Extravascular Blood Augments Myogenic Constriction of Cerebral Arterioles: Implications for Hemorrhage-Induced Vasospasm

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Background—Subarachnoid hemorrhage is a serious clinical condition that impairs local cerebral blood flow perfusion and consequently initiates neuronal dysfunction. Pressure-sensitive myogenic vasomotor regulation is an important mechanism involved in the regulation of cerebral blood flow. We hypothesized that extravascular hemolyzed blood enhances arteriolar myogenic constriction, which in vivo may contribute to the reduction of local cerebral blood flow after subarachnoid hemorrhage.

Methods and Results—Arterioles isolated from the middle cerebral artery (MCA arterioles) of mice were cannulated in a perfusion chamber. Arteriolar diameters in response to step increases in intraluminal pressure (20–120 mm Hg) were measured in various experimental conditions. In response to increases in intraluminal pressure, all MCA arterioles exhibited myogenic vasoconstrictions. Compared with controls, the pressure-induced constriction was significantly enhanced in arterioles (in vitro) exposed to extravascular hemolyzed blood or different concentrations of extracellular erythrocyte lysate (1%, 10%, and 20%) for different exposure durations (1–6 hours). The magnitude of enhancement was proportional to the lysate concentration and exposure duration. In in vivo experiments, 10 µL of autologous blood lysate were injected into the mouse subarachnoid space on the surface of the left MCA. Two hours later, MCA arterioles were isolated and left MCA arterioles displayed enhanced myogenic responses compared with the right MCA. The enhanced myogenic response was prevented by scavenge of superoxide in both in vitro and in vivo experiments.

Conclusions—Extravascular hemolyzed blood, perhaps by promoting vascular production of superoxide, augments myogenic constriction of cerebral arterioles, which plays a crucial role in the subarachnoid hemorrhage–induced cerebral ischemia. (J Am Heart Assoc. 2018;7:e008623. DOI: 10.1161/JAHA.118.008623.)

Key Words: cerebral arterioles • myogenic response • oxidative stress • subarachnoid hemorrhage • vasospasm
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Clinical Perspective

What Is New?

- This study demonstrates, for the first time, that exposure of cerebral arterioles to extravasated blood—a model that clinically imitates hemorrhagic stroke, such as subarachnoid hemorrhage—initiates an augmented pressure-induced myogenic vasoconstriction.
- This response is proportional to the duration of blood exposure and contributes significantly to the development of delayed vasospasm/ischemia after cerebral hemorrhage.
- This altered myogenic vasoconstriction is reversible by scavenging vascular superoxide.

What Are the Clinical Implications?

- The surgical removal of extravasated blood is necessary to prevent the development of delayed vasospasm/ischemia, which can be elicited and potentiated by prolonged blood exposure even without active bleeding.
- Interventions such as local application of antioxidants during surgery may reduce cerebral vasospasm and normalize arteriolar tone.

specific mechanism behind vasospasm actions, even after removal of the extravasated blood, has not been addressed. To this end, we hypothesized that short-term exposure of isolated cerebral arterioles to extravascular hemolyzed blood (EHB) or extravascular erythrocyte lysate (EEL) augments the myogenic constrictor response of cerebral arterioles at a given intraluminal pressure. In addition, we addressed the potential involvement of reactive oxygen species (ROS) in EHB/EEL-induced enhanced myogenic constriction, inspired by previous findings showing that augmentation of myogenic response was mediated by ROS in various pathologic conditions. To test our hypothesis, mouse cerebral arterioles branching off from the middle cerebral artery (MCA arterioles) were isolated and cannulated. Pressure–diameter curves were obtained before and after exposure of the arterioles to EHB, EEL, or plasma. After washout of blood lysates, their pressure–diameter curves were measured again. To extrapolate our findings to clinical conditions, in a separate group of experiments, EHB was in vivo injected into the subarachnoid space, and then EHB-exposed arterioles were isolated, followed by assessing their myogenic response. In this study, MCA arterioles (~100 \( \mu \text{m} \) in diameter) were used because they contribute significantly to the control of vascular resistance and CBF. It is important to note that changes in diameter of these small arterioles are unable to be detected during surgery or by using imaging modalities, such as computed tomography or magnetic resonance imaging. Consequently, it becomes necessary to in vitro investigate arteriolar responses to changes in intraluminal pressure by assessing their pressure–diameter relationship in various experimental conditions.

Materials and Methods

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Animals

Experiments were conducted on 12- to 15-week-old male C57BL/6 mice. All protocols were approved by the institutional animal care and use committee of New York Medical College and conform to the guidelines of the National Institutes of Health and the American Physiological Society for the care and use of laboratory animals.

Preparation of Whole Blood Lysate, Erythrocyte Lysate, and Plasma

Mice were anesthetized by inhalation of isoflurane. Approximately 800 \( \mu \text{L} \) of blood were withdrawn from the inferior vena cava. Autologous blood was lysed by undergoing 3 cycles of freezing (in liquid nitrogen)/thawing (in 37°C) and vortexing (30 seconds at highest speed). To prepare plasma and erythrocyte lysate, the collected blood was centrifuged at 2000g (4°C) for 15 minutes to separate the plasma and erythrocytes. The separated erythrocyte fraction was washed with saline 3 times and then lysed by adding an equal amount of saline and proceeding with the freeze/thaw/vortex cycles to become erythrocyte lysate. Only freshly prepared blood lysates were used.

Isolation of Arterioles

After collecting blood, the brain was carefully removed without damaging surface blood vessels and placed in a petri dish containing cold (0–4°C) 3-(N-morpholino)propanesulfonic acid–buffered (pH 7.4) physiological salt solution (PSS). Cerebral arterioles (89.6±14.6 \( \mu \text{m} \) in active diameter at 80 mm Hg with 200–300 \( \mu \text{m} \) in length) isolated from the middle cerebral artery (MCA) were cannulated in a vessel chamber. Arterioles were perfused with PSS (containing, in mmol/L: NaCl, 117; KCl, 4.7; MgSO4, 1; KH2PO4, 1.2; glucose, 10; NaHCO3, 24; CaCl2, 2.5; and EDTA, 0.2) at 37°C and gassed with air plus 5% CO2 to maintain pH at 7.4. Cannulated arterioles were equilibrated at 60 mm Hg intraluminal pressure for 1 hour. During this period, all arterioles developed spontaneous tone, which was \( \approx 75\% \) of their passive diameter obtained in Ca\(^{2+}\)-free PSS solution.
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Temperature of mice was maintained at 37°C using a homeothermic monitor. After scalp midline incision, the areas of left parietal and temporal skull were exposed, and 2 holes with 0.5 mm of diameter were made using a handheld Foredom MH145 drill. The first hole on the left temporal skull was made ∼0.5 mm anteroposterior (bregma) and ∼3.5 mm dorsoventral, and 10 μL autologous EHB or saline (as vehicle/osmotic control) were injected into the subarachnoid space (with a Hamilton syringe fixed on the stereotaxic and advanced to the hole at an angle of 28°). The second hole near the middle portion of left parietal skull was used to balance/release intracranial pressure when EHB was given. In separate experiments, 10 μL EHB mixed with 1 μL of 10⁻⁵ mol/L Tempol (in a final concentration of 10⁻⁴ mol/L) was administered. During the injection period, the Hamilton syringe needle tip was fixed on the skull with bone wax and then sealed with a drop of 3M Vetbond. After injection, the stereotaxic apparatus was left-lateral tuned for 45°, and the animal was maintained in the lateral position under a stable anesthetized condition for 2 hours. After that, the mouse was euthanized, and the brain was removed. Cerebral arterioles were isolated from corresponding ipsilateral (left MCA exposed to EHB or saline, as vehicle controls) and contralateral (without exposure to EHB, as normal controls) brain regions of the same mouse and then cannulated for measurement of myogenic response.

Myogenic Response

As described previously,² after equilibration, intraluminal pressure was lowered to 20 mm Hg and subsequently increased to 120 mm Hg in 20-mm Hg steps. Each pressure step was maintained for 5 to 10 minutes to allow the vessels to reach a stable condition. Selecting 20 mm Hg as a starting point was based on our preliminary results showing that these arterioles did not display remarkable myogenic responses when intravascular pressure was <20 mm Hg (Figure 1). Changes in MCA-arteriolar diameter in response to incremental increases in intraluminal pressure were recorded in the absence (control condition) and presence of EHB, plasma, or EEL. At the conclusion of each experiment, the chamber solution was changed to a Ca²⁺-free PSS containing 10⁻³ mol/L EGTA. Vessels were incubated for 30 minutes, and passive diameter (PD) at each pressure step was recorded.

Vascular Superoxide Production

As described previously,³ isolated MCAs were incubated with dihydroethidium (10⁻⁵ mol/L) for 1 hour, during which the superoxide in the vessels reacted with dihydroethidium to form 2-hydroxyethidium, which was detected by high-performance liquid chromatography/fluorescence detector. After incubation with dihydroethidium, the vessels were washed and homogenized in acetonitrile/water (1:1) and then centrifuged for 10 minutes. The supernatant fraction was collected for high-performance liquid chromatography analysis, and the precipitate was used for protein measurement using the Bio-Rad Protein Assay. The final concentration of superoxide in each sample was normalized to the protein contents of the corresponding vessels and expressed as picomoles of superoxide (2-hydroxyethidium) per milligram of protein.

In Vivo Administration of EHB Into the Subarachnoid Space

Two drops (≈40 μL) of blood were obtained from the right facial vein (submandibular vein) of anesthetized mice by pricking the vein with a 25-G needle, followed by the hemolytic procedure described earlier. The mouse was then mounted on a KOPF stereotaxic apparatus with the bregma and lambda points in a same horizontal plane. The body temperature of mice was maintained at 37°C using a homeothermic monitor. After scalp midline incision, the areas of left parietal and temporal skull were exposed, and 2 holes with 0.5 mm of diameter were made using a handheld Foredom MH145 drill. The first hole on the left temporal skull was made ∼0.5 mm anteroposterior (bregma) and ∼3.5 mm dorsoventral, and 10 μL autologous EHB or saline (as vehicle/osmotic control) were injected into the subarachnoid space (with a Hamilton syringe fixed on the stereotaxic and advanced to the hole at an angle of 28°). The second hole near the middle portion of left parietal skull was used to balance/release intracranial pressure when EHB was given. In separate experiments, 10 μL EHB mixed with 1 μL of 10⁻⁵ mol/L Tempol (in a final concentration of 10⁻⁴ mol/L) was administered. During the injection period, the Hamilton syringe needle tip was fixed on the skull with bone wax and then sealed with a drop of 3M Vetbond. After injection, the stereotaxic apparatus was left-lateral tuned for 45°, and the animal was maintained in the lateral position under a stable anesthetized condition for 2 hours. After that, the mouse was euthanized, and the brain was removed. Cerebral arterioles were isolated from corresponding ipsilateral (left MCA exposed to EHB or saline, as vehicle controls) and contralateral (without exposure to EHB, as normal controls) brain regions of the same mouse and then cannulated for measurement of myogenic response.

Experimental Procedures

Changes in myogenic response following exposure to EHB, EEL, or plasma

In the first series of experiments, the pressure–diameter relationship of arterioles was assessed before (control conditions) and after exposure of vessels to EHB, EEL or plasma, respectively, to identify which components of blood were responsible for modulating the myogenic response of arterioles. After the control pressure–diameter curve was
obtained, 200 μL of EHB, EEL, or plasma was added into the vessel chamber (1.8 mL in volume), where arterioles were incubated with different components of blood lysate for 1 hour. After incubation, vessels were washed 3 times by replacing the given lysate containing suffusion solution with the PSS, and then they were allowed to be stabilized for an additional 15 minutes. After that, the pressure–diameter curve was obtained once more.

Changes in myogenic response following exposure of different concentrations of EEL

Based on results obtained from the first protocol, the second protocol assessed myogenic response of arterioles before and after exposure to different concentrations of EEL (1%, 10%, and 20%). After control experiments, arterioles were separately incubated with each dose of EEL for 1 hour, and the myogenic response was recorded again.

Changes in myogenic response as a function of EEL exposure period

In this series of experiments, arterioles were divided into 3 groups and incubated with 10% EEL for 1, 2, and 6 hours, respectively. After incubation, vessels were washed and stabilized (as described earlier). The pressure–diameter relationship was measured, and then vessels were stabilized in PSS for recovery. The recovery period for each group varied; therefore, the myogenic response was repeated 2 or 3 times with an interval of at least 1 hour, during which the chamber solution (PSS) was refreshed every 30 minutes.

Roles of the endothelium and superoxide in EEL exposure–induced increases in myogenic response

In this series of experiments, the pressure–diameter relationship of arterioles that were exposed to 10% EEL for 1 hour was assessed to evaluate the roles of the endothelium and superoxide in EEL-induced increase in myogenic response; the pressure–diameter relationship was assessed before and after endothelial removal or, in another group of experiments, before and after administration of Tempol, a superoxide scavenger. Endothelial denudation was accomplished by injection of air into the vessel lumen, as described previously. Tempol (10−4 mol/L) was added into the vessel chamber 30 minutes before the administration of EEL, which was washed out after 1-hour incubation (as described earlier), whereas Tempol was present in the chamber solution throughout the experiment.

Changes in myogenic response of isolated cerebral arterioles after exposure to EHB in subarachnoid space

In the final protocol, myogenic response was assessed in arterioles isolated from the right MCA (normal controls) and the left MCA (EHB treatment) of the same brain. In the left hemisphere of the brain, EHB was administered into the subarachnoid space, so the arterioles were in vivo exposed to EHB for 2 hours, followed by isolation of the arterioles. In separate experiments, arterioles of the left MCA were in vivo exposed simultaneously to EHB and 10−4 mol/L Tempol for 2 hours. After isolation of the arterioles, Tempol was present in the vessel chamber throughout the experiment.

Statistical Analyses

Changes in diameter, as a function of changes in intraluminal pressure, were normalized to their corresponding PD and expressed as percentage of PD. Data are expressed as mean±SE, and the sample size refers to the number of mice. Using GraphPad Prism 6 software, all data were analyzed by 2-way ANOVA (repeated measures), followed by Sidak multiple comparisons, except for data of superoxide measurements in MCA, which were analyzed by 1-way ANOVA followed by Tukey multiple comparisons. Statistical significance was considered at the level of P<0.05.

Results

Threshold of Myogenic Constriction in Response to Increases in Intraluminal Pressure

Figure 1 shows that in the control condition, the myogenic constriction of MCA arterioles did not occur until intraluminal pressure increased up to 20 mm Hg, followed by further constrictions in response to increases in pressure from 40 to 120 mm Hg.

EHB and EEL Enhance Myogenic Constriction of Cerebral Arterioles

Figure 2 shows changes in diameter of MCA arterioles as a function of step increases in intraluminal pressure in control arterioles and in arterioles that were exposed to 10% EHB (Figure 2A), 10% plasma (Figure 2B), and 10% EEL (Figure 2C). After a passive increase in diameter at lower pressure steps, all arterioles developed constriction at ≈20 mm Hg, as shown by significantly reduced vessel diameter compared with their corresponding PD at the same pressure step (20 mm Hg; myogenic response). In response to further increases in pressure from 40 to 120 mm Hg, arterioles exhibited further constrictions. Compared with control responses, the myogenic constriction was significantly greater (expressed as a downward shift of the pressure–diameter curve) in arterioles exposed to EHB and EEL (Figure 2A and 2C), whereas exposure of arterioles to plasma did not change their myogenic responses (Figure 2B). To exclude the possibility that changes in arteriolar response to increases in pressure were attributed
to different PDs among different experimental groups, data were normalized to their corresponding PDs and summarized in Figure 2D. This graph shows that the myogenic response/constriction was significantly enhanced by exposure of the arterioles to both EHB and EEL, but it was not significantly affected if they were exposed to plasma.

**EEL Potentiates Myogenic Vasoconstriction in a Dose-Dependent Manner**

In another group of experiments, increasing doses of EEL were used to study further changes in the characteristics of myogenic responses. Normalized data showing changes in the magnitude of myogenic response as a function of different concentrations of EEL are depicted in Figure 3C. The graph shows that arterioles displayed a positive correlation between the magnitude of myogenic constriction and the concentrations of EEL exposed. In other words, compared with the control condition, there was a consecutive dose-dependent downward shift of the pressure–diameter curves in EEL-treated arterioles. Original data from the each group of arterioles treated with 1%, 10%, and 20% EEL are presented in Figure 3A and 3B (1% and 20%) and Figure 2B (10%), respectively.
Effects of the Duration of EEL Exposure on the Magnitude and Recovery Time of Myogenic Response

To assess whether duration of EEL exposure time-dependently potentiates the magnitude of myogenic constriction, arterioles were incubated with 10% EEL for 1, 2 and 6 hours, respectively. Figure 4 shows that the enhanced myogenic response initiated by 1-hour incubation of vessels with EEL was not further enhanced by extending the exposure time up to 6 hours, as shown by the 3 overlapping pressure–diameter curves obtained from vessels with 3 different exposure periods.

Next, we investigated the time necessary for arteriolar recovery from enhanced myogenic response, in terms of returning the response to the control level, as a function of different exposure duration (10% EEL for 1, 2, and 6 hours). As shown in Figure 5, arterioles exposed to EEL for 1 hour needed 2 hours for the myogenic response to fully return to the control level, although a progressive recovery was observed in the first hour (Figure 5A). Similarly, vessels that were exposed to EEL for 2 hours needed longer recovery periods (up to 4 hours) to reverse the enhanced myogenic response back to the control level (Figure 5B). Figure 5C shows that following incubation of vessels with EEL for 6 hours, the arteriole response was not fully restored during a 6-hour recovery period; in particular, there was no observable recovery in the first hour (as was observed with 1-hour incubation in Figure 5A). In summary, these results suggest a

Figure 3. Pressure–diameter curves of isolated arterioles branching off from middle cerebral arteries in vitro incubated with (A) 1% and (B) 20% erythrocyte lysate (EEL) and (C) normalized pressure–diameter curves of 1%, 10% (obtained from Figure 2C), and 20% EEL-treated and control arterioles. n=7 for each group. *Significant difference between the curves.

Figure 4. Normalized pressure–diameter curves of isolated arterioles branching off from middle cerebral arteries in control condition and after in vitro incubation with 10% erythrocyte lysate (EEL) for 1, 2, and 6 hours, respectively. n=7 for each group. *Significant difference from the control curve.
critical role for the duration of extravasated blood exposure in the development of so-called delayed ischemia after SAH or hemorrhagic stroke.

Role of Endothelium in the Increased Myogenic Constriction Following EEL Exposure

In this series of experiments, pressure-diameter relationships were obtained in arterioles treated with 10% EEL for 1 hour without (as controls) and with removal of the endothelium. Figure 6A shows that endothelial removal did not affect the EEL-induced enhanced myogenic response, suggesting that the enhanced constriction is endothelium-independent in nature. The efficacy of endothelial removal and the intact vasomotor function of smooth muscle were validated by the loss of arteriolar dilation to the endothelium-dependent vasodilator acetylcholine (10^{-6} mol/L) and maintained dilation to the nitric oxide (NO) donor (NONOate, 10^{-6} mol/L), respectively (Table).
Roles of ROS in Increased Myogenic Constriction Following EEL Exposure

Next, pressure–diameter relationships were obtained in MCA arterioles in the absence and presence of Tempol (10^{-4} mol/L) during their exposure to 10% EEL. As shown, the EEL-induced augmentation of myogenic response (Figure 6B) was concomitantly associated with a significant increase in vascular superoxide production (Figure 6C). Tempol (10^{-4} mol/L), which did not significantly affect the vascular superoxide level in normal vessels, prevented EEL augmentation of arteriolar myogenic constriction (Figure 6B) and reversed their increased superoxide production, confirming the superoxide-dependent nature.

In Vivo Exposure of Arterioles to EHB Via Subarachnoid Administration

To translate the finding of in vitro experiments, we mimicked SAH by in vivo exposure of MCA arterioles to autologous EHB or saline (as vehicle controls) that was injected into the subarachnoid space of anesthetized mice. As shown in Figure 7, arterioles of MCA that had been in vivo exposed to EHB displayed significantly greater myogenic constrictions than those of contralateral arterioles without exposure to EHB (normal controls). Moreover, in vivo arterioles simultaneously exposed to EHB and Tempol (10^{-4} mol/L) significantly reduced the enhanced myogenic constriction, supporting the in vitro observation (Figure 6B). In particular, the specificity of in vivo injection of EHB-induced alteration of myogenic response was verified by the result showing the comparable myogenic responsive pattern in arterioles of contralateral normal and ipsilateral vehicle controls.

Discussion

This study has several salient findings. First, in vitro exposure of MCA arterioles to EHB and EEL significantly enhanced their myogenic response in a concentration- and exposure duration-dependent manner. Second, enhancement of myogenic response was independent of arteriolar endothelium. Third, enhancement was prevented by a superoxide dismutase A

|                | ACh (10^{-6} mol/L) | NONOate (10^{-6} mol/L) |
|----------------|---------------------|-------------------------|
| EC+            | 80.7±13.2           | 86.8±13.1               |
| EC−            | −8.6±13.5*          | 87.7±7.2                |

*A Significant difference from EC+ vessels. n=7 in each group.

Table. Changes in Diameter (µm) of Isolated Cerebral Arteriolar to ACh and NO Donor NONOate Before and After Removal of Endothelium

Figure 6. Changes in normalized pressure–diameter curves (A and B) and vascular superoxide levels (C), as a function of incubation with 10% erythrocyte lysate (EEL) for 1 hour in (A) endothelium-intact (EC+) and endothelium-denuded (EC−) middle cerebral arterioles (n=6), (B) endothelium-intact arterioles in the absence and presence of 10^{-4} mol/L Tempol (n=6), and (C) endothelium-intact arterioles treated with dihydroethidium (10^{-5} mol/L; n=7). *Significant difference between 2 curves. †Significant difference from both controls. ‡Significant deference from 10% EEL treatment.
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This presents an important dilemma for the physician, namely, abnormal coagulation, traumatic brain injury, spinal rupture of a cerebral aneurysm, hypertensive crisis, conditions is SAH, which is predominately caused by spontaneous rupture of a cerebral aneurysm, hypertensive crisis, abnormal coagulation, traumatic brain injury, or spinal vessel abnormalities. The acute effect of SAH, which is defined as primary ischemia due to the loss of tissue perfusion as a direct result of hemorrhage, occurs within 1 to 3 days of initial bleeding. The second/delayed cerebral ischemia following the primary ischemic period can last much longer (weeks), during which cerebral edema, hydrocephalus, and vasospasm contribute significantly to the failure of adequate CBF to meet the metabolic demand of the cerebral tissue, leading to permanent brain parenchyma ischemia and potential cerebral infarction. In this regard, the reduction in CBF, as a function of delayed ischemia, becomes one of the most devastating consequences. Vasospasm, characterized as extended constriction of the large extraparenchymal arteries throughout the cerebral circulation, has attracted considerable attention and is believed to be one of the most important causes of CBF deficiency. To date, there are no effective treatments for delayed ischemia because of a lack of understanding about the mechanisms involved. Moreover, during hemorrhagic shock and cerebrovascular spasm, patients who have higher blood pressure usually have worse prognosis. This presents an important dilemma for the physician, namely, should systemic pressure be reduced to perhaps prevent a secondary hemorrhagic stroke? Or should it not be reduced so as to provide a greater pressure gradient and thus force for blood flow through those arteries with reduced diameters caused by increased pressure-induced myogenic constriction (Figures 2 through 5)? In this respect, understanding the underlying mechanisms of postbleeding vasospasm may help to develop optimal therapeutic modalities.

A unique feature of the cerebral circulation is the autoregulation of CBF controlled primarily by the myogenic mechanism, which is sensitive to changes in intraluminal pressure and aims to maintain constant blood flow. This mechanism, in particular, is well developed in small arteries and arterioles and may play crucial roles in the development of vasospasm observed in clinical conditions. During surgery, vasospasm can be observed only in the large arteries (>300 μm in diameter), but substantial vasospasm may also be present predominantly in smaller arteries (≈200 μm) and arterioles (<100 μm in diameter), which is indiscernible during operations. In pathological conditions, altered autoregulation of CBF as a function of increases in myogenic constriction may be responsible for vasospasm-induced brain injury.

Enhanced Myogenic Response as a Function of Extravasated Blood Lysates

We found that exposure of MCA arterioles to extravascular plasma did not enhance myogenic response, whereas both EHB and EEL did, as shown by the significant downward shift of pressure–diameter curves compared with controls (Figures 2 and 7), in a concentration-dependent manner (Figure 3). Consequently, it seems that EEL plays key roles in the mediation of enhanced myogenic response. We also found that the exposure of vessels to EEL or EHB for 1 hour elicited significantly greater myogenic constrictor responses (Figure 2); however, this response was not further enhanced by extending the duration of exposure up to 6 hours (Figure 4). More important, the recovery period necessary for returning enhanced myogenic constriction back to the normal level was proportional to the duration of EEL exposure (Figure 5). These findings may be extrapolated to 3 important clinical conditions. First, during hemorrhage or SAH, 1-hour exposure to extravasated blood is sufficient to elicit vasospasm, given enhanced myogenic constriction (even if the intraluminal/systemic blood pressure is unchanged); however, this is reversible if hematoma is removed or hemolyzed blood is washed out during this time period. Second, if blood lysates are not removed within a limited time frame, the maintained augmentation of myogenic constriction may initiate the development of longer lasting ischemia. Third, the enhanced myogenic response (vasoconstriction) is proportional to the
duration of blood lysate exposure—the phenomenon that provides mechanistically based explanations for the clinical observation that earlier removal of hematoma ensures a better prognosis of patients.

**Superoxide-Dependent Mediation of Enhanced Myogenic Constriction in Response to Blood Lysates**

Multiple molecular and cellular mechanisms play roles in augmented myogenic constriction and cerebral arterial vasospasm. Among others, the enhanced production of vasoconstrictors (e.g., endothelin), which are known to be capable of increasing calcium influx or reducing the release of vasodilators (e.g., NO), can increase the contractile properties of smooth muscle. We demonstrated previously that increases in smooth muscle Ca²⁺ level contribute significantly to the basilar cerebral artery constriction in response to hemolyzed blood. To date, the specific mechanism responsible for superoxide-dependent altered myogenic constriction is proposed primarily as impaired NO bioavailability, as a function of superoxide scavenging of NO. In the present study, however, enhanced myogenic constriction behaved in an endothelium-independent manner that was reversible with Tempol (Figure 6), suggesting that the effects of superoxide on arteriolar myogenic constriction are not predominantly caused by its scavenging of NO or simply independent of NO. Previous studies indicated that hemorrhage elicited oxidative stress, and we also found a ROS-dependent potentiation of myogenic response in a variety of pathological conditions. In this regard, it is logical to speculate that scavenging of ROS is able to prevent the EHB/EEL-induced enhancement of myogenic constriction. Indeed, both in vitro (Figure 6) and in vivo (Figure 7) administration of Tempol to vessels that were exposed to blood lysates prevented the augmentation of myogenic response and reversed increases in vascular superoxide. Aligning with our results, the overexpression of SOD1 (superoxide dismutase) has been demonstrated to be able to prevent hyperglycemia-induced cerebral myogenic dysfunction. Based on these findings, we suggest that an increase in myogenic vasospasm is attributable, at least in part, to the hemorrhage-induced increase in vascular superoxide that elicits increases in smooth muscle Ca²⁺ to promote contractility. This hypothesis is supported by the study showing that superoxide enhances Ca²⁺ entry through L-type channels in renal arterioles.

**Perspectives and Conclusions**

The translational aspect of our study is that we administered a small amount of blood into the subarachnoid space of mouse brain to simulate clinical conditions, such as SAH, and then assessed the myogenic response of isolated arterioles. We found similar myogenic responsive patterns in both in vivo and in vitro conditions that were clinically reminiscent of, first, adjusting high systemic blood pressure in patients after hemorrhagic stroke. Interestingly, a recent clinical trial indicated that an acute reduction of systemic blood pressure did not significantly improve CBF to surrounding brain areas after hemorrhagic shock. The outcomes correspond to our findings showing that the enhanced myogenic constriction in response to exposure of arterioles to hemolyzed blood was present in a relatively lower pressure range (corresponding to 40–120 mm Hg systemic blood pressure), implying that lowering systemic blood pressure may not be sufficient to overcome enhanced myogenic constriction after hemorrhage. Second, even in the absence of active bleeding, the surgical removal of cummulate blood may prevent the development of delayed vasospasm/ischemia, a response that can be elicited by altered myogenic vasoconstriction and potentiated by prolonged blood exposure. To this end, some local interventions are perhaps preferable, such as immediate debridging of extravasated blood and local application of antioxidants during surgery, therapies that may reduce cerebral vasospasm and prevent possible development of delayed ischemia.

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**Disclosures**

None.

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