Intracerebral Hemorrhage Elicits Aberration in Cardiomyocyte Contractile Function and Intracellular Ca²⁺ Transients

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Background and Purpose—The sequelae of intracerebral hemorrhage involve multiple organ damage including electrocardiographic alteration, although the mechanism(s) behind myocardial dysfunction is unknown. The aim of this study was to examine the impact of intracerebral hemorrhage on cardiomyocyte contractile function, intracellular Ca²⁺ handling, Ca²⁺ cycling proteins, I kappa B beta protein (IκB) phosphorylation, hypoxia-inducible factor 1α (HIF-1α), and nitrosative damage within 48 hours of injury.

Methods—Mechanical and intracellular Ca²⁺ properties were evaluated including peak shortening (PS), maximal velocity of shortening/relengthening (±dL/dt), time-to-PS (TPS), time-to-90% relengthening (TR90), fura-2 fluorescence intensity (FFI), and intracellular Ca²⁺ decay.

Results—Myocytes from intracerebral hemorrhage rats exhibited depressed PS, ±dL/dt, prolonged TPS and TR90, as well as declined baseline FFI and slowed intracellular Ca²⁺ decay between 12 and 24 hours after injury. Most of these aberrations returned to normal levels 48 hours after hemorrhage with the exception of ±dL/dt and TR90. Myocytes from 24-hour posthemorrhage rats exhibited a steppe negative staircase in PS with increased stimulus frequency. Cardiac expression of sarco(endo)plasmic reticulum Ca²⁺-ATPase 2a and phospholamban was enhanced, whereas that of Na⁺/Ca²⁺ exchanger and voltage-dependent K⁺ channel was decreased. IκB phosphorylation, HIF-1α, inducible NO synthase, and 3-nitrotyrosine were enhanced 12 hours after injury.

Conclusions—These data demonstrated that intracerebral hemorrhage initiates cardiomyocyte contractile and intracellular Ca²⁺ dysregulation possibly related to altered expression of Ca²⁺ cycling proteins, nitrosative damage, and myocardial phosphorylation of IκB. (Stroke. 2006;37:1875-1882.)

Key Word: calcium ■ cardiac myocytes ■ contraction ■ intracerebral hemorrhage

Clinical and experimental evidence has suggested that traumatic injury of brain leads to ECG changes, elevation in cardiac enzyme, myocardial dysfunction, and arrhythmias. A significant portion of patients with subarachnoid hemorrhage (40% to 70%) and intracerebral hemorrhage (60% to 70%) present with mild to moderate ECG abnormalities without coronary events during early stage of brain injury and may die eventually of cardiac sequelae, suggesting a likelihood cerebrogenic cardiac disturbance. Similar ECG changes have also been found in patients with head injury, brain tumors, and meningitis, including prolonged QT intervals, depressed ST segments, inverted T waves, and ectopic ventricular beats. These ECG changes, which usually evolve within days after injury, are associated with a high risk of sudden cardiac death. Up to date, the main mechanism speculated for cerebrogenic cardiac disturbance focuses on increased sympathetic nervous discharge (catecholamine spillover) and reduced parasympathetic nervous activity. This “neurogenic theory” of myocardial dysfunction was substantiated by the observation that sympathetic blockade nullifies brain injury–induced myocardial defects. Nevertheless, whether intracerebral hemorrhage exerts any direct influence on myocardial function is unknown. The aim of this study was to examine the impact of intracerebral hemorrhage on cardiomyocyte contractile function and intracellular Ca²⁺ homeostasis. Adult rat ventricular myocytes were used to avoid potential neurohormonal regulation of cardiac function. Protein expression of Ca²⁺ cycling proteins, including sarco(endo)plasmic reticulum Ca²⁺-ATPase (SERCA), phospholamban (PLB), Na⁺/Ca²⁺ exchanger (NCX), voltage-dependent K⁺ channel (Kv1,2), I kappa B beta protein (IκB), the negative regulator of transcription factor nuclear factor κB (NF-κB), hypoxia-inducible factor 1α (HIF-1α), and nitrosative damage (inducible NO synthase [iNOS] and 3-nitrotyrosine formation), were also determined to better understand the mechanism of action behind intracerebral hemorrhage–elicited cardiomyocyte contractile dysfunction.

Materials and Methods
Induction of Intracerebral Hemorrhage
All animal procedures were in accordance with our institutional guidelines. Intracerebral hemorrhage was induced by stereotaxic intrastriatal administration of bacterial type IV collagenase. In brief, after anesthesia with pentobarbital (50 mg/kg IP), adult female...
Sprague-Dawley rats (~300 g) were placed onto a stereotaxic frame instrument (David Kopf Instruments). A burr hole was made and a 26-gauge Hamilton syringe needle was inserted into the striatum (location 3.0 mm right lateral to the midline, 0.2 mm posterior to bregma, and 6 mm in depth below the skull). Hematoma and sham rats received 5 µL collagenase (0.2 U/µL) or saline, respectively. After the injection, the needle was removed with a 5-minute delay to prevent reflux, and the skin was sutured. During the recovery period, rats were assessed for forelimb flexion and contralateral circling to confirm intracerebral hemorrhage procedures. No seizure was observed during this procedure. After recovery from anesthesia, rats were maintained under a 12-hour light/dark circadian circle with free access to food and water.

**Isolation of Cardiomyocytes and Incubation of Bacterial Type IV Collagenase**

At 6, 12, 24, and 48 hours after intracerebral hemorrhage, rats were euthanized, and the hearts were removed and perfused (at 37°C) with a Krebs–Henseleit bicarbonate buffer containing 223 U/mL collagenase II and 0.1 mg/mL hyaluronidase for 30 minutes. After perfusion, left ventricles were minced to disperse cardiomyocytes.¹¹ Fresh isolated cardiomyocytes were used within 6 hours for mechanical and intracellular Ca²⁺ recording. It is possible that the bacterial collagenase administered via stereotaxic frame “leaks” through blood–brain barrier into circulation and damages cardiomyocytes. To this end, we incubated normal cardiomyocytes with 0.06 U/mL and 0.6 U/mL type IV collagenase for 12 hours using a culture system developed in our laboratory.¹¹ The concentration of type IV collagenase (0.06 U/mL) was calculated based on injected enzyme (1.0 U) normalized to blood volume of ~300-g rats (58 mL/kg), assuming all administered collagenase leaks into circulation. A ×10 concentration (0.6 U/mL) was also used for comparison.

**Cell Shortening/Relengthening and Intracellular Ca²⁺ Fluorescence**

Mechanical and intracellular Ca²⁺ properties were assessed using edge detection and fura-2 (0.5 µmol/L).¹¹ Cell shortening and relengthening were assessed using the after indices: peak shortening (PS), which indicates ventricular contractility or height of QRS complex; time-to-PS (TPS), which indicates systolic duration or

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**Figure 1.** Contractile properties of cardiomyocytes from control (Cont), sham, and 6, 12, 24, and 48 hours after intracerebral hemorrhage rat hearts. A, Resting cell length. B, PS. C, Maximal velocity of cell shortening (+dL/dt). D, Maximal velocity of relengthening (−dL/dt). E, TPS. F, TR90. Mean±SEM; n=115 to 119 cells per group; *P<0.05 vs control group.
width of QRS complex; time-to-90% relengthening (TR90), which indicates diastolic duration or QT interval; and maximal velocity of shortening/relengthening (\(dL/dt\)), which depicts maximal velocity of contraction and relaxation. Qualitative changes in intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) were inferred from the ratio of the fura-2 fluorescence intensity (FFI) at 2 wavelengths (360/380). Intracellular Ca\(^{2+}\) fluorescence decay constant (both single and biexponential decay curve fit) was calculated as an indication of the intracellular Ca\(^{2+}\) clearing rate. All measurements were performed at 25°C.

Western Blot Analysis of SERCA2a, PLB, NCX, Kv1.2, I\(_\alpha\)-B, iNOS, and HIF-1\(\alpha\)
Ventricular tissues were sonicated and lysed, followed by centrifugation. Protein samples (50 \(\mu\)g) were separated by polyacrylamide gel electrophoresis using 10% sodium dodecyl sulfate–polyacrylamide gels. Proteins were transferred and membranes were blocked and incubated overnight at 4°C with anti-SERCA2a (1:1000), anti-PLB (1:5000), anti-Kv1.2 (1:1000), anti–I\(_\alpha\)-B (1:1000), anti–phospho-I\(_\alpha\)-B (Ser32) (1:1000), anti-NCX (1:1000), anti-iNOS (1:1000), anti–HIF-1\(\alpha\) (1:1000), and anti–\(\beta\)-actin (1:5000) antibodies. After washing, blots were incubated for 1 hour with a horseradish peroxidase–conjugated secondary antibody (1:5000). The film was scanned, and the intensity of immunoblot bands was detected with a Bio-Rad calibrated densitometer.\(^{11}\)

Nitrotyrosine Determination
Tissues were homogenized and centrifuged, and supernatants (100 \(\mu\)g protein) were applied to disposable microtiter plates before overnight incubation at 4°C. The plate was then incubated with polyclonal anti-nitrotyrosine antibody (1:2000) followed by horseradish peroxidase–conjugated anti-rabbit IgG secondary antibody (1:2000). The peroxidase reaction product was generated using 100 \(\mu\)L tetramethylbenzidine (Abbott Diagnostics). Plates were incubated for 20 minutes in the dark at room temperature, and optical density was measured at 450 nm.\(^{12}\)

Statistical Analysis
Data were expressed as mean±SEM. Statistical analysis was performed by ANOVA followed by Newman–Keuls post hoc test. Significance was defined as \(P<0.05\).

Results
Effect of Intracerebral Hemorrhage on Cell Shortening and Relengthening
Intracerebral hemorrhage did not exert any effect on cardiomyocyte cell length until 12 hours after injury. The resting

Figure 2. Intracellular Ca\(^{2+}\) transient properties in cardiomyocytes from control (Cont), sham, and 6, 12, 24, and 48 hours after intracerebral hemorrhage rat hearts. A, Baseline intracellular Ca\(^{2+}\) FFI. B, Increase of intracellular Ca\(^{2+}\) FFI (AFFI) in response to electrical stimuli. C, Intracellular Ca\(^{2+}\) transient single exponential decay rate. D, Intracellular Ca\(^{2+}\) transient biexponential decay rate. Mean±SEM; n=50 to 69 cells per group; \(\ast P<0.05\) vs control group.

Figure 3. PS amplitude of cardiomyocytes from control (Cont), sham, and 6, 12, 24, and 48 hours after intracerebral hemorrhage rat hearts at different stimulus frequencies (0.1 to 5.0 Hz). Each point represents PS amplitude normalized to that of 0.1 Hz. Mean±SEM; n=21 to 32 cells per group; \(\ast P<0.05\) vs control group.
cell length was significantly shortened at 12 and 24 hours after intracerebral hemorrhage. However, the shortened cell length returned to control level by 48 hours after injury (Figure 1A). PS normalized to resting cell length, and +dL/dt were significantly reduced by intracerebral hemorrhage but only after 12 hours of brain injury. Reduced PS and +dL/dt were restored at 24 and 48 hours after injury (Figure 1B and 1C). Intracerebral hemorrhage significantly depressed −dL/dt and prolonged TR90 at 12 hours and beyond (Figure 1D and 1F). TPS was also significantly prolonged at 12 and 24 hours but not at 48 hours after injury (Figure 1E). Sham operation (12 to 24 hours after surgery) did not exhibit any significant effect on mechanical indices tested.

**Effect of Intracerebral Hemorrhage on Intracellular Ca^{2+} Transients**

We used fura-2 to evaluate intracellular Ca^{2+} property in myocytes from control, sham, and intracerebral hemorrhage rats. The fluorescence measurements revealed that intracerebral hemorrhage significantly decreased resting FFI and prolonged intracellular Ca^{2+} extrusion (both single and biexponential) at 12 and 24 hours after injury, whereas these defects returned to control levels by 48 hours. The electrically stimulated increase of Ca^{2+} (ΔFFI) was not altered by intracerebral hemorrhage; nor did sham operation affect any of intracellular Ca^{2+} properties (Figure 2). These results revealed abnormalities in cardiomyocyte intracellular Ca^{2+} handling after intracerebral hemorrhage.

**Figure 4.** Contractile and intracellular Ca^{2+} properties of normal cardiomyocytes incubated with bacterial type IV collagenase (0.06 and 0.6 U/ml) for 12 hours. A, PS amplitude normalized to resting cell length. B, Maximal velocity of shortening/relengthening (+dL/dt). C, TPS. D, TR90. E, Increase of intracellular Ca^{2+} FFI (ΔFFI) in response to electrical stimuli. F, Intracellular Ca^{2+} transient single exponential decay rate. Mean±SEM; n=31 to 50 cells per group.
Effect of Intracerebral Hemorrhage on Myocyte Shortening With Increasing Stimulus Frequencies

To understand possible derangement of cardiomyocyte contractile function after intracerebral hemorrhage, we incrementally enhanced the stimulus frequency from 0.5 Hz (used in Figures 1 and 2) to 5.0 Hz (300 bpm). Cells were initially stimulated to contract at 0.5 Hz for 5 minutes to ensure the steady state before commencing the frequency study. All recordings were normalized to PS obtained at 0.1 Hz from the same cell. Figure 3 displays comparable negative staircases in PS with increasing stimulus frequency in myocytes from control, sham-operated, and 6, 12, and 48 hours after injury groups. However, myocytes from the 24-hour group displayed a significantly greater reduction in PS at stimulating frequencies of 1.0 Hz or higher, suggesting that intracellular Ca\(^{2+}\) resequetration may be dampened at this time point.

Effect of Type IV Collagenase on Mechanical and Intracellular Ca\(^{2+}\) Transient in Cardiomyocytes

To examine whether alterations of cardiomyocyte contractile properties in the bacterial collagenase–induced intracerebral hemorrhage model resulted simply from an artifact attributable to “leakage” of collagenase into systemic circulation, we incubated normal cardiomyocytes for 12 hours with type IV collagenase at either “actual” (0.06 U/mL) or a 10-fold higher level (0.6 U/mL). Results shown in Figure 4 indicated that neither cell shortening (PS, \(\pm dL/dt\), TPS, and TR\(_{90}\)) nor intracellular Ca\(^{2+}\) (ΔFFI and intracellular Ca\(^{2+}\) decay rate) was significantly affected by bacterial collagenase. These data did not favor any direct injury of type IV collagenase on cardiomyocyte function in our experimental setting.

Effect of Intracerebral Hemorrhage on SERCA2a, PLB, NCX, Kv\(_{1.2}\), IκB, and Phosphorylated IκB Expression

To delineate the mechanisms responsible for intracerebral hemorrhage–induced change in myocyte contractile and intracellular Ca\(^{2+}\) properties, protein expressions of key intracellular Ca\(^{2+}\) cycling proteins SERCA2a, PLB, NCX, as well as Kv\(_{1.2}\), were evaluated in myocardium from control and posthemorrhage rats. These proteins are closely associated with myocardial mechanical properties. Our results indicated that cardiac expression of SERCA2a was significantly elevated at 12 and 24 hours after injury but was decreased at 48 hours after injury. Cardiac expression of PLB was upregulated after 6 hours of injury and remained elevated throughout the rest of duration studied. Intracerebral hemorrhage downregulated NCX expression at 6 hours and reduced Kv\(_{1.2}\) abundance at \(\geq 24\) hours after injury (Figure 5). Our study further evaluated phosphorylation of the proinflammatory cytokine NF-κB inhibitor IκB, which triggers NF-κB activation in posthemorrhage rat hearts. Although the total expression of IκB was not affected by intracerebral hemorrhage, phosphorylation of IκB (both absolute levels and phosphorylated IκB [p-IκB]-to-IκB ratio) was significantly enhanced 12 hours after the injury and remained elevated throughout 48 hours.

Figure 5. Western blot analysis of protein expression of SERCA2a (A), PLB (B), NCX (C), and Kv\(_{1.2}\) potassium channel (D) in myocardium from control (Cont), sham, and 6, 12, 24, and 48 hours after intracerebral hemorrhage rats. Inset, Representative gel blots of SERCA2a, PLB, NCX, and Kv\(_{1.2}\) using specific anti-SERCA2a, anti-PLB, anti-NCX, and anti-Kv\(_{1.2}\) antibodies. Mean±SEM; n=4 to 6; *P<0.05 vs control group.
hours after injury (with the exception that p-IκB was non-statistically elevated at 24 hours after injury; Figure 6). These data indicated that intracerebral hemorrhage activates NF-κB in myocardium, which may contribute to altered cardiomyocyte function in postintracerebral hemorrhage hearts.

**Effect of Intracerebral Hemorrhage on HIF-1α, iNOS, and 3-Nitrotyrosine Formation**

Figure 7 depicted that intracerebral hemorrhage upregulated HIF-1α, a master transcriptional regulator of local cellular response to hypoxia, between 6 and 24 hours after injury. Our data also revealed upregulated iNOS expression and 3-nitrotyrosine formation at 6 or 12 hours after the brain injury (Figure 7). These data indicated possible involvement of hypoxic transcription factor HIF-1α and nitrosative damage in intracerebral hemorrhage–induced cardiomyocyte dysfunction.

**Discussion**

Our present study reported for the first time a direct link between intracerebral hemorrhage and cardiomyocyte dysfunction accompanied with altered cardiac Ca2+ cycling proteins, activation of NF-κB (attributable to higher phosphorylation of IκB), nitrosative damage, and upregulation of HIF-1α. The major mechanical abnormalities were decreased cell shortening, reduced velocity of shortening and relengthening, prolonged duration of shortening and relengthening, reduced baseline intracellular Ca2+, and slowed intracellular Ca2+ decay at 12 and 24 hours after injury. Most of these mechanical aberrations returned to normal levels by 48 hours, with the exception of −dL/dt and TR90. Myocytes also displayed reduced ability to pace with increased stimulus frequency at 24 hours after intracerebral hemorrhage but not at any other time point tested. Our immunoblot analysis revealed that expression of essential Ca2+ cycling protein SERCA2a was enhanced at 12 and 24 hours after injury, whereas the level of it was declined by 48 hours after injury. Similarly, expression of the SERCA locker protein PLB was significantly enhanced after 6 hours of hemorrhage. Our data also revealed depressed levels of NCX and Kv1.2 at 6 hours or >24 hours after injury. Our data suggest that alteration of certain Ca2+ cycling proteins, activation of NF-κB, nitrosative damage, and HIF-1α expression contributes to the impaired cardiomyocyte function after intracerebral hemorrhage.

Myocardial dysfunction is not uncommon after brain injury. Patients with spontaneous intracranial hemorrhage or brain injury display segmental left ventricular dysfunction and ECG alteration.5,15–17 These ECG changes are often ischemic-like in nature, including QT prolongation, ST segment shift, and late ventricular potentials.5 Because ischemic-like ECG changes are mainly direct consequences of cerebral condition, absence of these changes can usually rule out cerebrogenic cardiac abnormalities in intracerebral hemorrhage.6 Nonetheless, these ECG changes are considered to be highly sensitive but poorly specific. Appearance of ECG abnormalities, especially QT prolongation, may often result

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**Figure 6.** Western blot analysis of protein expression of IκB and p-IκB in myocardium from control (Cont), sham, and 6, 12, 24, and 48 hours after intracerebral hemorrhage rats. A, Representative gel blots of IκB and p-IκB using specific anti-IκB, anti–p-IκB, and anti–β-actin antibodies. B, IκB expression. C, p-IκB expression. D, p-IκB-to-IκB ratio. Mean±SEM; n=4 to 6; *P<0.05 vs control group.
from pre-existing coronary artery disease, which makes it rather difficult to assess the sole effect of intracerebral hemorrhage on myocardial function. Using single cardiomyocytes devoid of neurohormonal regulation, we were able to evaluate the effect of intracerebral hemorrhage on the hearts. The most prominent cardiac defects after intracerebral hemorrhage seen in our current study are reduced cell shortening, maximal velocity of shortening/relengthening (which depicts poor contractility), and prolonged duration of contraction and relaxation duration (which reflects prolonged duration of systole and diastole or QT prolongation) associated with diminished stress tolerance of myocyte shortening with increased stimulus frequency. Several potential mechanisms may be speculated for impaired mechanical functions, including reduction in either abundance or function of cardiac contractile proteins (eg, actin, myosin) and intracellular Ca\(^2+\) mishandling. Our intracellular Ca\(^2+\) measurement confirmed a decrease in intracellular Ca\(^2+\) level and a decline in intracellular Ca\(^2+\) extrusion in myocytes from hemorrhagic rats, although other machineries remain to be explored. The fact that the rise in intracellular Ca\(^2+\) (ΔFFI) is comparable between control and hemorrhagic groups indicates that loss or impaired function of these proteins may directly contribute to contractile dysfunction in myocytes from hemorrhagic rats. The mechanism of enhanced SERCA2a levels at 12 and 24 hours after hemorrhage is unclear but may serve as a compensatory mechanism for impaired intracellular Ca\(^2+\) homeostasis (prolongation of relaxation duration and reduction in −dL/dt). The decreased SERCA2a level at 48 hours after injury may reflect an eventual decompensation of SERCA2a protein. PLB was elevated in posthemorrhagic group, consistent with slowed intracellular Ca\(^2+\) clearing, reduced −dL/dt, and prolonged relaxation duration. Our finding of enhanced myocardial IkB phosphorylation (correlates with an enhanced NF-κB activation) after intracerebral hemorrhage provided a causal relationship between cytokines and onset of cardiomyocyte dysfunction (12 hours). Activation of cytokines such as NF-κB is consistent with enhanced iNOS expression and nitrotyrosine formation, which is indicative of cardiac nitrrosative damage after intracerebral hemorrhage. Upregulated iNOS and nitrrosative damage are associated myocardial morphological and functional abnormalities. The enhanced cardiac HIF-1α expression is consistent with the increased brain HIF-1α levels after intracerebral hemorrhage. Although the impact of HIF-1α on cardiomyocyte contractile function has not been elucidated, constitutive expression of HIF-1α was found to elicit cardioprotection by reducing infarct size and improve echocardiographic performance after myocardial infarction. It is possible that enhanced HIF-1α serves as a compensatory mechanism against myocardial ischemia as a result of intra-
cerebral hemorrhage. Further study is warranted to understand the relationship between HIF-1α and cardiomyocyte function under both physiological condition and pathophysiological environment. Last but not the least, our short-term incubation of cardiomyocytes with type IV collagenase did not favor possible “contamination” of collagenase to hemorrhage-induced cardiomyocyte dysfunction. In fact, the level of collagenase we used to disperse cardiomyocytes (∼72 U/mL) is ∼1200 times higher than that used to induce intracerebral hemorrhage.11

In summary, this study provides direct evidence of altered cardiac contractile function at cardiomyocyte level under experimental intracerebral hemorrhage. Our data support the existence of a “myogenic theory” of intracerebral hemorrhage–associated myocardial damage, which may work in concert with the “neurogenic” mechanism to deteriorate myocardial performance. It is not clear at this time why some of the compromised cardiomyocyte functions reverse; future studies will be required to elucidate cell death and survival mechanism in cardiomyocytes after intracerebral hemorrhage.

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Disclosures
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References
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