Cerebral Ischemia Elicits Aberration in Myocardium Contractile Function and Intracellular Calcium Handling

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Key Words

Cerebral ischemia • Intracellular calcium • Ca^{2+} cycling proteins

Abstract

The mechanisms of myocardial dysfunction and calcium handling disturbance underlying cerebral ischemia remain obscure. Here we for the first time report that acute cerebral ischemia significantly increased left ventricular end diastolic pressure (LVEDP), but decreased $+dP/dt$, $-dP/dt$, and left ventricular systolic pressure (LVSP). Significant increase in either the resting or KCl-induced $[\text{Ca}^{2+}]_i$ in ventricular myocytes was also detected by scanning confocal microscopy at 2 and 24 hours after cerebral ischemia. Verapamil as a blocker of $I_{\text{Ca,L}}$, ryanodine as a specific inhibitor of RyR, thapsigargin as a highly specific inhibitor of sarco(endo)plasmic reticulum Ca^{2+} -ATPase 2a (SERCA2a) and SEA0400 as a selective NCX inhibitor changed the area under the curve of averaged ratio of fluorescence (F/F_0) induced by KCl. Cardiac expression of $\text{Ca}_v1.2$ was significantly up-regulated at 2 and 24 hours after cerebral ischemia, whereas cardiac expression of SERCA2a and Na^+ - Ca^{2+} exchanger (NCX) was significantly down-

regulated at the same time period after cerebral ischemia. Cardiac expression of phospholamban (PLB) was significantly elevated at 2 hours after cerebral ischemia but was restored to about normal level at 24 hours after injury. These data suggest that acute cerebral ischemia may specifically disturb cardiac function and calcium homeostasis, which are related to increase of $\text{Ca}_v1.2$ and decrease of through up-regulating $\text{Ca}_v1.2$ and PLB, down-regulating SERCA2a and NCX, subsequently leading to Ca^{2+} overload by the enhancement of Ca^{2+} influx and inhibition of intracellular Ca^{2+} extrusion and cerebral ischemia-induced myocardial dysfunction.

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Introduction

Ischemic cerebrovascular disease is a major public health issue that affects millions of patients worldwide and remains the third leading cause of mortality and morbidity in low-income and middle-income population. It is estimated that stroke caused about 5.7 million deaths in 2005; the number of global deaths is predicted to rise to 6.5 million in 2015 and to 7.8 million in 2030,

respectively, without intervention [1]. One of the important reasons causing a higher incidence and mortality of ischemic cerebrovascular disease is a cardiac dysfunction during the initial period of cerebral ischemia, which is revealed by changes in the ECG, elevation in cardiac enzyme, and life-threatening arrhythmias [2-4]. It has been well documented that prolonged QT intervals, depressed ST segments, inverted T waves, and ectopic ventricular beats were frequently observed in patients with ischemic cerebrovascular disease [5-6], suggesting that cardiac dysfunction may be the case that, at least in part, leads to death of those patients. Even though the imbalance between sympathetic and parasympathetic activity was found in those patients with cardiovascular disturbances induced by cerebrovascular diseases [7-8], the full understanding of underlying mechanisms of cerebral ischemia-induced cardiovascular disturbances has never been discovered. Our previous studies have demonstrated that I_{Na^+} , I_{to} , and $I_{Ca,L}$ channelopathy and the increase in $[Ca^{2+}]_i$ in ventricular myocytes isolated from cerebral ischemic rats, which might be one of the major ionic mechanisms underlying cerebrogenic cardiac arrhythmias [9-10]. Nevertheless, whether cerebral ischemia plays any direct role in affecting myocardial function and how the calcium handling changes during this pathophysiological condition should be elucidated.

It has been known that cardiac contraction is triggered by the elevation of the Ca^{2+} concentration in the cytoplasm of the cardiomyocyte, which is mediated by Ca^{2+} -release channels (Ryanodine Receptors, RyRs) that taps the Ca^{2+} store in the lumen of the SR, and/or plasma-membrane Ca^{2+} channels (mainly L-type calcium channels) that taps the high concentrations of Ca^{2+} in the extracellular space [11-12]. Cardiac relaxation is triggered by the lowering of the cytosolic Ca^{2+} concentration through the combined activity of SERCA2a, Plasma-Membrane Ca^{2+} -ATPases (PMCA) and Na^+/Ca^{2+} Exchangers (NCXs) that replenish the SR and extracellular Ca^{2+} stores [13-14].

The present study was therefore designed to examine the direct impact of cerebral ischemia on cardiomyocyte contractile function and intracellular Ca^{2+} homeostasis. Adult rat ventricular myocytes were used to avoid potential neuro-hormonal regulation of cardiac function. Protein expressions of Ca^{2+} cycling proteins were also determined to better understand the mechanism of action behind cerebral ischemia-elicited cardiomyocyte contractile and diastole dysfunction. These data have been the first time to demonstrate that the direct link between cerebral ischemia and cerebral ischemia-induced cardiac

dysfunction, which confirmed by the up-regulation of $Ca_v1.2$ and PLB expressions, and the down-regulation of SERCA2a and NCX expressions, respectively, in the initial period of cerebral ischemia, leading to Ca^{2+} overload and subsequent cardiac dysfunction.

Materials and Methods

Preparation of cerebral ischemia model by middle cerebral artery occlusion

Wistar rats (250 ± 20 g, male from Experimental Animal Center of Harbin Medical University, Harbin, China) were used. All rats were kept at the animal facility with room temperature at $23 \pm 1^\circ\text{C}$, humidity of $55 \pm 5\%$, and 12 h dark/light cycle and allowed unlimited food and water. All experimental protocols were pre-approved by the Experimental Animal Ethic Committee of Harbin Medical University, China. The rats were anesthetized with chloral hydrate (300 mg/kg). The procedure for occluding the middle cerebral artery (MCA) using an intraluminal suture was described in detail previously [15]. In brief, we exposed the right common carotid artery, internal carotid artery, and external carotid artery surgically. A 4-0 monofilament nylon suture (Beijing Sunbio Biotech Co Ltd) with a rounded tip was aseptically introduced into the right carotid artery, in an anterograde fashion towards the carotid bifurcation. It was then directed distally up the right internal carotid artery to a distance of 17.5 ± 0.5 mm from the carotid bifurcation to occlude the origin of the MCA for 2 h and 24 h. Body temperature ($37 \pm 1^\circ\text{C}$) was monitored and maintained by a rectal thermometer and a heating pad. In sham-operated rats, the thread was immediately removed as soon as the origin of the MCA was reached.

Identification of cerebral infarction and evaluation of neurological deficit after MCA occlusion

The neurological deficits were tested by single blind method after MCAO, which was previously developed [16] and the evaluation of neurological deficits can be made by counting cumulative scales from scale 0 to 4, representing no visible neurological deficits as the scale 0, forelimb flexion as the scale 1, contralateral forelimb grips weakly as the scale 2, circling to the paretic side only when pulled by the tail (the animal was allowed to move about freely) as the scale 3, and spontaneous circling as the scale 4, respectively. For identification of the cerebral infarction, the brain was then removed carefully and dissected into coronal 2-mm sections after the animal was tested for neurological deficits, subsequently immersed into a 2% solution of 2,3,5-triphenyltetrazolium chloride (TTC) in normal saline at 37°C for 30 min, and then fixed in 4% paraformaldehyde solution at 4°C .

Measurements of Hemodynamic Function

For terminal study, rats from 2 h and 24 h after MCAO were anesthetized and a standard lead II ECG from both healthy and MCAO rats were recorded using (BL-420, Chengdu TME Technology Co, Ltd, China). Left ventricular systolic pressure

(LVSP), left ventricular end diastolic pressure (LVEDP), and time derivatives of pressure change were measured during contraction ($+dP/dt$) and relaxation ($-dP/dt$) using a polygraph as described previously [17].

Isolation of Rat Ventricular Myocytes

The heart was quickly excised from rats after anesthesia. The excised heart was retrogradely perfused (~ 7 ml/min) with Ca^{2+} -containing Tyrode's solution by using a Langendorff perfusion system for 5 min at 37°C . The perfusate was then switched to a nominally Ca^{2+} -free Tyrode solution for 5 min, followed by the same solution containing collagenase (type II, 100–150 kU/L) and 1% bovine serum albumin. The left ventricular tissue was then excised from the softened hearts, minced, and placed in a KB medium at 4°C for about 1 h before experiments. This procedure typically yielded 50% rod-shaped Ca^{2+} -tolerant cardiomyocytes. Only single rod-shaped cells with clear cross-striation and without spontaneous contraction were selected for experiments.

Measurement of Intracellular Ca^{2+} Concentration ($[\text{Ca}^{2+}]_i$)

Intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) was assessed using Fluo-3/AM (5 $\mu\text{mol/L}$) [18]. Pluronic F-127, a mild detergent, is used as a dispersing agent to facilitate the loading. Final concentration in the loading medium is 0.04% Pluronic F-127 to load fluo-3. Fluorescent changes of Fluo-3/AM-loaded cells were detected using laser scanning confocal microscope (Olympus FV-300) with 488 nm for excitation from an argon ion laser and 530 nm for emission. The $[\text{Ca}^{2+}]_i$ was further investigated in Fluo-3/AM loaded cardiac myocytes at $23\text{--}24^\circ\text{C}$ and 30 mmol/L KCl in standard Tyrode's solution. KCl was added between 1st and 2nd scan. For some experiments, verapamil (10 $\mu\text{mol/L}$) as a blocker of $\text{I}_{\text{Ca,L}}$, ryanodine (100 $\mu\text{mol/L}$) as a specific inhibitor of RyR, thapsigargin (10 nmol/L) as a highly specific inhibitor of SERCA (Sigma), SEA0400 (1.0 $\mu\text{mol/L}$) as a selective NCX inhibitor (SEA0400 was dissolved in dimethyl sulfoxide (vehicle), Sigma-Aldrich (St. Louis, MO, USA), were applied to bath solution before KCl. The fluorescent intensities before (FI_0) and after (FI) drug administration were both recorded. Qualitative changes in $[\text{Ca}^{2+}]_i$ were inferred from the ratio of FI/FI_0 .

Real-time Quantitative Polymerase Chain Reaction

The expression levels of $\text{Ca}_v1.2$, RyR₂, SERCA2a, and NCX mRNA were evaluated by using conventional real-time RT-PCR. Primers for $\text{Ca}_v1.2$ mRNA were 5'-TCT GCT CTG CCT GAC TCT GA-3' (forward) and 5'-AAC CCA TTA GGA ACA TTG AAA C-3' (reverse). Primers for RyR₂ mRNA were 5'-AAC CCA TTA GGA ACA TTG AAA C-3' (forward) and 5'-TTG GTC TCT GAG TTC TCC AAA AGC-3' (reverse). Primers for SERCA2a mRNA were 5'-ATT GAG ATC ACA GCT ATG ACT GGT G-3' (forward) and 5'-GCA TTG CAC ATC TCT ATG GTG ACT AG-3' (reverse). Primers for NCX mRNA were 5'-GGC AGA AAC AGG ACC AAA TG-3' (forward) and 5'-GAC TTC TAT AGA GGA CAT AAA CC-3' (reverse). Primers for GAPDH mRNA were 5'-AAG AAG GTG GTG AAG CAG GC-3' (forward) and 5'-TCC ACC ACC CTG TTG CTG TA-3' (reverse). The total

RNA samples from rat hearts post-MCAO were isolated using TRIzol reagent (Invitrogen, USA) and first-strand cDNA was synthesized by reverse transcriptase kit (Invitrogen, USA) according to manufacturers' instruction. Detection Kit (Ambion, USA) in conjunction with real-time PCR with SYBR Green I, using total RNA samples isolated from individuals from different groups, was performed with the expression level of GAPDH as an internal control.

Western blot analysis

The protein samples were extracted from rat heart after MCAO, the procedures for total protein preparation have been described previously [19]. The rat left ventricles were quickly rinsed in PBS buffer (pH7.4) containing protease inhibitors and snap frozen in liquid nitrogen and stored at -80°C for Western blot analysis. Frozen tissue was homogenized with 1000 μl solution contained 40% SDS, 60% RIPA and 1% protease inhibitor in each 100 mg cardiac tissue. The homogenate was then centrifuged at 13,000 rpm for 30 min and the supernatants (containing cytosolic and membrane fractions) were collected. Protein concentrations were measured spectrophotometrically using a BCA kit (Universal Microplate Spectrophotometer; Bio-Tek Instruments, Winooski, VT, USA). Protein samples were fractionated by SDS-PAGE (10% polyacrylamide gels for $\text{Ca}_v1.2$, SERCA2a and NCX, 15% polyacrylamide gels for PLB, 5% polyacrylamide gels for RyR₂) then transferred to PVDF membrane. The primary anti-SERCA2a, NCX, RyR₂ antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-phospholamban antibody (MA3922, ABR Affinity BioReagents, USA) and primary antibody against $\text{Ca}_v1.2$ (Alomone Labs, Jerusalem, Israel) were used with GAPDH (Kangcheng, Shanghai, China) as an internal control. The HiMark™ Pre-Stained High Molecular Weight Protein Standard (Catalog no. LC5699, Invitrogen, USA) was used to determine the apparent molecular weight of high molecular weight RyR₂ proteins.

Data analysis

Group data are expressed as mean $\pm$ SEM and analyzed using SPSS 13.0 software. Statistical comparisons among multiple groups were performed using analysis of variance. A two-tailed $P < 0.05$ was considered to be statistically significant.

Results

Verification of cerebral infarction and evaluation of motor deficit after Middle Cerebral Artery Occlusion

After MCA occlusion, the behavioral deficits were observed as shown in Fig. 1A. Motor deficit scores for 24 h after MCAO (3.42 ± 0.25) were higher than that for 2 h after MCAO (2.39 ± 0.16) (Fig. 1B, $P < 0.01$, $n = 18$ for MCAO 2 h, $n = 12$ for MCAO 24 h). Meanwhile, the morphological observations of brain sections with MCAO were shown in Fig. 1C with 1% TTC, and white-colored areas represented the infarction regions in these sections.

Fig. 1. Representative diagram of the volume of cerebral infarction and results of the neurological deficit in middle cerebral artery occlusion (MCAO). (A, B) The neurological deficit behavior after MCAO, motor deficit scores for 24 h after MCAO were higher than that for 2 h after MCAO (** $P < 0.01$, $n=18$ for MCAO 2 h, $n=12$ for MCAO 24 h). (C) The verification of infarction is shown in a coronal section. Red represents normal tissue. White represents infarct tissue.

The infarction incidences in both model groups accorded with our previous reports [9].

Effects of cerebral ischemia on cardiac Hemodynamic Parameters

Hemodynamic parameters measured in the three groups of rats were shown in Table 1. Compared with the sham animals, heart rate (HR) was slightly increased in MCAO group, but there was no statistical significance ($P > 0.05$). However, the LVEDP, $+dP/dt$ and $-dP/dt$ as well as LVSP were dramatically changed in MCAO rats. As indicated in Table 1, the LVEDP was significantly increased and $+dP/dt$ and $-dP/dt$ were both significantly decreased, while the LVSP was also decreased as well in MCAO groups compared with sham group ($P < 0.01$). These data suggested that either systolic or diastolic function of the hearts were severely impaired after cerebral ischemia.

Effect of cerebral ischemia on intracellular Ca^{2+} concentration

The $[Ca^{2+}]_i$ was further investigated in ventricular cardiomyocytes loaded with Fluo-3/AM before and after stimulation with KCl (30 mmol/L) in standard Tyrode's

Fig. 2. The intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) in cardiac myocytes after MCAO (A) Sequences of fluorescent images in cells after KCl (30 mmol/L) exposures during 290 s with 10 s interval. (B) A linear graph showed comparison of all groups. These linear graphs showed the difference in FI/FI_0 among sham group ($n = 30$), MCAO 2 h ($n = 39$) and MCAO 24 h ($n = 16$). Statistical significance symbols are not shown for clarity. (C) Area under the curve of $[Ca^{2+}]_i$ values of each group, (** $P < 0.01$, vs sham group). (D) Baseline intracellular Ca^{2+} FFI of each group (** $P < 0.01$ for MCAO 2 h, * $P < 0.05$ for MCAO 24 h, vs sham group).

solution (Fig. 2A). Our data indicated that the averaged ratio of fluorescence was increased 5.71 ± 0.25 times in cardiac myocytes after 2 h of MCAO (Fig. 2B, $n = 39$ from 8 rats) and 5.10 ± 0.33 times after 24 h of MCAO ($n = 16$ from 8 rats), but the ratio was only increased 3.93 ± 0.14 times in myocytes of sham-operated rats ($n = 30$ from 6 rats). The area under the curve of averaged ratio of fluorescence (FI/FI_0) was increased to 714.50 ± 38.11 in cardiac myocytes after 2 h of MCAO and 752.40 ± 62.39 after 24 h of MCAO compared with 485.02 ± 25.37 observed in sham group (Fig. 2C, $P < 0.01$). The peaks of fluorescent intensity were appeared at ~ 40 -50 s after KCl and there on different among groups, but the maximal peak of fluorescent intensities were increased from baseline after 2 h and 24 h of MCAO (Fig. 2D, $P < 0.01$ for MCAO 2 h; $P < 0.05$ for MCAO 24 h). These results revealed considerable abnormalities of intracellular Ca^{2+} handling in cardiomyocytes after cerebral ischemia.

Effect of L-type calcium channel on the $[Ca^{2+}]_i$ increase induced by cerebral ischemia

It is well known that the termination of Ca^{2+} entry by shutting off Ca^{2+} channels is crucial for the

Fig. 3. Effect of L-type calcium channel on the $[Ca^{2+}]_i$ increase induced by cerebral ischemia. (A) Effect of verapamil on increasing $[Ca^{2+}]_i$ induced by KCl among sham group ($n = 8$), MCAO 2 h ($n = 20$) and MCAO 24 h ($n = 10$). The elevated extent is larger in cardiac myocytes of cerebral ischemic rats than control. (B) Area under the curve of $[Ca^{2+}]_i$ values of each group after verapamil treatment, (** $P < 0.01$, vs sham group). (C) Inhibition of area under the curve of $[Ca^{2+}]_i$ values induced by verapamil of each group (* $P < 0.05$, vs sham group). (D, E) The relative quantification of $Ca_v1.2$ protein levels was attained by normalizing the band densities to GAPDH, followed by further normalization to the values from control hearts (* $P < 0.05$, vs sham group, $n = 8$ for each group). (F) The relative quantification of $Ca_v1.2$ mRNA levels was attained by normalizing the band densities to GAPDH, followed by further normalization to the values from sham hearts, (** $P < 0.01$, vs sham group, $n = 8$ for each group).

normalization of intracellular Ca^{2+} during diastole. To delineate the mechanisms responsible for cerebral ischemia-induced change in contractile of cardiac myocytes and intracellular Ca^{2+} properties, we firstly verified the contribution of $I_{Ca,L}$ to the $[Ca^{2+}]_i$ increase induced by cerebral ischemia through inhibition of $I_{Ca,L}$ by verapamil (10 $\mu\text{mol/L}$). The averaged ratio of fluorescence was 3.40 ± 0.16 times in cardiac myocytes after 2 h of MCAO ($n = 20$ from 6 rats) and 2.49 ± 0.42 times in cardiac myocytes after 24 h of MCAO ($n = 10$ from 6 rats), whereas the ratio was only 1.86 ± 0.08 times in myocytes of sham-operated rats, respectively (Fig. 3A, $n = 8$ from 4 rats). The area under the curve of averaged ratio of fluorescence (FI/FI_0) in the presence of verapamil was 469.38 ± 35.19 in cardiac myocytes after 2 h of MCAO and 522.81 ± 57.31 after 24 h of MCAO compared with 354.82 ± 19.25 in sham group (Fig. 3C, $P < 0.01$). These data confirmed that the increased $[Ca^{2+}]_i$ was triggered predominantly by transmembrane Ca^{2+} entry through $I_{Ca,L}$ in cerebral ischemic myocytes. Furthermore, the $\alpha_{1C}/Ca_v1.2$ protein and mRNA levels were also examined. Interestingly, the protein expression levels of $\alpha_{1C}/Ca_v1.2$ represented as the ratio of $Ca_v1.2/\text{GAPDH}$ were significantly increased in myocardium of cerebral ischemic rats (Fig. 3C, 1.22 ± 0.11 for MCAO 2 h; 1.33 ± 0.09 for MCAO 24 h) compared with control group ($P < 0.05$, $n = 8$). Meanwhile, the mRNA expression levels of $\alpha_{1C}/Ca_v1.2$ represented as the ratio of α_{1C}/GAPDH were

significantly increased as well in myocardium of cerebral ischemic rats (Fig. 3D, 3.70 ± 0.24 for MCAO 2 h, 6.98 ± 0.60 for MCAO 24 h) compared with control group ($P < 0.01$, $n = 8$).

Effect of ryanodine receptors on the $[Ca^{2+}]_i$ increase induced by cerebral ischemia

The Ca^{2+} release channels (ryanodine receptors, RyRs) of the sarcoplasmic reticulum (SR) play a fundamental role in the cardiac excitation-contraction coupling, we then determined what parts RyRs take in the $[Ca^{2+}]_i$ increase induced by cerebral ischemia by ryanodine, a specific inhibitor of RyR. The averaged ratio of fluorescence induced by KCl without the incubation of ryanodine in cardiac myocytes of both MCAO 2h ($n = 10$ from 6 rats) and MCAO 24h ($n = 9$ from 6 rats) was higher compared with that in cardiac myocytes of sham-operated rats, respectively (Fig. 4A and B, $n = 9$ from 4 rats). The areas under the curve of averaged ratio of fluorescence (FI/FI_0) after incubation with ryanodine were not significantly different among three groups (Fig. 4C, 286.76 ± 14.35 for MCAO 2 h, 330.94 ± 38.86 for MCAO 24 h, and 242.32 ± 12.33 for sham group, $P > 0.05$). Our data also confirmed that both protein levels (Fig. 4D and E) and mRNA (Fig. 4F) of RyR_2 were not significantly changed in myocardium of cerebral ischemic rats after 2h and 24 of MCAO compared with control group ($P > 0.05$, $n = 8$).

Fig. 4. Effect of ryanodine receptors on the $[Ca^{2+}]_i$ increase induced by cerebral ischemia.

(A) Effect of ryanodine on increasing $[Ca^{2+}]_i$ induced by KCl among sham group ($n = 9$), MCAO 2 h ($n = 10$) and MCAO 24 h ($n = 9$). The elevated extent is larger in cardiac myocytes of cerebral ischemic rats than control. (B) Area under the curve of $[Ca^{2+}]_i$ values of each group after ryanodine treatment, ($**P < 0.01$, vs sham group). (C) Inhibition of area under the curve of $[Ca^{2+}]_i$ values induced by ryanodine of each group ($P < 0.05$, vs sham group). (D, E) The relative quantification of RyR₂ protein levels was attained by normalizing the band densities to GAPDH, followed by further normalization to the values from control hearts ($P > 0.05$, vs sham group, $n = 8$ for each group). (F) The relative quantification of RyR₂ mRNA levels was attained by normalizing the band densities to GAPDH, followed by further normalization to the values from sham hearts, ($P > 0.05$, vs sham group, $n = 8$ for each group).

Fig. 5. Effect of SERCAs on the $[Ca^{2+}]_i$ increase induced by cerebral ischemia.

(A) Effect of thapsigargin on increasing $[Ca^{2+}]_i$ induced by KCl among sham group ($n = 9$), MCAO 2 h ($n = 10$) and MCAO 24 h ($n = 9$). The elevated extent is larger in cardiac myocytes of cerebral ischemic rats than control. (B) Area under the curve of $[Ca^{2+}]_i$ values of each group after thapsigargin treatment, ($*P < 0.05$, vs sham group). (C) Enhancement of area under the curve of $[Ca^{2+}]_i$ values induced by thapsigargin of each group ($**P < 0.01$, vs sham group). (D) The relative quantification of SERCA2a protein levels was attained by normalizing the band densities to GAPDH, followed by further normalization to the values from control hearts ($*P < 0.05$ for MCAO 2 h, $**P < 0.01$ for MCAO 24 h, vs sham group, $n = 8$ for each group). (E) The relative quantification of SERCA2a mRNA levels was attained by normalizing the band densities to GAPDH, followed by further normalization to the values from sham hearts, ($**P < 0.01$, vs sham group, $n = 8$ for each group). (F) The relative quantification of PLB protein levels was attained by normalizing the band densities to GAPDH, followed by further normalization to the values from control hearts ($**P < 0.01$ for MCAO 2 h, $P > 0.05$ for MCAO 24 h, vs sham group, $n = 8$ for each group).

Effect of sarco(endo)plasmic reticulum Ca^{2+} -ATPase 2a on the $[Ca^{2+}]_i$ increase induced by cerebral ischemia

SERCAs play an important role for the accumulation of Ca^{2+} within the endoplasmic reticulum, which removed most of the Ca^{2+} from the cytosol. In the following experiment, the effects of thapsigargin, a highly specific inhibitor of SERCA on the intracellular calcium

concentration, were also tested. The averaged ratio of fluorescence induced by KCl before incubation with thapsigargin in cardiac myocytes of both MCAO 2h ($n = 10$ from 6 rats) and MCAO 24h ($n = 9$ from 6 rats) was higher compared with that in cardiac myocytes of sham-operated rats, respectively (Fig. 5A, B, $n = 9$ from 4 rats). Obviously the area under the curve of averaged ratio of fluorescence (FI/FI_0) was increased by

Fig. 6. Effect of NCX on the $[Ca^{2+}]_i$ increase induced by cerebral ischemia. (A) Effect of SEA0400 on increasing $[Ca^{2+}]_i$ induced by KCl among sham group ($n = 9$), MCAO 2 h ($n = 9$) and MCAO 24 h ($n = 10$). The elevated extent is larger in cardiac myocytes of cerebral ischemic rats than control. (B) Area under the curve of $[Ca^{2+}]_i$ values of each group after SEA0400 treatment, ($*P < 0.05$, vs sham group). (C) Enhancement of area under the curve of $[Ca^{2+}]_i$ values induced by SEA0400 of each group ($**P < 0.01$, vs sham group). (D, E) The relative quantification of NCX protein levels was attained by normalizing the band densities to GAPDH, followed by further normalization to the values from control hearts ($*P < 0.05$ for MCAO 2 h, $**P < 0.01$ for MCAO 24 h, vs sham group, $n = 8$ for each group). (F) The relative quantification of NCX mRNA levels was attained by normalizing the band densities to GAPDH, followed by further normalization to the values from sham hearts, ($**P < 0.01$, vs sham group, $n = 8$ for each group).

Group	HR (beats/min)	LVSP (mmHg)	LVEDP (mmHg)	+dP/dt (mmHg/s)	-dP/dt (mmHg/s)
Sham	373 ± 8.7	94.1 ± 4.0	2.8 ± 0.5	4025 ± 177	3706 ± 163
MCAO 2h	389 ± 9.5	67.5 ± 1.9**	13.9 ± 2.3**	2373 ± 263**	2426 ± 253**
MCAO 24 h	404 ± 10.3	52.6 ± 2.1**	14.1 ± 3.1**	1418 ± 116**	1388 ± 72**

Table 1. Effect of cerebral ischemia on hemodynamic parameters of hearts in rat. HR: heart rate; LVSP: left ventricular systolic pressure; LVEDP: left ventricular end diastolic pressure; $\pm dP/dt$ max: time derivatives of pressure. All values are mean $\pm$ SEM. $n = 8$ in each group. $*P < 0.05$ vs. Sham group; $**P < 0.01$ vs. Sham group.

thapsigargin in different groups. However, the enhancement of area under the curve of averaged ratio of fluorescence was decreased (Fig. 5C, 72.35 ± 3.33 for MCAO 2 h; 105.82 ± 7.94 for MCAO 24 h) compared with that in sham group (162.47 ± 6.92 , $P < 0.01$). Especially, the decrease in the area under the curve of average ratio of fluorescence was the most significant after 2h of MCAO. However, the mRNA and protein levels of SERCA were both down-regulated with the prolongation of cerebral ischemia (Fig. 5D and E, $P < 0.01$, $n = 8$). It has been demonstrated that phospholamban (PLB) monomers interact with SERCA2a and reversibly inhibit the Ca^{2+} transport activity of the pump, which interacts with SERCA molecules to lower the apparent affinity of SERCA2a for Ca^{2+} . As shown in Fig. 5F, cardiac expression of PLB was significantly elevated at 2 h after cerebral ischemia but was restored to about baseline at 24 h after cerebral ischemic injury.

Effect of Na^+ - Ca^{2+} exchanger On the $[Ca^{2+}]_i$ increase induced by cerebral ischemia

The Na/Ca exchanger plays a key role in the Ca^{2+} balance within cardiac myocytes, which is the major Ca^{2+} efflux pathway to remove Ca^{2+} influx during excitation-contraction coupling. The $[Ca^{2+}]_i$ was tested in the presence of KCl alone or conjugation with SEA0400, a selective NCX inhibitor. Our results showed that the inhibition of NCX with SEA0400 did increase the averaged ratio of fluorescence in different groups (Fig. 6A, B) and partial inhibition of NCX would lead to a net gain of $[Ca^{2+}]_i$. However, the enhancement of area under the curve of averaged ratio of fluorescence was decreased along with cerebral ischemia duration (Fig. 6C, 72.35 ± 7.11 for MCAO 2 h and 56.47 ± 2.75 for MCAO 24 h) compared with that in sham group (130.02 ± 4.90 , $P < 0.01$). To our interest, cardiac protein expression of NCX was significantly down-regulated after 2 h of cerebral ischemia and remained at even lower level after

Fig. 7. Summary of intracellular calcium handling in cardiovascular myocytes induced by cerebral ischemia.

24 h of cerebral ischemia (Fig. 6D and E, $P < 0.05$ for MCAO 2 h vs sham; $P < 0.01$ for MCAO 24 h vs sham). Meanwhile, the reduction of mRNA expression levels of NCX represented as the ratio of NCX/GAPDH were further confirmed in myocardium of cerebral ischemic rats (Fig. 6F, 0.66 ± 0.02 for MCAO 2 h and 0.48 ± 0.02 for MCAO 24 h) compared with shamed-control group ($P < 0.01$, $n = 8$).

Discussion

In the present study, the plausible mechanisms were investigated between cerebral ischemia and cardiomyocyte dysfunction accompanied with $[Ca^{2+}]_i$ increase via different Ca^{2+} cycling proteins, activation of L-type Ca^{2+} channel (contributing to Ca^{2+} influx to intracellular stores), inhibition of SERCA2a (preventing the relaxation of cardiac muscle, as it sequesters cytosolic Ca^{2+} by active transport into intracellular stores), down-regulation of Na/Ca exchange (reducing the Ca^{2+} efflux from the cell through NCX).

The ideal model of stroke by MCAO for preclinical therapeutic studies should be highly reproducible and have a low mortality, it should be mentioned that our measurements of infarction with TTC have been shown to correlate well with each other and the motor deficit scores after MCAO also confirmed effective occlusion of the artery [20] (Fig. 1). There were increasing evidences that ischemic cerebrovascular diseases played crucial roles in the prediction of sudden cardiac death associated with ventricular arrhythmia and left ventricular hypertrophy (LVH) accompanied with cerebral

ischemia/infarction [21–22]. The major mechanical abnormalities indicates that the cardiac contractile and relaxation functions have been impaired in myocytes from cerebral ischemic rats, consistent with increased intracellular Ca^{2+} and LVEDP, and reduced $\pm dP/dt$ and LVSP, and increased baseline intracellular Ca^{2+} (Table 1, Fig. 2). To delineate the mechanisms responsible for cerebral ischemia-induced changes in cardiomyocyte contractile and intracellular Ca^{2+} profiles, roles and expressions of key intracellular Ca^{2+} cycling proteins such as Cav1.2, RyR₂, SERCA2a, PLB, and NCX were evaluated in myocardium from control and cerebral ischemic rats simply because these proteins are closely associated with mechanical properties of myocardium [13].

The L-type Ca^{2+} channel expressed in cardiac muscle plays an important role in determining the intracellular Ca^{2+} ($[Ca^{2+}]_i$). Our previous studies [9, 10] demonstrated that $I_{Ca,L}$ density recorded from ventricular cardiomyocytes in cerebral ischemic rats was much higher than those from sham-operated rats. In the present study, verapamil was selected to inhibit $I_{Ca,L}$ in order to further estimate the changes in $[Ca^{2+}]_i$ that was expressed by the area under the curve of averaged ratio of fluorescence (FI/FI_0 , Fig. 3). Increased mRNA and protein expressions of $\alpha_{1C}/Ca_v1.2$ have consequence in higher function of this channel properties, which led to the increase in the current density of $I_{Ca,L}$. In the present study, the cerebral ischemia-induced $[Ca^{2+}]_i$ increase (Fig. 2) suggested that the theory of “ Ca^{2+} -induced Ca^{2+} release (CICR)” [23] might be involved in the cardiac dysfunction after cerebral ischemia.

It has been well documented that ryanodine receptors (RyR) function as Ca^{2+} channels that regulate

Ca²⁺ release from intracellular stores to control a diverse array of cellular processes [24]. To clearly distinguish between Ca²⁺ influx and Ca²⁺ release from the sarcoplasmic reticulum, the Ca²⁺ store was blocked with 100 μmol/L ryanodine. However, our results did not provide any specific roles that RyR Ca²⁺ release channels take part in the [Ca²⁺]_i increase induced by cerebral ischemia (Fig. 4), which do not correspond to the changes of [Ca²⁺]_i triggered by caffeine in our previous study [10], therefore the protein and mRNA expressions of RyR₂ were detected by Western Blot and Real time PCR techniques (Fig. 4E and F) and these results further confirmed that the ryanodine receptor (RyR) was not involved in the cardiomyocyte dysfunction induced by cerebral ischemia in rats. To further understand the mechanisms of Ca²⁺ release and reuptake from the sarcoplasmic reticulum, 10 nmol/L thapsigargin was used to inhibit SERCA in the following experiments.

Several studies have shown that disturbed excitation-contraction coupling processes may underlie disturbed myocardial function in various animal models and human conditions [25-28]. Ca²⁺ uptake and release by the sarcoplasmic reticulum (SR) have been found to be altered and the ratio of phospholamban/SR Ca²⁺ ATPase is thought to play a key role in the pathophysiology of heart failure [29]. Our results suggested that the abundance of SR Ca²⁺ ATPase protein has been consistently found to be reduced in the hearts after 2 h of cerebral ischemia. However, the up-regulated protein expression of PLB could contribute to further inhibition of SR Ca²⁺ accumulation, which may reflect a major defect in excitation-contraction coupling in cerebral ischemic rat hearts (Fig. 5). Whereas, the decrease in PLB expression level after 24 hours after injury may be due to the compensation of PLB protein. These data indicated that cerebral ischemia activates PLB in myocardium, which then alters cardiomyocyte function in cerebral ischemic hearts (Fig. 5F). So, the up-regulation of PLB and down-regulation of SERCA2a might contribute to the alterations in the PLB: SERCA2a ratio, then might depress SR Ca²⁺ uptake, leading to increased diastolic Ca²⁺ levels. Potential therapeutic strategies are to increase SERCA2a levels or to attenuate the PLN inhibitory effect on SERCA2a in cardiac myocytes after cerebral ischemia. These studies also supported the idea that a decreased expression of SERCA2a could disturb intracellular Ca²⁺ handling by increasing the relative ratio of PLB to SERCA2a. SERCA2a was reduced in post cerebral ischemic group and this evidence consistent well

with slowed intracellular Ca²⁺ clearance, reduced $+dP/dt$, prolonged relaxation duration, and increased LVEDP (Table 1) after cerebral ischemia.

The Na⁺/Ca²⁺ exchanger (NCX) takes an important part in cardiac Na⁺ and Ca²⁺ homeostasis and plays an essential role for Ca²⁺ extrusion from cardiomyocytes during Ca²⁺ recycling. Altered NCX function may also lead to an abnormal Ca²⁺ release from the sarcoplasmic reticulum (SR) and impaired cardiac electrical activity, and contractility in cardiovascular diseases such as arrhythmias, myocardial ischemia, hypertrophy and heart failure [30-31]. Our observation showed that the functional expression of NCX was reduced in cardiac tissue after cerebral ischemia, suggesting that the loss or impaired function of this protein may also contribute to contractile dysfunction in cardiomyocytes from cerebral ischemic rats (Fig. 6), this result was consistent with the increase in intracellular Ca²⁺ and LVSP, and the decrease in $+dP/dt$ (Table 1).

In summary, this study provides direct evidence of altered cardiac contractile function at cardiomyocyte level under the experimental condition of cerebral ischemia. Our data suggest that the disturbance of Ca²⁺ handlings associated myocardial damage triggered by cerebral ischemia. The expression changes and dysfunction of L-type Ca²⁺ channel, SERCA2a, NCX deteriorate myocardial injury after MCAO (Fig. 7). However, the potential neuro-hormonal regulation may take part in the accident of myocardial damage to negatively regulate the role of calcium handling proteins in the myocardial dysfunction after MCAO and our findings do not lead us to reveal whether other protein trafficking and ion channels function were normal or not for long time. Certainly, much more experiment will be indeed required to certify the changes of calcium handling proteins to elucidate cell death and survival mechanism in future studies.

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