischemic brain damage.

The ischemia-induced effect on the contractile activity of MCAs appears specific for the myogenic response and
Figure 4. Proposed mechanism of ischemia-induced damage to the cerebrovascular smooth muscle and its effect on stroke outcome. Longer periods of ischemia disrupt VSM actin filaments, which causes loss of myogenic tone. The loss of tone promotes autoregulatory loss and postischemic hyperemia, which in turn causes blood-brain barrier (BBB) disruption and edema formation. The cause of ischemia-induced actin filament disruption is not known but may include oxygen free radicals (O2·), nitric oxide (NO), diminished ATP, or an increase in the actin binding protein actin depolymerizing factor (ADF).

pressure-induced contraction. In our previous studies, MCAs that were ischemic for 2 hours and reperfused for 24 hours developed little or no tone and did not respond myogenically to pressure, but they contracted to serotonin with the same sensitivity and reactivity as nonischemic control arteries, strongly indicating that ischemia can specifically affect myogenic contraction.23 Myogenic contraction can also be specifically disrupted by the actin depolymerizing agent cytochalasin B, without interfering with agonist-induced constriction. Previous studies have demonstrated that isolated cerebral arteries with tone at 75 mm Hg dilate and lose tone when exposed to 0.3 μmol/L cytochalasin B but contract vigorously to the protein kinase C activator (−jindolactam-V, indicating that pressure-induced myogenic contraction is more dependent on an intact and dynamic actin cytoskeleton than other mechanisms of contraction (eg, agonist induced).8 Myogenic contraction involves the transduction of a mechanical stimulus (pressure) into a cellular response (contraction). The actin cytoskeleton is in a unique position to both sense and respond to pressure with contraction. In fact, reorganization of the actin cytoskeleton in response to pressure or stretch is an important mechanotransduction process for many cell types.25,26 The exact mechanism of pressure-induced contraction is still unknown; however, since both ischemia and actin filament depolymerization produce a loss of tone, and ischemia promotes actin filament disruption, it is likely that the ischemia-induced effect on myogenic tone is due to a specific disruption of the actin cytoskeleton of cerebral artery VSM.

The MCAO model used in these studies has been increasingly used for the study of ischemic stroke. In fact, this model has greatly improved our understanding of stroke and will likely continue to do so. While most investigators use this model to study damage to brain tissue, we have used it as a way of inducing different periods of ischemia and reperfusion on brain arteries. While we have found this method quite useful, there are certain considerations that should be addressed. First, since we are introducing a filament into the ICA, it is possible that this procedure induces mechanical damage (eg, denudation) to the ICA that could affect the MCA on reperfusion. We have minimized this effect by using a silicone-coated monofilament. The coating makes the filament very slick and therefore likely does not cause damage to the ICA. Second, it is possible that there is a gradient of damage along the MCA distal to the ICA. To eliminate variability due to this effect, we have gone to great lengths to be consistent in using the same segment of MCA in our studies. Lastly, it is possible that the MCA itself could be damaged in this procedure. This is unlikely since the monofilament is not inserted into the MCA but is advanced only until it occludes the bifurcation at the ICA (Figure 1). We are therefore confident that the loss of tone and F-actin noted in the MCA at longer periods of ischemia is due to ischemic damage and not to the use of a filament in this model.

In conclusion, myogenic tone is considerable and widespread in cerebral arteries, contributing significantly to autoregulation of CBF and CVR.1–3 We have demonstrated that longer periods of ischemia are associated with both loss of myogenic tone and disruption of VSM F-actin, with the threshold duration between 15 and 30 minutes. While the causality of these events is not clear, loss of autoregulation and diminished CVR during postischemic reperfusion are known to promote significant brain damage, including edema.5 Understanding how ischemia and reperfusion affect the structure and function of the cerebral arteries seems critical to understanding the pathophysiological mechanisms of stroke. Future treatments that target cerebrovascular protection may be beneficial and could minimize secondary brain tissue damage during reperfusion.

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References


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