Myosin Light Chain Phosphorylation and Contractile Proteins in a Canine Two-Hemorrhage Model of Subarachnoid Hemorrhage

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Background and Purpose—Subarachnoid hemorrhage (SAH) impairs both contraction and relaxation response in cerebral arteries. We tested the hypothesis that cerebral vasospasm might be ATP-independent contraction, such as latch state, and protein synthesis might be substantially downregulated due to ATP consumption after long-lasting contraction.

Methods—Chronic cerebral vasospasm was induced in the canine 2-hemorrhage model of SAH. The normal and spastic basilar arteries were stabilized in Krebs-Henseleit solution, and contraction was induced by 30 μmol/L prostaglandin F2α (PGF2α) in vitro and in vivo. Before and at 15 minutes and 1 hour after the treatment with PGF2α, the levels of phosphorylated 20-kDa myosin light chain (MLC20) were measured. The time course of expression of contraction proteins actin and MLC20, and contraction-inhibiting proteins h-caldesmon and calponin was determined by immunoblotting techniques.

Results—A significant vasospasm occurred in the basilar artery during days 4 to 21, most prominently on days 7 and 14. There were no significant differences in the baseline levels of phosphorylated MLC20 between normal and spastic basilar arteries. The increase in MLC20 phosphorylation by PGF2α was significantly attenuated in the spastic basilar artery in vitro and in vivo (P<0.05). The immunoreactivity for actin, h-caldesmon, and calponin in the spastic basilar arteries was progressively decreased until day 14 and returned to the normal level on day 21. In contrast, protein levels of MLC20 did not significantly change during days 0 to 21.

Conclusions—Chronic cerebral vasospasm closely resembles the latch state, and temporary deficiencies of contractile proteins may result from increased destruction and inhibition of protein synthesis. (Stroke. 1998;29:2149-2154.)

Key Words: cerebral ischemia, transient ■ myosin light chain ■ phosphorylation ■ protein synthesis ■ dogs

Delayed cerebral vasospasm following subarachnoid hemorrhage (SAH) has been generally attributed to sustained tonic contraction.1–4 Intracellular Ca2+ increases in smooth muscle cells during cerebral vasospasm,5–8 and this increase in Ca2+ also activates calmodulin and myosin light chain kinase (MLCK).9–11 Phosphorylation of 20-kDa myosin light chain (MLC20) by MLCK is considered to be the first step in vascular contraction.9–11 Although MLC20 phosphorylation is observed in the acute phase of contraction, it does not last a long time.9–11 In addition, a progressive decrease in the ATP level is observed concomitant with the development of vasospasm.12–14 In the absence of ATP, the crossbridges formed by the N-terminal (catalytic) head of myosin are attached to actin filaments, forming the characteristic rigor pattern.9,10 The mechanism of this high-force, low-phosphorylation, and low-energy-consumption state, named “latch,” is not known; in a 2-state contractile model, it results from an increase in the ratio of crossbridge attachment/detachment constant.10 Dephosphorylation, although necessary for the initiation of relaxation, is not its only rate-limiting step under all conditions, as it can be significantly faster than relaxation.10 Any contribution to latch by the thin-filament-associated proteins h-caldesmon15 and calponin16 must be auxiliary, because smooth muscle can be fully relaxed by dephosphorylating MLC20.10 However, time courses of MLC20 phosphorylation and of protein levels have not been demonstrated in the same model of SAH. Therefore, the purpose of our study was twofold: first, to investigate the time course of MLC20 phosphorylation after SAH; and second, to measure the expression of the contraction proteins actin and myosin and the contraction-inhibiting proteins h-caldesmon and calponin. This was an observational study to determine the state of myosin phosphorylation in vasospasm and the state of contractile proteins.

Materials and Methods
The animals were cared for in accordance with the Guidelines for Animal Experiments in the Mie University School of Medicine.
Seventy-eight adult mongrel dogs of both sexes, weighing 12 to 16 kg, were randomly assigned to 2 groups, with 23 in the normal control group and 55 in the SAH group.

On day 0, each dog in the SAH group was anesthetized by intramuscular injection of ketamine hydrochloride (10 mg/kg) and intravenous injection of pentobarbital sodium (15 mg/kg). The animals were intubated, and a peripheral venous line was placed. An intravenous injection of pentobarbital sodium (20 mg/kg per hour) maintained anesthesia, and pancuronium bromide (0.05 mg/kg per hour) maintained paralysis. A 2:1 mixture of N2O and O2 on a ventilator (SN-480—3, Shimano Co) was used to maintain the end-tidal CO2 (ETCO2) level at approximately 40 mm Hg during continuous ETCO2 monitoring (POET 601, Criticare System, Inc.). Body temperature was maintained at 37°C with a heating blanket, and the mean arterial blood pressure and pulse rate of the femoral artery were continuously monitored. Vertebral angiography was carried out via the catheter through the femoral artery. During angiography, exposure factors were maintained constant, and a radiopaque control standard was used for correction to constant magnification. SAH was induced by injections of 0.5 mL/kg fresh autologous arterial blood into the cisterna magna 48 hours apart. Vertebral angiography was repeated on days 4, 7, 14, and 21 after the first injection of blood. The caliber of the basilar artery was measured on the angiograms at 3 locations: close to the vertebro-basilar junction, at the midpoint, and close to the basilar tip. The changes in the diameters were expressed as percentages of the baseline value before SAH.

### Tissue Preparation for Measurement of MLC20 Phosphorylation

After the second angiography, the basilar artery from vertebral union to basilar bifurcation was excised via the transclival approach on days 4 (n=5), 7 (n=11), 14 (n=3), and 21 (n=3). Eleven normal basilar arteries were also removed by the same surgical procedures. In the SAH group, after the blood clot around the basilar artery and its branches were carefully removed, the basilar artery was immersed in Krebs-Henseleit solution (KHS, mmol/L: NaCl 115.0, KCl 4.7, CaCl2 2.5, MgCl2 1.2, NaHCO3 25.0, KH2PO4 1.2, and 37°C for 1 hour). The unfixed basilar arteries were weighed, cut into small pieces, and homogenized for immunoblotting analysis with the specific antibodies of actin, h-caldesmon, calponin, and MLC20 and developed by the enhanced chemiluminescence method (Amerham). Since 2 smooth muscle–specific actin isoforms designated as α- and γ-SM and 2 nonmuscle actin isoforms (β- and γ-NM) are expressed in vascular smooth muscle cells, we used anti-actin antibody to all actin isoforms (Sigma). Anti-h-caldesmon and anti-calponin polyclonal antibodies were provided by Dr J.T. Stull (University of Texas Southwestern Medical Center, Dallas, Tex). The region containing MLC20 was visualized as dark blue bands, using 4-chloro-1-naphthol. The extent of MLC20 phosphorylation was calculated by dividing the area of phosphorylated MLC20 by the total area of both phosphorylated and nonphosphorylated MLC20.

### Measurements of Contractile Proteins

The unfixed basilar arteries were weighed, cut into small pieces, and homogenized for 2 minutes at 4°C in 10 μL buffer/sample containing 30 mmol/L Tri-HCl (pH 7.0), 500 mmol/L NaCl, 5 mmol/L ATP, 2 mmol/L EGTA, 4 mmol/L EDTA, 1 μmol/L DTT, 10 μg/ml leupeptin, 1 mmol/L benzamidine, and 10 mmol/L α-PMPS. The polypeptide control standards were purchased from Sigma and used for correction to constant magnification. SAH was induced by injections of 0.5 mL/kg fresh autologous arterial blood into the cisterna magna 48 hours apart.

Vertebral angiography was repeated on days 4, 7, 14, and 21 after the second angiography, the basilar artery from vertebral union to basilar bifurcation was excised via the transclival approach on days 4 (n=5), 7 (n=11), 14 (n=3), and 21 (n=3). Eleven normal basilar arteries were also removed by the same surgical procedures. In the SAH group, after the blood clot around the basilar artery and its branches were carefully removed, the basilar artery was immersed in Krebs-Henseleit solution (KHS, mmol/L: NaCl 115.0, KCl 4.7, CaCl2 2.5, MgCl2 1.2, NaHCO3 25.0, KH2PO4 1.2, and 37°C for 1 hour. The in vitro basal phosphorylation levels of MLC20 were determined in the spastic basilar arteries from day 7 animals after SAH (n=5). To test the additional phosphorylation of MLC20 by agonist, other spastic basilar arteries from day 7 animals after SAH were further treated with 30 mmol/L PGE2, for 15 minutes or 1 hour. At the end of incubation, the arterial segments were frozen in liquid nitrogen and stored for biochemical examinations.

After the second angiography, the basilar arteries were exposed via the transclival route on days 4 (n=3), 7 (n=9), and 14 (n=3). The blood clots around the basilar artery were carefully removed, and the cranial window was superfused with KHS aerated with 5% CO2 and 95% O2 at 37°C for 1 hour. The in vivo basal phosphorylation levels of MLC20 were determined in the spastic basilar arteries from day 7 animals after SAH (n=5). To test the additional phosphorylation of MLC20 by agonist, other spastic basilar arteries from day 7 animals after SAH were further treated with 30 mmol/L PGE2 for 15 minutes or 1 hour. During the treatment, ETCO2 was adjusted to 40 mm Hg. For the determination of phosphorylation levels of MLC20, liquid nitrogen and frozen acetone containing 10% TCA and 10 mmol/L DTT were directly added into the cranial window, and the frozen basilar arteries were quickly excised.

### Tissue Preparation for Measurements of Contractile Proteins

The dogs were killed by exsanguination and infusion of saline into the left ventricle on days 4 (n=6), 7 (n=6), 14 (n=3), or 21 (n=3) after the initial SAH. The brain was removed, and the blood clots around the basilar artery were carefully removed. The basilar artery was quickly frozen in liquid nitrogen and stored for biochemical examination.

### Measurements of MLC20 Phosphorylation

The extent of MLC20 phosphorylation was measured through separation of phosphorylated and nonphosphorylated forms by urea-glyceral gel electrophoresis followed by electrophoretic transfer of the proteins to a nitrocellulose membrane. The relative amount of each form was quantified by an immunoblot procedure described in the next section. The arterial segments were frozen by immersion in aceton containing 10% TCA and 10 mmol/L DTT cooled with liquid nitrogen. The frozen tissues were washed twice with aceton containing 10 mmol/L DTT to remove the TCA and then dried, weighed, and cut into small pieces. The pieces were homogenized for 2 minutes at 4°C in 10 μL urea buffer/sample containing 8 mol/L urea, 10 mmol/L DTT, 18 mmol/L Tris base, 20 mmol/L glycine, 250 mmol/L sucrose, and 0.004% bromphenol blue with use of a Potter Teflon homogenizer. These urea-solubilized samples (20 μL) were subjected to urea-glyceral gel electrophoresis and immunoblot analysis, using the specific MLC20 antibody as documented. Anti-MLC20 rabbit polyclonal antibodies were provided by Dr J.T. Stull (University of Texas Southwestern Medical Center, Dallas, Tex). The region containing MLC20 was visualized as dark blue bands, using 4-chloro-1-naphthol. The extent of MLC20 phosphorylation was calculated by dividing the area of phosphorylated MLC20 by the total area of both phosphorylated and nonphosphorylated MLC20.

### Statistical Analysis

All values are given as mean±SD. Statistical comparison was made by the Student t test and intergroup comparisons by ANOVA. The level of significance of all tests of comparison was P<0.05.

### Results

#### Changes in the Basilar Artery Diameters

After the induction of SAH, the angiographic diameters of the basilar arteries gradually decreased, as shown in Figure 1. The percentage of the diameters from baseline values were as follows: day 4, 70.6±11.9%; day 7, 55.2±6.1%; day 14, 51.8±3.7%; and day 21, 66.9±5.6%. The narrowing of the basilar artery was most prominent on days 7 and 14. Mild vasospasm was observed on days 4 and 21 to the same degree.
Levels of MLC<sub>20</sub> Phosphorylation

The time course of baseline MLC<sub>20</sub> phosphorylation is shown in Figure 2. The percentages of phosphorylated MLC<sub>20</sub> in the spastic basilar arteries in vitro were as follows: day 0 control, 8.2 ± 2.8%; day 4, 9.2 ± 4.1%; day 7, 9.5 ± 2.1%; day 14, 5.4 ± 3.8%; and day 21, 4.3 ± 5.6%. There were no significant differences in phosphorylation levels at any stage of measurements, regardless of vasospasm. The percentages of phosphorylated MLC<sub>20</sub> in the in vivo spastic basilar arteries were as follows: day 0 control, 55.3 ± 7.5%; day 4, 60.1 ± 9.6%; day 7, 58.7 ± 7.3%; and day 14, 53.8 ± 8.1%. Again, there were no significant changes at any stage of measurements.

MLC<sub>20</sub> Phosphorylation After PGF<sub>2α</sub> Treatments

After incubation with 30 μmol/L PGF<sub>2α</sub> in vitro, the changes of MLC<sub>20</sub> phosphorylation in the spastic basilar artery and control are shown in Figure 3. In the control artery, the MLC<sub>20</sub> phosphorylation significantly increased from 8.2 ± 2.8% to 37.7 ± 2.7% at 15 minutes and 34.4 ± 4.1% at 1 hour. In the spastic basilar arteries, the MLC<sub>20</sub> phosphorylation increased from 9.5 ± 2.1% to 19.8 ± 2.5% at 15 minutes and 20.1 ± 1.9% at 1 hour. The levels of MLC<sub>20</sub> phosphorylation significantly increased from the baseline level in spastic basilar arteries at 15 minutes and 1 hour (P < 0.05 by the Student t test). However, the increases in the phosphorylation levels were significantly attenuated in the spastic basilar arteries compared with control values (P < 0.01 by ANOVA).

The MLC<sub>20</sub> phosphorylation in vivo after superfusion with 30 μmol/L PGF<sub>2α</sub> is shown in Figure 4. In the control artery, topical application of 30 μmol/L PGF<sub>2α</sub> induced a sustained contraction during superfusion. As the contraction developed, the degree of MLC<sub>20</sub> phosphorylation significantly increased from 55.3 ± 7.5% to 84.6 ± 6.1% at 15 minutes (P < 0.05 by the Student t test) and not significantly to 72.3 ± 9.0% at 1 hour. In the spastic artery, there were no significant changes in MLC<sub>20</sub> phosphorylation levels after PGF<sub>2α</sub> application. The increases in the phosphorylation levels were significantly attenuated in the spastic basilar arteries compared with control values (P < 0.05 by ANOVA).
Changes in the Expression of Contractile Proteins

The immunoblotting for actin, h-caldesmon, calponin, and MLC 20 is shown in Figure 5. The intensities of the immunoreactive bands for contractile proteins were decreased during days 7 to 14 and recovered at day 21 (panel A). The immunoreactive band for calponin was not detected in 3 of the 6 arteries removed on day 7 and 2 of the 3 arteries removed on day 14. In addition, proteolysis of h-caldesmon and calponin was observed in the spastic basilar arteries harvested on days 7 and 14 (panel A). Changes in the densitometric quantities of immunoreactive bands are shown in panel B. Although MLC 20 decreased during days 7 to 14, it was not significant (panel B). h-Caldesmon, actin and calponin were significantly decreased in the spastic basilar arteries harvested on days 7 and 14 (P<0.05) (panel B). On day 21, all contractile proteins recovered to the control levels.

Discussion

The present study demonstrated that the degree of MLC 20 phosphorylation in the basilar arteries did not exhibit significant changes at any stage of measurement in vitro and in vivo. Second, the increase in MLC 20 phosphorylation induced by PGF 2α was significantly attenuated in the spastic basilar arteries (P<0.05 by ANOVA). *P<0.01 versus control; †P<0.05 between two groups (Student t test). The lower photographs show immunoblots of MLC 20 in the normal and spastic basilar arteries before and after superfusion with PGF 2α.

Reduced cross-bridge cycling rates.9–11 Phosphorylation of MLC 20 initiated by an increase in intracellular Ca2+ is correlated with the rate of the actin-myosin interaction in the acute phase of contraction.9–11 In the force maintenance phase, intracellular Ca2+ is decreased, and at the same time the MLC 20 phosphorylation declines to a steady intermediate level.9–11 Any contractile force developed may be maintained by the presence of an attached phosphorylation-independent noncycling cross-bridge (latch bridge).9–11 A number of studies have demonstrated that activation of the PKC system may play a role in the latch bridge, inducing a potent and sustained contraction without affecting MLC 20 phosphorylation.1–4 Low levels of MLC 20 phosphorylation have been associated with force maintenance.9–11 The ATP contents showed little or no significant change during tonic contractions.9–11 One recent study demonstrated that muscle fibers are commonly observed in 3 states.20 (1) In rigor (ie, rigor mortis, the state obtained in the absence of ATP), all myosin heads are bound rigidly with actin in a configuration thought to resemble that found at the end of the power stroke. (2) In relaxation, in the presence of ATP, myosin heads are largely detached from actin and may be bound in a helical array around the thick filament or may be disordered and not bound to either filament. (3) In active muscle contraction, in the presence
of Ca²⁺ and ATP the myosin heads are undergoing a cyclic interaction, with actin producing force. ATP binding was assumed to be a prerequisite for detachment of dephosphorylated actomyosin and relaxation. Indeed, the ATP content decreased in parallel with the progression of vasospasm.

Our results do not agree with the findings that MLC₂₀ phosphorylation significantly increased in spastic anterior spinal arteries. MLC₂₀ phosphorylation increased in the early phase, but was undetectable at the later phase of vasospasm using a femoral artery model. Differences in the experimental models and animal species as well as the sites of arteries may contribute to the differences in MLC₂₀ phosphorylation levels between the present study and other studies.

The present study determined the MLC₂₀ phosphorylation of the basilar artery in vivo and demonstrated that the mean control values for MLC₂₀ phosphorylation in vivo were much higher than the basal values in vitro. Under physiological conditions, the artery is exposed to hemodynamic stress and constantly contracts to maintain vascular resistance and regulate blood flow. Phosphorylation of MLC₂₀ may take place in real time in vivo.

Calpain, a calcium-activated protease, has been reported to be activated in the spastic basilar artery. Recently, protein synthesis has been impaired in smooth muscle cells of spastic cerebral arteries. In our study, the contractile proteins all progressively decreased whereas there was not a significant decrease in MLC₂₀. However, these proteins returned to the normal levels on day 21. These changes were consistent with the time course of the angiographic vasospasm. Although some studies have demonstrated that immunoreactivity for actin is unchanged at the time of vasospasm, other studies have shown a decrease of actin on day 7 of SAH. In the present study, actin significantly decreased on days 7 and 14. Myosin was substantially decomposed on day 7 in the canine 2-hemorrhage model; however, it was unchanged in the monkey model of SAH. Our study demonstrated that immunoreactivity for myosin decreased through days 4 to 21 but not significantly. It is likely that varying degrees of protein degradation may reflect the degree of cell degeneration.

The immunoreactivity of the contraction-inhibiting proteins h-caldesmon and calponin were significantly decreased during days 4 to 14 after SAH. Both h-caldesmon and calponin inhibit actin-myosin interaction without influencing the phosphorylation state of myosin. Phosphorylation of h-caldesmon or calponin by PKC and Ca²⁺-calmodulin–dependent kinase causes a loss of their ability to relax smooth muscle cells. In the present study, immunoblots showed that bands of h-caldesmon and calponin were significantly decreased on days 7 and 14. Interestingly, the decreases in h-caldesmon and calponin recovered on day 21, at the resolution stage of vasospasm. Taken together with the decrease in actin levels, temporary deficiencies of h-caldesmon and calponin may result from the increase in destruction and inhibition of protein synthetic activity.

In conclusion, chronic cerebral vasospasm closely resembles the latching state, an ATP-independent contraction.

Acknowledgments

This work was supported by grants-in-aid for scientific research (C) and (B) from the Ministry of Education, Science, Sports and Culture, and a grant-in-aid (1996–1997) from the Mie Medical Research Foundation (Dr Kanamaru). We thank Dr J.T. Stull for providing the anti-myosin light chain antibody and Dr M.P. Walsh for providing the h-caldesmon and calponin antibodies.

References

Sun et al test the hypothesis that vasospasm is an ATP-independent contraction like the latch state. The latch state is one theoretical mechanism that has been postulated to underlie the tonic phase of smooth muscle contraction. Second, they suggest that protein synthesis may be downregulated because of ATP consumption after long-lasting contraction. They found that there was no difference between normal and vasospastic arteries 4 to 21 days after subarachnoid hemorrhage in the basal levels of myosin light chain (MLC) phosphorylation. When contracted with PGF\textsubscript{2α}, however, vasospastic arteries 7 days after subarachnoid hemorrhage showed a smaller increase in MLC phosphorylation in vitro. Second, a semiquantitative assay showed that there was less actin, caldesmon, and calponin in vasospastic arteries 7 and 14 days after hemorrhage compared with control arteries. These findings do not specifically test and therefore do not directly support or refute their hypotheses.

The double-hemorrhage dog model of vasospasm is widely used. The vasospasm that occurs is reliable and severe, but it differs from that observed in humans. Two blood injections are required, and the vasospasm was still significant 21 days after the first injection. Vasospasm usually resolves within 14 days of subarachnoid hemorrhage in humans. Why the vasospasm lasts longer in dogs is unknown. It is interesting that the decreases in actin, caldesmon, and calponin occurred 7 and 14 days after hemorrhage and then recovered by day 21, despite the persistence of angiographic arterial narrowing. Thus, there is not really a good correlation between the changes in contractile proteins and vasospasm. The authors suggest that there is a decrease in protein synthesis during vasospasm and that this results from a decrease in ATP. Others\textsuperscript{1} have shown a decrease in ATP in vasospastic arteries, but this was not confirmed in the present experiments. Furthermore, the decrease in protein levels may be caused by proteolysis rather than decreased protein synthesis.

The authors also report that MLC phosphorylation levels in vasospastic arteries are the same as in control arteries tested under the same conditions. This is in keeping with prior studies which have shown that acute smooth muscle contraction is associated with an increase in MLC phosphorylation; when, however, the muscle remains tonically contracted, the level of MLC phosphorylation falls to near-basal levels. The authors discuss several postulated mechanisms, including latch state, rigor, or the action of some other smooth muscle contractile regulatory process. Their results do not determine which mechanism(s) is active in vasospasm. They suggest that vasospasm resembles a latch state, although the data do not rule out other mechanisms, and the latch state is usually not associated with proteolysis. In summary, the authors have documented several biochemical alterations in vasospastic arteries. The cause and importance of the changes remain to be determined. It is probably only with further detailed measurements of the changes in the known components of the contractile apparatus before, during, and after vasospasm that the mechanisms of vasospasm will be defined.

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Stroke. 1998;29:2149-2154
doi: 10.1161/01.STR.29.10.2149
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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