Middle Cerebral Artery Function After Stroke
The Threshold Duration of Reperfusion for Myogenic Activity
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Background and Purpose—Myogenic activity of the cerebral arteries is an important contributor to autoregulation of cerebral blood flow. Previous studies have demonstrated that increasing periods of ischemia diminished the amount of myogenic tone in cerebral arteries. In the present study, we investigated the effect of different periods of posts ischemic reperfusion on the myogenic behavior of middle cerebral arteries (MCAs). We measured both the amount of spontaneous myogenic tone that developed at 75 mm Hg and the contractile response to increased transmural pressure (TMP), ie, myogenic reactivity.

Methods—The MCA occlusion model was used in male Wistar rats (n=45) to induce 30 minutes of temporary ischemia, followed by different periods of reperfusion (0 or sham; 30 minutes; and 6, 12, 18, 20, and 22 hours), confirmed by laser Doppler flowmetry. MCAs were studied in vitro using an arteriograph system that allowed control of TMP and measurement of lumen diameter. After equilibration for 1 hour at 75 mm Hg, TMP was increased stepwise in 25–mm Hg increments to 125 mm Hg and lumen diameter measured at each pressure. The amount of spontaneous myogenic tone was determined in both ischemic and contralateral arteries for each reperfusion period and compared with the right and left MCAs in the sham group. Arteries were then fixed with 10% formalin pressed 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 using confocal microscopy.

Results—MCAs from the sham-operated control group possessed considerable myogenic tone (35%). However, the amount of tone in ischemic MCAs progressively diminished as the reperfusion duration increased. In addition, sham-operated control arteries responded myogenically to increases in TMP, decreasing diameter as pressure increased. There was a similar response in arteries exposed to 30 minutes and 6 hours of reperfusion, all producing a negative slope on the pressure-diameter curve; however, myogenic reactivity was diminished at the longer periods of reperfusion, producing a positive slope of the graph. The slopes of the pressure-diameter curves were as follows: −0.10±0.06 (sham), −0.07±0.12 (30 minutes), −0.08±0.11 (6 hours), +0.09±0.09 (12 hours), +0.25±0.16 (18 hours), +0.38±0.09 (20 hours), and +0.57±0.09 (22 hours). F-actin content was significantly less only in ischemic MCAs at 6 and 12 hours of reperfusion.

Conclusions—These results demonstrate that longer periods of reperfusion significantly diminish myogenic activity of MCAs. Understanding how different periods of ischemia and reperfusion affect the function of the cerebral circulation may promote more effective treatment of ischemic stroke. (Stroke. 2002;33:2094-2099.)

Key Words: cytoskeleton ■ middle cerebral artery ■ reperfusion injury ■ rats
response to neuronal, endothelial, or metabolic factors.\textsuperscript{3,10} The cerebral circulation also responds to changes in pressure or stretch, a function that has been termed myogenic reactivity.\textsuperscript{4} In fact, the concept of a pressure-dependent myogenic response that can play a major role in the autoregulation of CBF has been around for a century now, originating from the observations of Bayliss in 1902.\textsuperscript{11}

There are certain conditions that result in both diminished myogenic reactivity and autoregulatory capacity. For example, the reperfusion period after occlusion of a cerebral artery is associated with autoregulatory failure and a period of vasomotor paralysis that often contributes to reperfusion injury and edema formation.\textsuperscript{12–14} In addition, treatment with tissue plasminogen activator, the only Food and Drug Administration–approved treatment for ischemic stroke, significantly diminished myogenic reactivity in isolated cerebral arteries, a result that was additive if arteries were exposed to ischemia.\textsuperscript{15} Given that tissue plasminogen activator treatment has been shown to produce edema and hemorrhage if given after longer periods of ischemia and reperfusion,\textsuperscript{16} understanding how different periods of ischemia and reperfusion affect the structure and function of the cerebral arteries may be particularly important to improving treatment given that the vascular system is directly exposed to thrombolytic agents.

Our own work recently demonstrated that a threshold duration of ischemia for myogenic activity exists.\textsuperscript{17} In this study, the threshold duration of ischemia for myogenic tone was between 15 and 30 minutes. The loss of myogenic tone was associated with a loss of filamentous (F)–actin in the vascular smooth muscle, suggesting that longer periods of ischemia affect the structure as well as the function of cerebral arteries. In the present study, we sought to determine the threshold duration of reperfusion for myogenic activity, keeping the ischemic duration constant at 30 minutes. We used the middle cerebral artery occlusion (MCAO) model to induce different periods of reperfusion in the MCA and then evaluated the amount of myogenic activity that was present, as well as F-actin content. Our results show that there is a progressive loss of myogenic activity as reperfusion duration increases.

Materials and Methods

MCAO Model

Male Wistar rats (280 to 300 g) were used for all experiments. Different periods of reperfusion were produced by temporary filament occlusion of the right MCA for 30 minutes.\textsuperscript{15,17,18} 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 coagulated distal to the bifurcation. After temporary ligature 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. Successful occlusion and reperfusion of the MCA was confirmed using laser Doppler flowmetry (Perimed). After initial anesthesia, a skin incision in the right temporoparietal area was made, the temporalis muscle was retracted, and the micropipet of the laser Doppler fiberoptic probe was glued to the skull with Krazy Glue. The probe was left in place and CBF recorded during the experiment.

Preparation of MCAs and Pressurized Arteriograph System

After the appropriate duration of reperfusion, the animals were reanesthetized and the brains quickly removed and placed in oxygenated physiological salt solution (PSS). MCAs from both the occluded side of the brain and the contralateral side were quickly dissected and mounted on 2 glass microcannula within a dual-chamber arteriograph (Living Systems) that was filled with PSS, as described elsewhere.\textsuperscript{15,17} In previous studies, we have found the architecture of the MCA to be consistent among the rats, with the segment most proximal to the circle of Willis containing 6 or 7 collaterals followed distally by a branch-free segment. This branch-free segment of the MCA was consistently used for experimentation because vessels with collaterals lead to a variable anatomy provided a consistent segment for study. The proximal cannulas of both chambers were connected via a manifold to an in-line pressure transducer and servomechanism that continually measured and adjusted transmural pressure (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 there was no flow through 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 using video dimensional analysis, as described previously.\textsuperscript{15,19}

Confocal Microscopy and Determination of F-Actin Content

F-actin content was determined as previously described.\textsuperscript{17} Briefly, arterial segments were fixed while cannulated and pressurized at 75 mm Hg subsequent to diameter measurements by addition of 1 mL of 37% formaldehyde 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 and stained for F-actin with phalloidin, using a staining technique described previously.\textsuperscript{17} The arteries were then viewed using a BioRad MRC 1000 confocal scanning laser microscope. Rhodamine fluorescence was detected using an excitation of 568 nm and emission of 605 nm. Arteries were imaged with a 20× objective, and z-axis lines representing ~1.5 µm thick and 1.0 µm apart in the z-axis lines (into and out of the 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 vascular smooth muscle. 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 using 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×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 using ANOVA and considered significant at $P<0.05$. A post hoc Bonferroni test was performed to determine between-group differences.

Experimental Protocol

The MCAO procedure was used to induce 30 minutes of ischemia, followed by different periods of reperfusion, as follows: 30 minutes (n=6), 6 hours (n=7), 12 hours (n=8), 18 hours (n=6), 20 hours (n=6), and 22 hours (n=6). After the MCAO procedure, both occluded and contralateral MCAs were dissected and mounted in the 2 chambers of the arteriograph bath. Arteries were equilibrated for 1 hour at TMP=75 mm Hg, during which time spontaneous myogenic tone developed. TMP was then increased stepwise in 25–mm Hg increments to 125 mm Hg and diameter recorded at each pressure after it stabilized (~15 minutes). After diameter measurement, TMP was returned to 75 mm Hg and formalin was 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.

Control Arteries
Arteries subjected to ischemia and reperfusion were compared with a group of vessels from sham-operated control animals (n=6) in which the animals underwent anesthesia and a midline neck incision, but without any impairment of CBF. In addition to the sham-operated control animals, MCAs contralateral to the ischemic side were dissected and mounted in the second arteriograph chamber and were used as an internal control.

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 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 because they could interfere with F-actin organization. Statistical significance in the amount of myogenic tone between ischemic and contralateral MCAs was determined using a paired t test given that both arteries were taken from the same animals. Differences in the amount of tone at different periods of reperfusion for either ischemic or contralateral compared with sham-operated control, as well as differences in diameters at each pressure and slopes between reperfusion groups, were determined using 1-way ANOVA with a post hoc Bonferroni test and considered significant at P<0.05.

Drugs and Solutions
PSS contained the following (in mmol/L): NaCl 119.0, NaCHO 3 24.0, KCl 4.7, KH₂PO₄ 1.18, MgSO₄ 1.6, EDTA 0.026, and glucose 5.5. Phalloidin was obtained from Molecular Probes and mixed fresh before staining.

Results
Myogenic Reactivity After Different Periods of Reperfusion
Figure 1 shows the diameter response of each group of arteries during equilibration at 75 mm Hg and after stepwise increases in pressure. The diameter of all groups of arteries was similar at the start of equilibration (at 24°C; P=0.05). During equilibration, all arteries developed spontaneous myogenic tone and decreased diameter. The amount of tone that developed varied depending on reperfusion duration (see below), as can be seen by the different diameters at 75 mm Hg. The response of arteries to pressure (ie, myogenic reactivity) was variable as well, with the longer reperfusion groups (that had developed the least amount of spontaneous myogenic tone) responding the least. Several groups of arteries (sham-operated control, 30-minute reperfusion, and 6-hour reperfusion) responded myogenically and constricted to the higher intravascular pressures when pressure was increased stepwise within the myogenic pressure range from 75 to 125 mm Hg. However, this response diminished in arteries exposed to longer periods of reperfusion (12, 18, 20, and 22 hours).

The slope of the pressure-versus-diameter curves (Figure 2) provides valuable information regarding how the arteries responded to pressure, in both direction and magnitude. For example, the graph shows that arteries exposed to ≤6 hours of reperfusion had a small negative slope, demonstrating that in general these arteries responded with contraction to increased pressure (ie, had myogenic reactivity). However, arteries exposed to ≥12 hours of reperfusion had a positive slope that increased in magnitude (ie, the slope became more positive) as reperfusion duration increased. This demonstrates that not only did longer periods of reperfusion diminish myogenic reactivity, but the response became more diminished as the duration of reperfusion increased (ie, the slopes became more positive).

Contralateral MCAs in all groups responded to the increase in TMP with contraction, producing a negative slope on the graph (data not shown). The slopes of the pressure-versus-diameter curves were the following: -0.33±0.12 (contralateral sham), -0.23±0.07 (30 minutes), -0.12±0.06 (6 hours), -0.23±0.14 (12 hours), -0.33±0.09 (18 hours), -0.30±0.05 (20 hours), and -0.16±0.10 (22 hours).
Myogenic Tone After Different Periods of Reperfusion

As mentioned above, all arteries developed spontaneous myogenic tone during equilibration at 75 mm Hg, that varied in magnitude depending on the duration of reperfusion. Figure 3 shows the amount of tone in ischemic and contralateral MCAs for each period of reperfusion compared with sham-operated control animals. In the ischemic arteries, the amount of tone was significantly diminished as early as 30 minutes of reperfusion and continued to decline at subsequent time points. Similarly, contralateral MCAs had significantly less tone at reperfusion durations ≥6 hours, suggesting that the effect of ischemia and reperfusion on myogenic tone may be global.

F-Actin Content in Ischemic and Contralateral MCAs After Different Periods of Reperfusion

Confocal microscopy was used to determine the amount of F-actin in both ischemic and contralateral MCAs given that the polymerization state of actin has been shown to relate to the amount of tone in cerebral arteries and is altered in response to pressure. In addition, because previous work demonstrated that increased ischemic duration significantly diminished the amount of F-actin, in this study we wanted to determine whether the reperfusion duration affected F-actin content. We compared the amount of F-actin in 3 ways. First, F-actin content was compared in the ischemic and contralateral arteries within these groups to determine whether the increased reperfusion duration affected F-actin content (that may correlate with the diminished myogenic tone). Second, we compared the F-actin content of arteries from the ischemic animals (from both the ischemic and contralateral sides) to the sham-operated control animals to determine whether F-actin was similar to a group of animals that were not ischemic. Third, we compared the F-actin content between ischemic and contralateral arteries within each reperfusion group. The Table shows that the amount of F-actin in the ischemic MCAs was significantly less after 6 and 12 hours of reperfusion compared with the contralateral MCAs. There was no difference in F-actin content between the groups compared with sham for either ischemic or contralateral MCAs, or within each group, suggesting that the diminished tone was not due to altered F-actin content. However, it is interesting that the time periods in which there was decreased F-actin content also constituted the threshold duration of reperfusion for myogenic tone.

Discussion

Myogenic behavior is an important function of cerebrovascular smooth muscle that can play a major role in the autoregulation of CBF. We have assessed the myogenic activity of MCAs after different periods of reperfusion in 2 ways: (1) The amount of spontaneous myogenic tone that developed at 75 mm Hg was measured as a percent decrease in diameter. (2) Myogenic reactivity was determined as the diameter response to step increases in pressure from 75 to
125 mm Hg. We found that both the amount of tone and myogenic reactivity were fairly normal after short durations of reperfusion but diminished after longer periods of reperfusion. For example, the amount of tone was significantly diminished after just 30 minutes of reperfusion, and myogenic reactivity was diminished after 6 hours and became progressively worse as the reperfusion duration increased. Therefore, the threshold duration of reperfusion for myogenic reactivity was ≥6 hours. Because myogenic reactivity underlies autoregulation of CBF, it is possible that autoregulatory failure occurs after 6 hours of reperfusion.

In normotensive adults, CBF is maintained at ≈50 mL/100 g of brain tissue per minute provided mean arterial blood pressure is in the range of 60 to 150 mm Hg. Above and below this limit, autoregulation is lost and CBF becomes dependent on mean arterial pressure in a linear fashion. However, certain conditions can alter the autoregulatory curve and shift the range to higher or lower pressures. For example, in chronically hypertensive patients, both the lower and upper limits of autoregulation are extended and the curve is shifted to the right.

During postischemic reperfusion, edema formation can raise intracranial pressure, which decreases cerebral perfusion pressure and shifts the autoregulatory curve to the lower levels. In the present study, we chose to study the myogenic activity of arteries between 75 and 125 mm Hg, pressures within the normal autoregulatory range, for several reasons. First, it is unlikely that the shift in the autoregulatory curve during edema formation is due to vascular remodeling (which does occur in hypertension); it is more likely due to ischemia. Ischemic injury to the cerebral vascular smooth muscle has been demonstrated in our own studies to be associated with a loss of F-actin. Because the state of actin polymerization has also been shown to be affected by pressure, we wanted to study all groups of arteries under the same pressurized conditions to detect changes due to ischemia and reperfusion. Second, it is not clear that the duration of ischemia used, 30 minutes, created edema formation, which would shift the autoregulatory curve. Autoregulation was not measured in this study, which was focused on cellular changes that affect the myogenic activity of the cerebral arteries. Third, by studying all groups of arteries within the normal pressure range, we can better understand how reperfusion alters myogenic activity compared with normal. In fact, there was a significant decrease in both myogenic reactivity and tone of arteries exposed to longer periods of reperfusion within this range of pressures.

For all the groups studied, the ischemic duration was held constant at 30 minutes. This was done because, in a previous study that investigated the threshold duration of ischemia for myogenic activity, we found that after 30 minutes of ischemia there was a significant loss of myogenic tone. Unlike the ischemic threshold in which there was a sharp drop in myogenic tone between 15 and 30 minutes, the response to increasing periods of reperfusion resulted in a more progressive loss of tone over time (Figure 3). Interestingly, there was a similar, although less pronounced, loss of tone in contralateral MCAs, suggesting the mechanism of disruption of tone to be both focal and global after stroke. The mechanism by which contralateral MCAs were affected is not clear, although global effects of ischemia have been reported that could affect the entire circulation. For example, production of NO and superoxide are produced in large quantity during ischemia and reperfusion. As this is an anastomotic circulation, diffusible vasoactive factors produced during reperfusion could affect other regions of the brain.

In addition to comparing responses to the side contralateral to ischemia, we also compared myogenic activity with that of a group of animals that underwent anesthesia and a midline incision in the neck, but did not have any interference of blood flow. We compared the response of these sham-operated control animals from both the right and left sides of the brain as a control for both the ischemic and contralateral arteries in the experimental groups. We found that the myogenic tone was ≈35% in arteries from both sides of the brain in sham-operated control animals. In ischemic animals, the amount of myogenic tone declined after just 30 minutes of reperfusion in the ischemic MCA and declined after 6 hours of reperfusion in the contralateral MCA (Figure 3), further demonstrating that the effect of ischemia and reperfusion on myogenic activity is partially global.

It is likely that the mechanism by which ischemia affects the cerebral circulation is different from that of reperfusion. During ischemia, there is a loss of ATP, increased NO production in both cerebral endothelial cells and neurons, and decreased protein synthesis. During reperfusion, however, the predominant events are generation of oxygen free radicals and immune response elements, including leukocyte adhesion and platelet activation. In addition, the increased NO production during ischemia is available to react with oxygen free radicals generated during reperfusion, forming peroxynitrite (ONOO−). Peroxynitrite is a highly reactive compound and vasoactive factor that has been shown to be increased during postischemic reperfusion. In rat brain, 2 hours of MCAO produced a time-dependent increase in nitrotyrosine, a marker of ONOO− production, in the cerebral cortex. The effect of ONOO− on myogenic activity of the cerebral circulation has been demonstrated and has been shown to reduce dilation to decreased intravascular pressure and cause vasomotor paralysis at high concentrations. Therefore, ONOO− generation during reperfusion may be a significant contributor to the loss of tone and myogenic reactivity during reperfusion.

Myogenic behavior has been shown to depend on an intact and dynamic actin cytoskeleton. For example, inhibition of actin polymerization by cytochalasin B causes loss of tone at high concentrations and diminishes myogenic activity at lower concentrations. In addition, depolymerization of actin filaments with cytochalasin D causes both a loss of tone and myogenic reactivity that correlates with increased globular monomeric actin. Ischemia has been shown to affect the state of actin polymerization in numerous cell types, including vascular smooth muscle. We previously demonstrated that loss of tone due to increased ischemic duration was associated with loss of F-actin. In the present study, there was no correlation between the polymerization state of actin and the amount of myogenic tone. In fact, we saw a loss of F-actin only at 6 and 12 hours of reperfusion. The discrepancy between this study and the
previous one may be a result of the mechanism of damage during ischemia being different from that during reperfusion. For example, F-actin may be more susceptible to ischemic injury by NO or ATP depletion; however, when the ischemic duration is kept constant and the reperfusion period increases, increased peroxynitrite production may be causing the loss of tone, as has been demonstrated in other studies.5,37

In conclusion, we have shown that both myogenic tone and reactivity to pressure are diminished as the reperfusion period increases. Although the mechanism for this is unclear, it appears to be different from ischemic damage in which the actin cytoskeleton was severely affected. Understanding both the threshold duration of both ischemia and reperfusion, as well as the mechanisms by which myogenic activity is lost, is important to understanding how stroke affects the cerebral circulation.

Acknowledgments

This study was supported by National Institute of Neurologic Disorders and Stroke (National Institutes of Health) Grants R55 NS37854 and RO1 NS40071 (both to M.J.C.) and by the Totman Center for Cerebrovascular Research (to M.J.C.).

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Stroke. 2002;33:2094-2099
doi: 10.1161/01.STR.0000020712.84444.8D
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0039-2499. Online ISSN: 1524-4628

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