Stroke Development in Stroke-Prone Spontaneously Hypertensive Rats Alters the Ability of Cerebrovascular Muscle to Utilize Internal Ca\(^{2+}\) to Elicit Constriction

John S. Smeda, PhD

**Background and Purpose**—The ability of middle cerebral arteries (MCAs) to utilize intracellular smooth muscle (SM) Ca\(^{2+}\) to produce constriction in response to pressure and agonists was assessed in relation to hemorrhagic stroke development in Wistar-Kyoto stroke-prone (SHRSP) and stroke-resistant (srSHR) spontaneously hypertensive rats.

**Methods**—MCAs were studied with the use of a pressure myograph at 100 mm Hg.

**Results**—MCAs from srSHR and prestroke SHRSP exhibited pressure-dependent constriction and constricted in response to vasopressin or serotonin in the presence of nifedipine or the absence of \([\text{Ca}^{2+}]_o\). MCAs from poststroke SHRSP lost the latter functions and could only constrict in response to vasopressin/serotonin in Krebs’ solution containing Ca\(^{2+}\) in the absence of nifedipine. This indicated that the SM could not utilize internal Ca\(^{2+}\) for constriction and maintained constriction by Ca\(^{2+}\) entry through L-type channels. The MCAs of poststroke SHRSP could not constrict to \([K^+]_o\)-induced depolarization, suggesting that the agonist-induced opening of the L-type channels occurred by mechanisms other than SM depolarization. Depletion of the sarcoplasmic SM Ca\(^{2+}\) stores of MCAs from srSHR with cyclopiazonic acid did not prevent pressure-dependent constriction.

**Conclusions**—Stroke in SHRSP produced a defect in the ability of MCAs to constrict in response to vasopressin or serotonin via the use of an intracellular source of Ca\(^{2+}\). This could be promoted by an inability of the SM to release intracellular Ca\(^{2+}\), by the depletion of internal Ca\(^{2+}\) stores, or by a decrease in the contractile sensitivity to Ca\(^{2+}\) released from the internal stores. (*Stroke. 2003;34:1491-1496.*)

**Key Words:** calcium ■ hypertension ■ muscle, smooth ■ sarcoplasmic reticulum ■ stroke ■ vasopressins

Cerebrovascular pressure-dependent constriction plays an important role in promoting cerebral blood flow autoregulation.\(^1\)\(^2\) Pressure-dependent constriction in the middle cerebral arteries (MCAs) and cerebral blood flow autoregulation in the cerebrum of Wistar-Kyoto stroke-prone spontaneously hypertensive rats (SHRSP) become defective before and after hemorrhagic stroke development.\(^3\)\(^-\)\(^5\) The loss of cerebral blood flow autoregulation in SHRSP before stroke could promote overperfusion of the brain under hypertensive conditions.

The role of intracellular smooth muscle (SM) Ca\(^{2+}\) in promoting pressure-dependent constriction remains controversial.\(^2\) Stretched cultured vascular SM cells and the SM of stretched aortic rings produce inositol triphosphate (IP\(_3\)),\(^6\)\(^7\) which can activate the release of sarcoplasmic Ca\(^{2+}\). Pressurization of dog renal arteries produces an increase in SM IP\(_3\) and diacylglycerol (DAG) levels.\(^8\) Fura-2 studies of isolated coronary arterial SM\(^9\) and pharmacological studies of cerebral arteries\(^10\)\(^11\) indicate that stretch produces an intracellular release of Ca\(^{2+}\) and phasic constriction in the absence of extracellular Ca\(^{2+}\). These responses are inhibited by phospholipase C (PLC) inhibitors and ryanodine, which depletes intracellular SM Ca\(^{2+}\) stores.\(^10\)\(^11\) PLC inhibition has also been shown to inhibit pressure-dependent constriction in rat cerebral arteries.\(^12\) Drugs such as thapsigargin and cyclopiazonic acid (CPA), which deplete SM sarcoplasmic stores of Ca\(^{2+}\) as well as the PLC inhibitor U-73122, inhibit pressure-dependent constriction in rat renal arterioles.\(^13\) These studies suggest that in some arterial systems, pressure-dependent constriction may involve the utilization of an internal SM store of Ca\(^{2+}\).

In the present study I tested the hypothesis that the MCAs of SHRSP and stroke-resistant spontaneously hypertensive rats (srSHR) utilized an internal SM store of Ca\(^{2+}\) to mediate pressure-dependent constriction and assessed the possibility that an altered ability to utilize this store after stroke development might be responsible in promoting the alterations in pressure-dependent constriction observed in the MCAs of SHRSP after hemorrhagic stroke development.

**Materials and Methods**

Experiments using SHRSP and srSHR were in compliance with the guidelines of the Canadian Council on Animal Care. At weaning (5
weeks of age), the rats were fed a Japanese-style diet (Zeigler Bros) containing 4% NaCl. SHRSP fed the diet develop hemorrhagic stroke at approximately 12 weeks of age and experience 100% mortality by 18 weeks of age, while srSHR fed the same diet do not develop stroke. The relationship of stroke development to blood pressure (BP) and the behavioral characteristics of stroke in SHRSP have been described elsewhere.3,14

Before sampling, the systolic BPs of unanesthetized rats were measured by a tail cuff compression method. Details of the BP measuring and arterial pressure myograph techniques have been described previously.3,5,13 Rats anesthetized with 65 mg/kg IP sodium pentobarbital were exsanguinated, and the brain was re-removed and placed in oxygenated (95% O2/5% CO2) Krebs’ physiological salt solution cooled with ice. A segment of the right or left MCA that crosses over the rhinalis fissure was excised and mounted in a pressure myograph.3,5 The distal end and the side branches of the artery were tied to form a closed artery filled with Krebs’ solution, and the exterior of the artery was suffused with oxygenated Krebs’ solution at 37°C. Through the use of a pressurized reservoir and reduction valves, any desired back pressure could be applied instantaneously to the arterial lumen. Arterial lumen changes were viewed with a microscope, video recorded, and measured at a magnification of ×322.

After the experiment, the brain was fixed in PO4 (85 mmol/L)–buffered 4% formaldehyde and 1% glutaraldehyde solution (pH 7.4), cut into 0.5-mm sections, and studied with a microscope to confirm the presence of intracerebral hemorrhage.

Pressure-dependent constriction in the MCAs was studied by a previously outlined protocol.3,5 Initially, the MCAs were pressurized to 100 mm Hg for 30 minutes, permitting the MCAs to develop pressure-dependent constriction. Subsequently, the pressure was decreased to 0 mm Hg for 6 minutes. The MCAs were then pressurized to 100 mm Hg, and the change in lumen diameter was measured from 1 second to 4 minutes after pressurization. The lumen diameter present at 1 second after pressurization to 100 mm Hg represents the diameter present before the engagement of pressure-dependent constriction. The MCA will constrict to a new steady state lumen diameter appropriate to the applied pressure within 3.5 minutes. The reduction in lumen diameter between 1 second and 4 minutes after pressurization to 100 mm Hg was used as a measure of pressure-dependent constriction.

All the pharmacological manipulations outlined in this report were performed on arteries at a pressure of 100 mm Hg. The chemicals used were purchased from Sigma Chemical Co.

The n values within the figures represent the number of rats sampled. Mean±1 SE values are shown. Results were considered significantly different at P<0.05. An ANOVA determined whether significant between-group differences existed in a given parameter, and an unpaired Student’s t test (compensated for multiple comparisons with the Bonferroni method) was used to determine subgroup differences. Linear regression analysis and Pearson product correlations were used to determine whether relationships existed between 2 parameters.

Results

The mean age of the rats was 14.2±0.5 weeks. Poststroke SHRSP had higher (P<0.05) mean systolic BPs (245±18 mm Hg) than either srSHR (213±14 mm Hg) or prestroke SHRSP (219±12 mm Hg).

The Table outlines the characteristics of pressure-dependent constriction observed in the MCAs of SHR. The MCA lumen diameters present 1 second after pressurization to 100 mm Hg were not different between prestroke SHRSP and srSHR, while MCAs from poststroke SHRSP had smaller lumen diameters than the latter groups. The MCAs of prestroke SHRSP and srSHR constricted in response to a pressure step of 100 mm Hg, whereas the MCAs of poststroke SHRSP did not. After 4 minutes of equilibration to 100 mm Hg, the MCAs of srSHR and prestroke SHRSP produced greater amplitudes of constriction and maintained a smaller lumen diameter than those of prestroke or poststroke SHRSP. The addition of 1 μmol/L nifedipine into normal Krebs’ solution or the presence of Ca²⁺-free/EGTA Krebs’ solution maximally relaxed the MCA. Under the latter conditions, pressure-dependent constriction was inhibited, and the MCA could not elicit any constriction in response to a pressure step of 100 mm Hg. Pressurization to 100 mm Hg for 1 second after equilibration to 0 mm Hg did not produce dilation of the lumen in the MCAs of poststroke SHRSP, indicating that these MCAs had basal tone, previously described as non-pressure-dependent tone,3,5 that was not modified by a decrease in pressure. Both pressure-dependent constriction and non-pressure-dependent tone were inhibited by 1 μmol/L nifedipine.

Figure 1 outlines the constriction of MCAs sampled from srSHR and SHRSP in response to vasopressin, serotonin, and 100 mmol/L K⁺, at a pressure of 100 mm Hg. Constriction was measured under control conditions of 2.5 mmol/L [Ca²⁺], in the presence of 1 μmol/L nifedipine, or in Ca²⁺-free/EGTA. Before each experiment, the MCAs were flushed with Krebs’ solution containing 2.5 mmol/L [Ca²⁺], to replenish the internal SM Ca²⁺ stores.

In the presence of 2.5 mmol/L [Ca²⁺], the MCAs of srSHR and prestroke and poststroke SHRSP constricted in response to 0.17 μmol/L vasopressin (Figure 1A) and 2.7 μmol/L serotonin (Figure 1B). The amplitude of constriction was slightly smaller in the MCAs of poststroke SHRSP than in the other 2 groups of SHR; however, this difference was not significant at P<0.05.

The MCAs of srSHR and prestroke SHRSP constricted in response to vasopressin or serotonin in the presence of...
nifedipine or in the absence of external Ca\textsuperscript{2+}, while those of poststroke SHRSP failed to respond to the agonists (Figure 1A and 1B). The amplitude of constriction observed in the MCAs of srSHR in response to each agonist was comparable or greater than that present in the MCAs of prestroke SHRSP. The response observed in the MCAs of poststroke SHRSP was not different from zero.

Vasopressin and serotonin constriction of the MCA in the presence of nifedipine or in Ca\textsuperscript{2+}-free/EGTA Krebs’ solution was likely mediated by a release of Ca\textsuperscript{2+} from an intracellular SM store. Therefore, the aforementioned results would predict that either the size of the internal Ca\textsuperscript{2+} pool used for contraction was reduced or absent or that the signal transduction mechanisms promoting the release of Ca\textsuperscript{2+} from the pool in response to the agonists were defective in the MCAs of poststroke SHRSP. Alterations in contractile sensitivity to Ca\textsuperscript{2+} may also have played a role in inhibiting constriction (see Discussion).

Constriction in response to 100 mmol/L [K\textsuperscript{+}] o in the MCAs was inhibited by 1 \mu mol/L nifedipine and did not occur in Ca\textsuperscript{2+}-free/EGTA Krebs’ solution, indicating that this response depended on the presence of external Ca\textsuperscript{2+} influx through SM L-type Ca\textsuperscript{2+} channels. In normal Krebs’ solution (2.5 mmol/L Ca\textsuperscript{2+}) lacking nifedipine, the response in prestroke SHRSP was not different from that measured in srSHR, while the MCAs of poststroke SHRSP were unresponsive to 100 mmol/L [K\textsuperscript{+}] (Figure 1C).

Both vasopressin and serotonin utilized identical pools of internal Ca\textsuperscript{2+} for constriction (Figure 2). The MCAs of srSHR produced a phasic constriction in Ca\textsuperscript{2+}-free/EGTA Krebs’ solution. Constriction disappeared in 5 minutes. Presumably the Ca\textsuperscript{2+} released from the internal Ca\textsuperscript{2+} pool by vasopressin diffused from the SM and was chelated by the EGTA. After vasopressin, serotonin could not constrict the MCA (pressure=100 mm Hg; n=5 srSHR).

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In addition to releasing internal SM Ca\textsuperscript{2+}, both serotonin and vasopressin opened L-type Ca\textsuperscript{2+} channels. As shown in Figure 3, despite the inability to elicit constriction in response to elevated [K\textsuperscript{+}] o, or to utilize an internal Ca\textsuperscript{2+} store for constriction (ie, to constrict in the presence of Ca\textsuperscript{2+}-free/
EGTA Krebs’ solution), the MCAs of poststroke SHRSP constricted in response to serotonin and vasopressin in the presence of 2.5 mM [Ca\(^{2+}\)]_o. Since the latter constriction was blocked by 1 µmol/L nifedipine, the results were interpreted to suggest that the agonists maintained constriction by Ca\(^{2+}\) entry into the SM through L-type Ca\(^{2+}\) channels. Since the same MCAs could not constrict in response to elevated [K\(^{+}\)]_o, (i.e., via depolarization), agonist constriction involving L-type channels may have occurred through a mechanism other than SM depolarization.

An attempt was made to deplete the internal SM Ca\(^{2+}\) pool released by vasopressin and serotonin and prevent its refilling by the use of a sarcoplasmic Ca\(^{2+}\) pump inhibitor, CPA. The ability of the MCA to elicit pressure-dependent constriction under the latter conditions was tested in srSHR. As shown in Figure 4, the experiment was started under conditions in which both the lumen and exterior of the MCAs of srSHR were bathed with Ca\(^{2+}\)-free/EGTA Krebs’ solution. The arteries dilated maximally and did not constrict in response to a pressure step of 100 mm Hg. Then 2.5 mM [Ca\(^{2+}\)]_o was introduced into the suffusate, and pressure-dependent constriction to a pressure step of 100 mm Hg indicated a 44% constriction. The MCAs, now pressurized to 100 mm Hg, were resuffused with Ca\(^{2+}\)-free/EGTA Krebs’ solution. Pressure-dependent constriction disappeared in the arteries. In the absence of external Ca\(^{2+}\), vasopressin was introduced into the bath and produced a potent phasic constriction of the MCA (see vasopressin peak 2 in Figure 4). After 10 minutes, the constrictor response to vasopressin disappeared, and the arteries dilated maximally. The subsequent introduction of 2.7 µmol/L serotonin failed to promote constriction, suggesting that the Ca\(^{2+}\) from the common internal SM pool released by serotonin and vasopressin was depleted from the arteries. The arteries were then flushed with Ca\(^{2+}\)-free/EGTA Krebs’ solution containing CPA (10 µmol/L) for 4 minutes and Krebs’ solution containing 2.5 mM [Ca\(^{2+}\)]_o, plus CPA for another 6 minutes. CPA inhibits the uptake of Ca\(^{2+}\) into the sarcoplasmic reticulum of arteries. The ability of the MCA to constrict to a pressure step of 100 mm Hg in the presence of CPA was tested (see 5. in Figure 4). Under conditions in which the sarcoplasmic Ca\(^{2+}\) should have been depleted and prevented from refilling, the MCAs of srSHR still exhibited approximately 24% constriction to the pressure step. However, the pressure-dependent constriction response was significantly less than the 44% pressure-dependent constriction observed before the applied treatment. An analysis of the absolute lumen diameter changes occurring at A and B of Figure 4 indicated that the reduction in the amplitude of pressure-dependent constriction that occurred under conditions in which the internal Ca\(^{2+}\) store was depleted was achieved by the presence of elevated vascular tone 1 second after 100 mm Hg pressure was applied to the arteries and by a decreased ability to achieve the same reduction in lumen diameter 4 minutes after the application of the pressure step.

In the last phase of the experiment, the ability of the MCAs to constrict to 100 mM/L [K\(^{+}\)]_o in the presence of CPA was tested. In some experiments (3 of 10), CPA totally abolished pressure-dependent constriction. However, such MCAs also failed to constrict to 100 mM/L [K\(^{+}\)]_o, indicating that functional changes other than the depletion of the sarcoplasm-
mic Ca²⁺ pool had occurred. In 1 of 10 experiments, the MCAs contracted to pressure (40%) after treatment but did not respond to elevated K⁺. MCAs that could not constrict to elevated K⁺ after CPA treatment were rejected from the experiment. The remaining arteries (6 MCA/6 srSHR) were used in the experiment outlined in Figure 4. In the presence of 2.5 mmol/L [Ca²⁺], there existed a direct relationship between the ability of the MCA to constrict to pressure after the depletion of the sarcoplasmic SM Ca²⁺ pool (B in Figure 4) and the ability of the MCA to constrict to 100 mmol/L [K⁺]. (constriction to pressure step of 100 mm Hg after internal SM Ca²⁺ depletion=96.1±3.51 times the percent constriction to 100 mmol/L [K⁺]; r=0.758, P<0.05). On the other hand, no significant relationship (r=0.097) was observed between constriction to pressure in 2.5 mmol/L [Ca²⁺] and subsequent peak constriction to 0.17 μmol/L vasopressin in Ca²⁺-free/EGTA Krebs’ solution (ie, A versus vasopressin peak 2. in Figure 4). These observations suggested that the decrease in pressure-dependent constriction observed before versus after the depletion of the SM sarcoplasmic Ca²⁺ was not due to the involvement of the Ca²⁺ stores in promoting pressure-dependent constriction but rather was the result of secondary changes in the MCA (ie, the inability to constrict to SM depolarization) associated with CPA treatment. The observation that pressure-dependent constriction cannot take place in the MCAs of srSHR or SHRSP in the presence of nifedipine (when [Ca²⁺] i is 2.5 mmol/L) or in the absence of external Ca²⁺, even when internal SM Ca²⁺ stores are full, further supports the hypothesis that an internal SM Ca²⁺ source is not used to mediate pressure-dependent constriction.

Discussion

After stroke development, the MCA of SHRSP lost the ability to elicit pressure-dependent constriction and could not utilize an intracellular source of Ca²⁺ to constrict in response to vasopressin or serotonin. The MCAs of poststroke SHRSP still contracted in response to serotonin and vasopressin, but such constriction took place only in the presence of extracellular Ca²⁺ and was blocked by nifedipine. This suggested that constriction was maintained by the movement of Ca²⁺ into the SM through L-type channels.

The signal transduction mechanisms promoting arterial constriction in response to serotonin or vasopressin involve SM PLC activation. This elevates intracellular IP₃, and DAG. IP₃ mediates the release of Ca²⁺ from an intracellular Ca²⁺ pool, and, through a signal cascade, DAG activation of protein kinase C (PKC) mediates sensitization of the contractile apparatus to Ca²⁺. The inability to utilize internal SM Ca²⁺ to elicit constriction may be indicative of defects in mechanisms mediating the release of sarcoplasmic Ca²⁺ or the possible depletion of the internal SM Ca²⁺ stores. A decrease in SM contractile sensitivity to Ca²⁺ could also contribute to this dysfunction. Constriction in response to phorbol dibutyrate in the presence of nifedipine (a PKC-mediated response) is abolished in the MCAs of poststroke SHRSP. Therefore, the activation of PKC by vasopressin or serotonin (secondary to DAG) might also be attenuated, resulting in the failure of the contractile apparatus to be sensitized to Ca²⁺. In such an instance, the increases in intracellular Ca²⁺ produced solely by the release of Ca²⁺ from the sarcoplasmic stores may be below the threshold needed to elicit constriction. Constriction of the MCA in the presence of [Ca²⁺], and the absence of nifedipine could have allowed additional Ca²⁺ entry through L-type channels, surpassing the higher than normal threshold needed to produce constriction.

I found no evidence indicating that pressure-dependent constriction in the MCAs of SHR utilized Ca²⁺ from an intracellular SM pool. In the absence of [Ca²⁺], or the presence of nifedipine, the MCAs of srSHR could not elicit even phasic constriction to a pressure step of 100 mm Hg. Pressure-dependent constriction also took place after the vasopressin/serotonin releasable intracellular Ca²⁺ pool was depleted and was prevented from refilling (via the use of CPA).

The MCAs of poststroke SHRSP could not constrict to 100 mmol/L [K⁺]. This defect cannot be accounted for by alterations in the ability of high [K⁺] o to produce SM depolarization and may be the result of a dysfunction in the voltage sensor in the L-type channel. In the MCAs of poststroke SHRSP, the channel behaved as though it was locked in a semi-open state and was unresponsive to alterations in SM membrane potential. If the latter channel was involved in promoting pressure-dependent constriction secondary to pressure-dependent depolarization, the inability of the arteries to constrict to depolarization would inhibit pressure-dependent constriction. Paradoxically, although the MCAs of poststroke SHRSP did not constrict to depolarization, they did constrict to vasopressin and serotonin via Ca²⁺ entry through L-type channels. This inconsistency may be explained by the observation that a variety of agonists can increase Ca²⁺ conductance into arterial SM cells through L-type channels when the membrane potential is constant, indicating that the channel can be opened in a voltage-independent manner.

A future challenge will be to link and critically assess the involvement of PLC and PKC in promoting arterial constriction to pressure. Both PLC and PKC inhibitors inhibit pressure-dependent constriction in cerebral arteries. However, paradoxes exist. One would expect that PLC activation in response to elevated pressure might lead to the production of IP₃, causing the release of sarcoplasmic Ca²⁺. However, studies have shown that PLC activation in cerebral arteries can form DAG with no measurable IP₃. This can occur by PLC cleavage of phosphatidylcholine. It is possible that pressure-dependent constriction in cerebral arteries is mediated by a phospholipase pathway that does not involve the breakdown of phosphatidylinositol and the production of IP₃ but (like the breakdown of phosphatidylcholine) still involves the production of DAG. If such was the case, PKC activation could take place without an IP₃-mediated release of Ca²⁺ from an internal SM pool. Other possibilities also exist. Studies have implicated 20-hydroxyicosatetraenoic acid (20-HETE) as an a signaling agent for pressure-dependent constriction in rat cerebral arteries. 20-HETE is a cytochrome P-450 metabolite of arachidonic acid that is increased in pressurized cerebral arteries. Preventing the production of 20-HETE or antagonizing its contractile actions with inhibitors prevents pressure-dependent constriction from occurring in cerebral
vessels in vitro and inhibits cerebral blood flow autoregulation in vivo. 20-HETE is capable of both depolarizing vascular SM (by decreasing K+ conductance), thus opening voltage-gated Ca2+ channels, and activating PKC. If the actions of 20-HETE did not involve the production of IP3, then this could provide a signal pathway for pressure-dependent constriction that involves SM PKC activation, depolarization, and the opening of voltage-gated Ca2+ channels but not the release of Ca2+ from a sarcoplasmic stores.

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References
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